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Population Structuring and Dispersal in the Highly Pelagic Leach's Storm-Petrel: Implications for the EU Population

By Anthony William James Bicknell

PhD

2011

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POPULATION STRUCTURING AND DISPERSAL IN THE HIGHLY PELAGIC LEACH'S STORM-PETREL: IMPLICATIONS FOR THE EU POPULATION

by

Anthony William James Bicknell

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in partial fulfilment for the degree of

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Population structuring and dispersal in the highly pelagic Leach's storm-petrel: implications for the EU population

Anthony William James Bicknell

Abstract

Dispersal is a fundamental process that shapes many aspects of population ecology and evolution. Dramatic local population declines brought about by anthropogenic-driven changes to ecological processes are unfortunately becoming ubiquitous and increasing the urgency to understand dispersal behaviour in many species. For species where direct methods of tracking movement and dispersal are unsuitable, other indirect methods need to be employed to understand and characterise these behaviours.

The steep population decline, due to predation, at the largest EU population of Leach's storm-petrels *Oceanodroma leucorhoa* (St Kilda, Scotland), and the potential part immigrant birds have in buffering this threat, highlighted the need to understand dispersal and connectivity of widely spaced colonies in this species. The implications for the persistence of local colonies and the wider population of Leach's storm-petrels could then be assessed.

A population genetic analysis of 9 colonies across the North Pacific and Atlantic distribution, using two DNA markers (mtDNA control region and microsatellites), revealed ocean basin differentiation (Global $\Phi_{ST} = 0.32$ $P < 0.0001$, Global $F_{ST} = 0.04$, $P < 0.0001$) but also identified a migrant individual in the Pacific population (STRUCTURE migrant assignment). The Atlantic population was found to be genetically homogenous, with patterns of historical and contemporary gene flow, indicating that long-distance effective dispersal is prevalent in Leach's storm-petrels within the ocean basin.

Bayesian stable isotope analysis of carbon and nitrogen ($\delta^{13}C$ & $\delta^{15}N$) provides evidence for natal dispersal as an important dispersal mechanism, and reveals movement of immature birds between colonies during the breeding season as a likely mechanism to promote inter-colony exchange and gene flow. Stable isotope comparison also identified mixing on wintering grounds as another possible influence on dispersal. The potential for immigrant birds to offset the loss caused by predation at the St Kilda colony is supported by these studies, and will likely help the persistence of the colony in the short-term. However, future viability is debateable considering the evidence for both avian and mammalian predation.

This research provides a better understanding of the extent and mechanism of dispersal in the Leach's storm-petrel, which is important to predict the potential impact of environmental change and, where possible, implement effective population management for this species.

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AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

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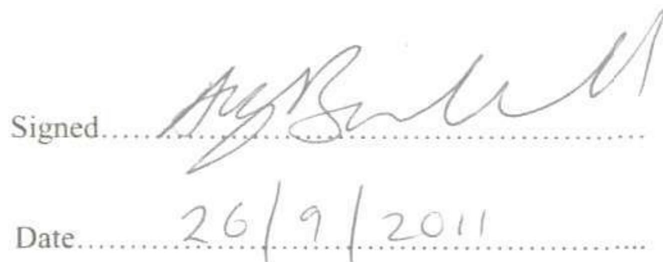

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Date.......26/9/2011.......

Chapter 1

Introduction

1. Introduction

Each chapter in this thesis has been written as an independent piece of research, intended to be integrated together and provide an understanding of dispersal in Leach's storm-petrel *Oceanodroma leucorhoa* and assess the vulnerability of a declining regionally important breeding colony. In this general introduction I will first briefly introduce the concepts and techniques underlying the work. I will then describe the Leach's storm-petrel, its' global population and the decline at the largest colony in the EU, which initiated this research. Finally I will provide an overview of each data chapter.

Concepts

Anthropogenic-driven environmental change is impacting the viability of global and local populations of many species and current rates of extinction have reached levels equivalent to previous global mass extinctions (Barnosky *et al.*, 2011). The capacity for species or populations to cope with these changes is central to their long-term sustainability and, therefore, needs to be assessed if declines and losses at both local and global scales are to be addressed.

Dispersal has been defined as the movement of individuals (or genes) from one breeding area or social group to a another (Clobert *et al.*, 2001). It is a critical ecological behaviour that impacts many aspects of population ecology and genetics, and can be crucial in population viability (Clobert *et al.*, 2001). Natal and breeding dispersal represent a change of location and ultimately the mechanism for genetic exchange, when it results in successful breeding (effective dispersal). Fragmented populations rely upon dispersal to connect sub-populations (demes) and reduce isolation, which may lessen extinction risk (Matthiopoulos *et al.*, 2005). Theory predicts that population genetic structure is an inverse function of gene flow (Wright, 1931) and therefore

genetic structure is negatively correlated with effective natal or breeding dispersal. Revealing patterns of dispersal in fragmented populations and understanding the mechanisms underlying it can therefore provide insights into population dynamics, genetic structuring and conservation of species. However, this requires the movements of individuals or populations to be tracked, which has proved technically problematic, particularly for small, highly vagile species. Studies using marked individuals have been used to estimate dispersal (Paradis *et al.*, 1998, Lebreton *et al.*, 2003) but the sample sizes and intensity required can be prohibitive. The attachment of electronic tracking devices has also advanced the understanding of dispersal and migration (regular bidirectional movement) in many taxa (e.g. Hanski *et al.*, 2000, Godley *et al.*, 2002, Ropert-Coudert and Wilson, 2005, Egevang *et al.*, 2010), but for some species the size of the device is a constraint (although this is rapidly changing). An alternative to unravelling dispersal behaviour and migration is to adopt an indirect approach using genetic markers and/or via the analysis of stable isotope ratios in tissues. These rely on small sample sizes and the relatively simple non-destructive extraction of blood or other organic material from individuals sampled on breeding or wintering grounds.

Genetic analysis

Genetic markers have greatly impacted population biology since the development of the polymerase chain reaction, or PCR (the amplification of specified stretches of DNA into useable concentrations), the application of evolutionarily conserved sets of PCR primers (to target specific genetic sequences) and the advent of routine DNA sequencing that enables the detection of nucleotide or fragment length differences between individuals (Sunnucks, 2000). Measuring heritable variation in genetic markers between species or populations can give estimates of genetic diversity, population structuring, divergence times and gene flow. Such estimates and inferences are generally based on the

assumption that the genetic marker being used is influenced only by the opposing forces of genetic drift and mutation (random events with predictable rates/strengths), and not by the directional force of natural selection (the neutral theory of molecular evolution (Kimura, 1968)). With the development of novel markers and analytical approaches for genetic data (reviewed in Sunnucks, 2000, Pearse and Crandall, 2004), their use has increased within studies interested in revealing dispersal. More sophisticated statistical approaches such as maximum likelihood coalescent methods, Bayesian probability theory and Monte Carlo Markov chain simulation (see: Pearse and Crandall, 2004), use more of the information in a data set than traditionally used statistics (e.g. F_{ST} & Φ_{ST}). These approaches are not restricted by the same assumptions associated with F_{ST} and its analogues (e.g. drift-mutation equilibrium, *n*-island model of dispersal), so are more robust, especially when considering migration rates or divergence time (Smith and Friesen, 2007). These have led to more detailed analysis of genetic structure to reveal historical and contemporary gene flow and dispersal in wild populations (Clobert *et al.*, 2001, Wakeley, 2004). Genetic distinction between populations can also enable individual assignment to populations and provide means for characterising dispersal among populations on ecological time scales (Manel *et al.*, 2005, Hall *et al.*, 2009). Despite the increase in genetic markers and analyses it still remains difficult to predict the extent of genetic differentiation in natural populations of seabirds. There is extensive variation among species in the degree of population genetic structure found, even within lineages that have similar distribution, ecology and life history traits (Alcidae: Moum and Arnason, 2001). Species that may have been expected to show a trend based on, apparent, high levels of dispersal or philopatry have been found to have the opposite expected population structure. (Diomedeidae: Burg and Croxall, 2001, Van Bekkum *et al.*, 2006). This demonstrates how multifaceted the process of differentiation is and how research is required to elucidate different barriers to gene flow, whether

physical, non-physical, ancient or contemporary, that shape this evolutionary process (reviewed in Friesen *et al.*, 2007a). Combining different types of genetic markers (e.g. mtDNA and microsatellites) has brought an increase in both resolution and analytical power (Burg and Croxall, 2001) and with the growth of these studies the knowledge of the processes influencing population genetic structure and dispersal patterns in seabird species will increase.

Studies combining molecular markers or different analytical techniques are becoming more common in the literature (Burg and Croxall, 2001, Kelly *et al.*, 2005, Gómez-Díaz and González-Solís, 2007). One such technique is stable isotope analysis, which when combined with genetic data can increase the studies explanatory power by inferring movement of individuals and possible mechanisms to gene flow.

Stable isotope analysis

Isotopes are atoms with the same number of protons and electrons but different numbers of neutrons in the nucleus. Stable isotopes are those that are energetically stable and do not decay. Due to the differing nuclear mass between stable isotopes of the same element they behave differently in biological and biochemical processes. This leads to naturally varying stable isotope abundance in the environment, which can be further influenced by anthropogenic factors, such as agriculture and pollution (Rubenstein and Hobson, 2004). Differences in relative abundance of these isotopes can be measured using a mass spectrometer and expressed as the ratio of the heavy to light form, which can then be standardized against international reference samples and reported in the delta (δ) notation as parts per thousand or per mil (‰). Over the last 20 years, analysis of the stable isotope ratios of a number of light elements has become a powerful tool in animal ecology (Hobson, 2005, Inger and Bearhop, 2008). The ability to trace movement of individuals, or assign them to populations using these markers is based on

the fact that stable isotope signatures in animal tissues reflect those of local food webs. The signatures can vary spatially and are passed onto the consumer within that food web. The isotopically distinct signature that is retained in the animal's tissues can therefore reflect feeding locations. The temporal resolution of feeding location is dependent on the elemental turnover rates for the tissue sampled, with metabolically inert keratinous tissues like feathers, nails or hair reflecting the location of synthesis and metabolically active tissues reflecting a range of a few days (e.g. blood plasma) to the lifetime of an individual (e.g. bone collagen) (Hobson, 1999). Establishing these distinct isotopic signatures in nature and choosing the correct tissue to reflect species movement over the appropriate temporal scale is critical to studies of dispersal. For marine studies the primary patterns of interest are, (1) a decline in $\delta^{13}C$ and $\delta^{34}S$ isotopes (heavy carbon and sulphur isotopes) between inshore or benthic food webs versus offshore or pelagic food webs (Hobson *et al.*, 1994, Hobson *et al.*, 1995); (2) the enrichment of $\delta^{15}N$ (heavy nitrogen isotope) at higher trophic levels (Cherel *et al.*, 2007) and (3) the negative correlation between latitudinal gradient and values of $\delta^{13}C$ in ocean waters (Cherel *et al.*, 2007, Paiva *et al.*, 2010). Many seabird studies have used these patterns of stable isotopes to infer, for example, trophic relationships (Thompson et al., 1995, Cherel et al., 2007), diet and feeding ecology (Hedd and Montevecchi, 2006), feeding specialisation (Furness et al., 2006), sex differences in foraging (Bearhop et al., 2006), moult origins and identification of foraging locations (breeding or wintering) (Cherel et al., 2000, Quillfeldt et al., 2005, Cherel et al., 2006). However, the number of studies addressing specifically the assignment of individuals to natal or breeding origin is limited for seabirds (shorebird: Wunder *et al.*, 2005, Gómez-Díaz and González-Solís, 2007).

Measuring the stable isotope ratios in tissues of consumers can be informative for studying foraging and wintering strategies in seabirds, for instance, Cherel et al (2000)

explained differences in carbon and nitrogen signatures found in feather samples in terms of differences in winter foraging areas based on the change in distinct regional signature north of an ocean front (Subtropical Front, STF). However, there are areas of discrepancy that makes assignment of individuals to a specific location problematic. Firstly, using feather samples relies on a sound knowledge of moult strategies in each species and the use of correct feathers that retain the location signature of interest. In many seabird species this is not well understood (especially for secondary feathers) (Bridge, 2006) and other tissues may give a more reliable location signal. Secondly, limited knowledge of isotopic signatures in foraging locations and possible small- scale or annual variation in values could lead to incorrect assignment (Gómez-Díaz and González-Solís, 2007). If assignment to location is based on general isotopic patterns then there is also the possibility of overlap with different gradients (Cherel and Hobson, 2007). Gómez-Díaz and González-Solís (2007) evaluated the use of stable isotopes as an effective assignment tool, using morphological, genetic and biogeochemical analysis to assign *Calonectris* shearwaters to breeding colony, breeding archipelago and taxonomic level within the Mediterranean and east Atlantic. They analysed $\delta^{13}C$ and $\delta^{15}N$ isotope signatures of innermost primary feathers (known to grow at breeding grounds) from birds of known breeding origin to construct a cladogram to determine any structuring that could be used for assignment. Although geographic gradients were found within regions these were too weak to reliably assign birds to colonies using isotopes alone. However, the predictive power was greatly improved when combined with trace elements, morphology or genetic data. This study highlights the difficulty of resolution at a local scale (colonies) when using biogeochemical markers in the highly variable marine environment, but also suggests that using a combined, evidence-based approach can be useful. Such a strategy has been used with some degree of success in other avian taxa (Kelly et al., 2005)

Leach's storm-petrel

The Leach's storm-petrel *Oceanodroma leucorhoa* belongs to the order Procellariiformes and the family Hydrobatidae (storm-petrels). The nominate subspecies *O. l. leucorhoa* (Vieillot) has a mainly temperate, northern hemisphere distribution with colonies across the north Pacific and Atlantic oceans. Predominately dark blackish-brown, it has a paler upper wing bar, white rump and distinctive forked tail. The wingspan is between 450-480 mm and it weighs ~45 grams (Huntingdon *et al.*, 1996). The other three subspecies (*O. l. champmani, O. l. socorroensis* and *O. l. cheimomnestes*) are only known to breed on a few small islands off Baja California, Mexico, and are generally smaller with variation of tail fork depth, rump patch and vocalisations (Huntingdon *et al.*, 1996).

Like all procellariiforms the Leach's storm-petrel is tied to land only during the breeding period, outside which it is highly pelagic and migrates south to warmer waters during the winter period. Age at first breeding is variable but is commonly at 5-7 years. One chick is raised in each annual breeding event, which lasts approximately three and a half months from egg laying to fledging (between late May and November; Huntingdon *et al.*, 1996). Nests are in burrows dug on wooded or treeless islands, or in crevices among rocks, and individuals show high fidelity to the same burrows each year (Blackmer *et al.*, 2004). Breeding monogamy is believed to be high, with no evidence for extra-pair fertilizations found in a study using DNA fingerprinting of chicks and parents at a small Canadian colony {Mauck, 1995 #202}. Breeding adults (and prospecting pre-breeding birds) are nocturnal visitors to the colonies, after foraging trips over and beyond continental shelves (Hedd and Montevecchi, 2006). A combination of regurgitates and stable isotope analyses revealed the pelagic diet, which reflects the available prey species in the region, with small fish and zooplankton being the major components (Watanuki, 1985b, Hedd and Montevecchi, 2006). Their ability to find prey

and foraging hotspots in such a large, and we perceive homogenous, seascape is believed to be a combination of social and olfactory cues (Nevitt, 2008). One study suggests their ability to detect (smell) dimethyl sulphide, a biogenic sulphur compound associated with primary production, may assist in locating areas of high prey abundance (Nevitt and Haberman, 2003). Unpublished genetic work suggests the Pacific and Atlantic populations are genetically distinct (Friesen *et al.*, 2007a) but a preliminary comparison of random amplified polymorphic DNA (RAPD) markers among 3 colonies in the northwest Atlantic, (Paterson and Snyder, 1999) was inconclusive as to the structure on a regional scale.

Global population and the St Kilda colony decline

Global estimates of the Leach's storm-petrel breeding population are around 9,000,000 - 10,600,000 pairs, split approximately evenly between the Atlantic and Pacific Ocean basins (Mitchell *et al.*, 2004). However, this does not include the juvenile and pre-breeding birds that would substantially increase an overall population estimate. The Pacific breeding colonies are located around the north Pacific Rim from Japan in the west to California in the east. The Aleutian Islands, Alaska hold the most substantial breeding colonies, totalling around 2,000,000 – 3,500,000 pairs (Mitchell *et al.*, 2004). The Atlantic population is of similar size but 97% of the breeding pairs are found on islands around Newfoundland, Canada, including the largest colony in the world on Baccalieu Island (~3,360,000 breeding pairs; Sklepkovych and Montevecchi, 1989). In Europe there are approximately 165,000 pairs, although the vast majority (~98%) are found in two small island archipelagos (Vestmanyjaer, Iceland ~115,000 bp; St Kilda, Scotland ~45,000 bp; Mitchell *et al.*, 2004).

The St Kilda colony represents ~94% of the European Union population and Leach's storm-petrel is included in the Annex 1 list of rare and vulnerable species of the EU

Birds Directive (EU, 2009), partly because such a high percentage of the population occurs at this one site. Between 1999 and 2006 there was >50% decline in the St Kilda colony (Mitchell *et al.*, 2004, Newson *et al.*, 2008), which coincided with intense predation by a large population of great skuas *Stercorarius skua* (Votier *et al.*, 2006). There had been a rapid expansion of the great skua population on St Kilda at that time, in part, due to immigration from other Scottish colonies (Phillips *et al.*, 1999a). This relatively recent colonist is estimated to have consumed between 15,000 (Phillips *et al.*, 1999b) and 21,000 (Miles, 2010) Leach's storm-petrels each year since the mid-1990s. In a closed population, the high level of predation should have lead to extirpation of the St Kilda colony, even considering the potentially large numbers of juvenile or pre-breeding birds attending the colony (Votier *et al.*, 2006, Miles, 2010). This suggests that immigration from other large colonies in the Atlantic may play a role in offsetting this decline. Although the skua population on St Kilda has recently stabilised, the predation rate remains high (Miles, 2010). Therefore the future prospect of the Leach's storm-petrel colony on St Kilda is in doubt.

Aims of the thesis

The movements and dispersal of Leach's storm-petrels have been little studied, during either breeding or wintering periods, and any existing evidence is scant and/or circumstantial. The behaviour of juvenile or pre-breeding birds is even less well known due to the difficulties in tracking them before they settle at breeding sites (Votier *et al.*, 2011). In light of the decline at the St Kilda colony, and the suggestion that immigration from other colonies may offset the losses caused by predation, there is a clear need to understand dispersal in Leach's storm-petrels and the degree of connectivity among distant colonies. In this thesis I aim to reveal the extent of gene flow between Leach's storm-petrel colonies and a potential mechanism of dispersal that promotes this

exchange, using a combination of genetic techniques and stable isotopes. I also aim to identify other stages of the annual cycle that may influence dispersal and assess the implications for a colony with high annual loss of individuals from predation (St Kilda). Additionally, I hope to highlight further potential threats at the breeding grounds.

Chapter 2 is a technical note on the characterisation of a number of microsatellite markers specifically designed for Leach's storm-petrels. In **Chapter 3** these newly developed markers are used as part of a study to investigate historical and contemporary gene flow among, and between, both Atlantic and Pacific colonies of Leach's storm-petrels. By examining population structure and individual assignment probabilities using both mitochondrial and nuclear DNA markers, the study aimed to understand the dispersal dynamics of Leach's storm-petrels over large spatial scales and assess the potential implications for the declining St Kilda colony.

A potential method to characterise variation in stable isotope values of marine ecosystems and to reconstruct Leach's storm-petrel movement is to analyse biological material collected via the Continuous Plankton Recorder (CPR – one of the longest running measures of plankton communities worldwide). This technique uses preservatives to store phytoplankton and zookplankton which may have implications for stable isotope analysis. **Chapter 4** describes an experiment that investigates the effect of formalin on the carbon and nitrogen stable isotope ratios in a copepod species to simulate storage in the CPR. **Chapter 5** incorporates these findings into a study that uses genetic markers to confirm gene flow between 3 colonies, and stable isotopes to investigate movement of immature Leach's storm-petrel among these colonies in the north Atlantic. This study uses a novel application of stable isotope mixing models in a Bayesian framework to identify potential immigrant birds attending colonies and considers this movement during the breeding season as a potential dispersal mechanism that may lead to gene flow among colonies. **Chapter 6** also investigates potential

mechanisms that may explain the high levels of gene flow in this species by examining evidence for mixing during the non-breeding period (an important predictor of genetic differentiation among populations of seabirds). Using corpses of birds found during wrecks in Canada and Europe, analysis of moult pattern enables the selection of appropriate feather tracts to determine breeding colony stable isotope signatures. Based upon these values it is possible to determine whether birds found dead in European waters during the winter months originated from multiple colonies. This could indicate mixing of individuals from different colonies outside the breeding season and highlight a behaviour that would potentially facilitate movement between colonies.

Chapter 7 focuses on the declining St Kilda colony and aims to highlight the resident field mouse population as another potential source of predation on Leach's storm-petrels.

The general discussion in **Chapter 8** attempts to bring together the main points of the thesis and provide an overview of how it has enhanced our knowledge of dispersal in Leach's storm-petrels and their capacity to cope with environmental change.

Chapter 2

Characterisation and predicted genome locations of Leach's storm-petrel (*Oceanodroma leucorhoa*) microsatellite loci (PROCELLARIIDAE, AVES)

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2. Technical Note

The Leach's storm-petrel *Oceandroma leucorhoa* is a small highly pelagic seabird of the Procellariformes family. We have characterised a microsatellite marker set in this species in order to investigate regional and global population structure and dispersal between colonies.

Existing microsatellite markers were tested for utility in the Leach's storm-petrel (LSP) including 7 loci isolated in a different storm-petrel species (Sun *et al.*, 2009) and 47 loci with primer sets of engineered and/or proven high cross-species utility (Dawson *et al.*, 2010; *Bb111-TG, HvoB1-TGG*, Klein et al. 2009; *Calex01, Calex08-ZEST, Pte24-CEST, HvoB1*, Dawson et al. unpublished data). A microsatellite-enriched LSP genomic library was also created. Blood (20 μl) was collected from LSP individuals and stored in 1.5ml of absolute ethanol. Genomic DNA was extracted using an ammonium acetate precipitation method. The genomic library was constructed from a single male LSP (SKB02) sampled at St Kilda, Scotland (Co-ordinates 57°48'N, 8°34'W) in 2008. We used the method of Armour *et al.* (1994) and enriched for the following motifs: $(GT)_n$, $(CT)_n$, $(GTAA)_n$, $(CTAA)_n$, $(TTTC)_n$ and $(GATA)_n$ and their complements, which had been bound to magnetic beads Glenn & Schable (2005). Transformant colonies were directly sequenced by the NERC Biomolecular Analysis Facility at the University of Edinburgh.

A total of 211 new unique LSP microsatellite sequences were isolated (EMBL accession numbers FR696377-FR696588). Primer sets were designed for 50 unique microsatellite sequences using PRIMER3 (Rozen and Skaletsky, 2000). The new LSP and existing loci were initially tested for amplification and polymorphism in 4-6 unrelated individuals sampled from Gull Island, Witless Bay, Canada (Co-ordinates: 47°15'N, 52°46'W) in 2008. The 4-6 individuals were amplified using a gradient of 12 different annealing temperatures (56–65°C). The temperature producing the cleanest and

strongest PCR product when observed on a 1.5% agarose gel stained with SYBRSafe was selected for amplification in 24 additional Gull island individuals. Each 2-µl PCR contained approximately 10 ng of lyophilised genomic DNA, 0.2 µM of each primer and 1 µl QIAGEN multiplex PCR mix (QIAGEN Inc.; Kenta *et al.*, 2008). The PCR program used was: 95°C for 15 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing temperature (Table 2.1) for 90 seconds, 72°C for 1 minute, and finally 60°C for 6 minutes. PCR amplification was performed using a DNA Engine Tetrad 2 thermal cycler (MJ Research, Bio-Rad, Hemel Hempstead, Herts., UK). Amplified products were loaded onto an ABI 3730 48-well capillary DNA Analyser (Applied Biosystems, California, USA) and allele sizes were assigned using GENEMAPPER v3.7 (Applied Biosystems, California, USA). Individuals were sex-typed with the 2550F/2718R (Fridolfsson and Ellegren, 1999), Z002A (Dawson, 2007) and Z-037B markers (Dawson, DA unpublished data).

Of the 47 pre-existing loci tested in 6 individuals, 2 loci did not amplify or produced non-specific products, 30 were monomorphic and 15 were polymorphic. Of the 50 new LSP loci tested in 4-6 individuals, 6 loci did not amplify or produced non-specific product, 18 were monomorphic and 26 were polymorphic.

Predicted chromosome locations were assigned by comparing the microsatellite sequences with the location of their homolog on the chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*) genome assembly (methods as in Dawson et al. 2006 & 2007). This allowed us to identify if any loci were located on the sex chromosomes or were physically linked.

Thirty-nine of the 41 polymorphic loci could be assigned a chromosomal location with a BLAST hit E-value of >1E-10 with a >100 base pair match (Table 2.1, Fig. 1.1). Three pairs of loci were less than 1Mb apart in the zebra finch genome (*Ole23:Ole10, Oc87B:Ole03 & Ole06:Ole22*) and therefore may be physically linked. However, after a

sequential Bonferroni correction (Rice, 1989), no pairs of loci showed evidence of linkage disequilibrium ($p<0.05$) in the LSP.

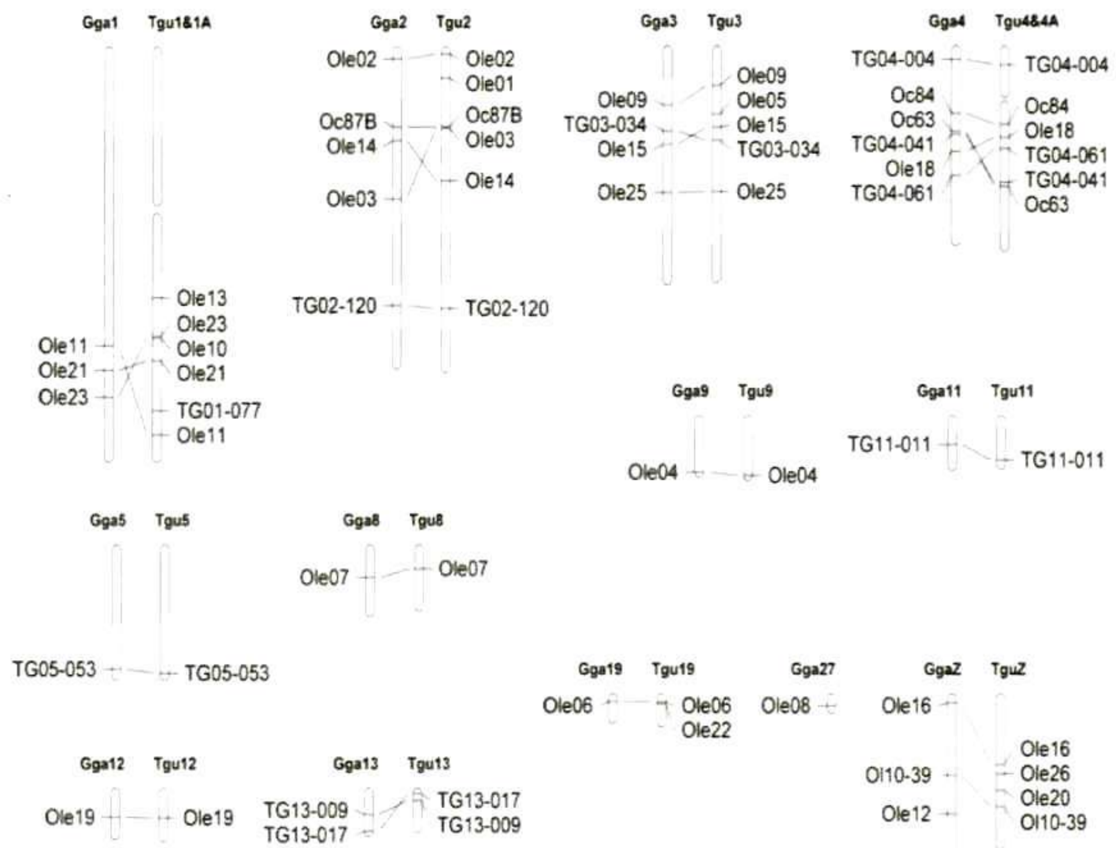

Figure 2.1 Predicted chromosome locations of microsatellite loci polymorphic in the Leach's storm-petrel *Oceanodroma leucorhoa*. Gga, chicken (*Gallus gallus*) chromosome name.Tgu, zebra finch (*Taeniopygia guttata*) chromosome name.

A combined total of 41 polymorphic loci were then typed in 24 unrelated individuals (14 male and 10 female) sampled at the Gull Island population. These displayed between 2 and 22 alleles (Table 2.1). Four loci displayed a genotype pattern consistent with linkage to the Z chromosome (Table 2.1) being homozygous (hemizygous) in all females (ZW) but heterozygous or homozygous in males (ZZ), in agreement with their Z chromosome location assignment (Figure 2.1). *Ole16* was assigned to the Z chromosome based on sequence homologue but displayed heterozygotes in females

suggesting it was autosomal in the LSP. A Fisher's Exact test comparing numbers of male and female homozoygotes supported that 3 loci were Z-linked (*Ol10-39, Ole12, Ole26*; P-values <0.001) but due to low heterozygozity in males *Ole20* was not significant, and therefore sex-linkage should be viewed with caution.

Observed and expected heterozygosities, and predicted null allele frequencies were calculated using CERVUS v3.0.3 (Kalinowski *et al.*, 2007). Tests for departures from Hardy-Weinberg equilibrium (HWE) and assessment of linkage disequlibrium were conducted in GENEPOP v3.4 (Rousset, 2008). Only males were used when Z-linked loci were analysed. Prior to a sequential Bonferroni correction, 6 loci deviated from HWE (P<0.001; Table 2.1). After correction only 2 loci (P<0.001; *Ole02 and TG05-053*) deviated. Eleven loci displayed a high estimated null allele frequency (above 0.10) including 9 loci that did not deviate from HWE (Table 2.1). When typed in 24 individuals, locus *Ole21* showed high levels of polymorphism (>20 alleles) and 6 loci included 1bp allele size increments (Table 2.1). The combined first parent non-exclusion probability for the 37 autosomal polymorphic markers is <0.0001.

Table 2.1 Characterisation of microsatellite loci in Leach's storm-petrel *Oceanodroma leucorhoa*

(a) Characterisation of 15 published microsatellite loci in Leach's storm-petrel *Oceanodroma leucorhoa*

Locus	EMBL acession number	Source species	Reference	Primer sequence (5'-3')	Fluoro label (F)	T_a	*N*	*A*	Exp. allele size (bp)	Obs. allele size range (bp)	H_O	H_E	HWE P-value	Est. null allele freq.	Chromosome, location (bp) (E-value)
Ol10-39¥	FJ238106	*Oceanodroma leucorhoa*	Sun *et al.* 2009	F:TTAAGAACAGAGCCTGACTTG R:ACAAAATCTCATGTCCTTGG	PET	56	22F 24M	3 3	135	134-138 134-138	0.00 0.58	- 0.64	- 0.449	- 0.044	TguZ, 53939951 (9.5e-12) GgaZ, 38227643 (1.1e-06)
Oc63	FJ238100	*Oceanodroma castro*	Sun *et al.* 2009	F:TCACACCAACCTCCATGAAA R:AACGGGGAATATGTGGTTCTT	VIC	58	24	4	190	174-188	0.67	0.65	0.508	-0.021	Tgu4, 22623887 (5.6e-23) Gga4, 39929604 (1.9e-34)
Oc87B	FJ238104	*Oceanodroma castro*	Sun *et al.* 2009	F:TTTAAGGAACGCAAGTCAGG R:GTGATTCTTGCAGTGGCTTT	PET	58	24	8	261	261-277	0.67	0.79	0.131	0.076	Tgu2, 57390462 (1.5e-69) Gga2, 37216829 (9.7e-63)
Oc84	FJ238103	*Oceanodroma castro*	Sun *et al.* 2009	F:CCTTTTTCCAGGCAGACAAA R:AGTTCAAGGGCAACCTTGTG	NED	56	24	3	298	302-306	0.58	0.59	0.055	-0.010	Tgu4, 8910815 (3.0e-61) Gga4, 30971263 (1.0e-45)
TG01-077	CK305147	*Taeniopygia guttata*	Dawson *et al.*2010	F:GTATGTCAGTTATCAAAAACAAGC R:AAATGGCAGGTAAGGATACTCTC	HEX	56	24	3	153	153-155‡	0.67	0.67	**0.007**	-0.006	Tgu1, 95581733 (3.3e-129) Gga1, 86284969 (1.2e-92)
TG02-120	DV945440	*Taeniopygia guttata*	Dawson *et al.*2010	F:TTGGGCAAAGATGATATGAATG R:AGCCAGGTCCAGTTTCTAAGC	6FAM	56	24	3	230	236-244	0.44	0.52	0.568	0.046	Tgu2, 127242053 (1.2e-107) Gga2, 125741749 (7.9e-92)
TG03-034	CK311260	*Taeniopygia guttata*	Dawson *et al.*2010	F:GAGATCGCCACCATCCTG R:AAGTCTACATTTCCCTTGTCTTGG	6FAM	56	23	2	178	177-179	0.09	0.16	0.131	0.289	Tgu3, 44507023 (9.5e-159) Gga3, 40012285 (4.9e-134)
TG04-004	DV946288	*Taeniopygia guttata*	Dawson *et al.*2010	F:CTGGAGCAGTATTTATATTGATCTTCC R:GAAGATGTGTTTCACAGCATAACTG	HEX	56	23	5	166	161-169	0.39	0.56	0.091	0.144	Tgu4A, 6999784 (7.3e-145) Gga4, 4186152 (1.1e-108)
TG04-041	CK316380	*Taeniopygia guttata*	Dawson *et al.*2010	F:CTGAATTGTTGACCTTTGCTTAC R:GTCCTTTTAGAAAGCAGCACAG	HEX	56	24	2	173	176-178	0.50	0.30	0.272	-0.142	Tgu4, 37987277 (1.0e-115) Gga4, 41461789 (1.0e-99)
TG04-061	CK235034	*Taeniopygia guttata*	Dawson *et al.*2010	F:GACAATGGCTATGAAATAAATTAGGC R:AGAAGGGCATTGAAGCACAC	HEX	56	24	3	186	202-204‡	0.46	0.52	0.813	0.051	Tgu4, 20910894 (1.3e-113) Gga4, 61990187 (2.5e-98)
TG05-053	CK314425	*Taeniopygia guttata*	Dawson *et al.*2010	F:GCATCATCTGGTTGAACTCTC R:ACCCTGTTTACAGTGAGGTGTT	6FAM	56	24	4	196	211-213‡	0.29	0.71	**<0.001***	0.417	Tgu5, 61276203 (2.3e-161) Gga5, 59348193 (7.6e-120)
TG08-024 (primer set1)	CK314428	*Taeniopygia guttata*	Dawson *et al.*2010	F: CACAAATCCTGAATTTCATATCC R: AACAACGACAGCTATGAAAGAAC	HEX	56	20	3	243	240-242‡	0.45	0.59	0.389	0.132	Tgu8, 21,095,625
TG11-011	CK308096	*Taeniopygia guttata*	Dawson *et al.*2010	F:CAAACTAAGTACATCTATATCTgAAG R:TAAATACAGGCAACATTGG	6FAM	56	22	10	223	213-230‡	0.55	0.71	**0.002**	0.158	Tgu11, 19380799 (3.5e-123) Gga11, 11693433 (3.7e-102)
TG13-009	DV948691	*Taeniopygia guttata*	Dawson *et al.*2010	F:TGTGGTGGGATAGTGGACTG R:CTGTAAAATGTGCAAGTAACAGAGC	HEX	56	23	3	195	194-197	0.30	0.34	0.240	0.028	Tgu13, 3672804 (2.2e-136) Gga13, 10457183 (4.7e-108)
TG13-017	CK313422	*Taeniopygia guttata*	Dawson *et al.*2010	F:GCTTTGCATCTTGCCTTAAA R:GGTAACTACAACATTCCAACTCCT	6FAM	56	24	4	300	204-210	0.58	0.50	0.911	-0.112	Tgu13, 3672804 (2.2e-136) Gga13, 18850515 (2.4e-143)

Table 2.1 *(b)* Characterisation of 26 newly isolated Leach's storm-petrel *Oceanodroma leucorhoa* microsatellite loci

Locus	EMBL acession number	Repeat motif	Primer sequence (5'-3')	Fluoro label (F)	T_m(°C)	T_a	N	A	Exp. allele size (bp)	Obs. allele size range (bp)	H_O	H_E	HWE P-value	Est. null allele freq.	Chromosome, location (bp) (E-value)
Ole01	FR696377	(ACAG)$_5$	F: CACCCTGAATCCCAGAACCAAGC	6FAM	F:57.59	60	24	2	218	213-217	0.25	0.28	0.501	0.053	Tgu2, 13058294 (1.2e-51)
			R: TCAGTGAAGGTCCACTCCGAATCC		R:57.93										No match (Gga)
Ole02	FR696378	(GAAA)$_{32}$	F: GGGTTCCTGTTACCAAAGGGCAG	HEX	F:57.52	60	17	12	489	412-485	0.47	0.89	**<0.001***	0.301	Tgu2, 1188159 (4.2e-06)
			R: CATAAGCACAAGGTTCTAGCTCCCTC		R:57.07										Gga2, 3443337 (8.4e-187)
Ole03	FR696379	(GAAA)$_{29}$	F: TCCTTCACCACCTCTTGCTGCC	HEX	F:58.76	60	24	12	451	421-462	0.92	0.87	0.795	-0.040	Tgu2, 37830396 (2.0e-39)
			R: ACGCATCTGTAGCTCACAAATCCCAG		R:59.14										Gga2, 72796221 (9.1e-31)
Ole04	FR696380	(ATTCT)$_{13}$	F: TGTTTATTGCACTGCCTGAAACTTGC	HEX	F:57.37	60	24	4	355	343-358	0.33	0.65	**0.002**	0.314	Tgu9, 27014609 (3.5e-61)
			R: TGTGCAACAGCGGTCACTGAG		R:57.62										Gga9, 25331698 (7.4e-49)
Ole05	FR696381	(GAATA)$_{10}$	F: TGGAATGGGATGGGATGGTTTGGG	HEX	F:58.66	60	23	10	299	297-353	0.61	0.75	0.083	0.086	Tgu3, 31402896 (1.5e-39)
			R: TGAGGCTGGAGGAGAATGCTGTG		R:58.39										No match (Gga)
Ole06	FR696382	(TATT)$_5$	F: GCCAACTGCTGTTCAGTGAGCC	6FAM	F:58.61	60	24	2	201	198-202	0.33	0.42	0.347	0.107	Tgu19, 1687821 (1.6e-75)
			R: GCCACAAACGCAAACATTCATAAACCC		R:58.48										Gga19, 1482233 (6.7e-86)
Ole07	FR696383	(CA)$_{10}$	F: CCCAAGGAGTTCCTGTGCGTGC	HEX	F:60.24	60	24	3	154	153-157	0.46	0.37	0.636	-0.125	Tgu8, 9452460 (1.3e-64)
			R: TGGCTCTGTGCTGCGCTTTACC		R:59.99										Gga8, 13826157 (3.6e-39)
Ole08	FR696384	(GA)$_8$	F: ACTCCACACGGACTCTGCACTG	HEX	F:58.53	60	24	2	218	212-214	0.38	0.31	0.551	-0.102	No match (Tgu)
			R: TGTGATTTGTTGTGAGGCGAGCG		R:58.45										Gga27, 3578087 (2.6e-118)
Ole09	FR696385	(CA)$_{10}$	F: GGCCTGGATTCTGCGTGTTGGG	6FAM	F:60.30	60	24	2	151	149-153	0.25	0.28	0.501	0.053	Tgu3, 17145330 (1.0e-88)
			R: CAGCTACAGGCCACGGGTGC		R:60.04										Gga3, 27034682 (7.2e-48)
Ole10	FR696386	(CA)$_{12}$	F: ACCAGCTCCTAACTGGAAGCCAC	6FAM	F:58.32	60	24	3	236	232-238	0.08	0.08	1.000	-0.012	Tgu1, 59413510 (1.4e-97)
			R: TGCAGGTAGGTTCTCAGTATGCAAAGG		R:58.64										No match (Gga)
Ole11	FR696387	(AG)$_{14}$	F: TCACAACCAGAGCTGAGACACAGAG	6FAM	F:58.20	60	23	2	474	468-472	0.44	0.43	1.000	-0.013	Tgu1, 107441638 (8.6e-79)
			R: AGCCAAGGCTCCAAGCTTATGAAAC		R:57.79										Gga1,145644036 (7.4e-280)
Ole12¥	FR696388	(AGAT)$_6$,	F: GCTCCTGTAGAGCTGGAAATGAACC	6FAM	F:57.41	60	22F	6	246	222-248	0.00	-	-	-	No match (Tgu)
		(AGAT)$_6$	R: GGCAGTGCTACCTGTGGATGC		R:57.80		25M	6		226-252	0.60	0.67	0.461	0.050	GgaZ, 57547873 (6.3e-31)
		AGA(TACA)$_9$													
Ole13	FR696389	(TG)$_9$	F: GGGCCAGACATGTATTTACTGGAGGG	HEX	F:58.69	60	24	3	244	242-246‡	0.46	0.41	1.000	-0.069	Tgu1, 39467860 (1.8e-56)
			R: GTGCATCCGAGAGCCGACGC		R:60.51										No match (Gga)

Table 2.1 *(b)* Continued

Locus	EMBL acession number	Repeat motif	Primer sequence (5'-3')	Fluoro label (F)	T_m (°C)	T_a	N	A	Exp. allele size (bp)	Obs. allele size range (bp)	H_O	H_E	HWE P-value	Est. null allele freq.	Chromosome, location (bp) (E-value)
Ole14	FR696390	$(AC)_8$	F: CCAGCCTGCAGGGCTTTCC R: CAGAGCTTTGCCTCTGTCTTAGTCTTC	6FAM	F:57.75 R:57.36	60	24	3	348	339-349	0.46	0.46	1.000	-0.005	Tgu2, 63946342 (5.6e-75) Gga2, 43905738 (1.6e-267)
Ole15	FR696391	$(TG)_{11}$	F: TCAGGAACAGCACTGGAAACTGGAC R: TGCCACTGACTTTCCATTCTCCCTC	HEX	F:58.87 R:58.46	60	24	2	235	232-234	0.04	0.04	NA	-0.004	Tgu3, 37963153 (4.9e-38) Gga3, 46474483 (4.9e-17)
Ole16	FR696392	$(CA)_{11}$	F: GCAGCCTCCAGCCGTGAGTG R: GTGATTGCAGCAGCTCCTGGTCG	HEX	F:60.04 R:60.36	60	24	4	232	219-232	0.75	0.71	0.134	-0.036	TguZ, 32967540 (7.5e-55) GgaZ, 2104154 (7.9e-65)
Ole17	FR696393	$(GT)_5$ GG $(GT)_5$	F: TGACATGACCACTTTCCATAGC R: AATGTCTGTGGTTCAAATGTGC	HEX	F:60.00 R:59.90	60	24	5	210	209-236	0.46	0.52	0.372	0.084	No matches
Ole18	FR696394	$(CA)_{13}$	F: TGGTTTGTATGTTGTGGGTTG R: GATCCACTTAGCCACCCTTG	6FAM	F:59.21 R:59.55	60	24	5	151	147-155	0.58	0.69	0.681	0.079	Tgu4, 15498065 (3.2e-32) Gga4, 49917509 (8.3e-19)
Ole19	FR696395	$(GA)_{10}$ CA $(GA)_7$	F: GAAGGCTCTCAAGGGTCAAG R: TTTGGGAGAAGTTCAGTAGAAGAAC	6FAM	F:59.01 R:59.39	60	23	8	242	227-377	0.56	0.80	**0.012**	0.164	Tgu12, 12215753 (1.2e-59) Gga12, 11880314 (1.9e-59)
Ole20€	FR696396	$(AGGC)_7$ ACG $(CA)_9$	F: AGGTCAGCAGCTAAAGCATACC R: AGGTCCCTTCCAATCCAAAC	HEX	F:59.94 R:60.17	60	21F 22M	1 2	345	344 344-346	0.00 0.09	- 0.09	- 1.000	- -0.014	TguZ, 45790217 (3.3e-48) GgaUnk, 6499150 (5.8e-41)
Ole21	FR696397	$(GA)_{11}$ $(A)_6$ $(GAAA)_{31}$	F: CCACGAGAAATGGACATACAAC R: TGAAGAACTTCCGAAATAACTGTG	HEX	F:59.36 R:59.70	60	24	21	330	316-418	0.92	0.96	0.573	0.011	Tgu1, 70616595 (9.9e176) Gga1,157866922 (1.1e-204)
Ole22	FR696398	$(CT)_9$	F: AGCAGAGGCCACTACATCAC R: AAAATATCATAGGGGAGTAAAGAGC	HEX	F:57.90 R:57.59	60	24	3	248	249-253	0.38	0.33	1.000	-0.096	No match (Tgu) Gga19, 2678502 (1.7e-144)
Ole23	FR696399	$(GT)_{18}$	F: TCTTTGGCATGCAATCTTTG R: CCCTGGTCAACTGAGAAAGC	6FAM	F:59.81 R:59.84	60	24	10	416	404-432	0.86	0.85	0.423	-0.014	Tgu1, 58566787 (3.5e-79) Gga1, 171173789 (4.9e-65)
Ole24	FR696400	$(ATCT)_9$	F: TGTGCTGACTTGTTTATTGTTCC R: GGCCATCTTGATACGGCTAC	HEX	F:59.19 R:59.56	60	24	5	159	156-176	0.88	0.76	0.911	-0.087	No matches
Ole25	FR696401	$(AGAT)_4$ & $(GAAA)_{16}$	F: CCTCCTCATCGTAGGGACTG R: TTAAAGGCAGCGATTCTGTTC	HEX	F:59.67 R:59.49	60	24	7	390	370-392	0.88	0.80	0.695	-0.059	Tgu3, 70012260 (3.3e-51) Gga3, 70378881 (1.4e-74)
Ole26¥	FR696402	$(TG)_{10}$	F: TCAGCTTCTGGTGCAGTTATG R: TCCTGCTGATGGATAGGTTATG	HEX	F:59.09 R:59.07	60	22F 25M	2 2	241	239-245 239-245	0.00 0.60	- 0.49	- 0.415	- -0.103	TguZ, 37567887 (2.2e-230) No match (Gga)

T_m, melting temperature; T_a, annealing temperature; N, number of unrelated Leach's storm-petrel *Oceanodroma leucorhoa* individuals; A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy-Weinberg equilibrium; Est. null allele freq., estimated null allele frequency; Tgu, zebra finch *Taeniopygia guttata* ; Gga, chicken *Gallus gallus*; **Bold**, Deviation from HWE; *Deviation from HWE after sequential Bonferroni correction; F, results of female individuals only; M, results of 24 male individuals genotypes for Z-linked loci. €, possibly Z linked (see text). ¥, Z linked locus; ‡, loci with 1 base pair size increment

Chapter 3

Long-distance dispersal and gene flow among seabird colonies: Implications for population persistence

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3.1 Introduction

Dramatic local population declines brought about by anthropogenic-driven changes to ecological processes are unfortunately becoming a ubiquitous global conservation problem (Thomas *et al.*, 2004). Predicting the potential effects of adverse change is becoming increasingly urgent in a world beset by habitat fragmentation, climate change and species introductions. Therefore, understanding dispersal dynamics and population structuring can provide key insights into the conservation of local populations and, ultimately, species (Bowler and Benton, 2005).

Dispersal is a fundamental process that shapes many aspects of population ecology and evolution (Clobert *et al.*, 2001, Bowler and Benton, 2005) . Although the dispersal and movement of individual animals can be estimated directly via capture-recapture studies (Lebreton *et al.*, 2003) or reconstructed in fine detail by attaching electronic tracking devices (Shaffer *et al.*, 2006, Sims *et al.*, 2008), for many taxa these approaches are impractical due to size, abundance and/or location of individuals. An alternative is to apply molecular techniques, which have been widely used to examine colonization patterns, population genetic structure, gene flow and individual immigrants (Rousset, 2001) and are therefore integral to our understanding of dispersal for a wide range of taxa (e.g. Knight *et al.*, 1999, Kelly *et al.*, 2001, Jehle *et al.*, 2005).

Seabirds of the order Procellariiformes (Fürbringer, 1888) are extremely vagile and have an extraordinary ability to travel huge distances at sea (Furness, 1987, Shaffer *et al.*, 2006). They also exhibit high levels of philopatry, sometimes returning to colonies to breed within a few meters of their natal nest (Huyvaert and Anderson, 2004). They therefore represent something of an enigma: their extreme vagility means that individuals can disperse freely between populations and thus theoretically maintain high levels of gene flow (Van Bekkum *et al.*, 2006), yet conversely, strong philopatry would be expected to lead to pronounced genetic differentiation (Dearborn *et al.*, 2003).

Understanding these dynamics is important since seabirds tend to nest at very high density in a small number of sites (fragmented), such that some locations contain a disproportionately large proportion of the population. Moreover the life-history traits of most seabirds (low annual fecundity, delayed sexual maturation, biparental care, long life, extended chick rearing period; Schreiber and Burger, 2002) mean that populations are unable to respond rapidly to change and are therefore particularly sensitive to changes in habitat suitability (Priddel *et al.*, 2006), food availability (Oro and Furness, 2002) and predation (Votier *et al.*, 2006). Indeed, seabirds are among the most threatened groups of birds in the world (BirdLife, 2008), in part because of threats to their breeding colonies. Understanding movement among seabird colonies is therefore an essential prerequisite for successful conservation and management.

Leach's storm-petrel *Oceanodroma leucorhoa* (Vieillot) is a colonial nesting seabird that breeds at a small number of widely spaced colonies across the North Atlantic and Pacific. This small (~45g) oceanic bird is inconspicuous at sea and only attends breeding colonies under the cover of darkness. Therefore, despite being extremely abundant (>10 million breeding pairs (bp) split approximately equally between the Atlantic and Pacific (Mitchell et al. 2004)) many aspects of their ecology remain unknown. There are four sub-species in the Pacific but only one (the nominate) found in both oceans (*Oceanodroma leucorhoa leucorhoa*; Huntingdon *et al.*, 1996), with 97% (4.8 million bp) of the Atlantic population breeding on a small number of islands in eastern North America (Huntingdon *et al.*, 1996, Mitchell *et al.*, 2004, Robertson *et al.*, 2006). In Europe there are approximately 165,000 pairs in ~20 colonies, although 98% are found in two small island archipelagos (Vestmanyjaer, Iceland ~115,000 bp; St Kilda, Scotland ~45,000 bp; Mitchell *et al.*, 2004). The St Kilda colony represents ~94% of the European Union population and Leach's storm-petrel is included in the

Annex 1 list of rare and vulnerable species of the EU Birds Directive (EU, 2009), partly because such a high percentage of the population occurs at a single site.

The vulnerability of these densely populated but isolated colonies has been highlighted recently by a steep population decline at the main colony on St Kilda (Dùn). Between 1999 and 2003 there was a 48% decline, with a further 12% decline between 2003 and 2006 (Mitchell *et al.*, 2004, Newson *et al.*, 2008). These declines have coincided with intense predation by a large population of great skuas *Stercorarius skua* (Votier *et al.*, 2006). This recent colonist is estimated to have consumed between 15,000 (Phillips *et al.*, 1999b) and 21,000 (Miles, 2010) Leach's storm-petrels each year since the mid-1990s. Such heavy predation should have lead to local extinction, even considering the large numbers of immature individuals in the population (Votier *et al.*, 2006, Miles, 2010). This suggests that immigration from the other large colonies in the Atlantic may play a role in offsetting this decline. Therefore, an understanding of movement between this colony and other very large colonies in the North Atlantic is necessary for the effective management of this regionally important population. Moreover, understanding the global dispersal dynamics of this species should provide insight into how they may be buffered against other effects associated with global change.

The only previously published genetic study of Leach's storm-petrels is a preliminary comparison of random amplified polymorphic DNA (RAPD) markers among 3 colonies in the northwest Atlantic, (Paterson and Snyder, 1999). We here report a more comprehensive study of breeding colonies using both mitochondrial DNA and microsatellite markers to investigate population genetic structure in nominate Leach's storm-petrels within the North Atlantic and between the North Atlantic and North Pacific. We investigate past colonization and gene flow in order to better understand the dispersal dynamics of Leach's storm-petrels, and used Bayesian analyses to characterize contemporary gene flow and movement. Based on these data we consider the

importance of gene flow among more widely distributed sites for Leach's storm-petrels in particular and for seabird conservation in general.

3.2 Materials and Methods

Sample collection and DNA extraction

Blood and tissue samples were obtained from nine colonies of Leach's storm-petrels across their North Atlantic and North Pacific ranges (Table 3.1; Figure 3.1). Between 10-20μl of blood was collected (under appropriate regional licences) from the brachial vein of breeding adults or chicks caught in nesting burrows at seven of these colonies. Blood was collected into microfuge tubes, mixed with 1.5ml of absolute ethanol and stored at -20°C in a spark-proof freezer. Burrow access on North Rona, Scotland was restricted so we sampled breeding birds caught in mist-nets visiting the colony at night. Although this approach may lead to the capture of some non-breeding birds (Furness and Baillie, 1981), we reduced this risk by categorising active breeders based on the following criteria: individuals caught in the highest density area of the colony without a chatter call lure; individuals in breeding condition (i.e. with a bare brood patch); individuals regurgitating prey items assumed to be food for a waiting chick; and/or individuals that had a ≥ 2 year old British Trust for Ornithology leg ring. Samples from Baccalieu Island, Canada consisted of striated muscle tissue from euthanized chicks. Tissue samples were stored in 1ml of absolute ethanol at -20°C in a spark-proof freezer. DNA was extracted using a standard proteinase-K ammonium acetate method (Nicholls *et al.*, 2000) and diluted to ~10 ng/μl with low TE buffer (Tris 1mM, EDTA 10mM, pH 8.0).

Table 3.1 Sampling site locations and numbers of Leach's storm-petrel individuals included in mitochondrial and nuclear microsatellite DNA analyses

Colony	Geographic Location	Abbreviation	Ocean Basin	Latitude / Longitude	Number of individuals	
					mtDNA	Microsatellite
Buldir Island	Western Aleutian Islands, Alaska	BUL	Pacific	52°21′N / 175°55′E	12	25
St Lazaria Islands	Gulf of Alaska, Alaska	LAZ	Pacific	56°59′N / 135°43′W	17	32
Baccalieu Island	Newfoundland	BACC	Atlantic	48°07′N / 052°48′W	12	25
Gull Island	Witless Bay, Newfoundland	GULL	Atlantic	47°15'N / 052°46'W	10	48
Bon Portage Island	Nova Scotia	BON	Atlantic	43°28'N / 065°25'W	12	40
Vestmannaeyjar	South Iceland	VEST	Atlantic	63°25′N / 020°17′W	11	25
Røst	Nordland, Norway	NOR	Atlantic	67°31′N / 012°05′E	7	-
North Rona	Western Isles, Scotland	RONA	Atlantic	59°07′N / 005°49′W	12	18
St Kilda	Western Isles, Scotland	STK	Atlantic	57°49′N / 008°35′W	10	32
Total	9 populations				103	245

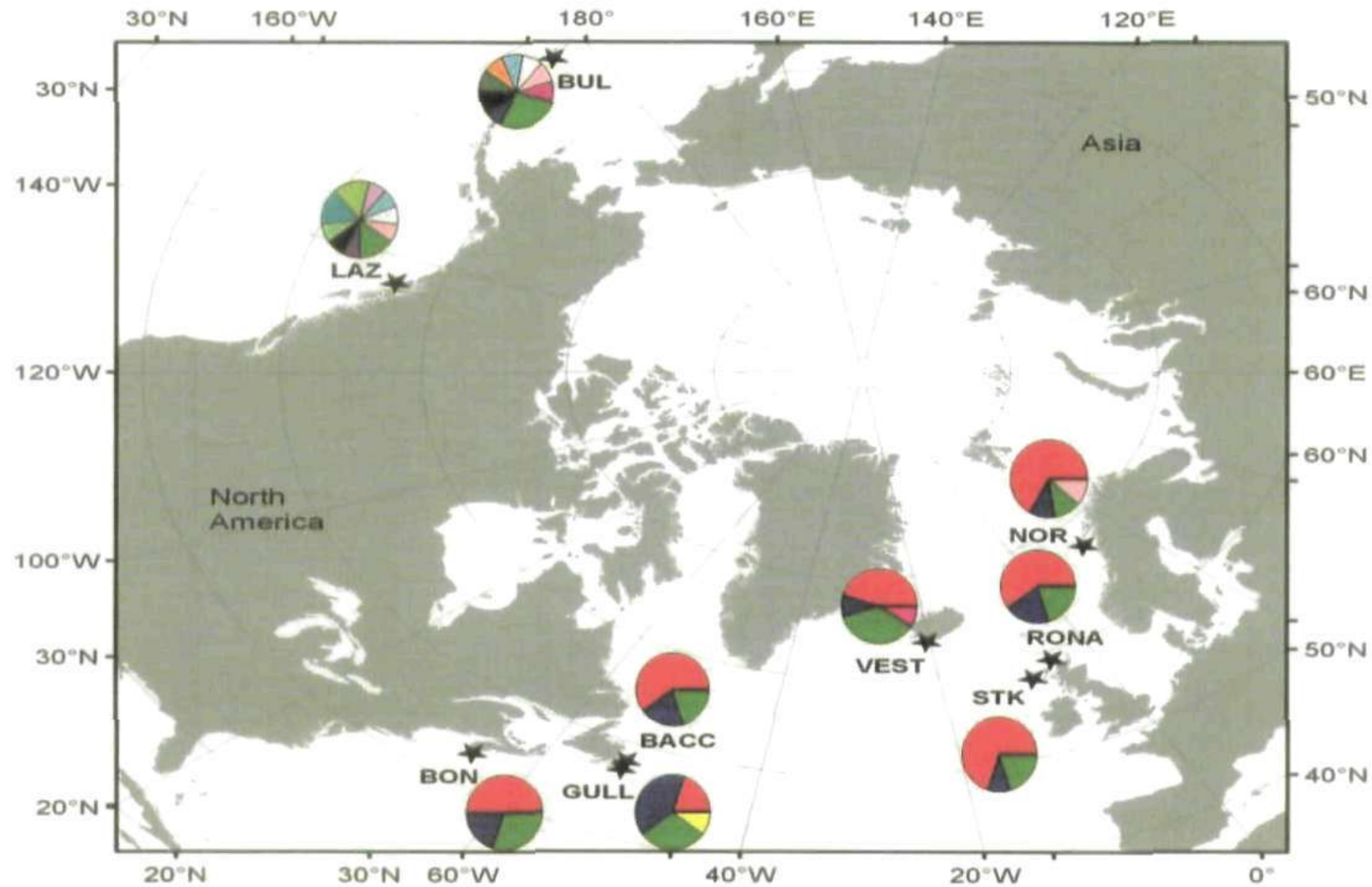

Figure 3.1 Locations of Leach's storm-petrel colonies sampled in the North Atlantic and Pacific. Colony abbreviations detailed in Table 1. Pie charts indicate mitochondrial DNA fragment haplotype frequencies in each colony. Each colour represents a single haplotype.

Mitochondrial DNA sequencing

A 360-base pair (bp) fragment of the mitochondrial control region (Domains I and II) was amplified from 103 Leach's storm-petrel samples using previously developed primers OcL61 (Smith *et al.*, 2007; band-rumped storm-petrel *Oceanodroma castro*) and H521 (Quinn and Wilson, 1993; generic). PCR was performed using either a G-STORM GS1 (Gene Technologies, Byfleet, UK) or an AB2720 (Applied Biosystems, Carlsbad, USA). PCR reactions were run using the GeneAmp Gold PCR reagent kit (Applied Biosystems, Carlsbad, US) in 25 μl reaction volumes containing 2.5 mM $MgCl_2$, 1 x reaction buffer (150 mM Tris-HCl and 500 mM KCl, pH 8.0), 0.2 mM each of the four dNTPs, 0.4 μM of each of the primers *OcL61* and *H521*, and 0.75 units of AmpliTaq Gold DNA polymerase (Roche Molecular Systems, Inc.). The PCR profile consisted of an initial denaturing step (90 s at 96°C), followed by 31 cycles of 30 s denaturation at 96°C, 30 s annealing at 60°C, and 1 min extension at 72°C, ending with a final 3 min extension step at 72°C. The amplified fragment was used as the template for 20 μL cycle sequencing reaction volumes using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's protocol and resolved by electrophoresis on an AB3130 Genetic Analyser (Applied Biosystems). Both forward and reverse strands were sequenced and aligned by eye using BioEdit version 7.0.9.0 (Hall, 1999).

Microsatellite genotyping

All samples were genotyped at 32 autosomal microsatellite loci. The loci used included 20 species-specific Leach's storm petrel loci (*Ole01, Ole03, Ole05-11, Ole13-18, Ole21-25*; Bicknell *et al.*, 2011), 5 loci previously developed for storm-petrels: (*Ol10-39, Oc51, Oc63, Oc84, Oc87B*; Sun *et al.*, 2009) and 7 loci engineered for cross-utility in avian species: (*TG02-120, TG03-034, TG04-041, TG04-061, TG05-053, TG13-017;*

Dawson et al., 2010, CAM12, F:TGGCARTAAWTCCAGAGATTACC, R:CTGRCATTTGTCTTAAGCGTG*)*. Loci were amplified in seven sets of multiplexed reactions with optimised primer concentrations and PCR annealing temperatures (Supplementary Data Table 3.1). Multiplexes 1-5 were prepared in 2 µl reaction volumes containing approximately 10 ng of lyophilised genomic DNA and 1 µl QIAGEN multiplex PCR mix (Qiagen Inc.; Kenta *et al.*, 2008). PCR amplification was performed using a DNA Engine Tetrad 2 thermal cycler (MJ Research, Bio-Rad) with the following program: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, annealing temperature (Supplementary Table 3.1) for 90 s, 72°C for 1 min, and finally 60°C for 6 min. Amplified products were resolved on an AB3730 48-well capillary DNA Analyser (Applied Biosystems). Multiplexes 6 and 7 were prepared in 10 µl reaction volumes containing ~10 ng/µl of sample DNA and Qiagen Core Kit reagents (25 mM $MgCl_2$, 10 x reaction buffer (Tris-HCl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7)), 0.1 mM of each of the four dNTPs and 0.5 units of Qiagen *Taq* DNA polymerase), and amplified with PCR conditions as described in Friesen *et al.*, (2007). PCR amplification was performed using either a G-STORM GS1 (Gene Technologies) or an AB2720 (Applied Biosystems) thermal cycler. Amplified products were resolved on an AB3130 DNA Analyzer. Failed samples were re-run to ensure a complete dataset and allele sizes for all multiplexed loci were assigned using GENEMAPPER v3.7 (Applied Biosystems). 10% of samples were re-run to estimate genotyping error.

Tests of assumptions and genetic variation

To test whether patterns of genetic variation in the mitochondrial DNA (mtDNA) control region sequence deviated from selective neutrality Ewens-Watterson (Ewens, 1972, Watterson, 1978) and Tajima's neutrality tests (Tajima, 1989) were performed in ARLEQUIN v3.5.1.2 (Excoffier and Lischer, 2010). Haplotypic diversity (h; Nei, 1987)

and nucleotide diversity (Tajima, 1983) were also calculated to assess levels of genetic variation within colonies. All microsatellite loci were tested for departures from Hardy-Weinberg equilibrium within each breeding colony and assessed for deviations from linkage disequilibrium using a Markov-chain method implemented in GENEPOP v3.4 (Rousset, 2008). Significance levels were adjusted for multiple comparisons using a Benjamini-Yekutieli corrections (Benjamini and Yekutieli, 2001). Estimated null allele frequencies were calculated using CERVUS v3.0.3 (Kalinowski *et al.*, 2007). Observed and expected heterozygosity and mean numbers of alleles were calculated for each colony and each subsequently identified population using ARLEQUIN v3.5.1.2. Allelic richness and private allelic richness were calculated using HP-RARE 1.0 (Kalinowski, 2005). Means are provided ± standard deviations unless stated. Samples from Røst, Norway, were not included in any microsatellite analyses due to the small number of samples available (n=7).

Population structure and demographic history

Genetic differentiation between all pairs of Leach's storm-petrel colonies was assessed by calculating pairwise F_{ST} and Φ_{ST} values (Whitlock, 2011). A hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was conducted to assess within Atlantic and between ocean differences (groupings based on pairwise comparison results). For mtDNA analyses, Kimura's two-parameter model of substitution (Kimura, 1980) with a shape parameter (α) for the gamma distribution of 0.47 (based on α estimates of Domain I, II and III of the control region in finches, *Fringilla* and *Carduelis spp.*; [Marshall, Baker, 1997]) was used. To determine whether estimates of genetic variation (see above) were associated with the longitude or latitude of colonies in the Atlantic linear regressions were used. Indices and tests were run in ARLEQUIN v3.5.1.2, except for linear regressions which were run in R v2.11.1 (R, 2011).

The mtDNA control region variation was used to calculate parameters of demographic history (i.e. population growth or decline) in ARLEQUIN v3.5.1.2, for colony groups as defined by the AMOVA results. Fu's Fs test of selective neutrality was performed as this has been shown to be sensitive to population expansion, indicated by significantly negative values (Hasegawa *et al.*, 1985). LAMARC 2.0 (Kuhner, 2006) was used to employ a maximum likelihood estimate based on coalescent theory to test for evidence of population growth. Significant population growth was determined by comparing twice the difference between the log likelihood at the maximum growth estimate and the log likelihood at zero growth with the critical χ^2 value for alpha = 0.05 and one degree of freedom. TRACER V1.5 was used to ensure that the search had reached stationary distribution and had searched parameter space efficiently (ESS for each population >200).

Microsatellite data were checked for evidence of a recent reduction of effective population size in the genetically defined populations using BOTTLENECK v1.2.02 (Cornuet and Luikart, 1996). If a population has experienced a recent bottleneck the observed heterozgosity will be larger than the heterozygosity expected from the observed number of alleles under the assumption of mutation/drift equilibrium. The exact mutation model of microsatellites is unknown (Bhargava and Fuentes, 2010) so three mutation models; step-wise mutation model (SMM), infinite allele model (IAM) and two-phase mutation model (TPM), were used and results compared. The TPM incorporates both other types of mutation models and was run assuming 95% step-wise mutations and 5% multiple step mutations (Piry *et al.*, 1999). The Wilcoxon signed-rank test was applied to determine heterozygosity excess across loci.

Estimating time since divergence ($t = T\Theta/2\mu$, where T is population divergence time in N_f generations, Θ is $2N_f\mu$ and μ is the mutation rate per year of the mitochondrial fragment) and gene flow (*M*, in number of females per generation) between any

genetically distinct populations was performed using IMa2 @ BioHPC (Nielsen and Wakeley, 2001, Hey and Nielsen, 2007), which implements a coalescent based Markov chain Monte Carlo (MCMC) analysis method under an isolation with migration model of population divergence. Unlike traditional methods (e.g. using δ; Wilson *et al.*, 1985) this analysis does not assume populations are in migration-drift and mutation-drift genetic equilibrium, which may be true in populations that share haplotypes, as has been suggested for other temperate seabird species with large population sizes (e.g. Morris-Pocock *et al.*, 2008). The program was run on the control region locus data only, assuming a finite site model (Hasegawa-Kishino-Yano model; Hasegawa *et al.*, 1985) that allows for multiple substitutions, particularly suitable for mtDNA. Three separate chains were run starting at different random seeds, with 1,000,000 burn-in and 3,000,000 Markov chain length to ensure convergence and robustness of results. T_{MAX} and M_{MAX} were set at 20 and 10, respectively, after exploratory runs had confirmed a posterior distribution that approximated a Poisson distribution. Point estimates and 95% credibility intervals for M and T were determined from the respective posterior distributions and log-likelihood tests were performed to test significance from zero (Nielsen and Wakeley, 2001, Hey, 2010). However, it is often not possible to estimate the upper bound of T if the likelihood curves approach zero very slowly (Nielsen and Wakeley, 2001), so the upper bound is referred to as 'undefined' in the results for these cases.

Microsatellite Bayesian cluster and assignment analyses

Bayesian clustering analysis implemented in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000, Falush *et al.*, 2003) was used to identify genetically distinct Leach's storm-petrel populations. This model-based method uses a MCMC simulation to assign individuals to genetic clusters (K) based on individual genotypes. Simulations were run both with

and without sampling location as prior population information to help check for weak population structure (Hubisz *et al.*, 2009). We performed 10 replicate runs for K = 1 on 8 genetic populations (number of sampled colonies) using the admixture model and assuming correlated allele frequencies (Falush *et al.*, 2003). A burn-in of 500,000 followed by 1,000,000 iterations was used following exploratory runs to confirm data convergence. We used a uniform prior for alpha (α), with initial value of 1.0 for all populations, and lambda (λ) set at 1.0. To evaluate the most probable number of genetic populations the posterior probability, Ln[P(X|K)], was compared directly (Pritchard *et al.*, 2000) and the standardized second order rate of change (ΔK) was examined as suggested by Evanno *et al.* (2005).

Two methods were used to identify dispersal between genetically distinct populations. Migrant individuals were identified using exclusion methods as implemented in GENECLASS2 (Piry *et al.*, 2004) with Bayesian estimation methods (Rannala and Mountain, 1997). This method calculates the probability of an individual originating from the population it was sampled in using the exclusion criteria L_h/L_{max} (where L_h is the likelihood of an individual originating from the population it was sampled in and L_{max} is the likelihood of an individual originating from any other population) and Paetkau's *et al.* (2004) resampling method to generate critical values for rejecting the null hypothesis that an individual was from its sampled population. The alpha value (expected type I error rate) was set at 0.001 and 0.01 to explore the effect on identification of possible immigrant individuals. The second method to identify dispersal was the assignment test implemented in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000, Falush *et al.*, 2003). This method detects putative migrants along with individuals with recent immigrant ancestry. This fully Bayesian approach uses geographical location as prior population information and the a prior probability that an individual was an immigrant was set at $\nu=0.01$ (Pritchard *et al.*, 2000). Models were performed

under the assumption of correlated allele frequencies and lambda was set to 1.0. MCMC simulations were run with a burn-in of 500,000 followed by 1,000,000 iterations.

3.3 Results

Tests of assumptions and genetic variation

Seventeen mtDNA haplotypes were obtained from 103 individuals sampled across 9 colonies. These were defined by 11 polymorphic sites all of which were substitution sites, including 10 transitions and 1 transversion. One haplotype was shared across all colonies (LSP_3) and 1 was found at high frequency only in the Atlantic (LSP_1). Gull Island (LSP_4), Buldir Island (LSP_9, LSP_{10}, LSP_{11}) and St Lazaria Island (LSP_{12}, LSP_{13}, LSP_{14}, LSP_{15}, LSP_{16}, LSP_{17}) had unique haplotypes, with the latter two colonies combining to produce 11 haplotypes only found in the Pacific (Figure 3.1; Table 3.2; Supplementary Table 3.3). Nucleotide diversity (π) was similar across all colonies (0.0055 ± 0.0008), but haplotype diversity (h) tends to be higher in the Pacific colonies (0.92 ± 0.012) compared to the Atlantic (0.66 ± 0.086) (Table 3.2). Ewens-Watterson (all $P > 0.32$) and Tajima's D (all $P > 0.28$) tests within all colonies were non-significant. Of the 31 microsatellite loci genotyped, 6 were removed from subsequent analyses due to significant deviations from the assumptions of Hardy-Weinberg or linkage equilibrium (*Ole13, Ole16, Ol10-39, Oc51, Oc84, TG05-053*). Seven further loci were also excluded due to high levels of null alleles (*TG03-034*) or inconsistent amplification leading to reduced confidence in genotype scoring (*Ole03, Ole05, Ole08, Ole11, Ole21,*

Ole23). All other loci (18) met both assumptions after Benjamini-Yekutieli corrections and showed low levels of null alleles. Only 5 genotypes across all samples and loci could not be reliable scored and the genotyping error rate, calculated from the re-scoring exercise, was <5%.

Mean observed heterozygosity at the microsatellite loci was similar across colonies (0.45 ± 0.02). A comparable pattern to the control region haplotypes was found for allelic and private allelic richness, with the Pacific colonies showing greater diversity than the Atlantic colonies (Table 3.2).

Table 3.2 Genetic variation at 18 microsatellite loci and a 357 bp fragment of the mitochondrial DNA control region in Leach's storm-petrel colonies in the Pacific and Atlantic ocean basins

Location	Mitochondrial Control Region				Microsatellite Data					
	N	H	*h*	π	*N*	*A*/locus	*A*	$A_{private}$	H_O	H_E
Pacific Basin	29	15	0.92	0.0054	57	5.06	3.42	0.88	0.48	0.47
Buldir Island	12	9	0.91	0.0054	25	4.33	3.38	0.21	0.47	0.47
St Lazaria Island	17	10	0.93	0.0054	32	4.50	3.45	0.27	0.48	0.47
Atlantic Basin	74	6	0.64	0.0054	188	4.67	3.11	0.57	0.44	0.45
Baccalieu Island	12	3	0.62	0.0048	25	3.82	3.02	0.05	0.46	0.48
Gull Island	10	4	0.78	0.0049	48	3.89	3.16	0.03	0.45	0.45
Bon Portage Island	12	3	0.67	0.0059	40	4.28	3.21	0.08	0.46	0.46
Vestmannaeyjar	11	4	0.71	0.0067	25	3.72	3.07	0.05	0.42	0.42
Røst	7	4	0.71	0.0069	-	-	-	-	-	-
North Rona	12	3	0.62	0.0048	18	3.39	2.91	0.01	0.41	0.41
St Kilda	10	3	0.51	0.0048	32	3.78	3.06	0.03	0.44	0.44

N, sample size; H, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; A/locus, mean number of alleles per locus; A, allelic richness; $A_{private}$, private allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity. Microsatellite data were not included for the Norwegian (Røst) colony due to small sample size

Population structure and demographic history

AMOVA indicated significant population structure from the mtDNA control region locus (Global Φ_{ST} = 0.32, P <0.0001). No significant colony pairwise Φ_{ST} values were found within ocean basins, but all pairwise comparisons between oceans were significant before Benjamini-Yekutieli correction (Table 3.3). After correction most St Lazaria comparisons were still significant and 3 remained for Buldir Island. The hierarchical AMOVA that best explained the variation was with colonies grouped by ocean (Φ_{CT} = 0.32, P <0.05). The microsatellite typing results generally support the patterns suggested by the mtDNA data (Global F_{ST} = 0.04, P <0.0001). All pairwise comparisons between St Lazaria Island and Buldir Island and Atlantic colonies were significant after Benjamini-Yekutieli corrections (Table 3). Colonies grouped by ocean basin was again the hierarchical AMOVA that best explained the variation (F_{CT} = 0.045, P <0.05).

There was no significant linear relationship between mtDNA diversity (both haplotype and nucleotide diversity) and geographic position (latitude and longitude) between Atlantic colonies (all P >0.05). Microsatellite heterozygosity (slope = -0.0007, Std. Error (SE) = 0.0002, r^2 = 0.66, $F_{1,4}$ = 10.9, P = 0.029) and mean number of alleles (slope = -0.009, SE = 0.003, r^2 = 0.58 $F_{1,4}$ = 8.1, P = 0.046) were significant in relation to colony longitudinal position. Heterozygosity also had a significant linear relationship to colony latitude (slope = -0.002, SE= 0.0006, r^2 = 0.72, $F_{1,4}$ = 14.266, P = 0.019) but A/locus was not significant, although, showed a similar trend (slope = -0.027, SE = 0.012, r^2 = 0.46, $F_{1,4}$ = 5.339, P = 0.082). The negative slopes of these relationships indicate a reduction in allelic variation moving from colonies in the northeast to southwest (i.e. Iceland/Scotland < Canada).

Table 3.3 Pairwise Φ_{ST} estimates based on mitochondrial DNA control region (above diagonal), and F_{ST} estimates based on nuclear microsatellite variation (below diagonal). Population abbreviations are given in Table 1. Dashes indicate pairwise comparisons not possible due to small sample size.

Location	BUL	LAZ	BACC	GULL	BON	VEST	NOR	RONA	STK
BUL		0.001	0.372**	0.157*	0.229*	0.140*	0.248*	0.372**	0.405**
LAZ	0.009*		0.407**	0.207**	0.280**	0.204*	0.308**	0.407**	0.439**
BACC	0.045**	0.039**		0.113	-0.048	-0.005	-0.096	-0.091	-0.048
GULL	0.046**	0.051**	0.004		0.005	-0.017	0.032	0.113	0.186
BON	0.039**	0.044**	0.005	-0.004		-0.080	-0.108	-0.048	-0.031
VEST	0.047**	0.053**	0.006	-0.001	0.004		-0.086	-0.005	0.015
NOR	-	-	-	-	-	-		-0.096	-0.094
RONA	0.056**	0.070**	0.017*	0.005	0.013*	0.005	-		-0.080
STK	0.046**	0.053**	0.005	-0.004	-0.003	-0.007	-	-0.002	

Pink shading * = significant before Benjamini-Yekutieli corrections; Red shading ** = significant after Benjamini-Yekutieli corrections.

The populations delineated by the AMOVA results (Atlantic and Pacific) show differing demographic histories (Supplementary Table 3.2). Fu's Fs test was significantly negative for the Pacific ($P < 0.001$), suggesting historical expansion, but was positive and not statistically significant for the Atlantic. Results from the LAMARC analysis also indicated the Pacific, but not the Atlantic population, had undergone significant population growth ($P < 0.001$; Supplementary Figure 3.1).

Significant microsatellite heterozygosity excess was observed in the Atlantic population using the IAM (BOTTLENECK, $P < 0.01$) but not when using either SMM or TPM (BOTTLENECK, $P > 0.05$). Heterozygosity excess was not observed using any mutation model for the Pacific population (BOTTLENECK, IAM, SMM and TPM, $P > 0.05$).

Assuming a mtDNA control region divergence rate of 21% per million years (Quinn, 1992) the Pacific and Atlantic populations are estimated to have diverged approximately 13,000 years ago [95% credibility interval: 8,250 – undefined]. After log-likelihood tests, gene flow was only significant from the Pacific to Atlantic population (and not vice-versa) with 1.96 females migrants per generation (~12 years) [95% credibility interval: 0.1172– 6.181].

Microsatellite Bayesian cluster and assignment analyses

Convergence of the STRUCTURE clustering analyses was confirmed by low variance in $\ln[P(X|K)]$ across replicate runs and visual inspection of likelihood and estimated parameters. When run with prior information of sampling location the $\ln[P(X|K)]$ and ΔK for different values of K indicated K=2 as the most likely number of clusters (Figure 3.2). When no prior information was used in the analysis K=3 was most likely, but the differences in Pr(K) between 2 and 3 clusters was small. Inference of K based on small differences in Pr(K) should be viewed with caution (Nielsen and Wakeley, 2001), and on inspection of Q (probability of membership) values for K=3 revealed that

individuals have low probability of assignment to any clusters (Supplementary Figure 3.1). When the extent of population genetic structure in one of the clearly defined populations is uncertain Pritchard *et al.* (2007) suggest running the analysis for only the individuals in that population. This analysis found no evidence of subdivision, with K=1 being the most likely number of clusters for these individuals (only Atlantic colonies; data not shown). Taken together these results support the K=2 model found when sampling location is used as prior information. The clustering of individuals into two populations clearly separates with the colony locations in the Atlantic versus Pacific (Figure 3.2), with no subdivision within ocean basins, in agreement with the patterns emerging from the mtDNA sequence data.

The methods to identify individual migrants could only be used between ocean basin populations due to the lack of genetic differentiation between Atlantic colonies. GENECLASS2 identified one individual as a first generation migrant (Pac02; P <0.001). This individual was sampled at the Buldir Island breeding colony. Using the strict alpha value (0.001) the null hypothesis (sampling location as place of origin) could not be rejected for any other individual. When the alpha value was relaxed (<0.01) two further individuals from St Lazaria Island (Pac37) and Bon Portage Island (Atl65) were identified as migrants. The same individual (Pac02) had the highest posterior probability (PP) of being an immigrant in the STRUCTURE analysis. When ν (prior probability) was set at 0.01 this was the only bird that had a PP >0.50 of being an immigrant. Both analyses identified this individual as having a high probability of being a migrant, suggesting movement from the Atlantic to the Pacific.

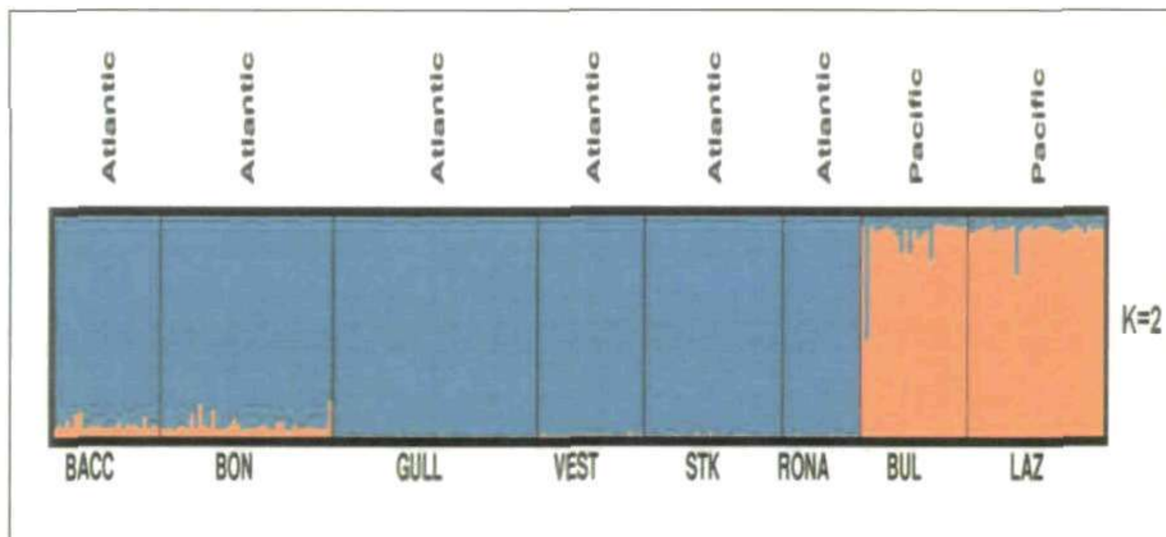

Figure 3.2 Probability of assignment (Q) of Leach's storm-petrel to two genetic clusters estimated using STRUCTURE with sampling location as prior information. Each line is a single individual and individuals are ordered by sampling location. Colours correspond to genetic clusters.

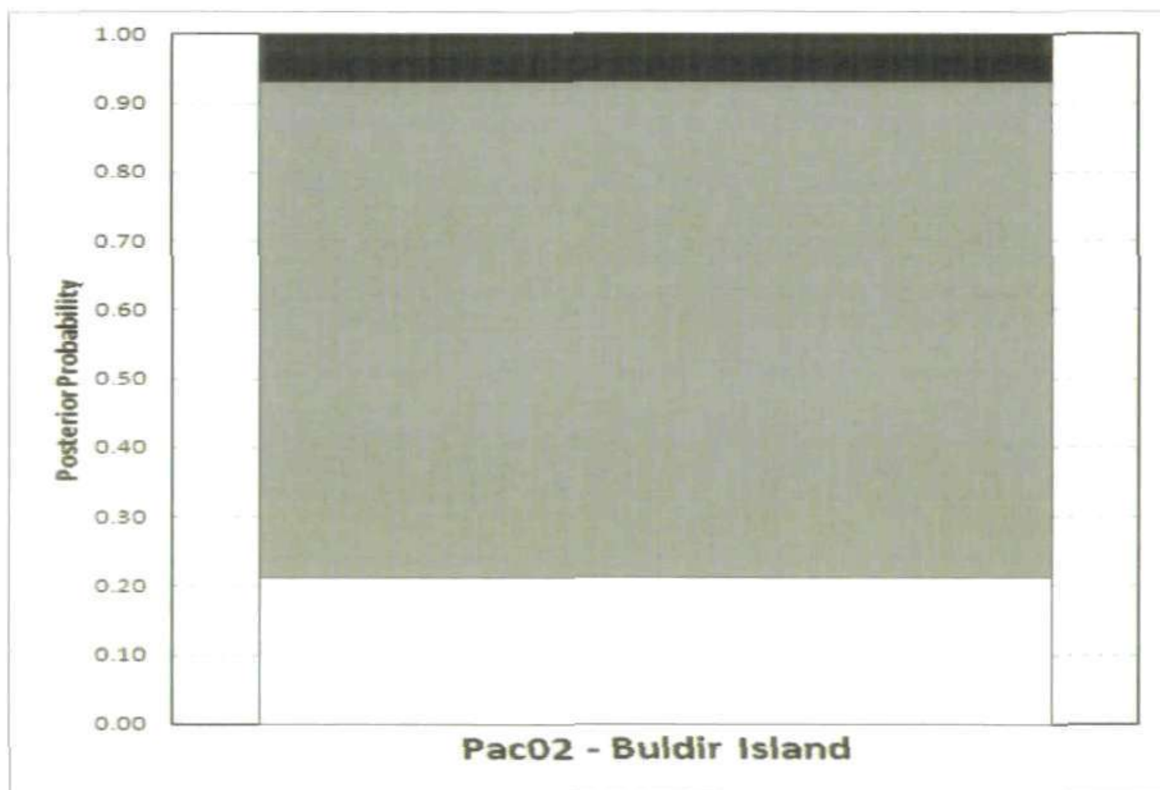

Figure 3.3 Probability estimates for the Leach's storm-petrel (Pac02) most likely identified as a migrant using the assignment test in STRUCTURE (ν=0.01). Colours correspond to three different categories: white = no immigrant ancestry; light grey = immigrant; dark grey = immigrant parent.

3.4 Discussion

Here we use mtDNA control region sequence data and 18 polymorphic autosomal microsatellites to investigate genetic structuring and gene flow among Leach's storm-petrel colonies within the Atlantic and between the Atlantic and Pacific basins. This is the first study of this species to benefit from the use of two molecular markers and complementary analyses (F_{ST}, Bayesian cluster and exclusion/assignment), and their concordance presents strong evidence for contemporary and historical gene flow among the Atlantic colonies and limited dispersal between ocean basins. The results are consistent with the evidence of gene flow found in an earlier introductory study in the North Atlantic (Paterson and Snyder, 1999). Contemporary and historical factors influencing gene flow and dispersal in this species, as well as their conservation implications are discussed below.

Patterns of gene flow and population history - ocean basins

Landmasses are major factors leading to population differentiation in seabirds (Friesen *et al.*, 2007a) and distinct genetic differences between the Pacific and Atlantic suggest that this is also the case for Leach's storm-petrel. There is little evidence for structuring within the Pacific (Aleutian Islands and northwest coast of America), although sampling only two colonies may have inhibited detection. Leach's storm-petrels breeding in the Pacific have higher genetic diversity at both mtDNA control region and microsatellite loci than those from the Atlantic (Table 3.2). This suggests no strong population contraction in the past, as further supported by no evidence for a recent bottleneck or founder event. The pattern of genetic diversity is characteristic of a Pacific source population and founder event in the Atlantic (Le Corre and Kremer, 1998, Caissie *et al.*, 2010). This is particularly noticeable for the control region sequences (but also for nuclear alleles) where all Atlantic haplotypes are nested within the Pacific variation

except the one at highest frequency, a pattern predicted by genetic theory (Page and Holmes, 2007). The microsatellite BOTTLENECK results show some support for this event but this is dependent on one mutation model (IAM), which may not be suitable for these markers (Shriver *et al.*, 1993, Bhargava and Fuentes, 2010). Divergence of the populations, as estimated from coalescent analysis (~13,000 ya), would place the founding event during the last glacial retreat when suitable habitat was becoming increasingly available for colonization and oceanic conditions were more favourable in the north Atlantic (Ruddiman and McIntyre, 1981).

Despite the clear genetic differences between Leach's storm-petrel populations in the Atlantic and Pacific, using three separate analysis (IMa2, STRUCTURE, GENECLASS) we also found evidence of contemporary gene flow and immigration between the Pacific and Atlantic. Coalescent analysis indicates low female-mediated gene flow has continued from the Pacific population but not to any significant degree in the opposite direction. More recent dispersal between oceans is found with one migrant Leach's storm-petrels identified in the Pacific. Given such a small number of samples relative to the population sizes in each ocean, this suggests the number of dispersers could be considerable, but still not enough to genetically homogenise the two populations.

North Atlantic Leach's storm-petrel colonies

The lack of population structure in the Atlantic (F_{ST} and Φ_{ST} analyses as lagging indictors; Whitlock and McCauley, 1999), suggests high historical gene flow among colonies. Population expansion from small refugial populations since the last Pleistocene glacial retreat (10,000-14,000 ya; Ruddiman and McIntyre, 1981) is well documented for many taxa (Hewitt, 2000) and has been inferred in other Atlantic seabird populations (Moum and Arnason, 2001, Morris-Pocock *et al.*, 2008). Our data suggest that Leach's storm-petrel may have colonised the Atlantic from a refugium in

northeast North America. The progressive decrease in allelic variation with increasing longitude and latitude (i.e. from Bon Portage to North Rona/Vestmannaeyjar, Figure 1) is indicative of sequential expansion (Pruett and Winker, 2005). The relatively short time since the estimated founding event and subsequent growth to the present large population size may not have allowed the Atlantic population to reach migration-drift equilibrium, as has been suggested for another temperate seabird species (common guillemots *Uria aalge*, Friesen *et al.*, 1996, Riffaut *et al.*, 2005, Morris-Pocock *et al.*, 2008). If migration-drift equilibrium has not been reached the population may still harbour some signature of ancestral variation and gene flow may be overestimated. Nonetheless, the consensus of evidence suggests high levels of gene flow amongst all colonies during and subsequent to colonization of new island habitats.

Mechanisms of gene flow

Our data provides strong evidence for contemporary gene flow among Atlantic colonies, but what are the mechanisms involved? Dispersal can be broken into three interdependent stages: emigration, inter-patch movement and immigration (Bowler and Benton, 2005). Emigration in Leach's storm-petrel is most likely via immature birds, since breeders exhibit high levels of philopatry after recruitment (Morse and Kress, 1984, Huntingdon *et al.*, 1996). The recapture of two ringed Leach's storm-petrels breeding at colonies 1000s of kilometres from their natal colony (Maine, US→ Iceland, Iceland→North Rona Scotland) provides some evidence for this (Y. Kolbeinsson, pers. comm.). The decision to emigrate can be influenced by many factors at the natal colony (e.g. colony density, food and habitat availability; Bowler and Benton, 2005), but population mixing on wintering grounds may also play an important role among pelagic seabirds (Friesen et al. 2007). The distribution of Leach's storm-petrels outside the breeding season is poorly known but large over-winter aggregations in the Bay of

Biscay (>2,000,000) far exceed the relatively small European population estimates (Hémery and Jouanin, 1988), suggesting that North American and European birds may share wintering grounds and through associations with conspecifics and/or following activity may lead to emigration .

Inter-patch movement appears to be an important part of selecting a place to breed among immature seabirds (Votier *et al.*, 2011), but the extent of this behaviour is poorly understood. This inter-patch movement may be more extensive in pelagic species compared with more inshore foragers, although the evidence for this difference influencing gene flow is equivocal (Burg and Croxall, 2001). Nevertheless it is interesting to note that, in comparison to the results presented here for the Atlantic Leach's storm petrel population, genetic structuring has been reported among Atlantic and Mediterranean populations in the closely related and inshore European storm-petrels *Hydrobates pelagicus* (Cagnon *et al.*, 2004).

Immigration can be influenced by various factors (Bowler and Benton, 2005), but the availability of suitable breeding habitat and mates is essential. It is possible that heavy predation, such as that recorded at St Kilda (Votier *et al.* 2006; Miles 2010), could increase the availability of nest sites and mates, but predation may quickly offset these benefits.

The dispersal dynamics of this species is clearly an area requiring further research to help elucidate the mechanisms involved in this multifaceted behaviour.

Conservation implications

Our findings indicate that a significant barrier to gene flow between Leach's storm-petrel colonies is large landmasses, although even these may be surmountable. For colonies within the same ocean basin long distance dispersal across open water appears to lead to high levels of gene flow. Therefore for conservation and management

purposes, Leach's storm petrel in the North Atlantic may function as a large meta-population (Esler, 2000) with dispersal occurring amongst all colonies. The disparity between persistence of the St Kilda colony and the exceptionally high predation estimates that would otherwise extirpate the local population are congruent with immigration from other colonies. Confident assignment of immigrants to a colony of origin and estimation of dispersal rates were not possible due to the lack of colony differentiation, but given the number of birds involved it is likely that the large colonies in Atlantic Canada are major contributors. We are also unable to determine whether the buffering occurs as a function of recruitment into the St Kilda breeding population, or inter-colony movement of prospectors that are then predated upon, or both.

It is unclear whether the current levels of immigration are able to buffer such predation in the long term, but the recent population declines suggest that this may not be the case. Although the great skua population on St Kilda has remained relatively stable in recent years, these generalist predators may show facultative switching to seabird prey in the face of declines in the availability of alternative foods such as fishery discards (Votier *et al.*, 2004). A selective cull of great skuas may offset heavy storm-petrel predation (Kokko and López-Sepulcre, 2006), but this may not be appropriate for a skua species that only breeds in Europe and for which St Kilda retains ~1.5% of the global population. To predict future population trends at St Kilda the combined effect of predation and dispersal on its demographic rates (i.e. growth and vital rates) need to be quantified. Although, genetic assignment has been previously used to estimate the impact of effective immigration on population growth in a seabird (Peery *et al.*, 2008) the genetic similarity and size of the Leach's storm-petrel colonies makes it impossible for this species. For highly vagile species with large populations, such as Leach's storm-petrels, other approaches to assessing dispersal rates are needed (e.g. stable isotopes and tracking technology), and used in combination with genetic and demographic data to

predict long-term population viability in the face of these impacts (Lowe and Allendorf, 2010).

This study shows that inter-colony movement over large spatial scales can affect colony resilience, thus impacts of dispersal need to be considered at both the local and meta-population scale (Hanski, 2001). The large non-breeding component of seabird populations has been shown to play an important role in buffering the effects of stochastic mortality with compensatory recruitment (Votier *et al.*, 2008), and our study suggests immigrant recruitment from distant populations could have similar effects. Understanding connectivity of disjunct populations of highly vagile colonial seabirds is vital to appropriately manage their populations and predict the effect of increasing levels of environmental change.

Chapter 4

Effects of formalin preservation on stable carbon and nitrogen isotope signatures in Calanoid copepods: Implications for the use of Continuous Plankton Recorder Survey samples in stable isotope analyses.

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4.1 Introduction

In marine ecosystems stable isotope analysis (SIA) has become a familiar technique for investigating trophic relationships (Petursdottir *et al.*, 2008), food web structure (Hobson *et al.*, 2002) and movement behaviour (Hobson, 1999, Hobson, 2007). The use of preserved and/or archived organic material offers huge potential for the conduct of retrospective analyses and investigate medium to long-term ecosystem changes. Most natural history museums and many other institutions hold large preserved collections that could be a major resource for such studies. However, there are uncertainties over the effect of different preservatives on the stable carbon and nitrogen isotope values, which is perhaps reflected in the scarcity of studies that utilise collections of preserved biological material. Understanding the effects of preservatives on stable isotope signatures is crucial for the appropriate interpretation of results from ecological studies.

Ideally the chemicals used to preserve biological material should not alter their molecular composition, but in many instances this is not the case (Carabel *et al.*, 2009, Ventura and Jeppesen, 2009, Syväranta *et al.*, 2011). The reported changes ($\delta^{13}C$: <3‰, $\delta^{15}N$: <1‰) lack consistency in these effects (Barrow *et al.*, 2008, Ventura and Jeppesen, 2009), and this precludes general conclusions being reached for particular preservatives. A range of preservatives has been used for short or long term collection storage and these preservatives have been shown to have different impacts (Barrow *et al.*, 2008), so require separate investigation before collection samples can be used in SIA.

The Sir Alister Hardy Foundation for Ocean Sciences (SAHFOS, Plymouth, UK) maintains the Continuous Plankton Recorder (CPR) Survey, which is one of the longest and geographically the most extensive measures of plankton communities worldwide. Since 1931 ships of opportunity (such as commercial and passenger vessels) have towed recorders for approximately 5.8 million nautical miles, continuously collecting

plankton. The core survey operates in the North Atlantic but it has grown in scope to include the North Pacific, the Southern Ocean and the Western Atlantic. CPR data has been integral to understanding how the ecosystem function is regulated in the North Atlantic (Beaugrand *et al.*, 2002, Beaugrand *et al.*, 2003, Edwards and Richardson, 2004, Frederiksen *et al.*, 2006) leading to further insights into the indirect effects of climate change on North Sea commercial fish stocks (Beaugrand and Kirby, 2010). The isotopic information available within the CPR Survey samples has great potential for large scale analysis of food web structures, historical ecosystem shifts and use as baseline marine ecosystem data. The CPR Survey uses a formalin solution to preserve plankton samples while vessels are at sea and later, when archived before analysis. In 2008 two formalin grades with different methanol content were used on survey routes to check the quality of sample preservation, but have subsequently returned to the original formalin mix. Before samples from this extensive plankton collection can be confidently used in stable isotope studies, the impact of formalin preservatives on stable isotope ratios need to be clarified.

Copepods dominate the global mesozooplankton biomass (Williams *et al.*, 1994) and are an integral part of the transfer of carbon from marine primary producers to higher trophic level species. Calanoid species are key components of marine ecosystems and their isotopic signatures have been valuable in investigating food web structures (Hobson and Welch, 1992, Hobson *et al.*, 2002) and predator-prey relationships (Gorokhova *et al.*, 2005), and they have great potential for isotopic characterisation of ocean regions for use in animal foraging or migration studies (Hobson, 2007, Votier *et al.*, 2010). To evaluate the impact of formalin preservation on stable carbon and nitrogen isotope ratios in calanoid copepods, fresh samples of *Calanus helgolandicus* were used in a controlled experiment. Here we report the effect of two types of formalin preservative (used on the CPR Survey) and freezing, over a 12 month period. We

discuss the impact that this has on the use of these and other CPR Survey samples in ecological stable isotope studies.

4.2 Materials and Methods

Study species

Calanus helgolandicus is a calanoid copepod found over a range of marine habitats (i.e. open ocean, coastal environments) in the North Atlantic and contributes between 6% and 93% to the mesozooplankton biomass in European waters (Bonnet *et al.*, 2005). These copepods consume a diverse diet of protozooplankton and phytoplankton (Fileman *et al.*, 2007) and are themselves an important source of food for juvenile fish (Beaugrand *et al.*, 2003). They are a temperate water species generally found in 9-20°C waters, with highest abundance from 13 to 17°C and the development to the adult form takes between 26 and 42 days (Bonnet *et al.*, 2005). In recent decades *C. helgolandicus* has become more abundant and widespread in the northeast Atlantic, which has coincided with a northward shift in the distribution of the cold-water congener *C. finmarchicus* (Bonnet *et al.*, 2005). The sensitivity of these two species to changes in water temperature apparently underlies this shift, and for this reason they are useful indicators of global change in marine environments.

Sampling method and preservation

Zooplankton samples were taken by the Dove Marine Laboratory (Newcastle University, Newcastle, UK) from the North Sea, close to the Northumberland coast of England (55°07'N, 01°20'W), using a 200 μm meshed WP2 plankton net in vertical hauls from 20m depth to the surface on 9th June 2009. Fresh *C. helgolandicus* from hauls were frozen and transported to Plymouth (UK) where they were placed in fresh seawater for several hours to allow gut evacuation. We removed CV and CVI adult *C.*

helgolandicus and sub-divided these into five different groups: those analysed immediately (control) and those preserved for 3 weeks, 2 months, 6 months and 12 months in 5 different treatments (see below). There were 4 replicates for the control group and a minimum of 3 replicates (3-6) for each treatment/time group. Each replicate contained between 10 and 15 individuals (to provide sufficient material for subsequent stable isotope analysis) and were stored in separate glass vials at room temperature (excluding the frozen treatment).

The formalin preservatives used for the treatments were supplied by SAHFOS and are currently in use (Type B), or have been used (Type A), on CPR Survey routes and for storage of collected samples. The majority of the chemical constituents of the mixtures were the same (Appendix I, Supplementary Material 4) but the supplier and amount of methanol in the formaldehyde were different: Type A = Fisher Scientific, Loughborough, UK (Technical grade, 37-41% formaldehyde containing 10-14% methanol), Type B = Alpha-Aesar, Heysham, UK (Technical grade, 37% formaldehyde containing 7-8 % methanol). The treatments involve both types of formalin preservatives and/or a simulation of the change in concentrations during the collection and storage of CPR samples. For the latter, a 3 week initial period of high concentration mix and seawater dilution represents the potential time between collection of samples and delivery of the CPR device back to SAHFOS for unloading. The treatments (preservation methods) are summarised in Table 4.1. The type of formalin used for each treatment were also analysed to determine their $\delta^{13}C$ values.

Lipid synthesis in organisms discriminates against ^{13}C (DeNiro and Epstein, 1977) and yields low $\delta^{13}C$ values in tissues with high lipid content when compared with those from an organisms dietary input (Tieszen *et al.*, 1983). The lipid content in marine copepods is highly variable between species (range of percentage dry weight: 2-73%) (Ventura, 2006), and can depend largely on their life history (Mauchline, 1998). To

eliminate the effect of lipids on the carbon isotope ratios in *C. helgolandicus* and to generate results with general relevance to other calanoid species, all samples were lipid-extracted. The samples were also decalcified to remove inorganic carbon from the copepods exoskeleton, since these carbonates tend to be increased in $\delta^{13}C$ and do not reflect dietary intake (DeNiro and Epstein, 1978). Other biochemical compounds contributing to $\delta^{13}C$ values such as proteins, carbohydrates and chitin have been found to be similar across zooplankton taxonomic groups and habitats (Ventura, 2006), and were therefore not altered in any way.

Once the samples had been freeze-dried and crushed, the lipids were extracted using 7% methanol in dichloromethane by volume (7% M DCM) and then dried overnight in a laboratory oven. The samples were added to 2N hydrochloric acid for 1 hour to remove inorganic carbonates and distilled water was used to rinse away acid and waste. The samples were oven-dried overnight in preparation for stable isotope analysis.

Table 4.1 Experimental treatments used to preserve *C. helgolandicus* samples.

Treatment ID	Description
T1	Frozen at -20°C
T2	Type A 40% formaldehyde mix with borax diluted to 4% with seawater (for first 3 weeks), then changed to Type A 4% formaldehyde mix
T3	Type A 4% formaldehyde mix
T4	Type B 40% formaldehyde mix with borax diluted to 4% with seawater (for first 3 weeks), then changed to Type B 4% formaldehyde mix
T5	Type B 4% formaldehyde mix

Sample analysis

Homogenised *C. helgolandicus* samples of approximately 0.70 mg were weighed and placed in tin capsules (5 x 3.5 mm). Formalin samples were placed in solid, flat bottomed tin capsules (5 mm x 2 mm) and cold-sealed with wire cutters. Each formalin sample was prepared less than two minutes before analysis, to minimise evaporation. Analyses were conducted at the East Kilbride Node of the Natural Environment Research Council Life Sciences Mass Spectrometry Facility via continuous flow isotope ratio mass spectrometry using a Costech (Milan, Italy) ECS 4010 elemental analyser interfaced with a Thermo Electron (Bremen, Germany) Delta XP mass spectrometer. Isotope ratios are reported as δ-values and expressed as ‰ according to the equation $\delta X = [(R_{sample}/R_{standard})-1] \times 1000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ and $R_{standard}$ is the ratio of the international references PDB for carbon and AIR for nitrogen. The standard deviation of multiple analyses of an internal gelatine standard in each experiment was better than 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$.

Data analysis

The effects of preservation method and time on the carbon and nitrogen stable isotope and element ratio values were analysed by comparing preserved samples with fresh samples (control) using analysis of variance (ANOVA) and Tukey's HSD post hoc multiple pairwise comparison tests. All data were tested for normality and homogeneity of variance. Statistical analyses were conducted using R version 2.12.0 (2011).

4.3 Results

Overall effect of formalin

In general formalin preservation and the amount of time for which the samples had been preserved influenced both the $\delta^{13}C$ (Treatment: $F_{4,77} = 20.66$, $P < 0.0001$, Time: $F_{4,77}$ =

25.07, $P <0.0001$) and the $\delta^{15}N$ values (Treatment: $F_{4,78} = 3.09$, $P = 0.02$, Time: $F_{4,78} = 5.96$, $P = 0.0003$) in *C. helgolandicus* samples. Compared with fresh samples all the formalin treatment samples (T2-T5) were significantly depleted in $\delta^{13}C$ (mean range = -0.48 – -0.67, all P values <0.05) and significantly enriched in $\delta^{15}N$ (mean range = 0.46 – 0.74, P values <0.05) (Table 4.2).

Effect of time stored in formalin on $\delta^{13}C$ and $\delta^{15}N$

The amount of time for which samples were preserved in formalin had a pronounced effect on $\delta^{13}C$ (Figure 4.1). $\delta^{13}C$ decreased significantly in all formalin treatments after 6 months and 12 months (all Tukey's HSD tests, $P <0.05$), except for treatment 5 where the value did not change significantly after 12 months (Tukey's HSD test, $P = 0.16$). In contrast, $\delta^{15}N$ values changed significantly when *C. helgolandicus* were stored in formalin however there was no obvious trend over time (Figure 4.2). After Tukey's multiple comparison tests, the $\delta^{15}N$ values from treatment 2 were not significantly different from those from fresh samples at different time intervals, although the overall enrichment was significant ($F_{1,20} = 5.18$, $P = 0.03$ [Table 4.2]). The increase of $\delta^{15}N$ was significant after 3 weeks in treatment 4 (Tukey's HSD test $P = 0.004$) and 2 months in treatments 3 and 5 (Tukey's HSD tests, T3: $P <0.001$, T5: P 0.034). This significant ^{15}N enrichment generally continued up to 12 months in treatments 4 and 5 but reduced for the final sampling period in treatment 3 (Tukey's HSD test, $P = 0.06$) (Figure 4.2).

Effect of freezing samples on $\delta^{13}C$ and $\delta^{15}N$

The frozen samples (T1) where not significantly different from fresh samples for either $\delta^{13}C$ ($F_{4,11} = 0.85$ $P = 0.52$) and $\delta^{15}N$ ($F_{4,11} = 0.87$, $P = 0.51$), but were significantly different from samples that had undergone formalin treatments (all Tukey's HSD tests, $P <0.001$) except for the $\delta^{15}N$ values from treatment 2 (Tukey's HSD test, $P = 0.18$).

The $\delta^{15}N$ values appeared to change over time for samples stored frozen (Figure 4.2), but these changes were not statistically significant.

Table 4.2 Mean (± 1 standard error) $\delta^{13}C$ and $\delta^{15}N$ values of fresh *C. helgolandicus* and the differences (± 1 standard error) from fresh values for treated samples over 12 months. *C:N* – ratio of carbon to nitrogen atoms, *%C* – percentage dry weight of carbon, *%N* – percentage dry weight of nitrogen. # and * indicates significant difference from fresh at $P < 0.05$ (ANOVA and Tukey's HSD post-hoc test respectively).

	Fresh (‰)	±S.E.	T1 (‰)	T2 (‰)	T3 (‰)	T4 (‰)	T5 (‰)
$\delta^{13}C$	-19.34	0.26					
3 weeks			0.07 ±0.06	-0.49 ±0.08	-0.43 ±0.08	-0.46 ±0.05	-0.26 ±0.13
2 months			0.10 ±0.13	-0.42 ±0.02	-0.39 ±0.06	-0.53 ±0.06	-0.29 ±0.13
6 months			-0.29 ±0.05	-0.99 ±0.03*	-0.79 ±0.11*	-0.83 ±0.04*	-0.77 ±0.11*
12 months			-0.15 ±0.10	-0.68 ±0.03*	-0.93 ±0.08*	-0.88 ±0.05*	-0.55 ±0.12
Overall			-0.07 ±0.06	-0.64 ±0.06#	-0.66 ±0.07#	-0.67 ±0.05#	-0.48 ±0.08#
$\delta^{15}N$	9.56	0.11					
3 weeks			0.48 ±0.17	0.57 ±0.08	0.33 ±0.12	0.75 ±0.09*	0.60 ±0.07
2 months			0.15 ±0.38	0.19 ±0.19	0.84 ±0.10*	0.92 ±0.10*	0.67 ±0.21*
6 months			0.09 ±0.25	0.77 ±0.12	0.73 ±0.11*	0.51 ±0.16	0.67 ±0.16*
12 months			-0.03 ±0.14	0.33 ±0.22	0.49 ±0.12*	0.67 ±0.16*	0.80 ±0.08*
Overall			0.17 ±0.12	0.46 ±0.09#	0.60 ±0.07#	0.73 ±0.07#	0.68 ±0.07#
C:N	3.95	0.13					
3 weeks			0.12 ±0.03	0.08 ±0.01	0.07 ±0.02	0.02 ±0.01	0.01 ±0.00
2 months			0.04 ±0.04	0.03 ±0.03	0.05 ±0.02	0.16 ±0.03	0.13 ±0.03
6 months			-0.01 ±0.04	-0.07 ±0.02	-0.03 ±0.02	0.02 ±0.02	-0.05 ±0.02
12 months			0.04 ±0.02	-0.08 ±0.07	-0.12 ±0.02	-0.01 ±0.04	-0.02 ±0.04
Overall			0.05 ±0.05	-0.01 ±0.02	-0.01 ±0.02	0.05 ±0.16	0.01 ±0.02
%C	46.02	0.74					
3 weeks			-0.57 ±0.18	-0.81 ±0.12	-1.45 ±0.37	-1.22 ±0.29	-1.39 ±0.11
2 months			0.37 ±0.33	-0.42 ±0.26	-1.10 ±0.26	-0.69 ±0.18	-0.93 ±0.20
6 months			-1.46 ±5.44	0.19 ±0.81	-1.46 ±0.33	-0.62 ±0.92	-3.09 ±0.86
12 months			1.22 ±4.44	1.51 ±1.63	1.89 ±1.82	0.23 ±2.07	-1.32 ±4.00
Overall			-0.11 ±1.53	0.14 ±0.50	-0.53 ±0.53	-0.57 ±0.54	-1.77 ±0.90
%N	11.66	0.24					
3 weeks			-0.50 ±0.13	-0.42 ±0.05	-0.55 ±0.15	-0.32 ±0.06	-0.36 ±0.03
2 months			0.00 ±0.19	-0.17 ±0.11	-0.42 ±0.09	-0.64 ±0.08	-0.61 ±0.14
6 months			-0.31 ±1.40	0.28 ±0.23	-0.25 ±0.11	-0.16 ±0.21	-0.59 ±0.19
12 months			0.21 ±1.14	0.60 ±0.54	0.84 ±0.46	0.13 ±0.43	-0.27 ±1.11
Overall			-0.15 ±0.40	0.07 ±0.18	-0.10 ±0.16	-0.25 ±0.14	-0.47 ±0.24

T1 = Treatment 1, T2 = Treatment 2, T3 = Treatment 3, T4 = Treatment 4, T5 = Treatment 5 (described in Table 1), ‰ = parts per thousand deviation from standard.

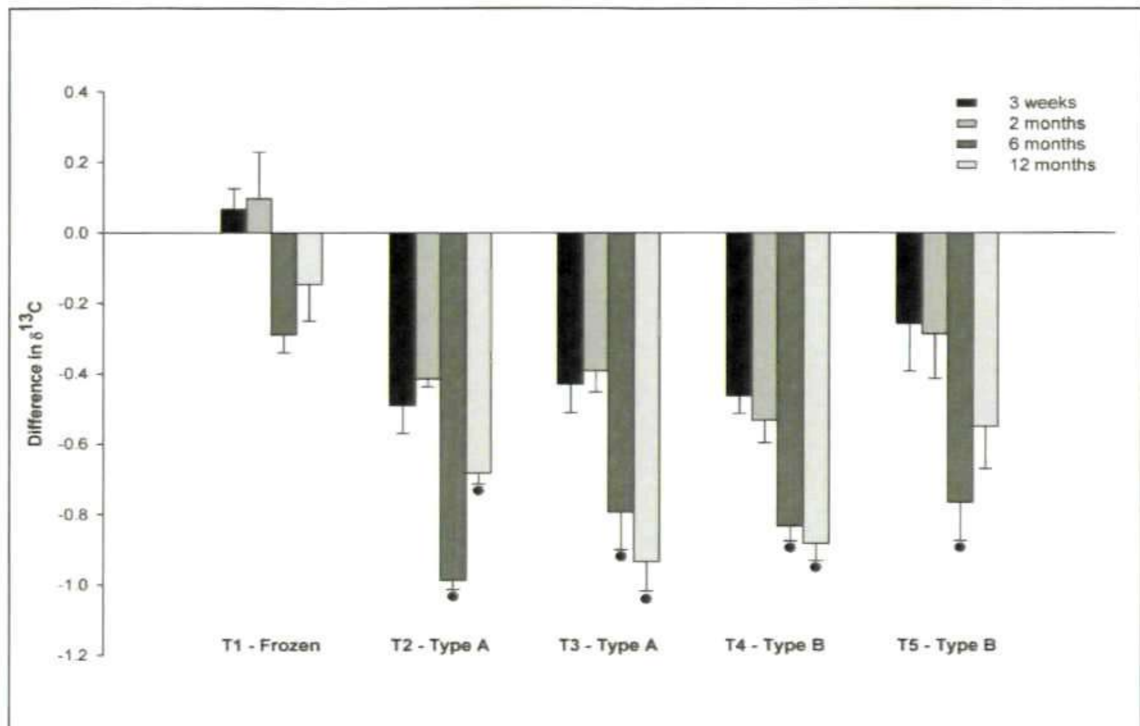

Figure 4.1 Mean (± 1 standard error) difference in $\delta^{13}C$ values after 3 weeks, 2 months, 6 months and 12 months for the 5 preservative treatments compared to the fresh control samples (0.0 on y axis). T1-T5 treatments as described in Table 1. Black spots represent significant differences from control samples (P <0.05).

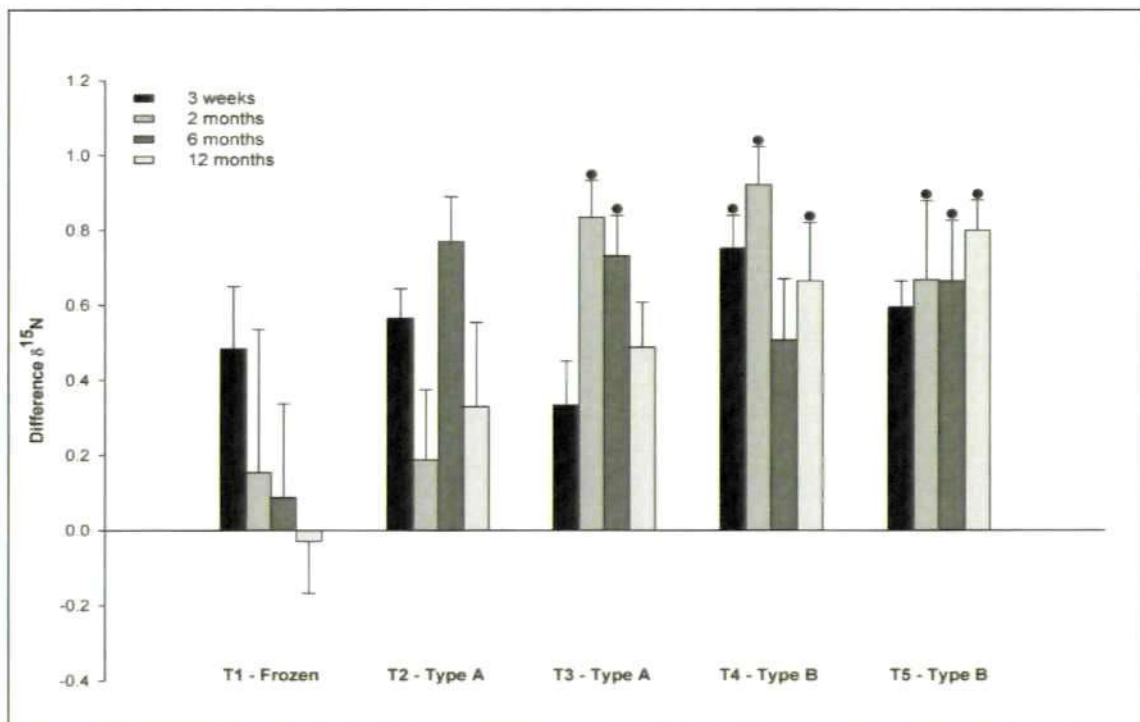

Figure 4.2 Mean (± 1 standard error) difference in $\delta^{15}N$ values after 3 weeks, 2 months, 6 months and 12 months for the 5 preservative treatments compared to the fresh control samples (0.0 on y axis). T1-T5 treatments as described in Table 1. Black spots represent significant differences from control samples (P <0.05).

%C, %N, C:N ratio and formalin

Change in the ratio of carbon to nitrogen (*C:N*) across treatments when compared with fresh sample values was minor (Table 4.2; all Tukey's HSD test, $P > 0.05$). Significant deviation from homogeneity of variance was found over the duration of the experiment in treatments 3 and 5 for percentage dry weight of carbon (*%C* Levene's test, T2: $F_{4,20} = 3.087$ $P = 0.04$, T4: $F_{4,16} = 4.699$ $P = 0.01$) and nitrogen (*%N* Levene's test, T2: $F_{4,20} = 3.093$ $P = 0.04$, T4: $F_{4,16} = 5.030$ $P = 0.01$). The mean $\delta^{13}C$ values (±1 SD) were similar for formalin preservative with the same chemical constituents across manufacturers: 1) 40% formaldehyde mix with borax diluted to 4% with seawater, Type A= -44.63 ±0.05‰, Type B= -44.07 ±0.56‰. 2) 4% formaldehyde mix, Type A= -29.52 ±0.24‰, Type B= -29.94 ±0.30‰.

4.4 Discussion

Our study provides evidence for a significant effect of formalin preservation on the stable carbon and nitrogen isotope values in *C. helgolandicus* over time, but there was no significant difference between batches of samples stored frozen and room temperature controls over the same period. The possible mechanisms involved in the isotopic alteration and the implications that these preservative effects have for the use of *C. helgolandicus* and other CPR zooplankton samples in stable isotope analyses, are discussed below.

Effects of formalin on $\delta^{13}C$ and $\delta^{15}N$

The decrease of $\delta^{13}C$ for *C. helgolandicus* samples stored in formalin is consistent with the majority of previous studies investigating the effect of formalin preservation on a variety of marine, freshwater and terrestrial species (Sarakinos *et al.*, 2002, Barrow *et*

al., 2008). However, the degree of depletion (~1‰) was considerably less than reported in the only other study on the effect of formalin on marine zooplankton (2-3‰)(Mullin *et al.*, 1984). The results also indicate that the isotope signatures continue to change with time, although it took approximately 6 months of preservation for this effect to become statistically significant. The only other previously observed depletion over time was in a freshwater clam *Corbicula fluminea*, although the fresh samples in this study were extremely depleted resulting in an overall enrichment of $\delta^{13}C$ (Syväranta *et al.*, 2011), and highlighting the difficulties in predicting these effects. Two mechanisms have been proposed to account for the depletion of ^{13}C. First, the formalin preservative promotes the leaching of compounds enriched in ^{13}C from the tissues and therefore modifies the apparent carbon isotope signature (Hobson *et al.*, 1997, Bosley and Wainwright, 1999). The formaldehyde hydrolyzes proteins that are enriched in ^{13}C compared with lipids, so depletion in tissue ^{13}C could reflect the relative increase in lighter lipid carbon. However, given that we extracted lipids from the *C. helgolandicus* samples before stable isotope analysis this seems unlikely. Secondly, the uptake of isotopically lighter carbon from the formalin into the tissues could shift the signature toward that of the preservative (Gloutney and Hobson, 1997, Hobson *et al.*, 1997, Bosley and Wainwright, 1999, Sarakinos *et al.*, 2002). The low $\delta^{13}C$ values found in the formalin used in this study (-29‰ and -45‰) would therefore suggest the incorporation of lighter carbon from the preservative is the most likely explanation for this effect. The composition and/or manufacture of the formalin preservative may contribute to the degree of carbon uptake into tissues (Sweeting *et al.*, 2004). Although the samples that were subjected to the lowest concentration of formaldehyde and methanol (T5 – Type B 4% formalin) showed slightly less overall ^{13}C depletion, there was little evidence for differences between the two manufacturers and formalin composition used in this

experiment. The comparable formalin $\delta^{13}C$ values between manufacturers may have resulted in these similarities.

Previous work has shown highly variable and somewhat contradictory impacts of formalin preservation on $\delta^{15}N$ (Barrow *et al.*, 2008), although generally the effects are slight with only one study showing an effect of > 1.0‰ (Bosley and Wainwright, 1999). Here we found a significant increase in $\delta^{15}N$ values for *C. helgolandicus* stored in formalin that is consistent with previous work on freshwater zooplankton (0.8‰ increase) (Feuchtmayer and Grey, 2003), but contrasts with a significant ^{15}N depletion shown for marine zooplankton (decrease <1‰) (Mullin *et al.*, 1984). Formalin does not contain nitrogen so uptake of any preservative fractions into tissues will have no effect on $\delta^{15}N$ values. Instead the effect of formalin is through disproportionate hydrolysis or leaching of isotopically lighter or heavier materials (Gloutney and Hobson, 1997, Hobson *et al.*, 1997, Bosley and Wainwright, 1999). For *C. helgolandicus* and the type of formalin used in our study this seems to be the loss of ^{14}N to the preservative with the resulting increase of $\delta^{15}N$, although an expected increase in the *C:N* ratio with loss of nitrogen was not observed. The increase was slightly higher in the Type B formalin mix, which is surprising because it contains slightly less formaldehyde (the hydrolysing agent) than the Type A mix, but may be a result of different manufacturing processes or raw materials (Bugoni *et al.*, 2008). It is apparent from this study and previous reports that the effect of formalin on $\delta^{15}N$ is not as predictable as for $\delta^{13}C$ and therefore requires species-specific experiments.

Use of CPR zooplankton in ecological stable isotope analyses

Significant changes in $\delta^{13}C$ and $\delta^{15}N$ values of *C. helgolandicus* stored in formalin could potentially bias stable isotope values if unchecked. The ~0.6‰ increase in $\delta^{15}N$ observed in this study could be regarded as minor in relation to the 2-3.5‰ shift found

between consumers and prey (Minagawa and Wada, 1984, Post, 2002), so may not be significant if used in stable isotope trophic position and food web studies. However, comparing preserved and unpreserved samples could produce misleading results and a simple correction factor should be considered on a species-specific basis in this situation (Edwards *et al.*, 2002). The increase in $\delta^{13}C$ per trophic level is much more conservative (<1‰) and more closely represents the primary carbon source of the marine ecosystem. An approximate 0.8‰ ^{13}C depletion in samples due to preservation could therefore lead to biased results if used in multiple isotope food web analysis or for regional baseline ecosystem signatures in migration studies. These analyses rely on appreciable distinction between $\delta^{13}C$ values and any bias will be most pronounced when the differences are relatively small. It has previously been proposed that as long as the $\delta^{13}C$ difference is more than 2.0‰ an equivalent depletion in preserved specimens (-2.0‰) will not obscure the results (Edwards *et al.*, 2002). For study systems where differences in carbon sources are considerable, e.g. C_3 and C_4 plants (>14‰) (O'Leary, 1988), or terrestrial vs marine (>7‰) (Fry and Sherr, 1989), or known through previous research, the small effect of formalin preservation may be unimportant, but $\delta^{13}C$ differences can be small and gradual over large spatial scales in marine systems (Graham *et al.*, 2010). The effect of preservation will be problematic if the results are ambiguous, and correction of $\delta^{13}C$ values would be appropriate in these instances.

The shifts of $\delta^{13}C$ and $\delta^{15}N$ in this study are consistent in direction across treatments but show variation in strength with time, making it difficult to predict the degree of change beyond the study period. The majority of archived samples collected by the CPR Survey have been preserved for much longer and the effect on these samples cannot be confidently estimated from this study. However, for *C. helgolandicus* samples collected by the CPR Survey within 12 months of analysis a correction factor specific to the type of formalin and time preserved may be appropriate to counter the effect and enable their

use in stable isotope studies. The extraction of lipids before analysis removed the high variation of this compound found in marine calanoid copepod species and should enable any corrections to be applied to other *Calanus* species, such as *C. finmarchicus*, collected and preserved by the CPR in the previous 12 months.

4.5 Conclusions

Our experiment was designed to establish whether the formalin preservative used to collect and store samples on CPR Survey routes significantly changes the signature of a zooplankton species over 12 months and how this may impact its use in ecological stable isotope studies. For *Calanus* copepods the preservative significantly affects carbon and nitrogen ratios but the lack of a predictable trend or stability in results does not allow estimation of the effect on preserved samples older than 12 months. However, application of specific correction factors may be appropriate for samples collected within this period to remove any bias in stable isotope analyses. The ubiquitous nature of this genus in the marine environment makes it ideal for use in large-scale ecological studies and the long-term, global collection of samples available through the CPR Survey could be a valuable resource to be used in contemporary and historical isotope analysis. However, our findings and contradictory results found in other studies investigating formalin preservation would suggest there is no general correction for this preservative and its effect needs to be experimentally established for individual species or genera specifically, over periods applicable to the preserved collection. Caution should be taken when deciding whether to use formalin preserved samples in stable isotope analyses. Preservation effect and isotopic differences within the study system need careful consideration when assessing the utility of the samples for the proposed research and, if used, whether or not correction factors are appropriate.

Chapter 5

Itinerant young: Movement of immature Leach's storm-petrels *Oceanodroma leucorhoa* leads to genetically homogenous populations across an ocean basin

5.1 Introduction

Highly fragmented populations are particularly reliant upon dispersal to maintain genetic and demographic connectivity (Bowler and Benton, 2005, Lowe and Allendorf, 2010), and isolation of these subpopulations (demes) can make them vulnerable to extinction via stochastic events (Matthiopoulos *et al.*, 2005) or prolonged environmental change. Establishing the extent of connectivity and the mechanism by which dispersal takes place is essential to predict population dynamics and the effect of increasing environmental change (Kokko and López-Sepulcre, 2006).

Dispersal can occur both through relocation of breeders (breeding dispersal) and the recruitment of young to non-natal breeding sites (natal dispersal) (Greenwood and Harvey, 1982). Natal dispersal is prevalent among birds and mammals (Greenwood, 1980), a likely consequence of the increased cost of movement for established breeders, such as, increased mortality while dispersing, loss of reproductive events and the risk of moving to a worse quality habitat (Clobert *et al.*, 2001, Danchin and Cam, 2002). A combination of genetic and environmental factors influence the likelihood of either natal or breeding dispersal, so understanding the system and degree of dispersal is important to assess the vulnerability of species to local adverse events (Clobert *et al.*, 2001).

The colonial breeding behaviour of seabirds naturally creates fragmented populations with the potential for vulnerable isolated colonies. Fidelity to breeding locations is known to be high in many seabirds (Schreiber and Burger, 2002, Newton, 2008) and suggests natal dispersal is important for inter colony exchange (Clobert *et al.*, 2001). The Leach's storm-petrel *Oceanodroma leucorhoa* (Vieillot, 1818) is a small (~45g) highly pelagic seabird with island breeding sites widely spread across the North Atlantic and Pacific (Huntingdon *et al.*, 1996). They are surface feeders, mainly consuming small fish and zooplankton caught over and beyond the continental shelf (Hedd and

Montevecchi, 2006). Although globally very abundant, they are particularly vulnerable because a very high proportion of the population depends on just a few breeding sites. Fidelity to breeding locations is high (Morse and Kress, 1984, Blackmer *et al.*, 2004) but recent work has revealed high levels of gene flow among colonies in the North Atlantic (Chapter 3). Connectivity within the Atlantic reveals populations are not isolated and the decline of a regionally important colony on St Kilda, Scotland (~54% decline between 1999-2006; Newson *et al.*, 2008) could potentially be buffered via immigration. Heavy predation by great skuas *Stercorarius skua* (between 15,000-21,000 a year; Phillips *et al.*, 1999b, Miles, 2010) on St Kilda, the likely cause of this reduction, far exceeds the observed population decline and it has been suggested that immigrants from other colonies may be eaten by skuas (Newson *et al.*, 2008; Chapter 3). Determining whether juvenile and pre-breeding birds (hereafter immatures) move between colonies in the North Atlantic would provide a potential dispersal mechanism (i.e natal dispersal) and substantiate this potentially important buffering effect. The life-history traits of seabirds (delayed sexual maturation, long life; Schreiber and Burger, 2002) produce large numbers of immatures that can comprise >50% of the population (Klomp and Furness, 1992). Therefore the large breeding population of Leach's storm-petrels in the North Atlantic (~5 million breeding pairs) should be associated with huge numbers of immatures. Although an important component of the population, virtually nothing is known about the dispersal and movement of immature Leach's storm-petrels once they leave the natal colony.

Dispersal between populations can be estimated directly using capture-mark-recapture methods (Paradis *et al.*, 1998, Lebreton *et al.*, 2003) or via bio-logging technology (Votier *et al.*, 2011). However these approaches are unsuitable for many species (Koenig *et al.*, 1996). For instance, some species may be too cryptic or have very low encounter rates for robust capture-recapture studies. Alternatively they may be too small

to carry tracking devices or recovery rates of devices too low to enable meaningful interpretation. Dispersal is often the behaviour of immature animals (Dobson, 1982, Greenwood and Harvey, 1982), which can be difficult to track and recapture (Daunt *et al.*, 2007), or identify with confidence. Leach's storm-petrels are too numerous to conduct an ocean wide capture-recapture study and too small for fine scale tracking. Therefore indirect techniques are required to characterise dispersal behaviour in this species.

Genetic markers (e.g. nuclear microsatellites and mitochondrial DNA[mtDNA] fragments), and an array of associated analytical approaches, can be used to investigate historical and contemporary gene flow (the consequence of effective dispersal). They provide evidence for gene flow among colonies by interpreting the spatial distribution of neutral genetic marker variation (Slatkin, 1987), where genetic differentiation is an inverse function of gene flow (Wright, 1931). Thus genetic homogeneity across demes implies high levels of effective dispersal. While this approach is extensively used, it does not reveal the mechanism behind any genetic exchange.

Stable isotope analysis (SIA) is used regularly in ecological studies of community structure (Post, 2002), diet reconstruction (Inger *et al.*, 2006), as well as to trace migration and dispersal (Hobson, 2007). This technique utilises naturally occurring gradients of elemental isotope ratios, which are reflected in consumer tissues. To reconstruct animal movements regional isotopic differences are important, and these differences must be characterised from known sources (Hobson, 2007). $\delta^{15}N$ shows a stepwise increase of 2.5-5‰ (Peterson and Fry, 1987) at each trophic level and ultimately reflects the nitrogen pools that support primary producers at the base of different food webs (Owens, 1987, Jennings and Warr, 2003). $\delta^{13}C$ values also increase at each trophic level (~1‰) but are more representative of the primary carbon source of a distinct food web and show a number of spatial gradients (such as inshore vs offshore,

pelagic vs benthic, and latitude (Hobson, 2007)). Different animal tissues are synthesised and replaced at different rates, thus reflecting the trophic position or food web signatures over a range of timescales. For example, blood plasma has a high turn-over rate and will yield information for the previous few days, while feathers will provide data spanning the period they were grown. Using tissue signatures, multi-source stable isotope mixing models offer the potential to infer movement of immature birds between regions and identify likely immigrants. This technique is typically used to estimate the proportional contribution of different prey types within consumer tissues (Inger *et al.*, 2006, Moreno *et al.*, 2010, Votier *et al.*, 2010). However it can be adapted to estimate the probability of immature birds originating from different colonies (reflecting different marine food webs). The Bayesian mixing model used in SIAR (Stable Isotope Analysis in R) offers advantages over earlier models by incorporating sources of natural variation and uncertainty to generate probability estimates of source proportions (Parnell *et al.*, 2010).

Following the previous study of Leach's storm-petrel (Chapter 3), we use two genetic markers to confirm gene flow among three colonies in the North Atlantic. In addition we use Bayesian stable isotope mixing models to determine whether immature birds attending colonies at night may have visited multiple sites, therefore testing the hypothesis that natal dispersal is the primary mechanism for gene flow in this population. Analysis of baseline primary consumers (zooplankton) and breeding Leach's storm-petrels provides known isotopic end-points, which are then used to infer movement of immature birds of unknown origin. We also consider the effect immature dispersal may have on the persistence of a declining population under threat from extreme predation.

5.2 Materials and Methods

Sample collection

For genetic and stable isotope analysis (SIA) blood samples were obtained during the breeding season from Leach's storm-petrel colonies at Gull Island, Canada (GULL) (47°15'N, 52°46'W) and St Kilda, Scotland (STK) (57°49'N, 08°35'W) in August 2008, and Vestmannaeyjar, Iceland (VEST) (63°25'N, 20°17'W) in August 2009 (Table 1; Figure 1). 10-20µl of blood was collected (under appropriate regional licences) from the brachial vein of breeding adults or chicks caught in nesting burrows and immature birds caught using a mist-net at night. For the SIA adult breeder samples were used for isotopic signatures of individuals from known locations/regions. Only confirmed breeders caught in burrows on chicks or eggs were used. To ensure the status of immatures only individuals that met all of the following criteria were included: caught away from the main breeding areas; attracted by a chatter call lure played on speakers close to the mist-net; did not regurgitate prey items when caught or handled; and had no obvious brood patch. Blood samples used in stable isotope analysis were separated into plasma and red blood cells (RBC), using a centrifuge within 2-3 hours of sampling, and stored frozen until preparation for analysis. The RBC isotope values reflect the dietary intake in the ~3-4 weeks prior to sampling (Bearhop *et al.*, 2002), and herein we use $\delta^{15}N$ and $\delta^{13}C$ values from this tissue in stable isotope analyses. Samples used in the genetic analysis were stored in absolute ethanol at -20°C.

For isotopic characterisation of the continental shelf slope and offshore oceanic areas (the main foraging areas) adjacent to the three Leach's storm-petrel colonies, we analysed zooplankton (primary pelagic consumers). These archived samples were collected by the Continuous Plankton Recorder Survey (CPR) managed by the Sir Alistair Hardy Foundation for Ocean Science. The CPR utilises merchant "ships of opportunity" that traverse the world's oceans and seas on regular monthly shipping

routes. Adult life stages (CV and CVI) of *Calanus helgolandicus* and *C. finmarchicus* individuals were removed from 27 formalin preserved "silks" (collection mesh representing 10km towed transects) ~8 months after they were collected on ship survey routes that passed close to each study colony during July 2008 (Figure 5.1). Adult copepods were analysed to ensure comparable feeding trophic levels between regions.

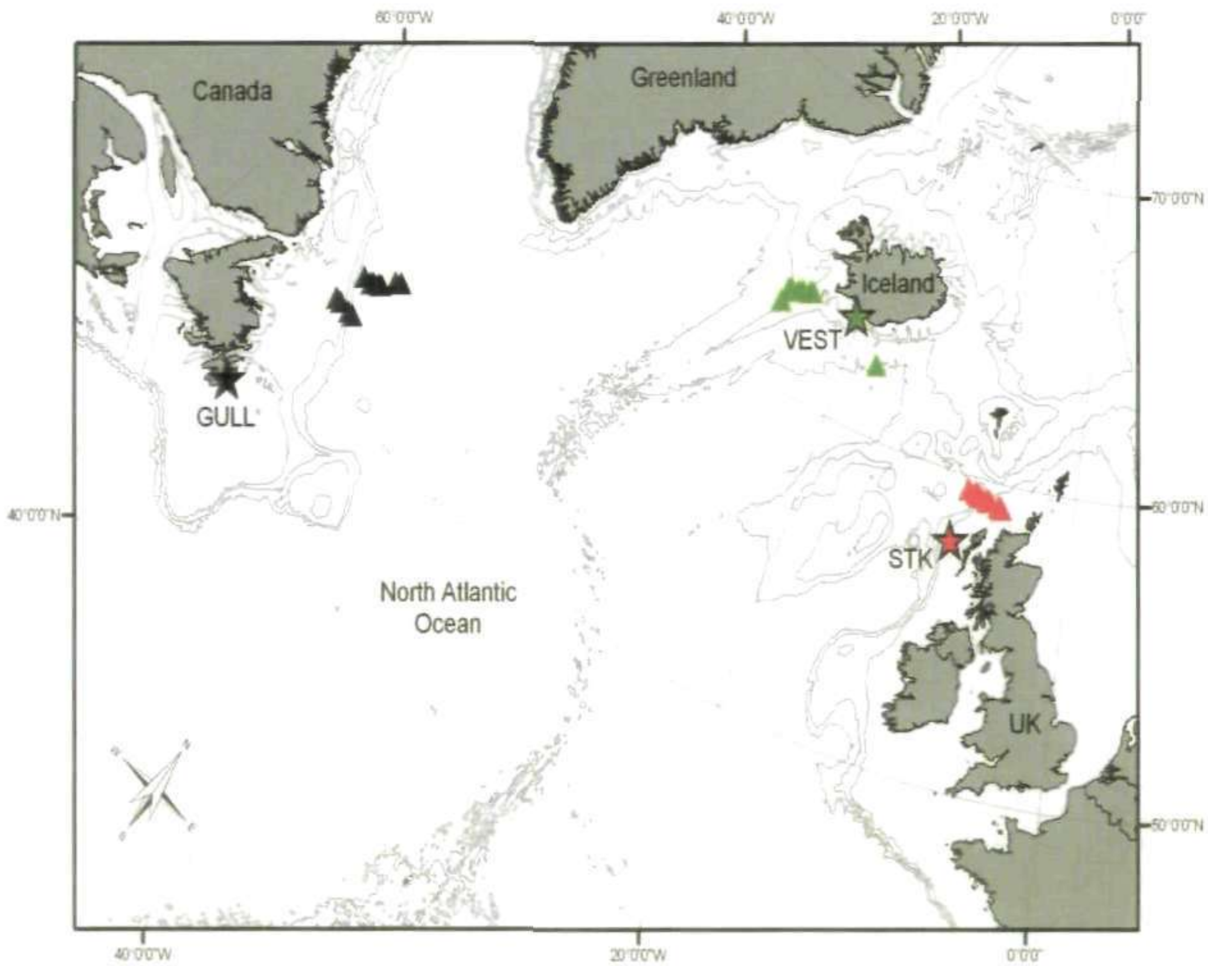

Figure 5.1 Locations of Leach's storm-petrel colonies and CPR sampling in the North Atlantic. Colony abbreviations detailed in Table 1. Black = Canada, Green = Iceland and Red = Scotland, Stars = colony locations, Triangles = CPR sample locations.

Table 5.1 Sampling site locations, species and numbers of samples (N) included in genetic and stable isotope (SIA) analyses.

Location	Abbreviation	Species	Type	N		
				Genetics (Whole blood)		SIA
				mtDNA	Microsatellite	
Colony						
Gull Island, Canada	GULL	*Oceanodroma leucorhoa*	Red blood cells	10	48	04 (b), 61 (imm)
Vestmannaeyjar, Iceland	VEST	*Oceanodroma leucorhoa*	Red blood cells	11	25	12 (b), 22 (imm)
St Kilda, Scotland	STK	*Oceanodroma leucorhoa*	Red blood cells	10	32	18 (b), 51 (imm)
Atlantic Region						
Newfoundland Shelf	Canada	*Calanus finmarchicus*	Whole organism	-	-	36*
Reykjanes Ridge/Iceland Basin	Iceland	*Calanus finmarchicus*	Whole organism	-	-	29*
North-East Rockall basin	Scotland	*Calanus helgolandicus*	Whole organism	-	-	18*

b = breeding adult Leach's storm-petrel, imm = immature Leach's storm-petrel, * = each sample represents 10-20 individual copepods.

Molecular analysis

Mitochondrial DNA and microsatellite loci

DNA was extracted from all Leach's storm-petrel blood samples using a standard proteinase-K ammonium acetate method (Nicholls *et al.*, 2000) and diluted to ~10ng/μl with 0.01M TE buffer. A 360-base pair (bp) fragment of the mitochondrial control region (Domains I and II) was amplified in 31 of the breeding adult or chick Leach's storm-petrel samples (i.e. known provenance) using the primers, PCR methods and sequencing as described in Chapter 3 (Quinn and Wilson, 1993, Smith *et al.*, 2007).

All samples were genotyped at 18 microsatellite loci previously developed for LSP (*Ole01, Ole04, Ole06-07, Ole09-10, Ole14-15, Ole17-18, Ole20, Ole22, Ole25;* Bicknell *et al.*, 2011, Chapter 2), storm-petrels (*Oc63, Oc87B;* Sun *et al.*, 2009) and cross-utility in avian species (TG04-041, TG13-017; Dawson *et al.*, 2010, CAM12; Ball, A. unpublished data). The multiplexes, PCR conditions and allele scoring are described in Chapter 3.

Tests of loci assumptions and genetic variation

To test whether patterns of genetic variation in the mitochondrial DNA (mtDNA) control region sequence deviated from selective neutrality Ewens-Watterson (Ewens, 1972, Watterson, 1978) and Tajima's neutrality tests (Tajima, 1989) were performed in ARLEQUIN v3.5.1.2 (Excoffier and Lischer, 2010). Haplotypic diversity (h; Nei, 1987) and nucleotide diversity (Tajima, 1983) were also calculated to assess levels of genetic variation within colonies. Microsatellite loci were tested for departures from Hardy-Weinberg equilibrium within each breeding colony and assessed for linkage disequilibrium using a Markov-chain method implemented in GENEPOP v3.4 (Rousset, 2008). Significance levels were adjusted for multiple comparisons using sequential Benjamini-Yekutieli corrections (Benjamini and Yekutieli, 2001). Predicted null allele

frequencies were calculated using CERVUS v3.0.3 (Kalinowski *et al.*, 2007). Observed and expected heterozygosities and mean numbers of alleles were calculated for each colony using ARLEQUIN v3.5.1.2. Allelic richness and private allelic richness were calculated using HP-RARE 1.0 (Kalinowski, 2005).

Genetic differentiation and Bayesian cluster analysis

Genetic differentiation between the Leach's storm-petrel colonies was assessed by calculating pairwise Φ_{ST} and F_{ST} values (Whitlock, 2011) for the mtDNA locus and microsatellite loci, respectively. Kimura's two-parameter model of substitution (Kimura, 1980) with a shape parameter (α) for the gamma distribution of 0.47 (based on α estimates of Domain I, II and III of the control region in finches, *Fringilla* and *Carduelis spp.*; [Marshall, Baker, 1997]) was used for mtDNA locus analysis.

Microsatellite Bayesian clustering analysis implemented in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000, Falush *et al.*, 2003) was used to identify genetically distinct colonies or populations. This model-based method uses a Markov chain Monte Carlo (MCMC) simulation to assign individuals to genetic clusters (K) based on individual genotypes, regardless of sampling location. We performed 5 replicate runs for K=1 to 3 genetic populations (number of sampled colonies) using the admixture model and assuming correlated allele frequencies (Falush *et al.*, 2003). A burn-in of 500,000 followed by 1,000,000 iterations was used following exploratory runs to confirm data convergence. We used a uniform prior for alpha (α), with initial value of 1.0 for all populations, and lambda (λ) set at 1.0. To evaluate the most probable number of genetic populations the posterior probability, Ln[P(X|K)], was compared directly (Pritchard *et al.*, 2000).

Stable isotope analysis

Sample preparation

All samples were freeze-dried, homogenised and ~0.7 mg weighed into a tin cup for analysis. Each zooplankton sample (tin cup) consisted of approximately 10-20 individuals. Lipid synthesis in organisms discriminates against ^{13}C (DeNiro and Epstein, 1977) and generally yields low $\delta^{13}C$ values in tissues with high lipid content when compared with an organisms dietary input (Tieszen *et al.*, 1983). Lipid content is highly variable between marine copepod species (Ventura, 2006) so to eliminate the effect of lipids on carbon ratios in *C. helgolandicus* and *C. finmarchicus* lipids were extracted prior to analysis. Inorganic carbonates were also removed from these samples, as these tend to increase $\delta^{13}C$ and do not reflect the organism's dietary intake (DeNiro and Epstein, 1978). Extraction and removal methods are described elsewhere (Bicknell *et al.*, In press, Chapter 4). The low lipid content of RBC does not necessitate lipid extraction (Cherel *et al.*, 2005).

Analyses were conducted at the East Kilbride Node of the Natural Environment Research Council Life Sciences Mass Spectrometry Facility via continuous flow isotope ratio mass spectrometry using a Costech (Milan, Italy) ECS 4010 elemental analyser interfaced with a Thermo Electron (Bremen, Germany) Delta XP mass spectrometer. Isotope ratios are reported as δ-values and expressed as ‰ according to the equation:

$$\delta X = [(R_{sample}/R_{standard})-1] \times 1000 \qquad \text{(Eq 1)}$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ and $R_{standard}$ is the ratio of the international references PDB for carbon and AIR for nitrogen. The standard deviation of multiple analyses of an internal gelatine standard in each experiment was better than 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$.

Regional characterisation

The $\delta^{15}N$ and $\delta^{13}C$ values in RBC and zooplankton samples were used to determine the degree to which breeding and immature birds caught at the three Atlantic colonies segregated isotopically and whether this could be a function of variation in foraging behaviour during the breeding season. In doing so, we assume that any potential differences in assimilation efficiency or physiology between breeders and immatures does not influence the relationship between isotope values in prey and blood. Breeding adults are restricted to regular returns to the colony to attend eggs or chicks during the breeding season (1-4 days; Ricklefs *et al.*, 1985), whereas immature Leach's storm-petrels have no such restriction to their movement and may show variation in their behaviour. Although lipid content varies between *Calanus* copepod species (Ventura, 2006), once lipids were extracted from *C. helgolandicus* and *C. finmarchicus* samples collected in the same ocean region no significant differences in $\delta^{13}C$ and $\delta^{15}N$ were found (Bicknell, A. unpublished). The formalin preservative used to archive material collected by the CPR Survey increases $\delta^{15}N$ and decreases $\delta^{13}C$ values in *C. helgolandicus*, therefore a correction factor of -0.9‰ for $\delta^{15}N$ and +1.0‰ for $\delta^{13}C$ was applied to all copepod sample values before subsequent analyses (Bicknell *et al.*, In press, Chapter 4).

To further describe any variation in food webs for geographic regions the trophic position of breeding adult Leach's storm-petrels was calculated using the following formula:

$$TL_{consumer} = \lambda + (\delta^{15}N_{consumer} - \delta^{15}N_{base}) / \Delta^{15}N \qquad \text{(Eq 2)}$$

where; λ is the trophic position of the organism used to estimate $\delta^{15}N_{base}$, $\delta^{15}N_{consumer}$ is measured directly and $\Delta\delta^{15}N$ is the nitrogen trophic enrichment factor (TEF) used for the food web of interest. For the three food webs in this study the following calculation was used:

$$TL_{bird} = 2 + (\delta^{15}N_{bird} - \delta^{15}N_{copepods}) / 2.75‰ \qquad \text{(Eq 3)}$$

where; 2 is the assumed trophic level of pelagic zooplankton (Hobson *et al.*, 1995, Sherwood and Rose, 2005, Petursdottir *et al.*, 2008) and a mean nitrogen TEF of +2.75‰ was taken from a recent review by Caut *et al.* (2009).

The TL_{bird} values were subsequently used to correct $\delta^{13}C$ for trophic level enrichment variation between food webs and allow further direct comparison of estimated primary carbon source values. This was calculated using the following formula:

$$\delta^{13}C_{TLcorrected} = \delta^{13}C_{consumer} - (TL_{consumer} \times \Delta^{13}C) \qquad \text{(Eq 4)}$$

where; $\delta^{13}C_{consumer}$ is measured directly, $TL_{consumer}$ is derived from Eq 2 and $\Delta^{13}C$ is the carbon TEF used for the food web of interest. A mean carbon TEF of +0.75‰ (Caut *et al.*, 2009) was used in this study.

Statistical analysis

To investigate segregation of $\delta^{13}C$ and $\delta^{15}N$ values in RBC of colony breeders multivariate analysis of variation (MANOVA) was conducted. Univariate analysis of variance (ANOVA) and Tukey's HSD post-hoc multiple pairwise comparison tests followed where significant differences were found. All statistical analyses were conducted using R version 2.12.2 (R, 2011).

SIAR isotope mixing models

The SIAR multi-isotope mixing model was used to estimate the proportion contribution of the three Leach's storm-petrel colony signatures (sources), to each individual immature birds RBC isotope signatures (mixture). The colony sources were defined using $\delta^{13}C$ and $\delta^{15}N$ of adult breeding Leach's storm-petrels RBC (see section above). Key to effective use of mixing models is comprehensive characterisation of possible end points. Here we examined, by eye, whether the isotope values of immature birds of

unknown origin were nested within isotope values from known populations. Mean (± SD) percentage dry weight of carbon and nitrogen in the adult breeder samples were used to incorporate concentration dependence in each colony source. As there is no trophic enrichment to be considered between RBC values of breeders and non-breeders, a TEF was not required in the mixing model. A low source proportion estimate for an individual non-breeders colony of capture would represent variation in the foraging region of a bird during the last ~3-4 weeks.

5.3 Results

Genetic analysis

Tests of loci assumptions and genetic variation

Only five mtDNA locus haplotypes were obtained from 31 individuals sampled over the 3 colonies. Three were shared across all locations with 1 each unique to GULL and VEST. Mean haplotype diversity was similar between GULL (0.78 ± 0.09) and VEST (0.71 ± 0.1), but lower in STK (0.51 ± 0.16). Mean nucleotide diversity (π) was similar between GULL (*0.0049 ± 0.004*) and STK (*0.0046 ± 0.003*), but slightly higher in VEST (0.0067 ± 0.004). Ewens-Watterson (all P >0.30) and Tajima's D (all P >0.50) neutrality tests within colonies were non-significant.

None of the microsatellite loci deviated from either Hardy-Weinberg or linkage equilibrium assumptions after Benjamini-Yekutieli corrections and showed low levels of null alleles. Mean number of alleles per locus, allelic and private allelic richness, and observed heterozygosity for the microsatellites were similar across colonies (*A*/locus, 3.9 ± 0.08; *A*, 3.1 ± 0.06; $A_{private}$, 0.04 ± 0.01; H_o, 0.44 ± 0.02) (Supplementary Table 5.1).

Genetic differentiation and Bayesian cluster analysis

No evidence for genetic differentiation was found among the three Leach's storm-petrel colonies; all pairwise Φ_{ST} and F_{ST} values were not statistically significant (Table 2).

Table 5.2 Pairwise Φ_{ST} estimates based on mitochondrial DNA control region (above diagonal), and F_{ST} estimates based on nuclear microsatellite variation (below diagonal). P>0.05 for all values. Population abbreviations are given in Table 5.1.

	GULL	VEST	STK
GULL		-0.017	0.186
VEST	-0.001		0.015
STK	-0.004	-0.007	

The convergence of the STRUCTURE Bayesian clustering analyses was confirmed by low variance in ln[P(X|K)] across replicate runs and visual inspection of likelihood and estimated parameters. The analysis also found no evidence of subdivision, with K=1 being the most likely number of clusters within the 105 individuals.

Stable isotope analysis

Regional characterisation from breeders and copepods

Breeding Leach's storm-petrel RBCs were isotopically segregated among colonies (MANOVA, Pillai, $F_{2,31}$ = 50.859, P <0.0001). Univariate analysis and post-hoc multiple pairwise comparison test (Tukey's HSD test) revealed $\delta^{15}N$ was significantly different between all colonies (ANOVA, $F_{2,31}$ = 55.182, P <0.0001; Tukey's HSD test, Table 3) and $\delta^{13}C$ was significantly different between VEST and the other colonies (ANOVA, $F_{2,31}$ = 46.052, P <0.0001; Tukey's HSD tests, Table 5.3), while GULL and STK values were similar.

The *Calanus* copepods were also isotopically segregated among the geographic regions (MANOVA, Pillai, $F_{2,80} = 93.173$, $P < 0.0001$), but in a different pattern to the breeding birds. Univariate analysis and post-hoc tests showed significant $\delta^{15}N$ differences between Iceland and the other regions (ANOVA, $F_{2,80} = 212.84$, $P < 0.0001$; Tukey's HSD tests, Table 5.3), while Scotland and Canada were similar. $\delta^{13}C$ differences were found among all regions (ANOVA, $F_{2,80} = 208.55$, $P < 0.0001$; Tukey's HSD tests, Table 5.3).

Table 5.3 $\delta^{13}C$ and $\delta^{15}N$ values for Leach's storm-petrels and *Calanus helgolandicus* copepods from three colonies and adjacent regions in the North Atlantic. The statistical results show the Tukey's HSD multiple pairwise comparisons tests, comparing: Leach's storm-petrel isotope values between breeders from different colonies (first column), between breeders and immatures within colonies (second column) and between immatures from different colonies (third column). Comparison of copepod values between regions are also shown in the first column.

Samples	$\delta^{13}C$	±SD	Statistical Results	$\delta^{15}N$	±SD	Statistical Results
Leach's storm-petrels						
GULL Breeders	-19.11	0.17	1, 7	14.66	0.12	4, 11
GULL Immatures	-19.15	0.14	7, 15	14.16	0.42	12, 17
STK Breeders	-19.10	0.23	1, 8	11.90	0.61	5, 13
STK Immatures	-19.21	0.35	8, 15	12.15	0.50	13, 18
VEST Breeders	-19.77	0.14	2, 9	13.07	0.42	6, 14
VEST Immatures	-19.64	0.14	10, 16	12.79	0.26	14, 19
Copepods						
Canada	-21.49	0.48	a	7.68	0.57	d
Scotland	-19.53	0.30	b	7.88	0.31	d
Iceland	-22.94	0.74	c	5.28	0.55	e

Same number or letter = not significantly different (P>0.05), different number or letter = significantly different (P<0.05), SD = standard deviation.

The trophic positions calculated using the copepod $\delta^{15}N$ as baseline values for each region suggest the breeders from GULL (TL = 4.9) and VEST (TL = 5.1) were feeding at approximately 1 trophic level higher than the STK breeders (TL = 3.8) (Figure 5.2). When $\delta^{13}C$ values were corrected for the different trophic levels, significant differences were found among all colonies (ANOVA, $F_{2,31} = 272.10$, $P < 0.0001$; All Tukey's HSD test $P < 0.0001$) (Figure 5.3) suggesting variation in carbon sources at the base of each food web.

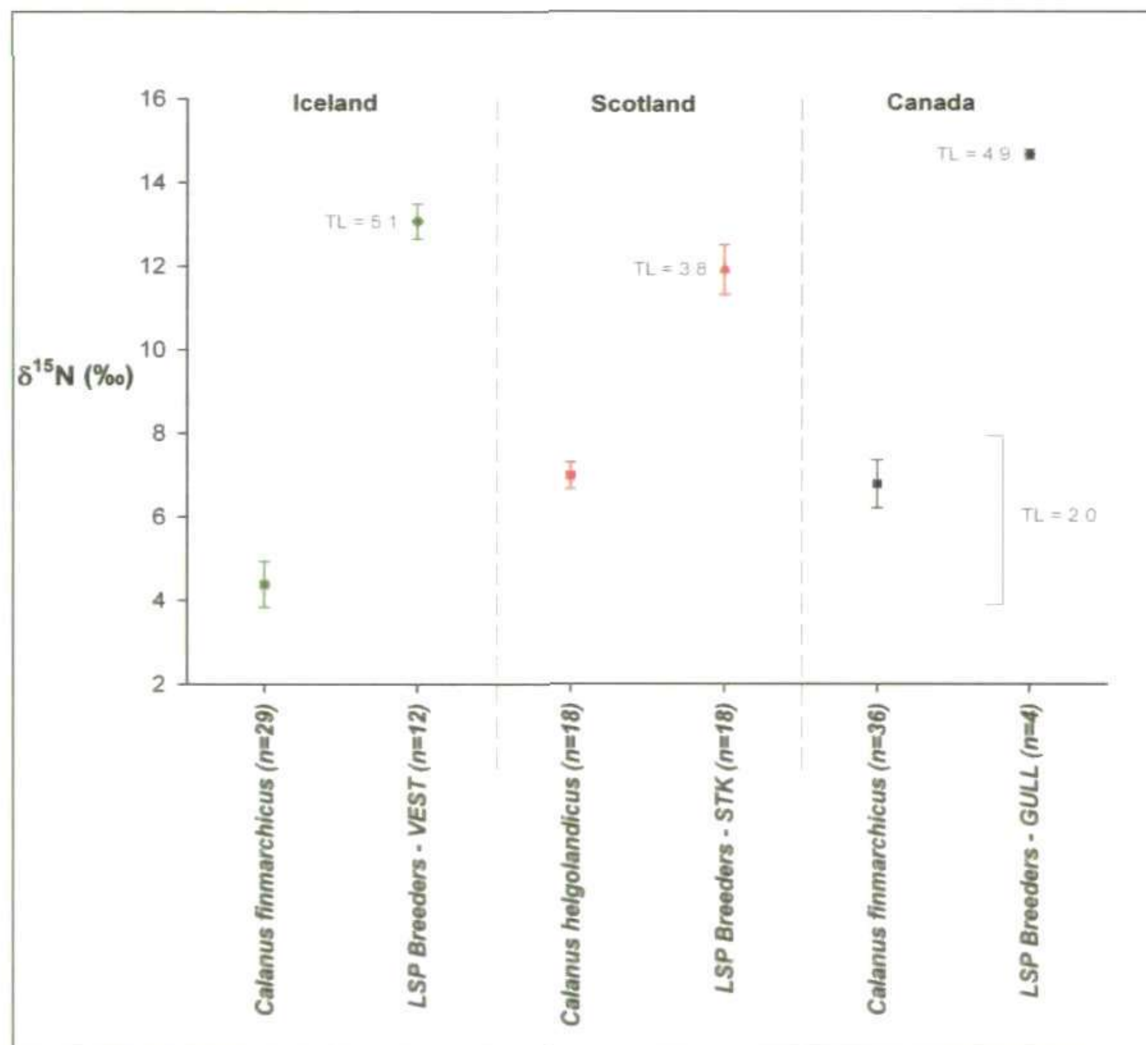

Figure 5.2 Nitrogen stable isotope plot (mean ± standard deviation) showing $\delta^{15}N$ values for breeding Leach's storm-petrels and *Calanus* copepods in each sampling region. TL represents assumed (copepod) and calculated (breeder) trophic levels. Closed squares = copepods, closed diamond, triangle and circle = breeding birds.

Immature birds

Immature Leach's storm-petrels showed similar patterns of regional mean stable isotopes as those found for breeders (Table 5.3; Figure 5.4), however $\delta^{13}C$ and $\delta^{15}N$ variance was significantly different (Levene's test, $\delta^{13}C$, $F_{2,133} = 12.76$, $P<0.0001$; $\delta^{15}N$, $F_{2,133} = 3.58$, $P = 0.03$), which was not the case for colony breeders (Levene's test, $\delta^{13}C$ and $\delta^{15}N$, $P > 0.05$).

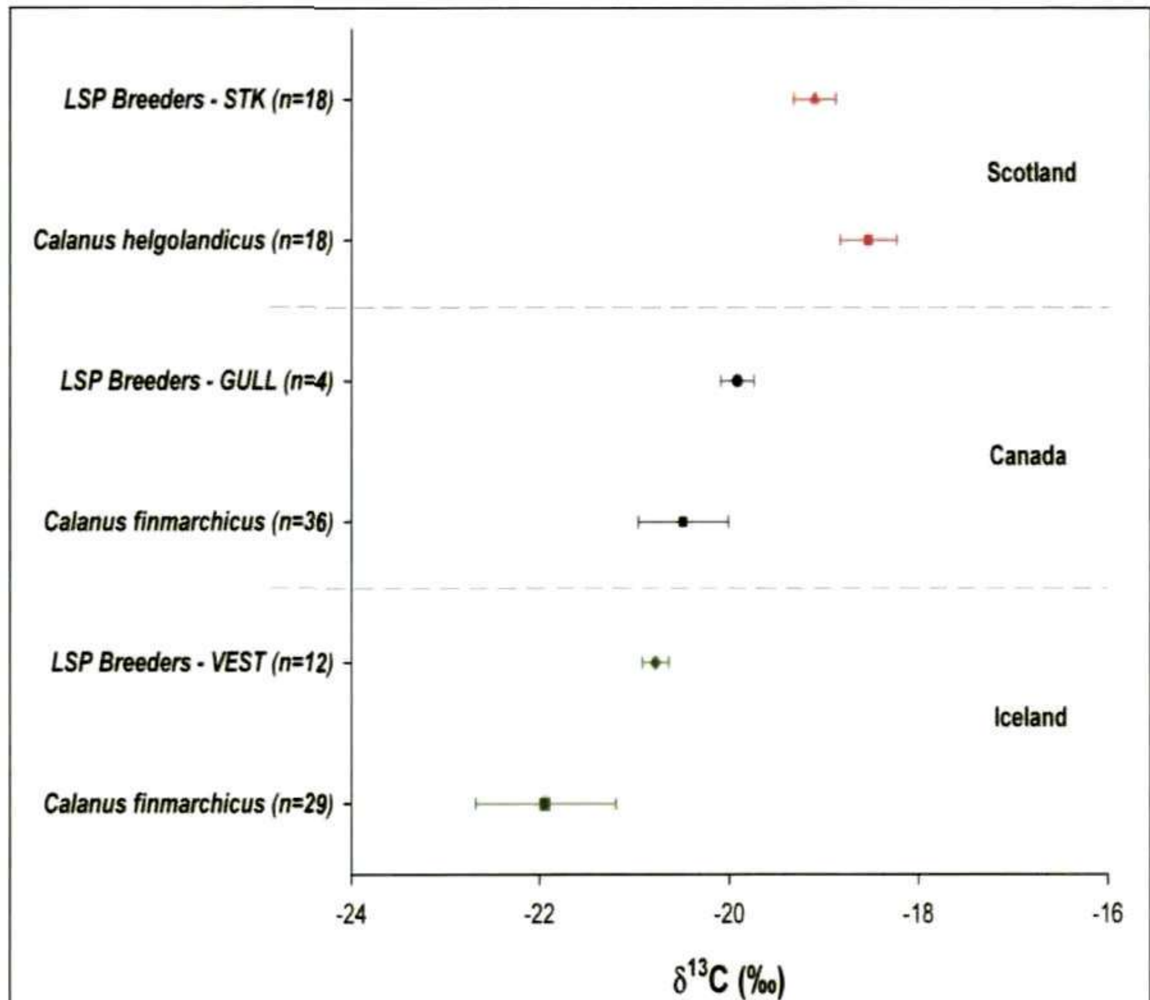

Figure 5.3 Carbon stable isotope plot (mean ± standard deviation) showing corrected $\delta^{13}C$ values for breeding Leach's storm-petrels and raw values for *Calanus* copepods in each sampling region. Closed squares = copepods, closed circles = breeding birds.

SIAR analysis

All but one of the isotope values for immatures were nested within the values for breeders (Figure 5.4), indicating a good characterization of possible isotopic sources. Of the 134 non-breeding Leach's storm-petrels analysed using the SIAR mixing model, 17 individuals had estimated proportions of <50% for their colony of capture (Figure 5.5), suggesting that they had recently arrived at the capture point from another Atlantic region. Of these 17 individuals, 2 were captured at GULL, 13 at STK and 2 at VEST, with proportions ranging from 3-44% for their presumed source colony (Figure 5.6).

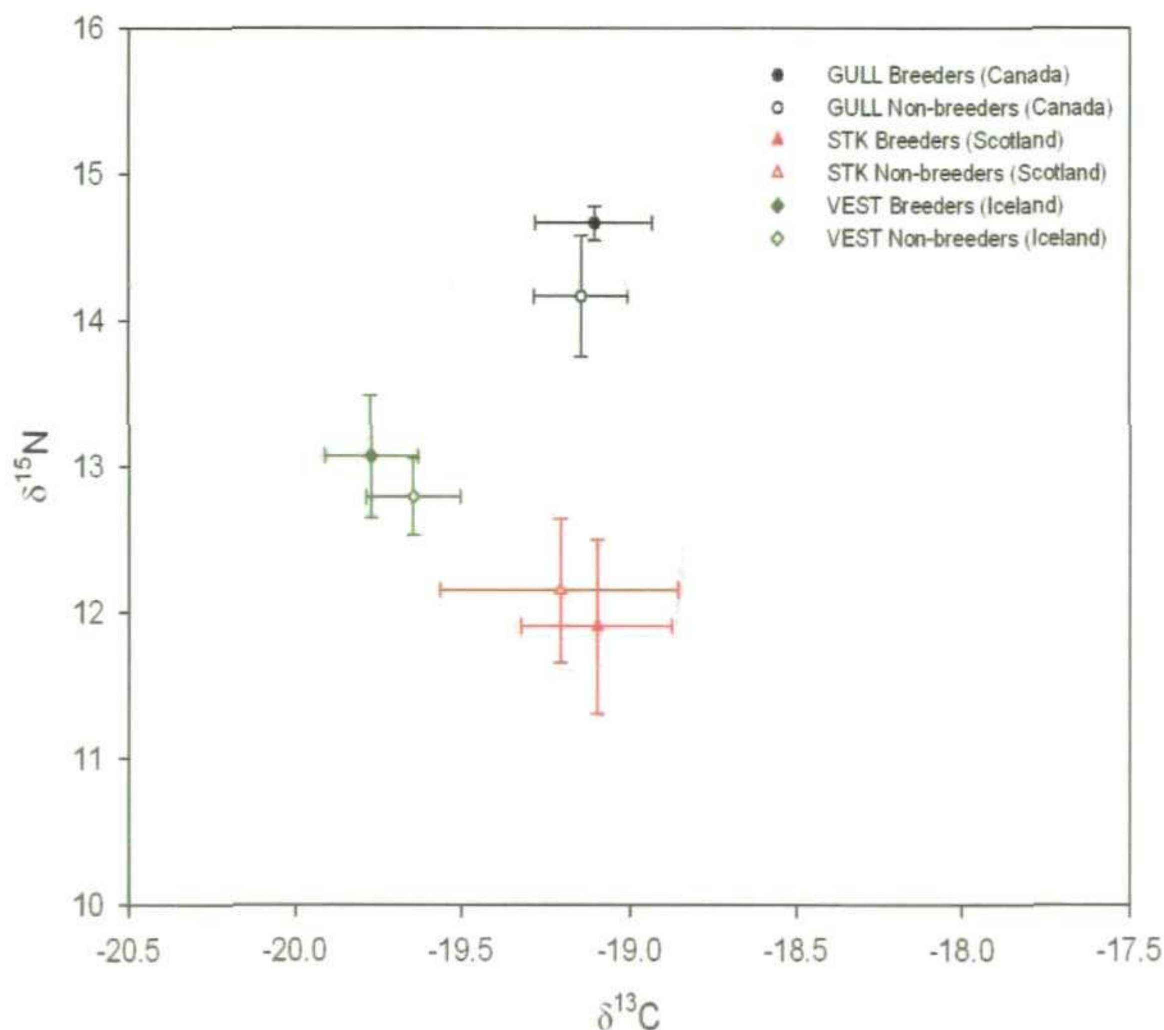

Figure 5.4 Dual stable isotope plots of nitrogen-carbon (mean ± standard deviation) showing the isotopic signature of breeding and immature Leach's storm-petrels sampled at three North Atlantic colonies. Dashed line = breeder variation, dotted line = immature variation.

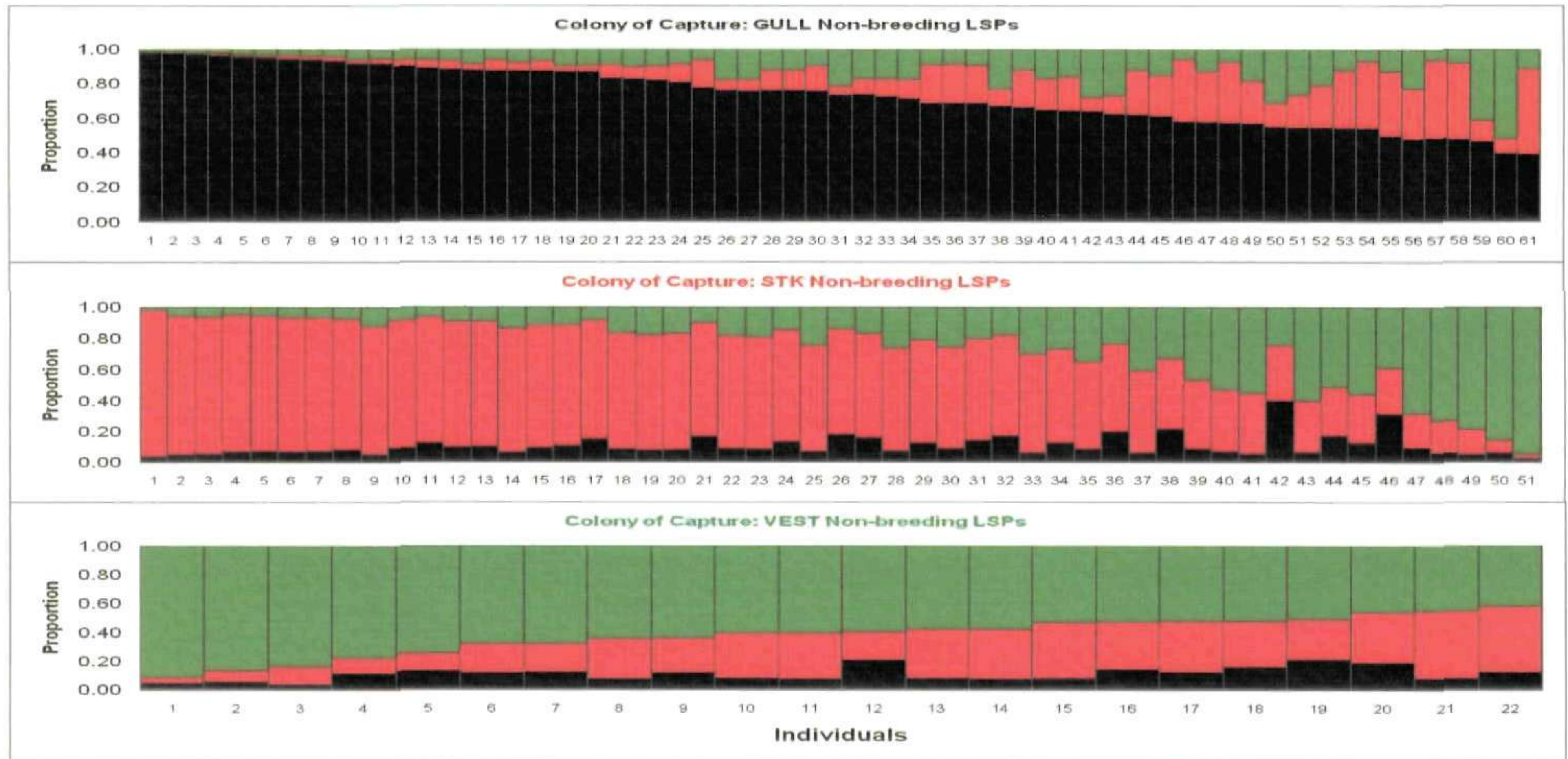

Figure 5.5 SIAR source proportion estimates for individual non-breeding Leach's storm-petrels caught at three colonies: GULL = Gull Island, STK = St Kilda, VEST = Vestmannaeyjar. Proportion colours: black = GULL, red = STK and green = VEST.

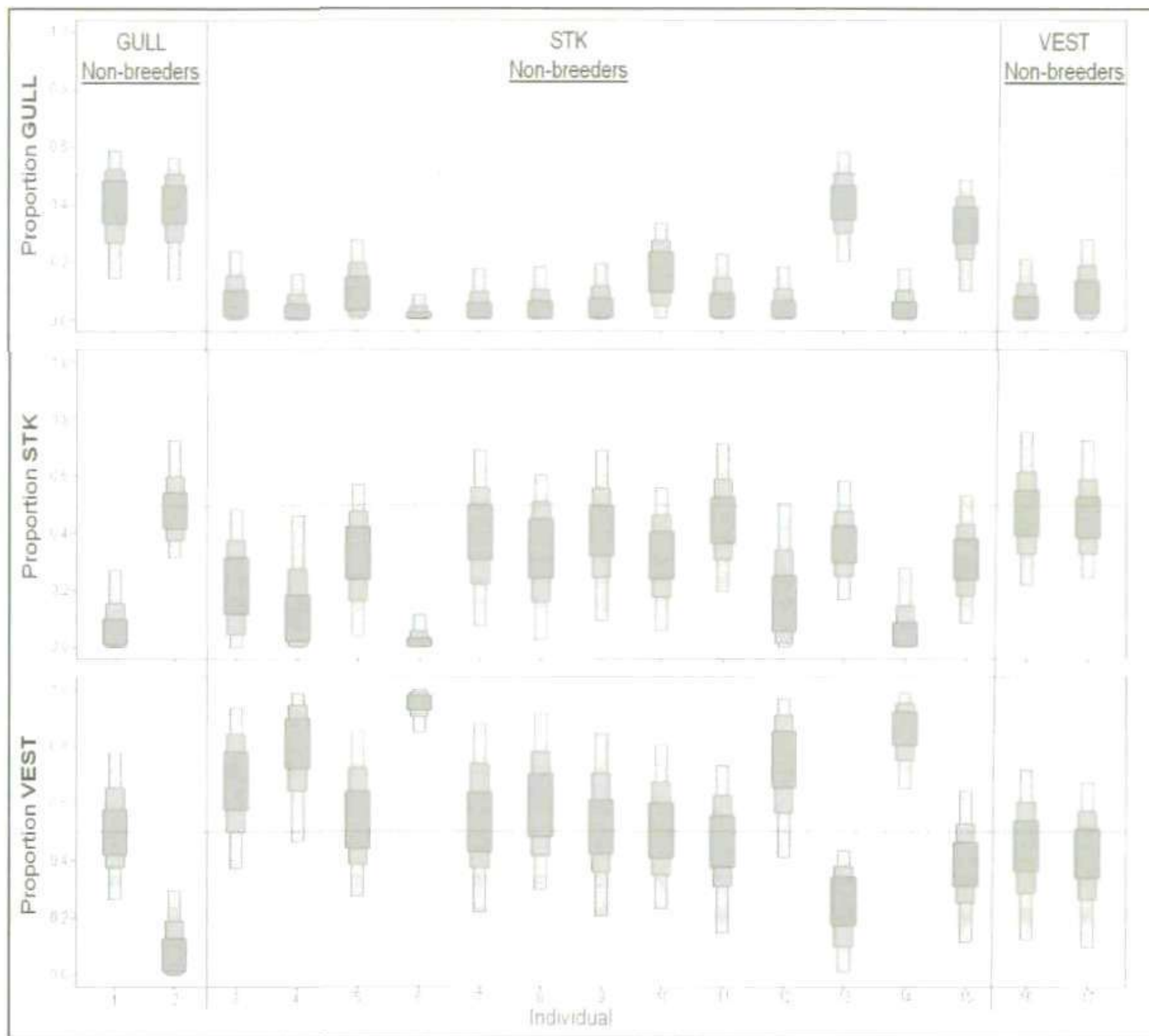

Figure 5.6 Results of SIAR mixing model (95, 75 and 50% credibility intervals) for individual non-breeding Leach's storm-petrels that were found to have proportion estimates <0.5 for their colony of capture. Dotted line = 0.5 proportion level.

5.4 Discussion

This study combines genetic and biochemical marker analyses to investigate gene flow and natal dispersal among three widely distributed Leach's storm-petrel colonies in the North Atlantic. The congruence of mtDNA and microsatellite loci analyses of breeding birds presents evidence for potentially high levels of contemporary gene flow among colonies. Moreover, Bayesian mixed model isotope analysis shows evidence for inter-colony movement of immatures during the breeding season, which may lead to the

observed gene flow. The findings and utility of the stable isotope analysis, as well as the likely implications for a Leach's storm-petrel colony under threat are discussed below.

Gene flow and dispersal of immature birds

The genetic evidence suggests the vast expanse of the North Atlantic ocean is no barrier to gene flow in Leach's storm-petrels and, from previous research, large continental landmasses are key physical barriers limiting long distance dispersal (Chapter 3). The lack of genetic differentiation or structure among colonies indicates both historical (FST and □ST analyses as lagging indicators; Whitlock and McCauley, 1999) and contemporary gene flow (Bayesian cluster analysis).

Breeding Leach's storm-petrel are believed to be highly site faithful (Mauck *et al.*, 2004) suggesting that natal dispersal is the most likely mechanism for gene flow. Our study presents evidence to support this. Based upon distinct isotopic sources (breeding Leach's storm-petrels of known provenance), we used Bayesian isotope mixing models to infer movement of immature Leach's storm-petrels among colonies. The majority of immatures (88%) had model estimates (>0.5: Figure 5.5) that strongly suggest feeding close to the colony of capture, consistent with tracking studies that show immature seabirds are site faithful to a single colony (Votier *et al.*, 2011). Of the remaining 17 birds, four (Individual 4, 6, 12, 14; Figure 5.6) had estimates that clearly indicated recent feeding in a different isotopic region (>0.7 proportion estimates for a colony not captured on). The further 14 birds included individuals that had high contributions for two regional sources, which meant their origin was not as clear. Consequently, less confidence can be associated with inferring movement of those individuals. Moving between isotopically distinct regions will not instantaneously lead to isotopic differences in consumer tissues, i.e. a dietary switch can take >30 days to reach isotopic

equilibrium in avian blood (Bearhop *et al.*, 2002). Therefore it is expected that there will be some intermediate values if individuals have changed locations very recently, and might explain the high estimates found for two sources in some individuals. However, an alternative explanation is the isotopic signatures reflect mid/central North Atlantic regions where immatures could have spent long periods before returning to the same colony. Although a possibility, it would seem reasonable to assume that immatures caught visiting colonies at night are prospecting for breeding opportunities, therefore in breeding condition. Spending large periods of time away from colonies would reduce their chance of recruitment and therefore is proposed to be an unlikely behaviour.

Characterizing all potential contributing sources to immature bird isotopic signatures is necessary to produce reliable estimates in this analysis and was the case for all but one individual. The much higher $\delta^{13}C$ of this particular STK bird suggests it had been foraging in a region influenced by warmer waters, which had not been included as a source in the model. It could potentially be a late migrant returning from warmer wintering waters with enriched ^{13}C isotope values (e.g. Gómez-Díaz and González-Solís, 2007).

Using the distinction between the North Atlantic regions the SIAR analysis provides persuasive evidence for the movement of immature Leach's storm-petrels during the breeding season and seems the likely mechanism promoting natal dispersal and ultimately gene flow. For species unsuitable for direct tracking there are few options to determine attendance of immigrant individuals at breeding locations. The SIAR mixing model approach provides an alternative indirect method to identify animals that show evidence of recent movement and are possible immigrant individuals.

Isotopic characterisation of regional food webs

Establishing isotopic differences between regional food webs was essential before using SIAR to infer movement of immature Leach's storm-petrels (Hobson, 1999). The $\delta^{13}C$ and $\delta^{15}N$ values of Leach's storm-petrel colony breeders and *Calanus* copepod samples separated well into isotopically distinct regions close to the colonies, suggesting both a difference in the primary carbon source for each food web but also a possible variation in food web structure or feeding behaviour (Figures 5.1, 5.2 & 5.3). All samples were collected during 2008, with the exception of Vestmannaeyjar, Iceland, which was sampled in 2009. Although annual variation in isotope signatures could influence our results, the copepod samples were broadly consistent and, therefore, we believe it reasonable to assume that any inter-annual regional variation is not large enough to significantly affect the between region differences found in the breeding Leach's storm-petrels.

The lower $\delta^{13}C$ values in Iceland and Canada are consistent with the tendency for depleted ^{13}C with decreasing temperature, a consequence of changes in dissolved carbon dioxide during carbon fixation (Lourey *et al.*, 2004, Lara *et al.*, 2010). These higher latitudes are influenced by colder Arctic waters (Afanasyev *et al.*, 2001, Astthorsson *et al.*, 2007) compared with the warmer waters from the Gulf stream around Scotland (Reid *et al.*, 2001).

Leach's storm-petrels breeding on St Kilda had lower $\delta^{15}N$ values than elsewhere in the North Atlantic (Figure 5.2). This may be due to birds feeding at a lower trophic level or because of variation in baseline nitrogen or food-web complexity among regions (Jennings and Warr, 2003). The similarity in $\delta^{15}N$ values for copepods in Canada and Scotland (Figure 5.2) suggests comparable nitrogen pools influencing the two regional food webs. Therefore the difference in $\delta^{15}N$ between breeding birds is more likely a trophic level effect. Assuming similar nitrogen pools the trophic disparity could either

be a change in foraging behaviour between regions or variation in food web structures. Small meso-pelagic fish and zooplankton species have been identified as the main prey of Leach's storm petrels at Pacific and Atlantic colonies (Watanuki, 1985b, Vermeer, 1988, Hedd and Montevecchi, 2006). The prey composition is thought to reflect the local marine food web (Hedd *et al.*, 2009), rather than a change in foraging behaviour between different regions. A more likely explanation for trophic variation could be different food web structures, as has been found in other aquatic systems (Matthews and Mazumber, 2003). Lower $\delta^{15}N$ could also be attributed to dominance of lower trophic prey in the diet, but is reliant on distinct differences in prey communities in Canada and Iceland versus Scotland. Data on Leach's storm-petrel diet in Europe and the meso-pelagic fish and zooplankton communities in the North Altantic are not comprehensive enough to determine the cause of regional trophic differences in breeding Leach's storm-petrels - this is an area where further studies are required. Although the cause(s) of the regional $\delta^{15}N$ change is unclear, there is clear isotopic distinction between the regions based on both $\delta^{13}C$ and $\delta^{15}N$, which can be used to infer movement of immature birds.

Colony implications

Previous evidence that the North Atlantic Leach's storm-petrel colonies function as a large meta-population (Chapter 1) is supported by both genetic and stable isotope analyses in this study. Inter-colony movement of immature Leach's storm-petrels prospecting for breeding opportunities is a likely mechanism for dispersal in this species and could provide an important buffering or "rescue effect" for colonies (Inchausti and Weimerskirch, 2002). The extreme level of predation found on St Kilda (Phillips *et al.*, 1999b, Miles, 2010) should have lead to local extinction if this was a closed population (Miles, 2010) but the colony still persists. The SIAR results imply approximately 1 in 4

of immature birds attending STK are potential immigrants from other regions of the North Atlantic. Caution needs to be taken with these estimates (as discussed) but based on estimates of attending natal immature birds (~35,000; Miles, 2010) this could indicate a substantial number of immigrants (>8,000) at any one time. Although exploratory, this does not seem unrealistic considering the continued persistence of the relatively small St Kilda breeding colony (~45,000 bp), despite annual losses of between 15,000-21,000 individuals. The immigrant birds could potentially buffer such loss (Klomp and Furness, 1992), but long term viability is questionable if high predation continues. With a predation threat, an increase in emigration rate might be expected (Clobert *et al.*, 2001), which may exacerbate the decline. However, the emergence of such anti-predator behaviour seems unlikely in the short-term given their long generation times (~12 years). It is difficult to confidently predict the future population trend for the STK colony, but regular breeder census and predation estimates would be the least required to monitor the long-term sustainability of this important European colony.

Leach's storm-petrel is an example of a mobile species with highly inter-connected spatially discrete colonies, subject to meta-population dynamics, i.e. source-sink colonies (Esler, 2000). This has led to local colony persistence through prospecting and/or recruitment of immature birds. Understanding the extent and mechanism of dispersal in such vagile species is important to predict the potential short- or long-term impact of environmental change and implement effective population management. Although some degree of breeding dispersal cannot be discounted in Leach's storm-petrels, especially after breeding failure or adverse conditions (Schmidt, 2004), this study indicates natal dispersal is most likely a mechanism to explain high levels of gene flow among North Atlantic colonies.

Chapter 6

Population mixing of Leach's storm-petrel during the non-breeding season: evidence from analysis of moult and stable isotopes in wrecked birds

6.1 Introduction

The mechanisms that facilitate, or limit, gene flow have important consequences for population dynamics and species conservation. The factors influencing gene flow may be multifaceted and many remain poorly understood (Friesen *et al.*, 2007a). Various stages of an animal's annual cycle may contribute to these factors and need to be considered. Seabirds are highly vagile and have the ability to disperse over huge distances (e.g. Gonzalez-Solis *et al.*, 2007, Egevang *et al.*, 2010), but physical (geographic) and non-physical barriers can still limit the extent of gene flow between populations. For example, population segregation during the non-breeding season was found to be strongly correlated with population structuring in seabirds (Friesen *et al.*, 2007a), highlighting the influence of over-wintering distribution and mixing for gene flow. Therefore, for migratory species, determining the extent to which individuals from different breeding colonies migrate to the same wintering grounds is important to understand the potential mechanisms that facilitate dispersal and, ultimately, gene flow (Webster *et al.*, 2002).

Colonial nesting of seabirds makes them relatively easy to study during the breeding season but collecting data to study migratory connectivity, i.e. the degree to which individuals from different breeding colonies mix on wintering grounds (Webster *et al.*, 2002), is more challenging. Ring recovery data of birds individually marked at breeding colonies can be used to infer migratory patterns (Clark *et al.*, 2009), but for pelagic seabirds the number of birds recovered may be low and there may also be bias associated with finding ringed birds (Wernham *et al.*, 2002). Alternatively, tracking devices (e.g. geolocators) can be used to re-construct migratory routes and provide important information on over-wintering location and behaviour (Shaffer *et al.*, 2006, Gonzalez-Solis *et al.*, 2007), but for some small species this is still currently impractical due to the size of devices. Also to establish connectivity, birds from multiple colonies would need to be tracked, which although possible, would greatly increase time and

cost. Another solution to reveal the over-winter behaviour is to use mass mortalities or wrecks of seabirds to provide specimens for analysis. Wrecks are the unexpected death of large numbers of individuals from apparent natural causes, which occur sporadically around the world (Newton, 2007). They are usually associated with severe weather and can provide samples from different periods of the annual cycle. These dead birds can be used to provide important information, such as biometrics, genetics, contaminants and stable isotopes. Where such metrics are known to vary among potential source populations, they can be used to determine origins (Gómez-Díaz and González-Solís, 2007) and mixing on migration or wintering grounds.

Natural variation in the stable isotopic composition of animal tissues reflect those in the local environment, and since feathers are metabolically inert after synthesis they reflect the birds diet during the time of growth (Thompson and Furness, 1995). Feathers are often used in stable isotope analyses to infer geographic origins of birds and to establish migratory connectivity between breeding, wintering and stop-over sites (Hobson, 2005). Therefore, understanding the timing and sequence of moult enables the use of appropriate feathers in these analyses. Isotopic distinction between regions or ecosystems is also a prerequisite to conduct such analyses and requires knowledge of natural isotopic gradients and/or collection of geographic baseline isotope data (Hobson, 1999).

Moult is an energetically demanding activity in birds (Murphy and King, 1992), and its pattern and timing are usually adjusted to minimize overlap with other demanding activities, e.g. reproduction and migration (Pietiäinen *et al.*, 1984, Espie *et al.*, 1996). In seabirds this often means that flight feather moult takes place outside the breeding season (Bridge, 2006). Therefore, because seabirds are generally at sea during this period, moult is rather poorly understood and more information is required to enable appropriate use of stable isotope analysis.

The Leach's storm-petrel *Oceandroma leucorhoa* is a small (~45g) highly pelagic seabird that is rarely seen close to land outside the breeding season and little is known about distribution and behaviour on the wintering grounds. Breeding colonies are on islands widely spread across the North Atlantic and North Pacific. Several thousand pairs breed in Europe, the majority of which are at two large colonies in Scotland (St Kilda) and Iceland (Vestmannaeyjar), but the vast majority of the Atlantic population nest in Newfoundland, Canada (>4 million breeding pairs). Previous work suggests high levels of gene flow among colonies within the Atlantic, and also evidence of migration between the Atlantic and Pacific (Chapter 2). Moreover analysis of stable isotopes in tissues of the abundant immature population (Leach's storm-petrel do not breed until age 5) suggests that natal dispersal is the likely mechanism connecting these distant colonies (Chapters 2 and 4). However given the importance of mixing on the non-breeding grounds for population structuring (Friesen *et al.*, 2007a), it is key to establish how strong migratory connectivity is for colonies in the North Atlantic.

Here we use Leach's Storm-petrel corpses associated with three wrecks (one in Canada and two in the north-east Atlantic) to better understand the extent of population mixing in the winter quarters. We firstly use corpses of wrecked birds to characterise the extent of flight feather moult to determine the most appropriate feather tract for isotopic analysis. We then compare the European wrecked birds' isotopic values with Canadian wrecked birds, and known regional isotopic end-points, to assess whether these originated from colonies in North America.

6.2 Materials and Methods

Wrecked Leach' storm petrels

Large numbers of Leach's storm-petrels were driven ashore following three separate storm events: (1) >200 birds during early September 2006 in Newfoundland (following part of Hurricane Florence), (2) unknown numbers (but potentially >500) in December

2006 in Britain and France (following an extra tropical cyclone that tracked across the Atlantic), and (3) again unknown numbers (but at least 100 were found in France), in November 2009 in Britain (following a series of intense low pressure systems in the north-east Atlantic). Many of these birds were discovered dead or dying by the general public and following requests, 18 corpses from the Newfoundland wreck and 18 corpses from the wrecks in Britain and France were available for feather moult and stable isotope analyses (Figure 6.1).

Moult

Before scoring, each bird was aged as either an adult (>1 year old) or juvenile (<1 year old) on the basis of feather wear. For each individual bird tail and flight feather moult was scored between 0 and 5, where 0 represents an old feather, 1-4 represent progressions of feather growth and 5 a fully grown new feather (Ginn and Melville, 2007). Wing feather tracts were numbered: Primaries, P1→ P10 ascending outwards from the carpel joint; secondaries, S1→S11 ascending inwards from carpel joint; and tertials, T1→T3 ascending inwards from last secondary towards the body. To enable close inspection of feather replacement and stage of growth of flight feathers, the underwing-coverts were removed before scoring. Tail feathers (rectrices) were numbered in pairs from the centre outwards, R1→R6.

Stable isotope samples and analysis

Preliminary analysis of the extent of moult indicated that tail feathers were completely replaced on the breeding grounds (see Results). Therefore stable isotope values from new tail feathers of wrecked birds were used to reflect the isotopic signature of the last breeding colony attended. The tip (~0.5 cm) of the outer most tail feather (R6) from all birds wrecked in the UK and France (hereafter European) was removed using sterile scissors. Not all Canadian wrecked birds had moulted R6 (instead these were retained

from the previous annual moult), so only 10 of the 18 were sampled in the same manner. Feather samples were washed in 1:2 chloroform:methanol solution to remove residual dirt and oil that might bias isotope values (Paritte and Kelly, 2009), and air dried for 24 hours.

For regional north Atlantic isotopic end-points we obtained blood samples from breeding adult Leach's storm-petrels at major colonies in Canada (Gull Island, 47°15'N, 52°46'W) and Scotland (St Kilda, 57°49'N, 08°35'W) during August 2008, and Iceland (Vestmannaeyjar, 63°25'N, 20°17'W) during August 2009 (Chapter 5). Between 10-20μl of blood was collected (under appropriate regional licences) from the brachial vein of breeding adults in nesting burrows. Blood samples were separated into plasma and red blood cells (RBC), using a centrifuge, within 2-3 hours of sampling, and were stored frozen until preparation for analysis. The RBC isotope values reflect the dietary intake in the ~3-4 weeks prior to sampling (Bearhop *et al.*, 2002).

All samples were freeze-dried, homogenised and ~0.7 mg weighed into a tin cup for analysis. The $\delta^{15}N$ and $\delta^{13}C$ values from feather and RBC samples were used in the stable isotope analysis.

Analyses were conducted at the East Kilbride Node of the Natural Environment Research Council Life Sciences Mass Spectrometry Facility via continuous flow isotope ratio mass spectrometry using a Costech (Milan, Italy) ECS 4010 elemental analyser interfaced with a Thermo Electron (Bremen, Germany) Delta XP mass spectrometer. Isotope ratios are reported as δ-values and expressed as ‰ according to the equation:

$$\delta X = [(R_{sample}/R_{standard})-1] \times 1000$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ and $R_{standard}$ is the ratio of the international references PDB for carbon and AIR for nitrogen. The standard deviation of multiple analyses of an internal gelatine standard in each experiment was better than 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$. One sample failed during analysis, which reduced the sample size for the 2006 European wreck to 17.

In addition, $\delta^{13}C$ and $\delta^{15}N$ data for tail feathers of breeding adult Leach's storm-petrels from Baccalieu Island, Canada (Figure 6.1) in 2001 and 2002 were obtained from Hedd and Montevecchi (2006), to provide further information on potential regional differences in isotopes.

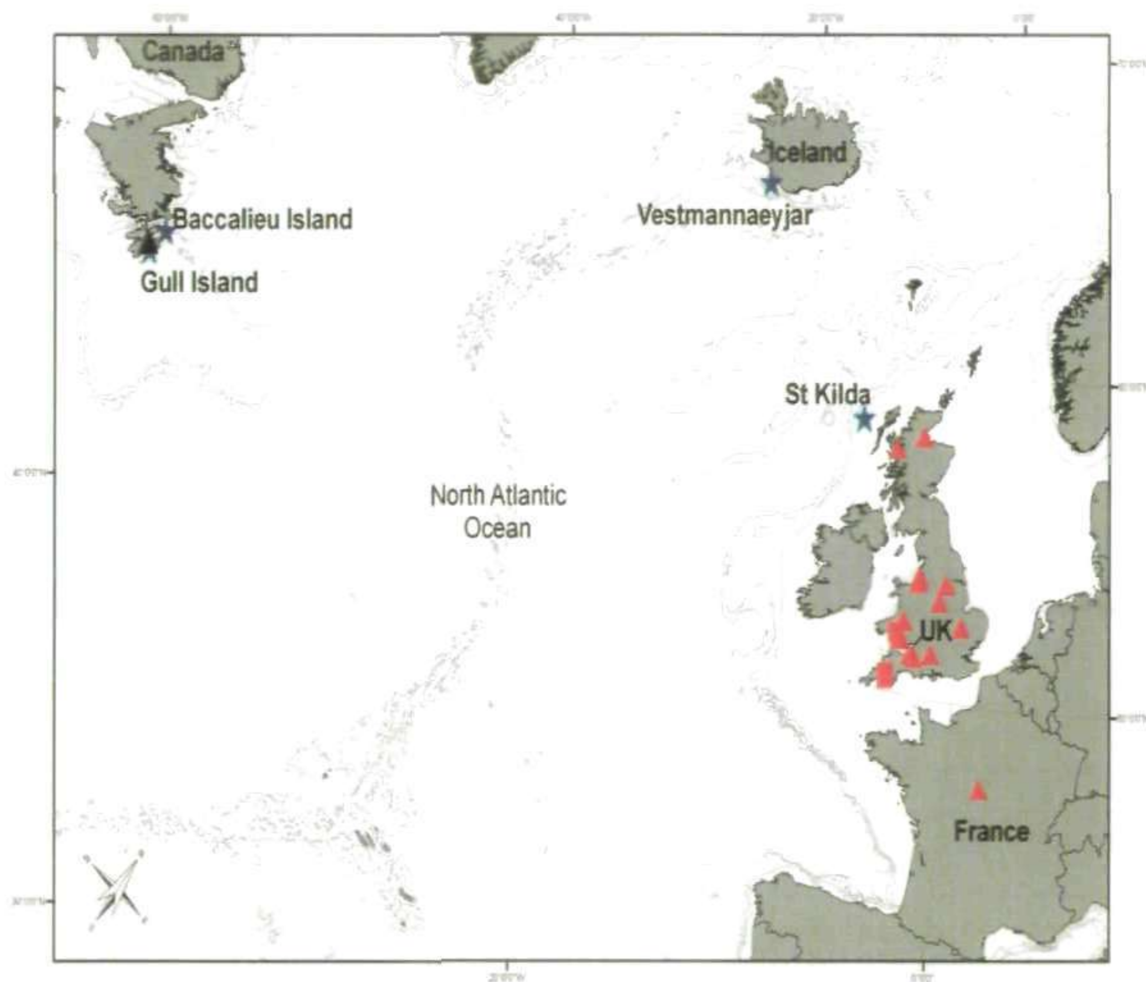

Figure 6.1 Locations of wrecked Leach's storm-petrels in 2006 and 2009, and the main breeding colonies in Canada and Europe. Black triangle = Canadian mass wreck 2006, red triangles = sites of wrecked birds in Europe 2006, red squares = sites of wrecked birds in Europe 2009.

Statistical analysis

To investigate segregation of $\delta^{13}C$ and $\delta^{15}N$ values in RBC of colony breeders multivariate analysis of variation (MANOVA) was conducted. Univariate analysis of

variance (ANOVA) and Tukey's HSD post-hoc multiple pairwise comparison tests followed where significant differences were found in a MANOVA. All statistical analyses were conducted using R version 2.12.2 (R, 2011).

6.3 Results

Moult of wrecked Leach's storm-petrels

All 36 wrecked birds were at least one year old with wing feathers appearing worn and brown with clear signs of abrasion.

Canada

No Canadian birds were in wing moult. Tail moult had started in 15 of the 18 Canadian birds. Many had only recently commenced tail moult and no birds had replaced all tail feathers (Figure 6.2). The tail moult is broadly symmetrical with suggestions that the inner and outer most feathers are replaced first.

Europe

All European birds had fresh completely moulted tail feathers, and were in active wing moult (Figure 6.3 a and b). Moult of primary feathers had started in all birds and was on average ~50% complete, with a clear symmetrical ascending progression on both wings. P1, P2 and P3 feathers had been replaced in all birds (Figure 6.3 a) and had completely re-grown. Moult of secondary feathers was not as advanced as primary feathers and one individual retained all old secondary feathers. Over 50% of birds had dropped S5 and S6 from the centre of the feather tract, and S11 from the proximal portion, suggesting possible focal points for secondary moult (Figure 6.3 a). Moult in tertial feathers had started in all birds but there was no obvious pattern or focal point (Figure 6.3).

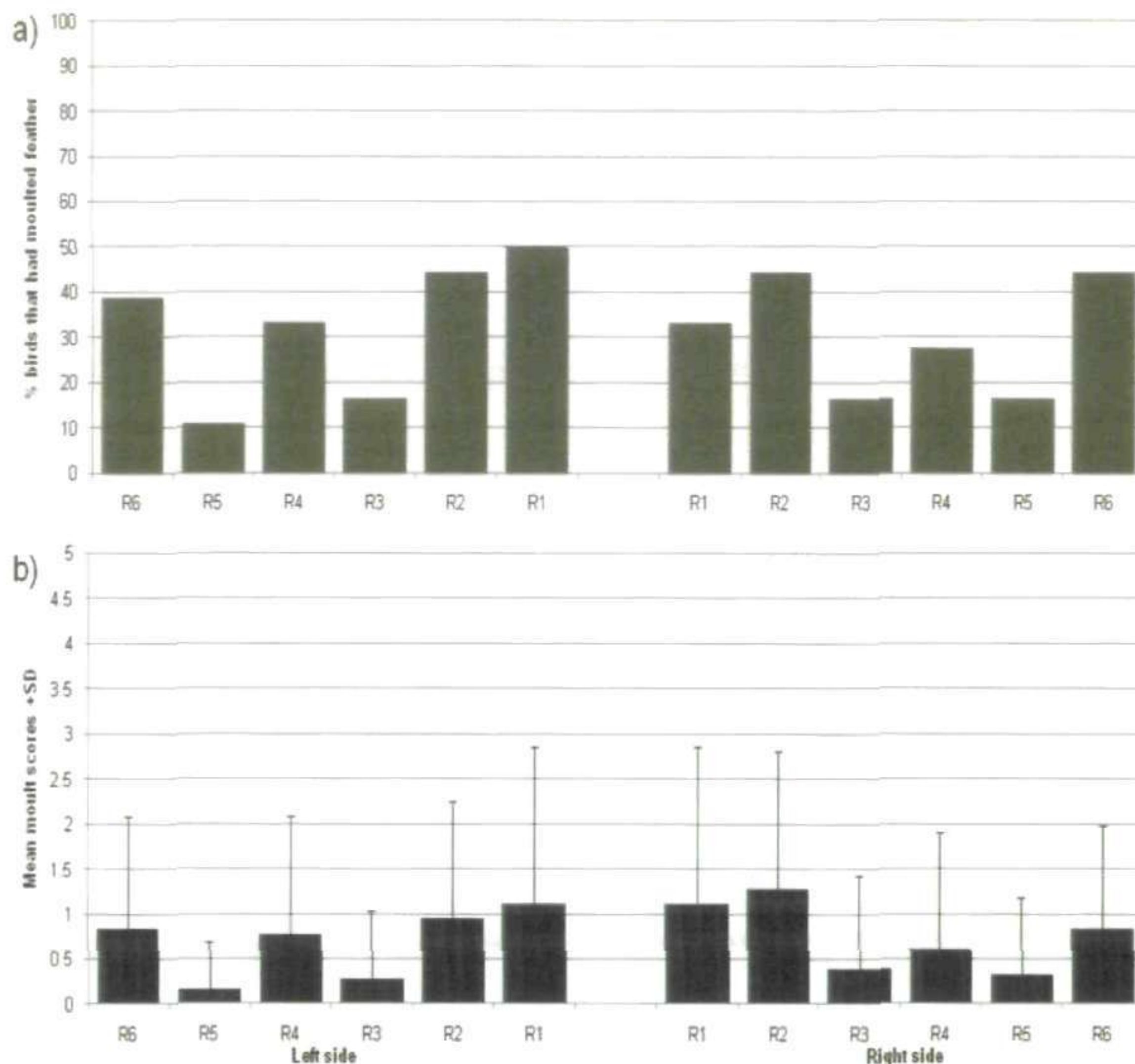

Figure 6.2 Tail feather moult in 18 Leach's storm-petrels wrecked in Newfoundland, Canada during September 2003. a) Percentage of birds that have moulted each tract, b) mean moult scores ± 1 standard deviation. Wrecked birds found in Europe during November and December had completed tail moult.

Stable isotope analysis of wrecked Leach's storm-petrels

$\delta^{13}C$ of tail feather (R6) values ranged from -20.71 to -17.62 and $\delta^{15}N$ values from 12.24 to 16.05 (Figure 6.4). With the exception of one individual with very high $\delta^{15}N$ and low $\delta^{13}C$, the birds found in Canada clustered within ~0.6‰ for $\delta^{13}C$ and ~1.5‰ for $\delta^{15}N$. Overall birds found in Europe had much more varied $\delta^{13}C$ and $\delta^{15}N$ values but 8 individuals clustered with similar isotopic values as the majority of Canadian birds

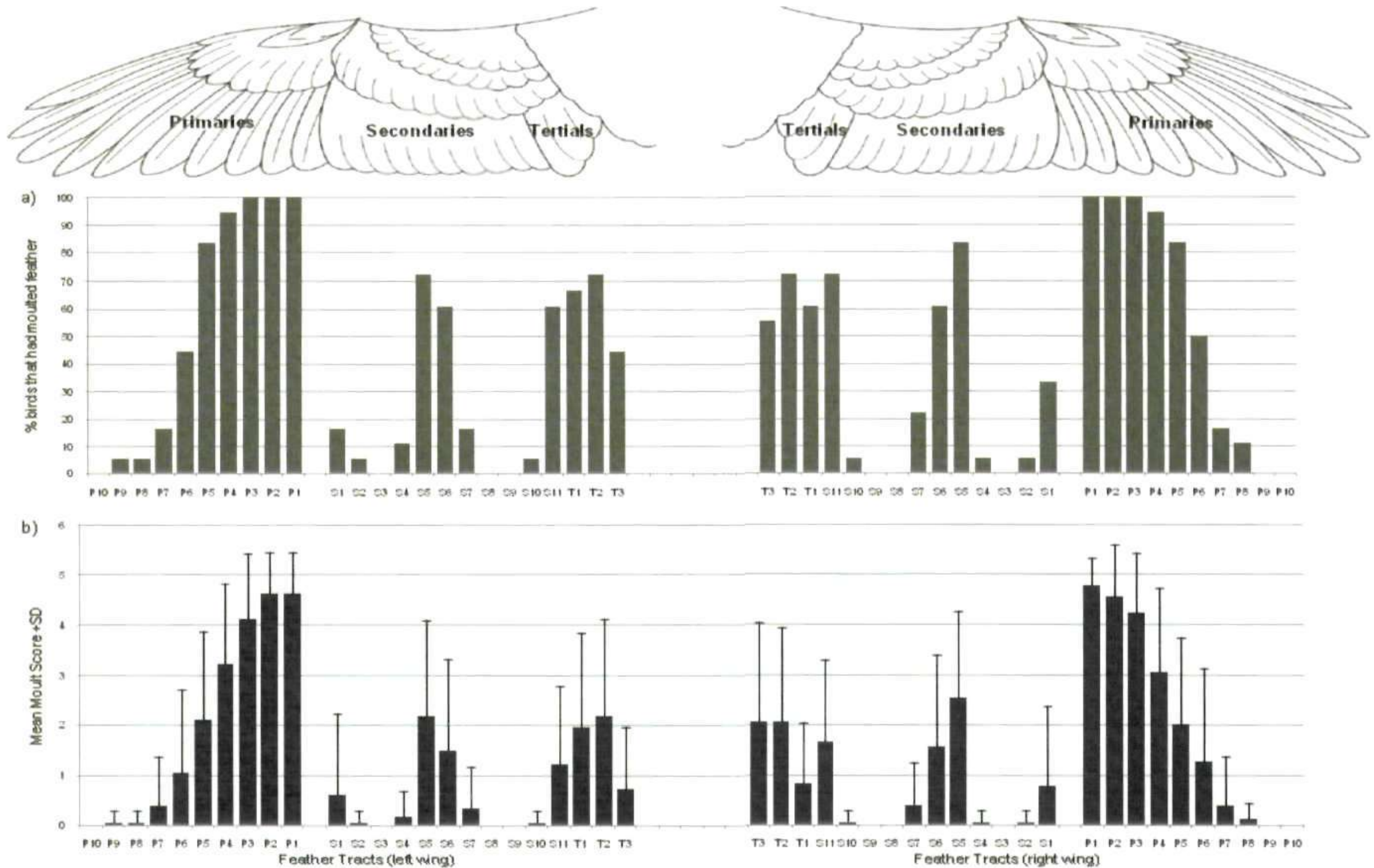

Figure 6.3 Wing moult in Leach's storm-petrels wrecked in Europe during November 2006. a) Percentage of birds that have moulted each feather, b) the mean moult scores ± 1 standard deviation. Sample of 18 wrecked birds.

(Figure 6.4). European birds wrecked in 2009 had higher $\delta^{13}C$ values than all other birds.

Stable isotope analysis of Leach's storm-petrels of known origin

Breeding Leach's storm-petrel RBC were isotopically segregated among three colonies in Scotland, Iceland and Canada (MANOVA, Pillai, $F_{2,31} = 50.859$, $P < 0.0001$; Figure 6.5), and in subsequent univariate analysis and post-hoc multiple pairwise comparison test (Tukey's HSD test) $\delta^{15}N$ was significantly different among all colonies (ANOVA, $F_{2,31} = 55.182$, $P < 0.0001$; Tukey's HSD test, $P < 0.0001$) and $\delta^{13}C$ was significantly different between Iceland and the other colonies (ANOVA, $F_{2,31} = 46.052$, $P < 0.0001$; Tukey's HSD tests, Iceland:Canada $P < 0.0001$, Iceland:Canada $P < 0.0001$), while Canada and Scotland values were similar (Figure 6.5). $\delta^{13}C$ and $\delta^{15}N$ mean ($\pm$ 1 standard deviation) values for tail feathers from breeders caught in 2001 and 2002 (Hedd and Montevecchi, 2006) had much larger variation in carbon compared to the RBC values from colony breeders in subsequent years (Figure 6.5). $\delta^{15}N$ is shown as the main regional distinction between Canada and Scotland, but it is $\delta^{13}C$ that distinguishes the Icelandic region (Figure 6.5).

Figure 6.4 $\delta^{13}C$ and $\delta^{15}N$ values of new outermost tail feathers in Leach's storm-petrels wrecked in Canada (10) and Europe in 2006 (14) and 2009 (3).

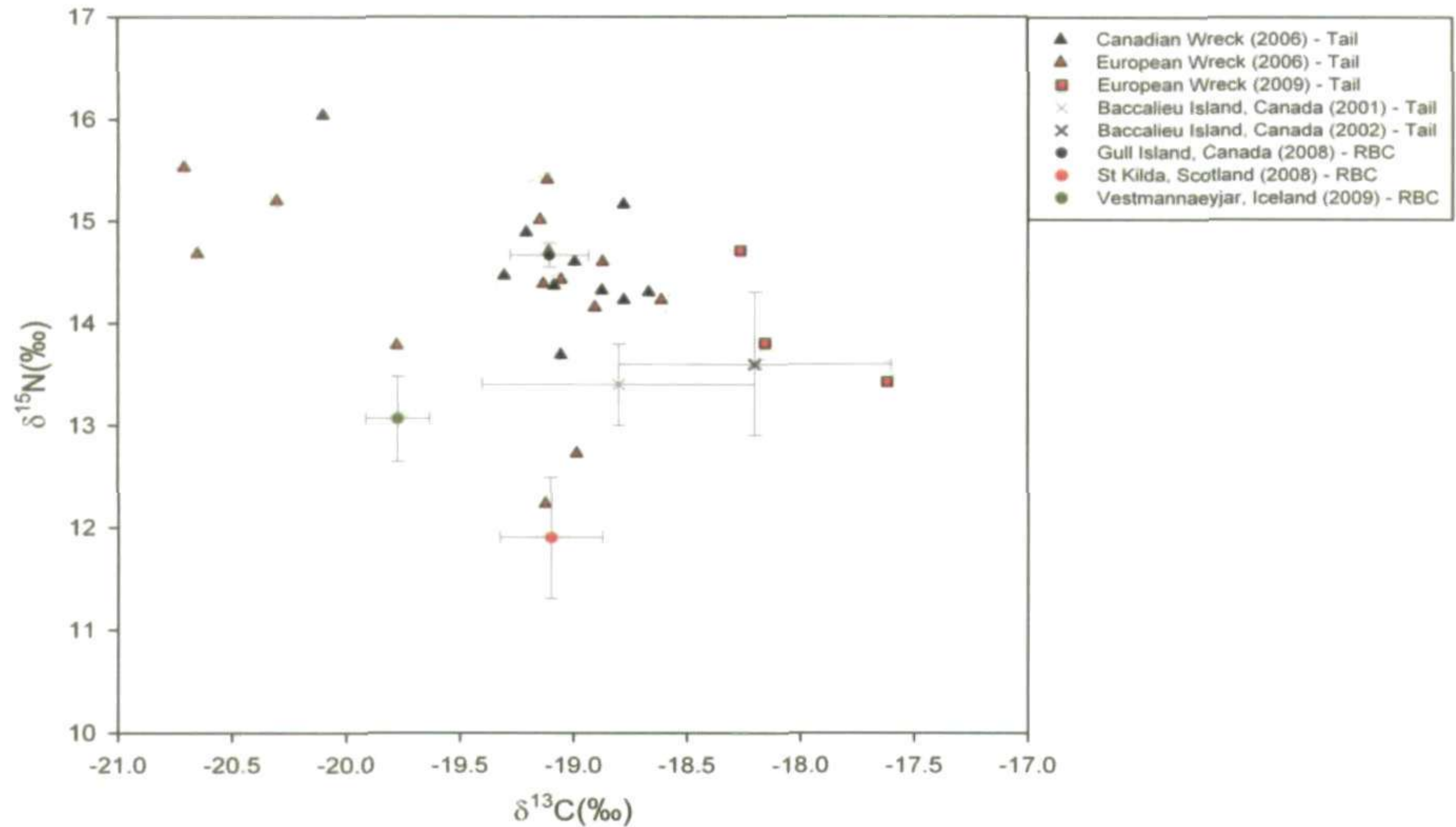

Figure 6.5 $\delta^{13}C$ and $\delta^{15}N$ values from Canadian and European wrecked Leach's storm-petrels (R6 tail feather) and breeding birds from four major North Atlantic colonies (tail feathers and red blood cells). (Wrecked birds clustered within ~0.7‰ ($\delta^{13}C$) and ~1.7‰ ($\delta^{15}N$) of each other in dashed eclipse).

6.4 Discussion

The isotope analysis presented here provides evidence that Leach's storm-petrels wrecked during mid-winter in Europe originated from a number of different colonies (including Canada) and therefore suggests population mixing during the non-breeding period. Flight feather moult was consistent with previous studies but also provided revealing evidence of replacement in the little studied secondary and tertial feathers. Moreover, the analysis established tail feathers as an appropriate tract to reflect the breeding colony in stable isotope analyses. The results of both moult and stable isotope analysis, and the trans-Atlantic movement of Leach's storm-petrels are discussed below.

Moult

The flight feather moult in wrecked Leach's storm-petrels are consistent with previous data for this species (Ainley *et al.*, 1976, Cramp and Simmons, 1977). Tail moult in birds wrecked in Canada must have started during the chick rearing period (late July to mid September; Huntingdon *et al.*, 1996), as had previously been observed at Newfoundland colonies (Hedd and Montevecchi, 2006). Wing moult was only found in birds wrecked in Europe after the breeding period, probably a strategy to reduce the overlap of these energetically demanding activities (Murphy and King, 1992, Bridge, 2006). Primaries were the first flight feathers to be dropped and showed a symmetrical ascending progression, which is important for aerodynamics and maneuverability (Thomas, 1993, Swaddle and Witter, 1997). Secondary and tertial moult in Leach's storm-petrel is poorly described (but see: Ainley *et al.*, 1976) and can be difficult to score reliably in live birds, so the opportunity to investigate this was of particular interest. The progression of growth in secondaries and tertials (S5, S6, S11, T1 and T2) suggests similar start of moult in these tracts. In general tertial and in particular secondary moult was complex. Secondary moult showed three centres of feather

replacement: S5, S11 and S1 (in respective order of growth). Progression is ascendant from S5 and S1, and descendant from S11, but showed some individual variation. Multiple focal points of moult in secondaries is a common pattern in non-passerines (Edelstam, 1984), including the closely related European storm-petrels *Hydrobates pelagicus* (Arroyo *et al.*, 2004). This moult pattern may reflect the need to reduce the size of gaps in this feather tract and limit its effect on flight capability (Edelstam, 1984). Wing symmetry in secondaries and tertials was not as high as in primaries, a pattern also found in the European storm-petrels (Arroyo *et al.*, 2004), presumably because of the relatively lower functional importance of flight feathers closer to the body (Møller and Swaddle, 1997).

The stress experienced by birds during the severe weather may have influenced progression of moult, and possibly caused loss of feathers in the wrecked birds. However, the overall patterns are consistent with other studies and therefore we believe they give a reliable snapshot of moult at these times.

Suitable feather tracts for stable isotope analysis

Knowledge of the pattern and timing of moult in a species is paramount to confidently use feather isotope signatures to infer geographic origins or movement. Although moult is well understood in many species, timing is less well known and there is much individual variation which may be problematic for isotope studies (Inger and Bearhop, 2008). Inspection of wrecked Leach's storm-petrels confirmed the onset of tail feather moult during the breeding season (Hedd & Montevecchi 2006), and therefore was assumed to reflect the isotopic signature of prey consumed at that time. Primary moult indicates that the main replacement of these feathers is during migration but timing is less clear. Outer tail and the innermost primary feather isotope signatures from the same individual are poorly correlated (Bicknell, A. unpublished data), indicating onset of

primary moult does not always take place at the breeding grounds. Variability in primary moult means caution should be taken using these feathers for isotope analyses, however it is reasonable to assume the outermost feathers (P8→P10) most likely reflect wintering areas (Cherel *et al.*, 2000). Because of the complexity both among and within individuals, application of secondary and tertiary feathers for isotope analysis would only be appropriate to broadly characterise the non-breeding period.

Stable isotope analysis

The most striking pattern found in the $\delta^{13}C$ and $\delta^{15}N$ of tail feathers was clustering of values from birds wrecked in Canada and Europe (Figure 6.4 & 6.5). This suggests these birds had been feeding in regions with similar isotopic signatures at the end of the breeding period and, potentially, that a number of Leach's storm-petrels wintering in European waters originated from Canadian colonies.

Trans-Atlantic movement of Leach's storm-petrels outside the breeding season provides evidence for potential mixing of individuals from distant colonies on migration and at winter grounds, with potential consequences for dispersal and gene flow (Webster *et al.*, 2002). Previous circumstantial evidence also hints at North American Leach' storm-petrels travelling to European waters. Estimates of birds wintering in the Bay of Biscay are far in excess of the known European populations, suggesting input from North America (Hémery and Jouanin, 1988). Therefore the high levels of gene flow between Leach's storm-petrel colonies (Chapter 2) may arise because of a combination of mixing of different populations on the wintering grounds or because of immature birds prospecting at multiple colonies during the breeding season lead to natal dispersal (Chapter 4). However, it is still unclear whether the birds wrecked in Europe and identified as of Northwest Atlantic origins were on migration, or had been displaced by

the severe weather systems. Wrecks (or observations) of native North American seabirds in Europe are extremely rare and suggests displacement of birds across the Atlantic is uncommon. Moreover, the previous genetic and isotopic evidence (Chapter 2 and 4) indicates trans-Atlantic movement occurs, providing further evidence that natural migration is most likely.

Caution needs to be taken with analyses, such as ours, that use samples of different tissues and years. Different tissue fractionation (the isotopic shift from prey to consumer) and inter-annual variation can potentially influence the results (Inger and Bearhop, 2008). However, the regional segregation seems to persist in different tissue types and the general pattern of high $\delta^{15}N$ in the Northwest Altantic was confirmed in both colony breeders and Canadian wrecked birds (Figure 6.5).

This opportunistic study of wrecked Leach's storm-petrels has helped increase our understanding of moult patterns and timing in this species, but also revealed the potentially importance of colony mixing out of the breeding season. In combination with the previous genetic and isotope analyses (Chapters 2 and 4) this presents evidence for a highly connected meta-population of Leach's storm-petrel colonies within the North Atlantic.

Chapter 7

Probable predation of storm-petrel eggs by the St Kilda field mouse

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7. Short Communication

The introduction of non-native mammals to island habitats has caused significant changes in species composition and ecosystem structure (Roemer *et al.*, 2002, Towns *et al.*, 2009), and may also lead to species extinction (Moors and Atkinson, 1984). Colonial nesting seabirds have been particularly badly affected by the introduction of ground predators, such as rats, *Rattus spp.*, feral cats *Felis catus* and North American mink *Mustela vison* (Burger and Gochfeld, 1994, Craik, 1997). As seabirds tend to breed on remote, predator free islands, they have evolved behavioural traits (burrow, ground or crevice nesting; limited chick defensive behavior) and life-history characteristics (low annual fecundity) that renders them especially vulnerable to introduced mammalian predators (Croxall and Rothery, 1991).

Introduced rats, *Rattus* spp., are considered to be the largest contributor to seabird population declines and extinctions (Jones *et al.*, 2008), facilitated by human colonization of remote islands. Mice share similar characteristics, and although were previously thought to have a negligible impact on island ecosystems (Chapuis *et al.*, 1994), recent studies on Gough Island, South Atlantic Ocean, suggest they can prey on seabird eggs and chicks at such a level to cause population declines (Wanless *et al.*, 2009).

The St Kilda island archipelago is one of the major seabird breeding stations in the North Atlantic, with internationally important populations of several species. For this reason, along with its unique cultural landscape and indigenous species, it has been designated a World Heritage Site by United Nations Educational, Scientific and Cultural Organization (UNESCO). It is home to the endemic St Kilda field mouse *Apodemus sylvaticus hirtensis*, a sub-species of the long-tailed field mouse or wood mouse *A. sylvaticus* (Berry, 1969), which has been living sympatrically with seabirds on St Kilda for at least 1000 years. Considering this is an endemic subspecies, resident on a

designated World Heritage Site, there is surprisingly little known about its breeding ecology, diet and population dynamics (but see; Harrisson and Moy-Thomas, 1933, Boyd, 1956). The two St Kilda islands known to be occupied by mice, Hirta and Dùn, have large colonies of burrow nesting Manx shearwater *Puffinus puffinus,* Atlantic Puffin *Fratercula arctica*, Leach's Storm-petrel *Oceanodroma leucorhoa* and European Storm-petrel *Hydrobates pelagicus*, but it is not known whether they form part of the mouse diet.

The Leach's Storm-petrel (hereafter LSP) population on St Kilda is of particular conservation concern due to the recent steep decline of the main colony on Dùn (a 48% decline between 1999-2003) (Newson *et al.*, 2008). This coincided with heavy predation of adult storm-petrels by a large population of Great Skuas *Stercorarius skua* (Votier *et al.*, 2006), estimated to consume approximately 15,000-21,000 adults each year (Phillips *et al.*, 1999b, Miles, 2010). It is unclear whether this level of predation will continue but declines in alternative food sources may lead to increased reliance on seabirds as prey (Votier *et al.*, 2004). Although globally numerous (~10 million breeding pairs), St Kilda colonies contain 94% of the EU's breeding LSP population. Identifying and understanding potential threats to the population is essential for effective conservation management at the European level. No formal study to determine the occurrence or impact of predation by the endemic mouse has been conducted, but during a detailed study of LSP breeding on Hirta and Dùn, evidence was obtained to indicate that they prey on eggs and possibly chicks. Here we discuss whether mouse predation could have significant implications for the sustainability of the population of LSPs on St Kilda.

Between late June and mid October 2008 45 LSP breeding burrows on Hirta, St Kilda (57°49'N, 08°35'W) were monitored using an endoscope on average every 10 days (SD

± 12 days). On each visit nest contents were recorded, as well as an assessment of possible causes of egg or chick loss.

Of the 45 nests, 11 failed at the egg stage, three at the chick stage and a further two failures at an unknown stage. This equated to an overall productivity (hatching success x fledging success; see Table 1) of 64%. Only one failure was clearly due to abandonment of the egg, while the other 15 either failed leaving a broken egg in the nest (6) or an empty nest chamber with no egg or chick (9). Broken egg remains were also found on three occasions close to or in the entrances of burrows not being monitored. Although there were no direct observations of predation, mice were regularly observed in and around LSP burrows on Hirta and Dùn, and one recorded egg showed chew marks consistent with those of a mouse (Blight and Ryder, 1999). LSPs are not known to remove broken eggs or dead chicks from burrows (Huntingdon *et al.*, 1996). Therefore our findings most likely represent predation or scavenging and as the only predatory terrestrial mammal on Hirta, this is almost certainly by the field mouse. Skuas are known predators of adult storm-petrels on St Kilda (Votier *et al.*, 2006) and although corvids have been observed excavating storm-petrel burrows (Huntingdon *et al.*, 1996), such behavior is not known on St Kilda, presumably because of the compacted substrate and long burrows.

Whether the behavior on Hirta is scavenging or predation is unclear but the ability of mice to consume seabird eggs or chicks is not in question (Wanless *et al.*, 2007). Although scavenging of unviable eggs probably occurs, the disappearance of an egg within two days of being brooded by an adult (observed once during monitoring) suggests predation of viable eggs. Egg neglect during the incubation period is not uncommon in LSPs (Wilbur, 1969) and has been observed for periods of up to three days in the Hirta colony (Money *et al.*, 2008), giving the field mouse ample time to find and remove viable eggs without encountering protective adults.

A comparison of the breeding success of LSP colonies where various mammalian and/or avian predators are present provides no clear evidence of population level effects (Table 7.1). The productivity of 64% for the Hirta colony is consistent with published estimates for colonies in North America and Japan (48-73%) with loss at the egg stage being the main cause of breeding failure (16-35%). However, mammalian and/or avian predators are present at all of the surveyed colonies, and we are unable to control for potential confounding effects of food availability, making it difficult to infer the likely impacts of predators.

Although the evidence for predation of LSP nests is circumstantial, the data suggest up to 15,000 nest failures could be a result of this behavior. Whether this level of egg/chick predation is likely to cause population decline on its own is uncertain, but a cumulative effect with high adult mortality needs to be considered. Active management of the St Kilda population to alleviate any such effects would be impractical due to the conservation status of the endemic mouse and globally restricted Great Skua population. Further research aimed at understanding the St Kilda field mouse population dynamics, behaviour and possible impacts on the LSP population is desirable. Moreover, our study further highlights the importance of mouse predation at seabird colonies and the need to consider the possible impact of these somewhat overlooked mammalian predators.

Table 7.1 Reproductive success and potential predator impact at breeding colonies of Leach's storm-petrels. Hatching success = % of laid eggs that hatched, Fledging success = % of hatched eggs that fledged, Productivity = Hatching success x Fledging success. Data from present study highlighted in bold.

Colony Site	Year(s)	Colony Size Estimate	Number of Nests Monitored	Hatching Success (HS)	Fledging Success (FS)	Productivity (P)	Mammalian Predators	Avian Predators
Kent I., New Brunswick	1955-1995	2,000-15,000[2,3] (BP)	10,041[1]	76%[1]	93%*[1]	73%*[1]	Muskrats *(Ondatra zibethicus)*(?)	Northern saw-whet owl (*Aegolius acadicus)* Herring Gull *(Larus argentatus)*
Little Duck I., Maine	1985-1989	4,000 (BP)[4]	1,253[1]	84%[1]	83%[1]	70%[1]		Herring gull *(L. argentatus)* Great Black-backed gull *(L. marinus)*
Daikoku I., Japan	1982	415,000 (BP)[5]	351[5]	75%[5]	92%[5]	69%[5]	Voles *(Clethrionomys rufocanus)*(?)	Slaty-backed gull *(Larus schistisagus)* Jungle crow *(Corvus macrorhynchos)*
Hirta, St Kilda	**2008**	**45,433 (AOS)**	**45**	**71%**	**91%**	**64%**	**Field mice *(A. sylvaticus hirtensis)***	**Great skua *(S. skua)* Snowy owl *(Nyctea scandiaca)*(?) Great Black-backed gull *(L. marinus)*(?)**
Petrel I. British Columbia	1983	10,666 (BP)[6]	86[6]	65%[6]	88%[6]	57%[6]	River otter *(Lutra canadensis)*	Northern saw-whet owl (*A. acadicus)* Northern raven *(C. corax)*(?) Northwestern crows *(C. caurinus)*(?)
Great I., Newfoundland	1982-1984	270,000 (BP)[7]	1,604[1]	68%[1]	72%[1]	48%[1]		Herring gull *(L. argentatus)* Northern raven *(C. corax)*(?) American crow *(C.brachyrhynchos)*(?)

* - n (for fledging success in years 1991-1993) = 160, BP – Breeding pairs, AOS – Apparently occupied sites, (?) – Potential predator, not documented. [1] (Huntingdon *et al.*, 1996), [2] (Cannell and Maddox, 1983), [3] (Wilbur, 1969), [4] (Chilelli, 1999), [5] (Watanuki, 1985a), [6] (Vermeer, 1988), [7] (Stenhouse and Montevecchi, 2000)

Chapter 8

General Discussion

8. Discussion

While each of the chapters in this thesis has been written as a separate piece of research in its own right, they may be integrated to provide a better understanding of Leach's storm-petrel *Oceanodroma leucorhoa* dispersal and the potential behaviours that facilitates the exchange of individuals (and genes) among distant colonies. They also intend to address the importance, and potential implications, of connectivity between naturally fragmented populations, specifically in the case of a regionally important colony that is experiencing predation from a recent colonist and a resident mammal. In this general discussion I aim to draw together the main points addressed in each chapter and discuss how the use of indirect methods has revealed aspects of Leach's storm-petrel movement and dispersal. I also consider how it may impact local and large-scale population dynamics and potentially influence this species' ability to cope with unfavourable change.

Population structure, gene flow and migrants

Our prior knowledge of genetic connectivity among Leach's storm-petrel colonies suggests gene flow within a regional (Nova Scotia and Newfoundland; Paterson and Snyder, 1999) and ocean scale (unpublished but see; Friesen *et al.*, 2007a) based on single genetic markers. In the present study we found no genetic structure across the entire north Atlantic population using two types of genetic marker, indicating potentially high gene flow and long-distance effective dispersal across the Atlantic (**Chapter 2 and 3**). Evidence strongly suggests the colonies in the Atlantic are not isolated, and the vast expanse of ocean is not a barrier to gene flow. Leach's storm-petrels would seem to exhibit a random dispersal pattern among breeding locations, which would allow gene flow to counter genetic drift more effectively and reduce population structure (Friesen *et al.*, 2007a). They are also pelagic feeders with the

potential to range widely across the oceans, especially as immature, and increase the possibility of common foraging areas that may promote interchange and reduce genetic differences between colonies (Burg and Croxall, 2004). The Pacific and Atlantic populations are genetically distinct but inter-ocean exchange was still evident with the identification of a first generation migrant. Although land is an effective barrier to gene flow among seabirds on a wider scale (Friesen *et al.*, 2007a), this migrant individual highlights this species remarkable ability to disperse between two ocean basins. The evidence of historical colonisation and gene flow also suggests movement from the Pacific to the Atlantic, followed by unobstructed colonisation and interchange within the Atlantic once suitable habitat became available. The exchange between ocean basins does beg the question "How?" Future technology may eventually answer this question directly, but currently we have to rely on several lines of indirect evidence to determine the most likely mechanisms leading to such wide-scale levels of gene-flow. The existence of two small southern hemisphere colonies off the South African coast and on the Chatham Islands, New Zealand (Huntingdon *et al.*, 1996) reveals the extent of their southern distribution and raises the possibility of movement around the southern tip of South America and Africa. Alternatively the potential for global wandering in immature birds during the "lost years" (Reich *et al.*, 2007) before breeding could also lead to wide-scale dispersal that could conceivably lead to movement between ocean basins.

Dispersal mechanisms

Genetic approaches to determine effective dispersal provide evidence for genetic connectivity and can identify individuals as potential immigrants (**Chapter 3**), but it does not reveal the dispersal mechanism that ultimately causes gene flow. Breeding site fidelity is common in seabirds in general (Schreiber and Burger, 2002, Newton, 2008) and for Leach's storm-petrels in particular (Blackmer *et al.*, 2004), and therefore this

suggests natal rather than breeding dispersal is the more likely mechanism. However, without extensive multi -year and -colony tagging studies (Dearborn *et al.*, 2003, Coulson and Coulson, 2008) it is difficult to distinguish between these two processes. Using a novel SIAR mixed model approach to estimate movement of immature Leach's storm-petrels, a proportion of these birds were found to be visiting more than one colony during a breeding season (**Chapter 4 and 5**), a likely prospecting behaviour to find a suitable breeding colony (Votier *et al.*, 2011). The approach provides evidence for dispersal and connectivity of the three sampled colonies, and given the high levels of gene flow and known breeding site fidelity (Blackmer *et al.*, 2004), suggests effective natal dispersal is the likely mechanism for gene flow in Leach's storm-petrels. However, the level of breeding dispersal was not studied in this thesis and some degree may occur, especially after breeding failure or during adverse conditions (Schmidt, 2004).

Population connectivity

There are various factors and behaviours during an organism's annual cycle that can influence population structuring and effective dispersal (Friesen *et al.*, 2007a). Prospecting of immature Leach's storm-petrels at different colonies during the breeding season (**Chapter 5**) is a behaviour that connects distant colonies in the north Atlantic and presents evidence for this to facilitate effective dispersal and gene exchange. Mixing of different populations during migration and on wintering grounds can also have an important influence on dispersal and connectivity (Webster *et al.*, 2002, Friesen *et al.*, 2007a). The evidence for mixing of Leach's storm-petrel from colonies in Canada and Europe on east Atlantic wintering grounds (**Chapter 6**) indicates that this is another potential factor that may influence population genetic structure. Conspecifics are thought to be play an important role in decision making in seabirds (Oro and Ruxton,

2001) and mixing of birds from various colonies on migration or wintering grounds presents opportunities for interactions and associations to form, which may lead to movement and prospecting at different colonies. These findings are also consistent with the limited evidence from ring recoveries and at-sea surveys. One chick and 1 breeding adult ringed at Atlantic coast colonies in North America were subsequently found dead in Spain and France in January 1988 providing clear evidence of transatlantic movement from west to east (Wernham *et al.*, 2002). Moreover counts from the Bay of Biscay at this time were estimated to be up to 2,000,000, which would far exceed the colony estimates in Europe and suggests extensive mixing of birds from other Atlantic colonies (Hémery and Jouanin, 1988). Mixing of juvenile grey-headed albatross *Thalassarche chrysostoma* from different colonies on foraging grounds has been proposed as the reason for lack of genetic structure in this species (Burg and Croxall, 2001), and mixing of immature Leach's storm-petrels on wintering grounds may be have a similar effect. The evidence provides strong support for high genetic connectivity in the north Atlantic population, potentially facilitated by behaviour of immature birds during the breeding period and mixing of birds from different colonies on the wintering grounds.

Implications for colony and population persistence

Leach's storm-petrel colonies in the north Atlantic are not genetically isolated and the movement of birds between distant locations may have important consequences for the persistence of individual colonies, as well as for the entire population. Site fidelity for species with spatially discrete breeding locations can have severe implications for growth of populations and the risk of extinction, if not coupled with dispersal to link locations or colonise new sites (Matthiopoulos *et al.*, 2005) The persistence of the St Kilda Leach's storm-petrel colony, despite the extremely high annual loss through predation, provided circumstantial evidence that this site was not isolated, and that

immigration of birds from other colonies may be offsetting the loss. The prospecting of immature Leach's storm-petrel during the breeding season could potentially have two effects at the St Kilda colony. Firstly, these birds may be consumed by great skuas reducing the numbers of breeders that are eaten. Conspicuous vocalisation during advertisement for a mate increases the risk of predation in petrels (Mougeot and Bretagnolle, 2000), and therefore prospecting birds looking to attract a partner may be more vulnerable compared with established and paired breeders. Secondly, immigrant birds may help buffer the decline with compensatory recruitment into the breeding population (Klomp and Furness, 1992, Votier *et al.*, 2008). In other words, a large pool of new recruits may offset losses of established breeders. Distinguishing between the two processes was not within the scope of this thesis but the regional isotopic distinction established in this work could potentially help. The great skua regurgitates that contain Leach's storm-petrel remains (e.g. indigestible feathers and bone), would retain isotopic signatures that may identify them as immigrant or natal birds. The proportions of immigrants would then give an indication of the extent this process plays in buffering breeder predation. The additional threat of egg predation by the St Kilda field mouse (**Chapter** 7), although not as devastating as adult mortality to seabird colonies, in combination with adult loss could increase the reliance on buffering or recruitment from natal or immigrant birds. It might also be expected that the predation threats would lead to emigration of individuals from the breeding site (Clobert *et al.*, 2001) and exacerbate the decline, but breeding site fidelity may constrain this species in the short term and mean breeding birds are unable to adapt to rapidly changing environmental conditions. The management options for alleviating the pressure of predation on St Kilda LSP population are limited due to the conservation status of the great skua and an endemic mouse, both breeding on a UNESCO World Heritage site partly designated for its wildlife. A selective cull of great skuas could benefit the petrel

population (Sanz-Aguilar *et al.*, 2009), but this may not be appropriate for a species that only breeds in Europe and for which St Kilda retains ~1.5% of the global population. To predict future viability of the St Kilda colony the combined effect of predation and dispersal on its demographic rates (i.e. growth and vital rates) need to be quantified. In the wider context of the Leach's storm-petrel Atlantic population, the connectivity and movement of birds between spatially discrete colonies suggest that it may be subject to meta-population dynamics, i.e. source-sink colonies, and the extremely large colonies in Canada have the potential to act as sources to help sustain unviable colonies, and reduce their extinction risk (Esler, 2000). These dynamics benefit sink colonies with high immigration but could also impact the source colonies (high emigration) and eventually affect the overall population persistence (Gundersen *et al.*, 2001).

Conclusions

For species that are unsuitable for direct methods of study, the application of indirect techniques is currently the only option to characterise dispersal and connectivity between populations. This approach is central to this thesis and proved valuable in revealing dispersal and movement in the Leach's storm-petrel. The genetic analysis provided the key evidence for effective dispersal and this was complemented by the stable isotope analyses to reveal a potential dispersal mechanism. Leach's storm-petrels are clearly travelling across ocean basins and only their inability to cross land (or ice) limits their movement between colonies. This immense capacity to disperse and potentially colonise new habitats could help the populations cope and persist in an increasingly changing world. It is difficult to predict the future of the St Kilda colony, but immigration from larger breeding colonies across the Atlantic may sustain it in the

short-term. However, if predation continues at the estimated levels it would seem unlikely it could be sustained in the long-term.

Appendix I

Supplementary Table 3.1 PCR conditions, fluorescent labels, allele sizes and optimized primer concentrations for microsatellite loci multiplexes. FW, forward primer; RV, reverse primer.

Multiplex	Annealing Temp (°C)	Locus	Fluorescent label	Observed allele size range (base pairs)	Concentration of FW and RV primers (μM)	PCR volume and reagents
1	61	Ole01	6FAM	213-217	0.1	2μL, Qiagen Q-mix
		Ole09	6FAM	149-153	0.1	
		Ole10	6FAM	232-238	0.1	
		Ole16	HEX	219-232	0.15	
		Ole22	HEX	249-253	0.1	
		TG03-034	6FAM	177-179	0.1	
2	61	Ole06	6FAM	198-202	0.1	2μL, Qiagen Q-mix
		Ole08	HEX	212-214	0.1	
		Ole15	HEX	232-234	0.05	
		Ole18	6FAM	147-155	0.1	
		Ole23	6FAM	404-432	0.4	
		Ole24	HEX	156-176	0.1	
3	65	Ole03	HEX	421-485	0.2	2μL, Qiagen Q-mix
		Ole05	HEX	297-353	0.2	
		Ole07	HEX	153-157	0.05	
		Ole11	6FAM	468-472	0.1	
		Ole13	HEX	242-246	0.1	
		Ole25	HEX	370-392	0.3	
4	56	Ole17	HEX	209-236	0.15	2μL, Qiagen Q-mix
		TGG12	6FAM	327-338	0.2	
		TG02-120	6FAM	236-244	0.1	
		TG04-041	HEX	176-178	0.15	
		TG13-017	HEX	204-210	0.1	
5	61	Ole14	6FAM	339-349	0.2	2μL, Qiagen Q-mix
		Ole21	HEX	316-418	0.2	
		TG04-061	HEX	161-169	0.1	
		TG05-053	6FAM	211-213	0.2	
6	56	Ol10-39	PET	134-138	0.25	10μL, Qiagen Core Kit
		Oc84	NED	302-306	0.15	
7	58	Oc63	VIC	174-188	0.1	10μL, Qiagen Core Kit
		Oc87B	PET	261-277	0.2	

Supplementary Table 3.2 Demographic history test results for Leach's storm-petrel populations in the Atlantic and Pacific oceans.

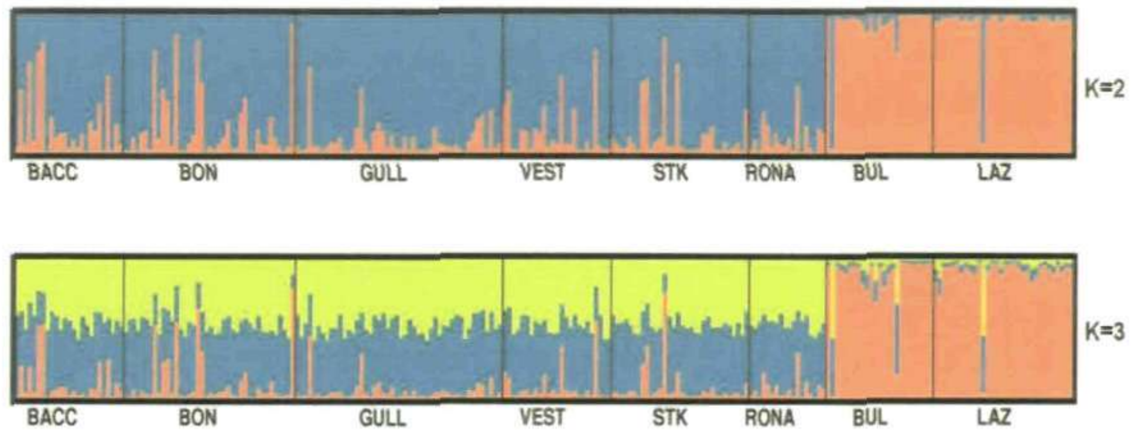
Population	Fu's F_S test	Growth (MLE)	χ^2 critical value comparison (1 df)
Atlantic	1.42	34.57	ns
Pacific	-10.88***	1150.83	<0.001***

ns, nonsignifficant; *, significant at 0.05 level; ***, significant at 0.001 level; SSD, sums of squared deviation; MLE, maximum likelihood estimate; χ^2, chi-squared; df, degrees of freedom.

Supplementary Table 3.3 Frequencies of mtDNA control region haplotypes and sample sizes (*N*) for Leach's storm-petrels from 9 colonies in the Pacific (P) and Atlantic (A) oceans. Population abbreviations are given in Table 1. **Bold** = allele present in population.

Allele	BUL	LAZ	BACC	GULL	BON	VEST	NOR	RONA	STK
N	12(P)	17(P)	12(A)	10(A)	12(A)	11(A)	7(A)	12(A)	10(A)
LSP_1	0.00	0.00	**0.58**	**0.20**	**0.50**	**0.45**	**0.57**	**0.58**	**0.70**
LSP_2	**0.08**	0.00	**0.25**	**0.40**	**0.17**	**0.09**	**0.14**	**0.25**	**0.10**
LSP_3	**0.33**	**0.18**	**0.17**	**0.30**	**0.33**	**0.36**	**0.14**	**0.17**	**0.20**
LSP_4	0.00	0.00	0.00	**0.10**	0.00	0.00	0.00	0.00	0.00
LSP_5	**0.08**	0.00	0.00	0.00	0.00	**0.09**	0.00	0.00	0.00
LSP_6	**0.08**	**0.06**	0.00	0.00	0.00	0.00	**0.14**	0.00	0.00
LSP_7	**0.08**	**0.06**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_8	**0.08**	**0.18**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_9	**0.08**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{10}	**0.08**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{11}	**0.08**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{12}	0.00	**0.06**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{13}	0.00	**0.06**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{14}	0.00	**0.18**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{15}	0.00	**0.12**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{16}	0.00	**0.06**	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSP_{17}	0.00	**0.06**	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Supplementary Figure 3.1 Proportional membership (Q) of Leach's storm-petrels to genetic clusters (K) for K=2 and K=3 as estimated by STRUCTURE with no prior information of sampling location. Each line is a single individual and individuals are ordered by sampling geographic sampling location. Colours correspond to genetic clusters.



Supplementary Material 4

Chemical composition of formalin preservatives used on the CPR survey and for storage of samples.

1. 40 % formaldehyde (100 % formalin)

Chemical constituents:

- 30 g sodium tetraborate (borax)
- 1000 ml of 37-41 % formaldehyde solution

This solution is used for filling the tank in the internal mechanisms before deployment. The borax is added to the formaldehyde for buffering purposes

2. Steedman's solution 'concentrate' approx. 13 % formaldehyde (32 % formalin)

Chemical constituents:

- 1200 ml 37-41 % formaldehyde solution
- 250 g sodium tetraborate (borax)
- 240 ml propylene phenoxytol (1 phenoxy-2 propanol)
- 2400 ml propylene glycol (propane-2, 2-diol)

This 'concentrate' mixture is used for making up the 4 % formaldehyde solution. Borax is dissolved in the 37-41 % formaldehyde solution and the propylene phenoxytol is dissolved in the propylene glycol. The formalin solution is added to the propylene solution and mixed well.

3. 4 % formaldehyde (10 % formalin)

Chemical constituents:

- Use 1 L of the 'concentrate' as described in 2.1.2
- Add 2 L of tap water and mix well

This 4 % solution is used to store unloaded routes, spray onto samples during the cutting process and when analysing samples.

Supplementary Table 5.1 Genetic variation at 18 microsatellites and a 357 bp fragment of the mitochondrial DNA control region in 3 Atlantic Leach's storm-petrel populations.

	Mitochondrial Control Region				Microsatellite Data					
Location	n	H	*h*	π	n	*A*/locus	*A*	$A_{private}$	H_O	H_E
Atlantic Colonies										
Gull Island	10	4	0.78	0.0049	48	3.89	3.16	0.03	0.45	0.45
Vestmannaeyjar	11	4	0.71	0.0067	25	3.72	3.07	0.05	0.42	0.42
St Kilda	10	3	0.51	0.0046	32	3.78	3.06	0.03	0.44	0.44

N, sample size; H, number of haplotypes; *h*, haplotype diversity; π, nucleotide diversity; A/locus, mean number of alleles per locus; A, allelic richness; $A_{private}$, private allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity.

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Probable predation of Leach's Storm-petrel Oceanodroma leucorhoa eggs by St Kilda Field Mice Apodemus sylvaticus hirtensis

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BTO

SHORT REPORT

Probable predation of Leach's Storm-petrel *Oceanodroma leucorhoa* eggs by St Kilda Field Mice *Apodemus sylvaticus hirtensis*

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Capsule Leach's Storm-petrels *Oceanodroma leucorhoa* may be depredated by endemic St Kilda Field Mice *Apodemus sylvaticus hirtensis*.

The introduction of non-native mammals to island habitats has caused significant changes in species composition and ecosystem structure (Roemer *et al*. 2002, Towns *et al*. 2009), and may also lead to species extinction (Moors & Atkinson 1984). Colonial nesting seabirds have been particularly badly affected by the introduction of ground predators, such as rats, *Rattus* spp., Feral Cats *Felis catus* and North American Mink *Mustela vison* (Burger & Gochfeld 1994, Craik 1997). As seabirds tend to breed on remote, predator-free islands, they have evolved behavioural traits (burrow, ground or crevice nesting; limited chick defensive behaviour) and life-history characteristics (low annual fecundity) that render them especially vulnerable to introduced mammalian predators (Croxall & Rothery 1991).

Introduced rats, *Rattus* spp., are considered to be the largest contributor to seabird population declines and extinctions (Jones *et al*. 2008), facilitated by human colonization of remote islands. Mice share similar characteristics, and although were previously thought to have a negligible impact on island ecosystems (Chapuis *et al*. 1994), recent studies on Gough Island, South Atlantic Ocean, suggest they can prey on seabird eggs and chicks at such a level to cause population declines (Wanless *et al*. 2009).

The St Kilda island archipelago is one of the major seabird breeding stations in the North Atlantic, with internationally important populations of several species. For this reason, along with its unique cultural landscape and indigenous species, it has been designated a World Heritage Site by the United Nations Educational, Scientific and Cultural Organization (UNESCO). It is home to the endemic St Kilda Field Mouse *Apodemus sylvaticus hirtensis*, a sub-species of the Long-tailed Field Mouse or Wood Mouse *A. sylvaticus* (Berry 1969), which has been living sympatrically with seabirds on St Kilda for at least 1000 years. Considering this is an endemic subspecies, resident on a designated World Heritage Site, there is surprisingly little known about its breeding ecology, diet and population dynamics (but see Harrisson & Moy-Thomas 1933, Boyd 1956). The two St Kilda islands known to be occupied by mice, Hirta and Dùn, have large colonies of burrow-nesting Manx Shearwater *Puffinus puffinus*, Atlantic Puffin *Fratercula arctica*, Leach's Storm-petrel *Oceanodroma leucorhoa* and European Storm-petrel *Hydrobates pelagicus*, but it is not known whether they form part of the mouse diet.

The Leach's Storm-petrel (hereafter LSP) population on St Kilda is of particular conservation concern due to the recent steep decline of the main colony on Dùn (a 48% decline between 1999 and 2003) (Newson *et al*. 2008). This coincided with heavy predation of adult Storm-petrels by a large population of Great Skuas *Stercorarius skua* (Votier *et al*. 2006), estimated to consume approximately 15 000 adults each year (Phillips *et al*. 1999). It is unclear whether this level of predation will continue but declines in alternative food sources may lead to increased reliance on seabirds as prey (Votier *et al*. 2004). Although globally numerous (~10 million breeding pairs), St Kilda colonies contain 94% of the EU's breeding LSP population. Identifying and understanding

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potential threats to the population is essential for effective conservation management at the European level. No formal study to determine the occurrence or impact of predation by the endemic mice has been conducted, but during a detailed study of LSP breeding on Hirta and Dùn, evidence was obtained to indicate that they prey on eggs and possibly chicks.

Here we discuss whether mouse predation could have significant implications for the sustainability of the population of LSPs on St Kilda.

Between late June and mid-October 2008 45 LSP breeding burrows on Hirta, St Kilda (57°49'N, 08°35'W) were monitored using an endoscope on average every 10 days (sd ± 12 days). On each visit nest contents were recorded, as well as an assessment of possible causes of egg or chick loss.

Of the 45 nests, 11 failed at the egg stage, 3 at the chick stage and a further 2 failures at an unknown stage. This equated to an overall productivity (hatching success × fledging success; see Table 1) of 64%. Only 1 failure was clearly due to abandonment of the egg, while the other 15 either failed leaving a broken egg in the nest (6) or an empty nest chamber with no egg or chick (9). Broken egg remains were also found on three occasions close to or in the entrances of burrows not being monitored. Although there were no direct observations of predation, mice were regularly observed in and around LSP burrows on Hirta and Dùn, and one recorded egg showed chew marks consistent with those of a mouse (Blight & Ryder 1999). LSPs are not known to remove broken eggs or dead chicks from burrows (Huntingdon *et al.* 1996). Therefore, our findings most likely represent predation or scavenging and as the only predatory terrestrial mammal on Hirta, this is almost certainly by field mice. Skuas are known predators of adult Storm-petrels on St Kilda (Phillips *et al.* 1999, Votier *et al.* 2006) and although corvids have been observed excavating Storm-petrel burrows (Huntingdon *et al.* 1996), such behaviour

Table 1. Reproductive success and potential predator impact at breeding colonies of Leach's Storm-petrels. Hatching success (HS) = % of laid eggs that hatched; fledging success (FS) = % of hatched eggs that fledged; productivity (P) = HS × FS. Data from present study are highlighted in bold.

Colony site	Year(s)	Colony size estimate	Number of nests monitored	HS	FS	P	Mammalian predators	Avian predators
Kent I., New Brunswick	1955–95	2000–15000[2,3] (BP)	10041[1]	76%[1]	93%*[1]	73%*[1]	Muskrats (*Ondatra zibethicus*) (?)	Northern Saw-whet Owl (*Aegolius acadicus*) Herring Gull (*Larus argentatus*)
Little Duck I., Maine	1985–89	4000 (BP)[4]	1253[1]	84%[1]	83%[1]	70%[1]		Herring Gull (*L. argentatus*) Great Black-backed Gull (*L. marinus*)
Daikoku I., Japan	1982	415000 (BP)[5]	351[5]	75%[5]	92%[5]	69%[5]	Voles (*Clethrionomys rufocanus*) (?)	Slaty-backed Gull (*Larus schistisagus*) Jungle Crow (*Corvus macrorhynchos*)
Hirta, St Kilda	**2008**	**45433 (AOS)**	**45**	**71%**	**91%**	**64%**	**Field Mice (*A. sylvaticus hirtensis*)**	**Great Skua (*S. skua*) Snowy Owl (*Nyctea scandiaca*) (?) Great Black-backed Gull (*L. marinus*) (?)**
Petrel I. British Columbia	1983	10666 (BP)[6]	86[6]	65%[6]	88%[6]	57%[6]	River Otter (*Lutra canadensis*)	Northern Saw-whet Owl (*A. acadicus*) Northern Raven (*C. corax*) (?) Northwestern Crow (*C. caurinus*) (?)
Great I., Newfoundland	1982–84	270000 (BP)[7]	1604[1]	68%[1]	72%[1]	48%[1]		Herring Gull (*L. argentatus*) Northern Raven (*C. corax*) (?) American Crow (*C. brachyrhynchos*) (?)

*n (for fledging success in years 1991–1993) = 160; BP – breeding pairs; AOS – apparently occupied sites, (?) – potential predator, not documented. [1]Huntingdon *et al.* (1996); [2]Cannell & Maddox (1983); [3]Wilbur (1969); [4]Chilelli (1999); [5]Watanuki (1985); [6]Vermeer (1988); [7]Stenhouse & Montevecchi (2000)

is not known on St Kilda, presumably because of the compacted substrate and long burrows.

Whether the behaviour on Hirta is scavenging or predation is unclear but the ability of mice to consume seabird eggs or chicks is not in question (Wanless *et al.* 2007). Although scavenging of unviable eggs probably occurs, the disappearance of an egg within two days of being brooded by an adult (observed once during monitoring) suggests predation of viable eggs. Egg neglect during the incubation period is not uncommon in LSPs (Wilbur 1969) and has been observed for periods of up to three days in the Hirta colony (Money *et al.* 2008), giving Field Mice ample time to find and remove viable eggs without encountering protective adults.

A comparison of the breeding success of LSP colonies where various mammalian and/or avian predators are present provides no clear evidence of population level effects (Table 1). The productivity of 64% for the Hirta colony is consistent with published estimates for colonies in North America and Japan (48–73%) with loss at the egg stage being the main cause of breeding failure (16–35%). However, mammalian and/or avian predators are present at all of the surveyed colonies, and we are unable to control for potential confounding effects of food availability, making it difficult to infer the likely impacts of predators.

Although the evidence for predation of LSP nests is circumstantial, the data suggest up to 15 000 nest failures could be a result of this behaviour. Whether this level of egg/chick predation is likely to cause population decline on its own is uncertain, but a cumulative effect with high adult mortality needs to be considered. Active management of the St Kilda population to alleviate any such effects would be impractical due to the conservation status of the endemic mice and globally restricted Great Skua population. Further research aimed at understanding the St Kilda Field Mouse population dynamics, behaviour and possible impacts on the LSP population is desirable. Moreover, our study further highlights the importance of predation by mice at seabird colonies and the need to consider the possible impact of these somewhat overlooked mammalian predators.

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Effects of formalin preservation on stable carbon and nitrogen isotope signatures in Calanoid copepods: implications for the use of Continuous Plankton Recorder Survey samples in stable isotope analyses

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Preserved and archived organic material offers huge potential for the conduct of retrospective and long-term historical ecosystem reconstructions using stable isotope analyses, but because of isotopic exchange with preservatives the obtained values require validation. The Continuous Plankton Recorder (CPR) Survey is the most extensive long-term monitoring program for plankton communities worldwide and has utilised ships of opportunity to collect samples since 1931. To keep the samples intact for subsequent analysis, they are collected and preserved in formalin; however, previous studies have found that this may alter stable carbon and nitrogen isotope ratios in zooplankton. A maximum ~0.9‰ increase of $\delta^{15}N$ and a time dependent maximum ~1.0‰ decrease of $\delta^{13}C$ were observed when the copepod, *Calanus helgolandicus*, was experimentally exposed to two formalin preservatives for 12 months. Applying specific correction factors to $\delta^{15}N$ and $\delta^{13}C$ values for similarly preserved Calanoid species collected by the CPR Survey within 12 months of analysis may be appropriate to enable their use in stable isotope studies. The isotope values of samples stored frozen did not differ significantly from those of controls. Although the impact of formalin preservation was relatively small in this and other studies of marine zooplankton, changes in isotope signatures are not consistent across taxa, especially for $\delta^{15}N$, indicating that species-specific studies may be required. **Copyright © 2011 John Wiley & Sons, Ltd.**

In marine ecosystems stable isotope analysis (SIA) has become a familiar technique for investigating trophic relationships,[1] food web structure[2] and movement behaviour.[3,4] The use of preserved and/or archived organic material offers huge potential for the conduct of retrospective analyses and investigate medium to long-term ecosystem changes. Most natural history museums and many other institutions hold large preserved collections that could be a major resource for such studies. However, there are uncertainties over the effect of different preservatives on the stable carbon and nitrogen isotope values, which is perhaps reflected in the scarcity of studies that utilise collections of preserved biological material. Understanding the effects of preservatives on stable isotope signatures is crucial for the appropriate interpretation of results from ecological studies.

Ideally, the chemicals used to preserve biological material should not alter their molecular composition, but in many instances this is not the case.[5–7] The reported changes ($\delta^{13}C$: <3‰, $\delta^{15}N$: <1‰) lack consistency in these effects,[6,8] and this precludes general conclusions being reached for particular preservatives. A range of preservatives has been used for short- or long-term collection storage and these preservatives have all been shown to have different impacts,[8] so require separate investigation before collection samples can be used in SIA.

The Sir Alister Hardy Fountain for Ocean Science (SAHFOS, Plymouth, UK) maintains the Continuous Plankton Recorder (CPR) Survey, which is one of the longest and geographically the most extensive measures of plankton communities worldwide. Since 1931 ships of opportunity (such as commercial and passenger vessels) have towed recorders for approximately 5.8 million nautical miles, continuously collecting plankton. The core survey operates in the North Atlantic but it has grown in scope to include the North Pacific, the Southern Ocean and the Western Atlantic. CPR data has been integral to understanding how the ecosystem function is regulated in the North Atlantic[9–12] leading to further insights into the indirect effects of climate change on North Sea commercial fish stocks.[13] The isotopic information available within the CPR Survey samples has great potential for large-scale analysis of food-web structures,

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historical ecosystem shifts and use as baseline marine ecosystem data. The CPR Survey uses a formalin solution to preserve plankton samples while vessels are at sea and later, when archived before analysis. In 2008 two formalin grades with different methanol content were used on survey routes to check the quality of sample preservation, but have subsequently returned to the original formalin mix. Before samples from this extensive plankton collection can be confidently used in stable isotope studies, the impact of formalin preservatives on stable isotope ratios need to be clarified.

Copepods dominate the global mesozooplankton biomass[14] and are an integral part of the transfer of carbon from marine primary producers to higher trophic level species. Calanoid species are key components of marine ecosystems. Their isotopic signatures have been valuable in investigating food web structures[2,15] and predator-prey relationships,[16] and they have great potential for isotopic characterisation of ocean regions for use in animal foraging or migration studies.[3,17] To evaluate the impact of formalin preservation on stable carbon and nitrogen isotope ratios in calanoid copepods, fresh samples of *Calanus helgolandicus* were used in a controlled experiment. Here we report the effect of two types of formalin preservative (used on the CPR Survey) and freezing, over a 12-month period. We discuss the impact that this has on the use of these and other CPR Survey samples in ecological stable isotope studies.

EXPERIMENTAL

Study species

C. helgolandicus is a calanoid copepod found over a range of marine habitats (i.e. open ocean, coastal environments) in the North Atlantic and contributes between 6% and 93% to the mesozooplankton biomass in European waters.[18] These copepods consume a diverse diet of protozooplankton and phytoplankton[19] and are themselves an important source of food for juvenile fish.[11] They are a temperate water species generally found in 9–20 °C waters, with highest abundance from 13 to 17 °C, and development to the adult form takes between 26 and 42 days.[18] In recent decades, *C. helgolandicus* has become more abundant and widespread in the northeast Atlantic, which has coincided with a northward shift in the distribution of the cold-water congener *C. finmarhicus*.[18] The sensitivity of these two species to changes in water temperature apparently underlies this shift, and for this reason they are useful indicators of global change in marine environments.

Sampling method and preservation

Zooplankton samples were taken by the Dove Marine Laboratory (Newcastle University, Newcastle, UK) from the North Sea, close to the Northumberland coast of England (55°07′N, 01°20′W), using a 200 µm meshed WP2 plankton net in vertical hauls from 20 m depth to the surface on 9 June 2009. Fresh *C. helgolandicus* from hauls were frozen and transported to Plymouth (UK) where they were placed in fresh seawater for several hours to allow gut evacuation. We removed CV and CVI adult *C. helgolandicus* and sub-divided these into five different groups: those analysed immediately (control) and those preserved for 3 weeks, 2 months, 6 months and 12 months in five different treatments (see below). There were four replicates for the control group and a minimum of three replicates (3–6) for each treatment/time group. Each replicate contained between 10 and 15 individuals (to provide sufficient material for subsequent samples) and were stored in separate glass vials at room temperature (excluding the frozen treatment).

The formalin preservatives used for the treatments were supplied by SAHFOS and are currently in use (Type B), or have been used (Type A), on CPR Survey routes and for storage of collected samples. The majority of the chemical constituents of the mixtures were the same (Appendix I, see Supporting Information) but the supplier and amount of methanol in the formaldehyde were different: Type A = Fisher Scientific, Loughborough, UK (Technical grade, 37–41% formaldehyde containing 10–14% methanol), Type B = Alpha-Aesar, Heysham, UK (Technical grade, 37% formaldehyde containing 7–8% methanol). The treatments involve both types of formalin preservatives and/or a simulation of the change in concentrations during the collection and storage of CPR samples. For the latter, a 3-week initial period of high concentration mix and seawater dilution represents the potential time between collection of samples and delivery of the CPR device back to SAHFOS for unloading. The treatments (preservation methods) are summarised in Table 1. The type of formalin used for each treatment were also analysed to determine their $\delta^{13}C$ values.

Lipid synthesis in organisms discriminates against ^{13}C[20] and yields low $\delta^{13}C$ values in tissues with high lipid content compared with those from an organism's dietary input.[21] The lipid content in marine copepods is highly variable between species (range of percentage dry weight: 2–73%),[22] and can depend largely on their life history.[23] To eliminate the effect of lipids on the carbon isotope ratios in *C. helgolandicus* and to generate results with general relevance to other calanoid species, all samples were lipid-extracted. The samples were also decalcified to remove inorganic carbon from the copepods exoskeleton, since these carbonates tend to be increased in $\delta^{13}C$ and do not reflect dietary intake.[24] Other biochemical compounds contributing to $\delta^{13}C$ values such as proteins, carbohydrates and chitin have been found to be similar across zooplankton taxonomic groups and habitats,[22] and were therefore not altered in any way.

Once the samples had been freeze-dried and crushed, the lipids were extracted using 7% methanol in dichloromethane by volume (7% M DCM) and then dried overnight in a laboratory oven. The samples were added to 2 N hydrochloric acid for 1 h to remove inorganic carbonates and distilled water was used to rinse away acid and waste. The samples were oven-dried overnight in preparation for stable isotope analysis.

Sample analysis

Homogenised *C. helgolandicus* samples of approximately 0.70 mg were weighed and placed in tin capsules (5 × 3.5 mm). Formalin samples were placed in solid, flat-bottomed tin capsules (5 mm × 2 mm) and cold-sealed with wire cutters. Each formalin sample was prepared less than 2 min before analysis, to minimise evaporation. Analyses were conducted

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Table 1. Experimental treatments used to preserve *C. helgolandicus* samples

Treatment ID	Description
T1	Frozen at −20 °C
T2	Type A 40% formaldehyde mix with borax diluted to 4% with seawater (for first 3 weeks), then changed to Type A 4% formaldehyde mix
T3	Type A 4% formaldehyde mix
T4	Type B 40% formaldehyde mix with borax diluted to 4% with seawater (for first 3 weeks), then changed to Type B 4% formaldehyde mix
T5	Type B 4% formaldehyde mix

Table 2. Mean (±standard error) $\delta^{13}C$ and $\delta^{15}N$ values of fresh *C. helgolandicus* and the differences (±1 standard error) from fresh values for treated samples over 12 months. *C:N* – ratio of carbon to nitrogen atoms, *%C* – percentage dry weight of carbon, *%N* – percentage dry weight of nitrogen. # and * indicates significant difference from fresh at $P<0.05$ (ANOVA and Tukey's HSD post-hoc test respectively)

	Fresh (‰)	±S.E.	T1 (‰)	T2 (‰)	T3 (‰)	T4 (‰)	T5 (‰)
$\delta^{13}C$	−19.34	0.26					
3 weeks			0.07 ± 0.06	−0.49 ± 0.08	−0.43 ± 0.08	−0.46 ± 0.05	−0.26 ± 0.13
2 months			0.10 ± 0.13	−0.42 ± 0.02	−0.39 ± 0.06	−0.53 ± 0.06	−0.29 ± 0.13
6 months			−0.29 ± 0.05	−0.99 ± 0.03*	−0.79 ± 0.11*	−0.83 ±0.04*	−0.77 ± 0.11*
12 months			−0.15 ± 0.10	−0.68 ± 0.03*	−0.93 ± 0.08*	−0.88 ±0.05*	−0.55 ± 0.12
Overall			−0.07 ± 0.06	−0.64 ± 0.06#	−0.66 ± 0.07#	−0.67 ±0.05#	−0.48 ± 0.08#
$\delta^{15}N$	9.56	0.11					
3 weeks			0.48 ± 0.17	0.57 ± 0.08	0.33 ± 0.12	0.75 ± 0.09*	0.60 ± 0.07
2 months			0.15 ± 0.38	0.19 ± 0.19	0.84 ± 0.10*	0.92 ± 0.10*	0.67 ± 0.21*
6 months			0.09 ± 0.25	0.77 ± 0.12	0.73 ± 0.11*	0.51 ± 0.16	0.67 ± 0.16*
12 months			−0.03 ± 0.14	0.33 ± 0.22	0.49 ± 0.12*	0.67 ± 0.16*	0.80 ± 0.08*
Overall			0.17 ± 0.12	0.46 ± 0.09#	0.60 ± 0.07#	0.73 ± 0.07#	0.68 ± 0.07#
C:N	3.95	0.13					
3 weeks			0.12 ± 0.03	0.08 ± 0.01	0.07 ± 0.02	0.02 ± 0.01	0.01 ± 0.00
2 months			0.04 ± 0.04	0.03 ± 0.03	0.05 ± 0.02	0.16 ± 0.03	0.13 ± 0.03
6 months			−0.01 ± 0.04	−0.07 ± 0.02	−0.03 ± 0.02	0.02 ± 0.02	−0.05 ± 0.02
12 months			0.04 ± 0.02	−0.08 ± 0.07	−0.12 ± 0.02	−0.01 ± 0.04	−0.02 ± 0.04
Overall			0.05 ± 0.05	−0.01 ± 0.02	−0.01 ± 0.02	0.05 ± 0.16	0.01 ± 0.02
%C	46.02	0.74					
3 weeks			−0.57 ± 0.18	−0.81 ± 0.12	−1.45 ± 0.37	−1.22 ± 0.29	−1.39 ± 0.11
2 months			0.37 ± 0.33	−0.42 ± 0.26	−1.10 ± 0.26	−0.69 ± 0.18	−0.93 ± 0.20
6 months			−1.46 ± 5.44	0.19 ± 0.81	−1.46 ± 0.33	−0.62 ± 0.92	−3.09 ± 0.86
12 months			1.22 ± 4.44	1.51 ± 1.63	1.89 ± 1.82	0.23 ± 2.07	−1.32 ± 4.00
Overall			−0.11 ± 1.53	0.14 ± 0.50	−0.53 ± 0.53	−0.57 ± 0.54	−1.77 ± 0.90
%N	11.66	0.24					
3 weeks			−0.50 ± 0.13	−0.42 ± 0.05	−0.55 ± 0.15	−0.32 ± 0.06	−0.36 ± 0.03
2 months			0.00 ± 0.19	−0.17 ± 0.11	−0.42 ± 0.09	−0.64 ± 0.08	−0.61 ± 0.14
6 months			−0.31 ± 1.40	0.28 ± 0.23	−0.25 ± 0.11	−0.16 ± 0.21	−0.59 ± 0.19
12 months			0.21 ± 1.14	0.60 ± 0.54	0.84 ± 0.46	0.13 ± 0.43	−0.27 ± 1.11
Overall			−0.15 ± 0.40	0.07 ± 0.18	−0.10 ± 0.16	−0.25 ± 0.14	−0.47 ± 0.24

T1 = Treatment 1, T2 = Treatment 2, T3 = Treatment 3, T4 = Treatment 4, T5 = Treatment 5 (described in Table 1).
‰ = parts per thousand deviation from standard.

at the East Kilbride Node of the Natural Environment Research Council Life Sciences Mass Spectrometry Facility via continuous flow isotope ratio mass spectrometry using a ECS 4010 elemental analyser (Costech, Milan, Italy) interfaced with a Delta XP mass spectrometer (Thermo Electron, Bremen, Germany). Isotope ratios are reported as δ-values and expressed as ‰ according to the equation $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ and $R_{standard}$ is the ratio of the international references Pee Dee Belemnite (PDB) for carbon and AIR for nitrogen. The standard deviation of multiple analyses of an internal gelatine standard in each experiment was better than 0.2‰ for $\delta^{15}N$ and $\delta^{13}C$.

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Data analysis

The effects of preservation method and time on the carbon and nitrogen stable isotope and element ratio values were analysed by comparing preserved samples with fresh samples (control) using analysis of variance (ANOVA) and Tukey's HSD post-hoc multiple pairwise comparison tests. All data were tested for normality and homogeneity of variance. Statistical analyses were conducted using R version 2.12.0.[25]

RESULTS

Overall effect of formalin

In general formalin preservation and the amount of time for which the samples had been preserved influenced both the $\delta^{13}C$ (Treatment: $F_{4,77} = 20.66$, $P < 0.0001$, Time: $F_{4,77} = 25.07$, $P < 0.0001$) and the $\delta^{15}N$ values (Treatment: $F_{4,78} = 3.09$, $P = 0.02$, Time: $F_{4,78} = 5.96$, $P = 0.0003$) in *C. helgolandicus* samples. Compared with fresh samples all the formalin treatment samples (T2–T5) were significantly depleted in $\delta^{13}C$ (mean range = −0.48 to −0.67, all P values <0.05) and significantly enriched in $\delta^{15}N$ (mean range = 0.46 to 0.74, P values <0.05) (Table 2).

Effect of time stored in formalin on $\delta^{13}C$ and $\delta^{15}N$

The amount of time for which samples were preserved in formalin had a pronounced effect on $\delta^{13}C$ (Fig. 1). $\delta^{13}C$ decreased significantly in all formalin treatments after 6 months and 12 months (all Tukey's HSD tests, $P < 0.05$), except for treatment 5 where the value did not change significantly after 12 months (Tukey's HSD test, $P = 0.16$). In contrast, $\delta^{15}N$ values changed significantly when *C. helgolandicus* were stored in formalin; however, there was no obvious trend over time (Fig. 2). After Tukey's multiple comparison tests, the $\delta^{15}N$ values from treatment 2 were not significantly different from those from the fresh samples at different time intervals, although the overall enrichment was significant ($F_{1,20} = 5.18$, $P = 0.03$; Table 2). The increase in $\delta^{15}N$ was significant after 3 weeks in treatment 4 (Tukey's HSD test $P = 0.004$) and 2 months in treatments 3 and 5 (Tukey's HSD tests, T3: $P < 0.001$, T5: P 0.034). This significant ^{15}N enrichment generally continued up to 12 months in treatments 4 and 5 but reduced for the final sampling period in treatment 3 (Tukey's HSD test, $P = 0.06$; Fig. 2).

Effect of freezing samples on $\delta^{13}C$ and $\delta^{15}N$

The frozen samples (T1) were not significantly different from fresh samples for both $\delta^{13}C$ ($F_{4,11} = 0.85$, $P = 0.52$) and $\delta^{15}N$ ($F_{4,11} = 0.87$, $P = 0.51$), but were significantly different from samples that had undergone formalin treatments (all Tukey's HSD tests, $P < 0.001$) except for the $\delta^{15}N$ values from treatment 2 (Tukey's HSD test, $P = 0.18$). The $\delta^{15}N$ values appeared to change over time for samples stored frozen (Fig. 2), but these changes were not statistically significant.

%C, %N, C:N ratio and formalin

Change in the ratio of carbon to nitrogen (*C:N*) across treatments compared with fresh sample values was minor (Table 2; all Tukey's HSD test, $P > 0.05$). Significant deviation from homogeneity of variance was found over the duration of the experiment in treatments 3 and 5 for percentage dry weight of carbon (%C Levene's test, T2: $F_{4,20} = 3.087$, $P = 0.04$, T4: $F_{4,16} = 4.699$, $P = 0.01$) and nitrogen (%*N* Levene's test, T2: $F_{4,20} = 3.093$, $P = 0.04$, T4: $F_{4,16} = 5.030$, $P = 0.01$). The mean $\delta^{13}C$ values (±1 SD) were similar for formalin preservative with the same chemical constituents across manufacturers: (1) 40% formaldehyde mix with borax diluted to 4% with seawater, Type A = −44.63 ± 0.05‰, Type B = −44.07 ± 0.56‰. (2) 4% formaldehyde mix, Type A = −29.52 ± 0.24‰, Type B = −29.94 ± 0.30‰.

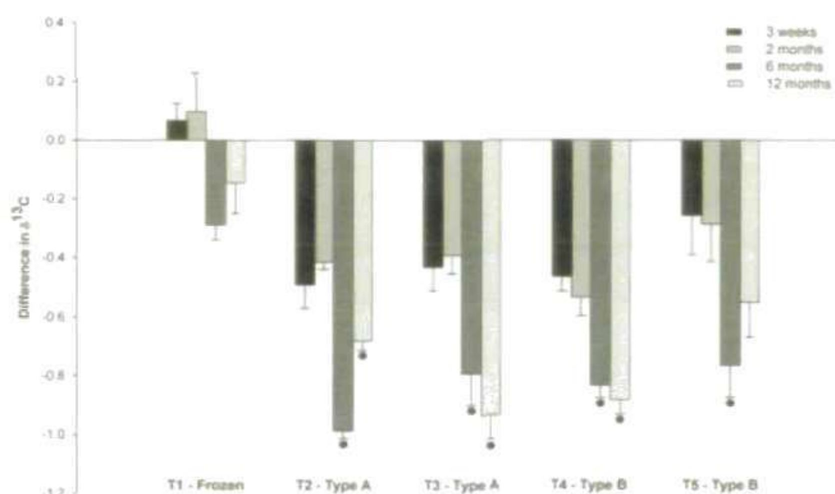

Figure 1. Mean (±standard error) difference in $\delta^{13}C$ values after 3 weeks, 2 months, 6 months and 12 months for the five preservative treatments from those of the fresh control samples (0.0 on y axis). T1–T5 treatments as described in Table 1. Black spots represent significant differences from control samples ($P < 0.05$).

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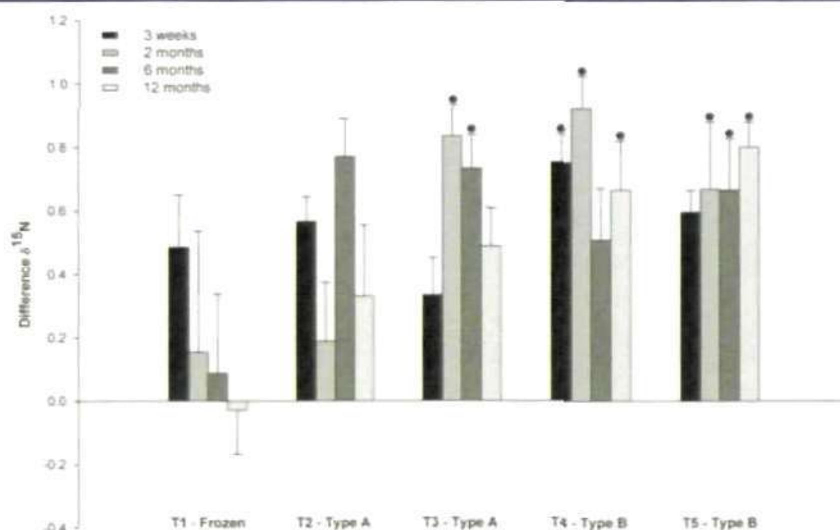

Figure 2. Mean (±standard error) difference in δ^{15}N values after 3 weeks, 2 months, 6 months and 12 months for the five preservative treatments from those of the fresh control samples (0.0 on y axis). T1–T5 treatments as described in Table 1. Black spots represent significant differences from control samples ($P < 0.05$).

DISCUSSION

Our study provides evidence for a significant effect of formalin preservation on the stable carbon and nitrogen isotope values in *C. helgolandicus* over time, but there was no significant difference between batches of samples stored frozen and room temperature controls over the same period. The possible mechanisms involved in the isotopic alteration and the implications that these preservative effects have for the use of *C. helgolandicus* and other CPR zooplankton samples in stable isotope analyses, are discussed below.

Effects of formalin on δ^{13}C and δ^{15}N

The decrease in δ^{13}C for *C. helgolandicus* samples stored in formalin is consistent with the majority of previous studies investigating the effect of formalin preservation on a variety of marine, freshwater and terrestrial species.[8,26] However, the degree of depletion (~1‰) was considerably less than reported in the only other study on the effect of formalin on marine zooplankton (2–3‰).[27] The results also indicate that the isotope signatures continue to change with time, although it took approximately 6 months of preservation for this effect to become statistically significant. The only other previously observed depletion over time was in a freshwater clam *Corbicula fluminea*, although the fresh samples in this study were extremely depleted, resulting in an overall enrichment of δ^{13}C[5] and highlighting the difficulties in predicting these effects. Two mechanisms have been proposed to account for the depletion of ^{13}C. First, the formalin preservative promotes the leaching of compounds enriched in ^{13}C from the tissues and therefore modifies the apparent carbon isotope signature.[28,29] The formaldehyde hydrolyses proteins that are enriched in ^{13}C compared with lipids, so depletion in tissue ^{13}C could reflect the relative increase in lighter lipid carbon. However, given that we extracted lipids from the *C. helgolandicus* samples before stable isotope analysis this seems unlikely. Secondly, the uptake of isotopically lighter carbon from the formalin into the tissues could shift the signature toward that of the preservative.[26,29–31] The low δ^{13}C values found in the formalin used in this study (−29‰ and −45‰) would therefore suggest the incorporation of lighter carbon from the preservative is the most likely explanation for this effect. The composition and/or manufacture of the formalin preservative may contribute to the degree of carbon uptake into tissues.[32] Although the samples that were subjected to the lowest concentration of formaldehyde and methanol (T5 – Type B 4% formalin) showed slightly less overall ^{13}C depletion, there was little evidence for differences between the two manufacturers and formalin composition used in this experiment. The comparable formalin δ^{13}C values between manufacturers may have resulted in these similarities.

Previous work has shown highly variable and somewhat contradictory impacts of formalin preservation on δ^{15}N,[8] although generally the effects are slight with only one study showing an effect of >1.0‰.[31] Here we found a significant increase in δ^{15}N values for *C. helgolandicus* stored in formalin that is consistent with previous work on freshwater zooplankton (0.8‰ increase),[33] but contrasts with a significant ^{15}N depletion shown for marine zooplankton (decrease <1‰).[27] Formalin does not contain nitrogen so uptake of any preservative fractions into tissues will have no effect on δ^{15}N values. Instead the effect of formalin is through disproportionate hydrolysis or leaching of isotopically lighter or heavier materials.[29–31] For *C. helgolandicus* and the type of formalin used in our study this seems to be the loss of ^{14}N to the preservative with the resulting increase of δ^{15}N, although an expected increase in the *C:N* ratio with loss of nitrogen was not observed. The increase was slightly higher in the Type B formalin mix, which is surprising because it contains slightly less formaldehyde (the hydrolysing agent) than the Type A mix, but may be a result of different manufacturing processes or raw materials.[34] It is apparent from this study and

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previous reports that the effect of formalin on $\delta^{15}N$ is not as predictable as for $\delta^{13}C$ and therefore requires species-specific experiments.

Use of CPR zooplankton in ecological stable isotope analyses

Significant changes in $\delta^{13}C$ and $\delta^{15}N$ values of *C. helgolandicus* stored in formalin could potentially bias stable isotope values if unchecked. The ~0.6‰ increase in $\delta^{15}N$ observed in this study could be regarded as minor in relation to the 2–3.5‰ shift found between consumers and prey,[35,36] so may not be significant if used in stable isotope trophic position and food-web studies. However, comparing preserved and unpreserved samples could produce misleading results and a simple correction factor should be considered on a species-specific basis in this situation.[37] The increase in $\delta^{13}C$ per trophic level is much more conservative (<1‰) and more closely represents the primary carbon source of the marine ecosystem. An approximate 0.6‰ ^{13}C depletion in samples due to preservation could therefore lead to biased results if used in multiple isotope food web analysis or for regional baseline ecosystem signatures in migration studies. These analyses rely on appreciable distinction between $\delta^{13}C$ values and any bias will be most pronounced when the differences are relatively small. It has previously been proposed that, as long as the $\delta^{13}C$ difference is more than 2.0‰, an equivalent depletion in preserved specimens (−2.0‰) will not obscure the results.[37] For study systems where differences in carbon sources are considerable, i.e. C_3 and C_4 plants (>14‰),[38] terrestrial vs. marine (>7‰),[39] or known through previous research, the small effect of formalin preservation may be unimportant, but $\delta^{13}C$ differences can be small and gradual over large spatial scales in marine systems.[40] The effect of preservation will be problematic if the results are ambiguous and correction of $\delta^{13}C$ values would be appropriate in these instances.

The shifts of $\delta^{13}C$ and $\delta^{15}N$ in this study are consistent in direction across treatments but show variation in strength with time, making it difficult to predict the degree of change beyond the study period. The majority of archived samples collected by the CPR Survey have been preserved for much longer and the effect on these samples cannot be confidently estimated from this study. However, for *C. helgolandicus* samples collected by the CPR Survey within 12 months of analysis, a correction factor specific to the type of formalin and time preserved may be appropriate to counter the effect and to enable their use in stable isotope studies. The extraction of lipids before analysis removed the high variation of this compound found in marine Calanoid copepod species and should enable any corrections to be applied to other *Calanus* species, such as *C. finmarchicus*, collected and preserved by the CPR in the previous 12 months.

CONCLUSIONS

Our experiment was designed to establish whether the formalin preservative used to collect and store samples on CPR Survey routes significantly changes the signature of a zooplankton species over 12 months and how this may impact on its use in ecological stable isotope studies. For *Calanus* copepods the preservative significantly affects the carbon and nitrogen ratios but the lack of a predictable trend or stability in results does not allow us to estimate the effect on preserved samples older than 12 months. However, the application of specific correction factors may be appropriate within this period to remove any bias in stable isotope analyses. The ubiquitous nature of this genus in the marine environment makes it ideal for use in large-scale ecological studies and the long-term, global collection of samples available through the CPR Survey could be a valuable resource to be used in contemporary and historical isotope analysis. However, our findings and the contradictory results found in other studies investigating formalin preservation would suggest there is no general correction for this preservative and that its effect needs to be experimentally established for individual species or genus specifically, over periods applicable to the preserved collection. Caution should be taken when deciding whether to use formalin-preserved samples in stable isotope analyses. The preservation effect and isotopic differences within the study system need careful consideration when assessing the utility of the samples for the proposed research and, if used, whether or not correction factors are appropriate.

SUPPORTING INFORMATION

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

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*Characterisation and predicted genome locations of Leach's storm-petrel (*Oceanodroma leucorhoa*) microsatellite loci (Procellariidae, Aves)*

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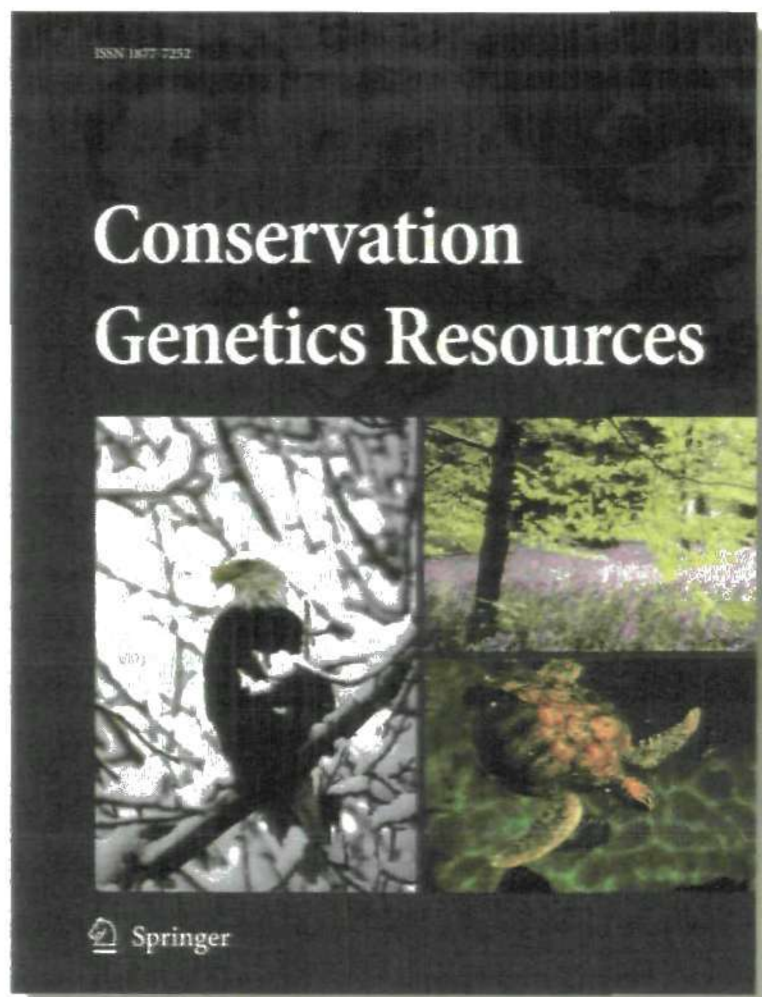

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TECHNICAL NOTE

Characterisation and predicted genome locations of Leach's storm-petrel (*Oceanodroma leucorhoa*) microsatellite loci (Procellariidae, Aves)

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Abstract Forty-one microsatellite loci were found to be polymorphic in the Leach's storm-petrel *Oceanodroma leucorhoa* when characterised in 24 unrelated individuals sampled from a population located at Gull Island, Newfoundland, Canada. Sequence homology was used to assign a predicted chromosome location for 39 of the polymorphic loci. Four polymorphic microsatellite loci were Z-linked based on the typing of known sex individuals and/or sequence homology. The set of 37 autosomal markers will be suitable for population and parentage studies of the Leach's storm-petrel (combined first parent non-exclusion probability <0.0001).

Keywords Aves · Leach's storm-petrel · Microsatellite · Predicted genome locations · Procellariidae · Z-linked loci

The Leach's storm-petrel *Oceandroma leucorhoa* is a small highly pelagic seabird of the Procellariformes family. We have characterised a microsatellite marker set in this species in order to investigate regional and global population structure and dispersal between colonies.

Existing microsatellite markers were tested for utility in the Leach's storm-petrel (LSP) including 7 loci isolated in a different storm-petrel species (Sun et al. 2009) and 47 loci with primer sets of engineered and/or proven high cross-species utility (Dawson et al. 2010; *Bb111-TG*, *HvoB1-TGG*, Klein et al. 2009; *Calex01*, *Calex08-ZEST*, *Pte24-CEST*, *HvoB1*, Dawson et al. unpublished data). A microsatellite-enriched LSP genomic library was also created. Blood (20 ul) was collected from LSP individuals and stored in 1.5 ml of absolute ethanol. Genomic DNA was extracted using an ammonium acetate precipitation method. The genomic library was constructed from a single male LSP (SKB02) sampled at St Kilda, Scotland (Co-ordinates 57°48'N, 8°34'W) in 2008. We used the method of Armour et al. (1994) and was enriched for the following motifs: $(GT)_n$, $(CT)_n$, $(GTAA)_n$, $(CTAA)_n$, $(TTTC)_n$ and $(GATA)_n$ and their complements, which had been bound to magnetic beads (Glenn and Schable 2005). Transformant colonies were directly sequenced by the NERC Biomolecular Analysis Facility at the University of Edinburgh.

A total of 211 new unique LSP microsatellite sequences were isolated (EMBL accession numbers FR696377–FR696588). Primer sets were designed for 50 unique microsatellite sequences using PRIMER3 (Rozen and Skaletsky 2000). The new LSP and existing loci were initially tested for amplification and polymorphism in 4–6 unrelated individuals sampled from Gull Island, Whitless Bay, Canada (Co-ordinates: 47°15'N, 52°46'W) in 2008. The 4–6 individuals were amplified using a gradient of 12 different annealing temperatures (56–65°C). The temperature producing the cleanest and strongest PCR product when observed on a 1.5% agarose gel stained with SYBRSafe was selected for amplification of the polymorphic loci in 24 additional Gull island individuals. Each 2 μl PCR contained approximately 10 ng of lyophilised genomic DNA, 0.2 μM of each primer and 1 μl QIAGEN multiplex PCR mix (QIAGEN Inc.; Kenta et al. 2008). The

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Table 1 Characterisation of microsatellite loci in Leach's storm-petrel *Oceanodroma leucorhoa*

Locus	EMBL accession number	Source species	Reference	Primer sequence (5′–3′)	Fluoro label (F)	T_a	N	A	Exp. allele size (bp)	Obs. allele size range (bp)	H_O	H_E	HWE P-value	Est. null allele freq.	Chromosome, location (bp) (E-value)
(a) Characterisation of 15 published microsatellite loci in Leach's storm-petrel Oceanodroma leucorhoa															
Ol10-39¥	FJ238106	*Oceanodroma leucorhoa*	Sun et al. (2009)	F: TTAAGAACAGAGCCTGACTTG	PET	56	22F	3	135	134–138	0.00	–	–	–	TguZ, 53939951 (9.5e-12)
				R: ACAAAATCTCATGTCCTTGG			24M	3		134–138	0.58	0.64	0.449	0.044	GgaZ, 38227643 (1.1e-06)
Oc63	FJ238100	*Oceanodroma castro*	Sun et al. (2009)	F: TCACACCAACCTCCATGAAA	VIC	58	24	4	190	174–188	0.67	0.65	0.508	–	Tgu4, 22623887 (5.6e-23)
				R: AACGGGGAATATGTGGTTCTT										0.021	Gga4, 39929604 (1.9e-34)
Oc87B	FJ238104	*Oceanodroma castro*	Sun et al. (2009)	F: TTTAAGGAACGCAAGTCAGG	PET	58	24	8	261	261–277	0.67	0.79	0.131	0.076	Tgu2, 57390462 (1.5e-69)
				R: GTGATTCTTGCAGTGGCTTT											Gga2, 37216829 (9.7e-63)
Oc84	FJ238103	*Oceanodroma castro*	Sun et al. (2009)	F: CCTTTTTCCAGGCAGACAAA	NED	56	24	3	298	302–306	0.58	0.59	0.055	–	Tgu4, 8910815 (3.0e-61)
				R: AGTTCAAGGGCAACCTTGTG										*0.010*	*Gga4, 30971263 (1.0e-45)*
TG01-077	CK305147	*Taeniopygia guttata*	Dawson et al. (2010)	F: GTATGTCAGTTATCAAAAACAAGC	HEX	56	24	3	153	153–155[‡]	0.67	0.67	**0.007**	–	Tgu1, 95581733 (3.3e-129)
				R: AAATGGCAGGTAAGGATACTCTC										0.006	Gga1, 86284969 (1.2e-92)
TG02-120	DV945440	*Taeniopygia guttata*	Dawson et al. (2010)	F: TTGGGCAAAGATGATATGAATG	6FAM	56	24	3	230	236–244	0.44	0.52	0.568	0.046	Tgu2, 127242053 (1.2e-107)
				R: AGCCAGGTCCAGTTTCTAAGC											Gga2, 125741749 (7.9e-92)
TG03-034	CK311260	*Taeniopygia guttata*	Dawson et al. (2010)	F: GAGATCGCCACCATCCTG	6FAM	56	23	2	178	177–179	0.09	0.16	0.131	0.289	Tgu3, 44507023 (9.5e-159)
				R: AAGTCTACATTTCCCTTGTCTTGG											Gga3, 40012285 (4.9e-134)
TG04-004	DV946288	*Taeniopygia guttata*	Dawson et al. (2010)	F: CTGGAGCAGTATTTATATTGATCTTCC	HEX	56	23	5	166	161–169	0.39	0.56	0.091	0.144	Tgu4A, 6999784 (7.3e-145)
				R: GAAGATGTGTTTCACAGCATAACTG											Gga4, 4186152 (1.1e-108)
TG04-041	CK316380	*Taeniopygia guttata*	Dawson et al. (2010)	F: CTGAATTGTTGACCTTTGCTTAC	HEX	56	24	2	173	176–178	0.50	0.30	0.272	–	Tgu4, 37987277 (1.0e-115)
				R: GTCCTTTTAGAAAGCAGCACAG										0.142	Gga4, 41461789 (1.0e-99)
TG04-061	CK235034	*Taeniopygia guttata*	Dawson et al. (2010)	F: GACAATGGCTATGAAATAAATTAGGC	HEX	56	24	3	186	202–204[‡]	0.46	0.52	0.813	0.051	Tgu4, 20910894 (1.3e-113)
				R: AGAAGGGCATTGAAGCACAC											Gga4, 61990187 (2.5e-98)
TG05-053	CK314425	*Taeniopygia guttata*	Dawson et al. (2010)	F: GCATCATCTGGTTGAACTCTC	6FAM	56	24	4	196	211–213[‡]	0.29	0.71	**<0.001***	0.417	Tgu5, 61276203 (2.3e-161)
				R: ACCCTGTTTACAGTGAGGTGTT											Gga5, 59348193 (7.6e-120)
TG08-024 (primer set1)	CK314428	*Taeniopygia guttata*	Dawson et al. (2010)	F: CACAAATCCTGAATTTCATATCC	HEX	56	20	3	243	240–242[‡]	0.45	0.59	0.389	0.132	Tgu8, 21,095,625
				R: AACAACGACAGCTATGAAAGAAC											
TG11-011	CK308096	*Taeniopygia guttata*	Dawson et al. (2010)	F: CAAACTAAGTACATCTATATCTgAAG	6FAM	56	22	10	223	213–230[‡]	0.55	0.71	**0.002**	0.158	Tgu11, 19380799 (3.5e-123)
				R: TAAATACAGGCAACATTGG											Gga11, 11693433 (3.7e-102)
TG13-009	DV948691	*Taeniopygia guttata*	Dawson et al. (2010)	F: TGTGGTGGGATAGTGGACTG	HEX	56	23	3	195	194–197	0.30	0.34	0.240	0.028	Tgu13, 3672804 (2.2e-136)
				R: CTGTAAAATGTGCAAGTAACAGAGC											Gga13, 10457183 (4.7e-108)
TG13-017	CK313422	*Taeniopygia guttata*	Dawson et al. (2010)	F: GCTTTGCATCTTGCCTTAAA	6FAM	56	24	4	300	204–210	0.58	0.50	0.911	–	Tgu13, 3672804 (2.2e-136)
				R: GGTAACTACAACATTCCAACTCCT										0.112	Gga13, 18850515 (2.4e-143)

Table 1 continued

Locus	EMBL accession number	Repeat motif	Primer sequence (5′–3′)	Fluoro label (F)	T_m (°C)	T_a	N	A	Exp. allele size (bp)	Obs. allele size range (bp)	H_O	H_E	HWE P-value	Est. null allele freq	Chromosome location (bp) (E-value)
(b) Characterisation of 26 newly isolated Leach's storm-petrel Oceanodroma leucorhoa microsatellite loci															
Ole01	FR696377	(ACAG)$_5$	F: CACCCTGAATCCCAGAACCAAGC	6FAM	F: 57.59	60	24	2	218	213–217	0.25	0.28	0.501	0.053	Tgu2, 13058294 (1.2e-51)
			R: TCAGTGAAGGTCCACTCCGAATCC		R: 57.93										No match (Gga)
Ole02	FR696378	(GAAA)$_{32}$	F: GGGTTCCTGTTACCAAAGGGCAG	HEX	F: 57.52	60	17	12	489	412–485	0.47	0.89	**<0.001***	0.301	Tgu2, 1188159 (4.2e-06)
			R: CATAAGCACAAGGTTCTAGCTCCCTC		R: 57.07										Gga2, 3443337 (8.4e-187)
Ole03	FR696379	(GAAA)$_{29}$	F: TCCTTCACCACCTCTTGCTGCC	HEX	F: 58.76	60	24	12	451	421–462	0.92	0.87	0.795	0.040	Tgu2, 37830396 (2.0e-39)
			R: ACGCATCTGTAGCTCACAAATCCCAG		R: 59.14										Gga2, 72796221 (9.1e-31)
Ole04	FR696380	(ATTCT)$_{13}$	F: TGTTTATTGCACTGCCTGAAACTTGC	HEX	F: 57.37	m	24	4	355	343–358	0.33	0.65	**0.002**	0.314	Tgu9, 27014609 (3.5e-61)
			R: TGTGCAACAGCGGTCACTGAG		R: 57.62										Gga9, 25331698 (7.4e-49)
Ole05	FR696381	(GAATA)$_{10}$	F: TGGAATGGGATGGGATGGTTTGGG	HEX	F: 58.66	60	23	10	299	297–353	0.61	0.75	0.083	0.086	Tgu3, 31402896 (1.5e-39)
			R: TGAGGCTGGAGGAGAATGCTGTG		R: 58.39										No match (Gga)
Ole06	FR696382	(TATT)$_5$	F: GCCAACTGCTGTTCAGTGAGCC	6FAM	F: 58.61	60	24	2	201	198–202	0.33	0.42	0.347	0.107	Tgu19, 1687821 (1.6e-75)
			R: GCCACAAACGCAAACATTCATAAACCC		R: 58.48										Gga19, 1482233 (6.7e-86)
Ole07	FR696383	(CA)$_{10}$	F: CCCAAGGAGTTCCTGTGCGTGC	HEX	F: 60.24	60	24	3	154	153–157	0.46	0.37	0.636	−0.125	Tgu8, 9452460 (1.3e-64)
			R: TGGCTCTGTGCTGCGCTTTACC		R: 59.99										Gga8, 13826157 (3.6e-39)
Ole08	FR696384	(GA)$_8$	F: ACTCCACACGGACTCTGCACTG	HEX	F: 58.53	60	24	2	218	212–214	0.38	0.31	0.551	−0.102	No match (Tgu)
			R: TGTGATTTGTTGTGAGGCGAGCG		R: 58.45										Gga27, 3578087 (2.6e-118)
Ole09	FR696385	(CA)$_{10}$	F: GGCCTGGATTCTGCGTGTTGGG	6FAM	F: 60.30	60	24	2	151	149–153	0.25	0.28	0.501	0.053	Tgu3, 17145330 (1.0e-88)
			R: CAGCTACAGGCCACGGGTGC		R: 60.04										Gga3, 27034682 (7.2e-48)
Ole10	FR696386	(CA)$_{12}$	F: ACCAGCTCCTAACTGGAAGCCAC	6FAM	F: 58.32	60	24	3	236	232–238	0.08	0.08	1.000	−0.012	Tgu1, 59413510 (1.4e-97)
			R: TGCAGGTAGGTTCTCAGTATGCAAAGG		R: 58.64										No match (Gga)
Ole11	FR696387	(AG)$_{14}$	F: TCACAACCAGAGCTGAGACACAGAG	6FAM	F: 58.20	60	23	2	474	468–472	0.44	0.43	1.000	−0.013	Tgu1, 107441638 (8.6e-79)
			R: AGCCAAGGCTCCAAGCTTATGAAAC		R: 57.79										Gga1,145644036 (7.4e-280)
Ole12¥	FR696388	(AGAT)$_6$,	F: GCTCCTGTAGAGCTGGAAATGAACC	6FAM	F: 57.41	60	22F	6	246	222–248	0.00	–	–	–	No match (Tgu)
		(AGAT)$_6$ AGA(TACA)$_9$	R: GGCAGTGCTACCTGTGGATGC		R: 57.80		25M	6		226–252	0.60	0.67	0.461	0.050	GgaZ, 57547873 (6.3e-31)
Ole13	FR696389	(TG)$_9$	F: GGGCCAGACATGTATTTACTGGAGGG	HEX	F: 58.69	60	24	3	244	242–246[‡]	0.46	0.41	1.000	−0.069	Tgu1, 39467860 (1.8e-56)
			R: GTGCATCCGAGAGCCGACGC		R: 60.51										No match (Gga)
Ole14	FR696390	(AC)$_8$	F: CCAGCCTGCAGGGCTTTCC	6FAM	F: 57.75	60	24	3	348	339–349	0.46	0.46	1.000	−0.005	Tgu2, 63946342 (5.6e-75)
			R: CAGAGCTTTGCCTCTGTCTTAGTCTTC		R: 57.36										Gga2, 43905738 (1.6e-267)
Ole15	FR696391	(TG)$_{11}$	F: TCAGGAACAGCACTGGAAACTGGAC	HEX	F: 58.87	60	24	2	235	232–234	0.04	0.04	NA	−0.004	Tgu3, 37963153 (4.9e-38)
			R: TGCCACTGACTTTCCATTCTCCCTC		R: 58.46										Gga3, 46474483 (4.9e-17)
Ole16	FR696392	(CA)$_{11}$	F: GCAGCCTCCAGCCGTGAGTG	HEX	F: 60.04	60	24	4	232	219–232	0.75	0.71	0.134	−0.036	TguZ, 32967540 (7.5e-55)
			R: GTGATTGCAGCAGCTCCTGGTCG		R: 60.36										GgaZ, 2104154 (7.9e-65)
Ole17	FR696393	(GT)$_5$ GG (GT)$_5$	F: TGACATGACCACTTTCCATAGC	HEX	F: 60.00	60	24	5	210	209–236	0.46	0.52	0.372	0.084	No matches
			R: AATGTCTGTGGTTCAAATGTGC		R: 59.90										
Ole18	FR696394	(CA)$_{13}$	F: TGGTTTGTATGTTGTGGGTTG	6FAM	F: 59.21	60	24	5	151	147–155	0.58	0.69	0.681	0.079	Tgu4, 15498065 (3.2e-32)
			R: GATCCACTTAGCCACCCTTG		R: 59.55										Gga4, 49917509 (8.3e-19)

Table 1 continued

Locus	EMBL accession number	Repeat motif	Primer sequence (5′–3′)	Fluoro label (F)	T_m (°C)	T_a	N	A	Exp. allele size (bp)	Obs. allele size range (bp)	H_O	H_E	HWE P-value	Est. null allele freq.	Chromosome, location (bp) (E-value)
Ole19	FR696395	$(GA)_{10}$ CA	F: GAAGGCTCTCAAGGGTCAAG	6FAM	F: 59.01	60	23	8	242	227–377	0.56	0.80	**0.012**	0.164	Tgu12, 12215753 (1.2e-59)
		$(GA)_7$	R: TTTGGGAGAAGTTCAGTAGAAGAAC		R: 59.39										Gga12, 11880314 (1.9e-59)
Ole20€	FR696396	$(AGGC)_7$	F: AGGTCAGCAGCTAAAGCATACC	HEX	F: 59.94	60	21F	1	345	344	0.00	–	–	–	TguZ, 45790217 (3.3e-48)
		ACG $(CA)_9$	R: AGGTCCCTTCCAATCCAAAC		R: 60.17		22M	2		344–346	0.09	0.09	1.000	−0.014	GgaUnk, 6499150 (5.8e-41)
Ole21	FR696397	$(GA)_{11}$ $(A)_6$	F: CCACGAGAAATGGACATACAAC	HEX	F: 59.36	60	24	21	330	316–418	0.92	0.96	0.573	0.011	Tgu1, 70616595 (9.9e176)
		$(GAAA)_{31}$	R: TGAAGAACTTCCGAAATAACTGTG		R: 59.70										Gga1,157866922 (1.1e-204)
Ole22	FR696398	$(CT)_9$	F: AGCAGAGGCCACTACATCAC	HEX	F: 57.90	60	24	3	248	249–253	0.38	0.33	1.000	−0.096	No match (Tgu)
			R: AAAATATCATAGGGGAGTAAAGAGC		R: 57.59										Gga19, 2678502 (1.7e-144)
Ole23	FR696399	$(GT)_{18}$	F: TCTTTGGCATGCAATCTTTG	6FAM	F: 59.81	60	24	10	416	404–432	0.86	0.85	0.423	−0.014	Tgu1, 58566787 (3.5e-79)
			R: CCCTGGTCAACTGAGAAAGC		R: 59.84										Gga1, 171173789 (4.9e-65)
Ole24	FR696400	$(ATCT)_9$	F: TGTGCTGACTTGTTTATTGTTCC	HEX	F: 59.19	60	24	5	159	156–176	0.88	0.76	0.911	−0.087	No matches
			R: GGCCATCTTGATACGGCTAC		R: 59.56										
Ole25	FR696401	$(AGAT)_4$ &	F: CCTCCTCATCGTAGGGACTG	HEX	F: 59.67	60	24	7	390	370–392	0.88	0.80	0.695	−0.059	Tgu3, 70012260 (3.3e-51)
		$(GAAA)_{16}$	R: TTAAAGGCAGCGATTCTGTTC		R: 59.49										Gga3, 70378881 (1.4e-74)
Ole26¥	FR696402	$(TG)_{10}$	F: TCAGCTTCTGGTGCAGTTATG	HEX	F: 59.09	60	22F	2	241	239–245	0.00	–	–	–	TguZ, 37567887 (2.2e-230)
			R: TCCTGCTGATGGATAGGTTATG		R: 59.07		25M	2		239–245	0.60	0.49	0.415	−0.103	No match (Gga)

T_m melting temperature, T_a annealing temperature, *N* number of unrelated Leach's storm-petrel *Oceanodroma leucorhoa* individuals, *A* number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, *HWE* Hardy–Weinberg equilibrium, *Est. null allele freq* estimated null allele frequency, *Tgu* zebra finch *Taeniopygia guttata*, *Gga* chicken *Gallus gallus*, *Bold* deviation from HWE

* Deviation from HWE after sequential Bonferroni correction, *F* results of female individuals only, *M* results of 24 male individuals genotypes for Z-linked loci, € possibly Z linked (see text), ¥ Z linked locus, ‡ loci with 1 base pair size increment

Author's

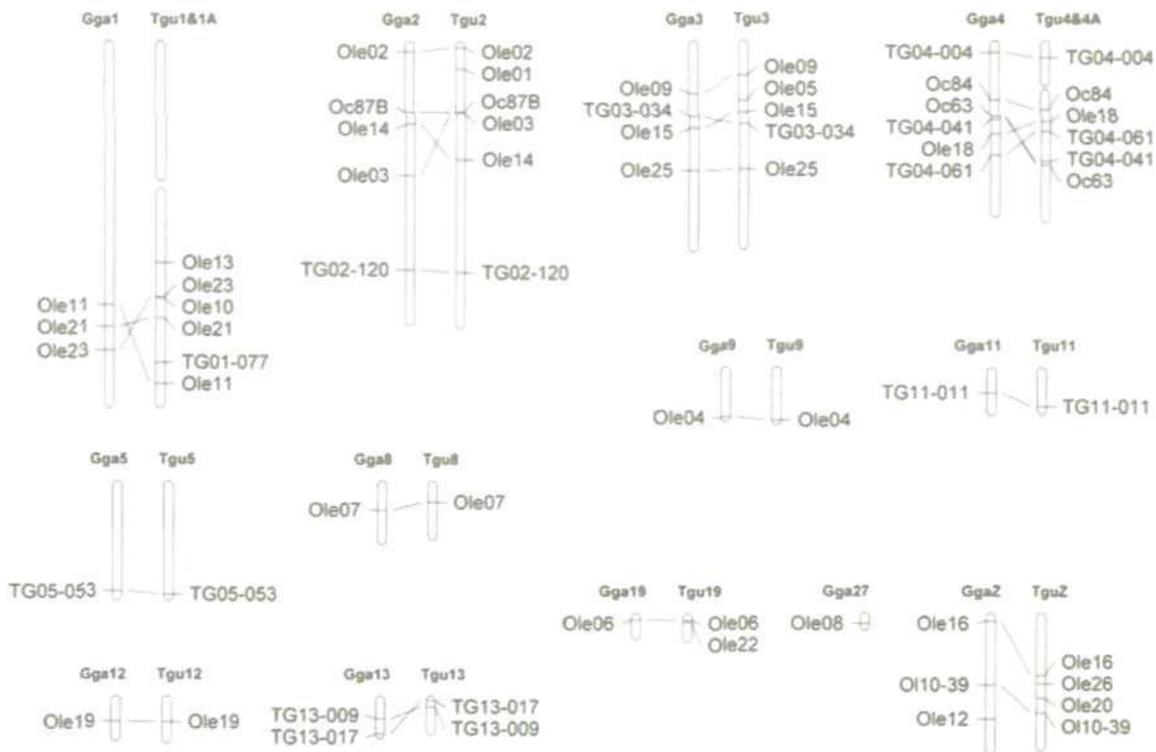

Fig. 1 Predicted chromosome locations of microsatellite loci polymorphic in the Leach's storm-petrel *Oceanodroma leucorhoa*. Tgu, zebra finch (*Taeniopygia guttata*) chromosome name. Gga, chicken (*Gallus gallus*) chromosome name

PCR program used was: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, annealing temperature (Table 1) for 90 s, 72°C for 1 min, and finally 60°C for 6 min. PCR amplification was performed using a DNA Engine Tetrad 2 thermal cycler (MJ Research, Bio-Rad, Hemel Hempstead, Herts., UK). Amplified products were loaded on an ABI 3730 48-well capillary DNA Analyser (Applied Biosystems, California, USA) and allele sizes were assigned using GENEMAPPER v3.7 (Applied Biosystems, California, USA). Individuals were sex-typed with the 2550F/2718R (Fridolfsson and Ellegren 1999), Z002A (Dawson 2007) and Z-037B markers (Dawson, DA unpublished data).

Of the 47 existing loci tested in 6 individuals, 2 loci did not amplify or produced non-specific products, 30 were monomorphic and 15 were polymorphic. Of the 50 new LSP loci tested in 4–6 individuals, 6 loci did not amplify or produced non-specific product, 18 were monomorphic and 26 were polymorphic.

Predicted chromosome locations were assigned by comparing the microsatellite sequences with the location of their homolog on the chicken (*Gallus gallus*) and zebra finch (*Taeniopygia guttata*) genome assembly (methods as in Dawson et al. 2006, 2007). This allowed us to identify if any loci were located on the sex chromosomes or were physically linked.

Thirty-nine of the 41 polymorphic loci could be assigned a chromosomal location with a BLAST hit E-value of > 1E-10 with a > 100 base pair match (Table 1, Fig. 1). Three pairs of loci were less than 1 Mb apart in the zebra finch genome (*Ole23:Ole10, Oc87B:Ole03 & Ole06:Ole22*) and therefore may be physically linked. However, after a sequential Bonferroni correction (Rice 1989), no pairs of loci showed evidence of linkage disequilibrium ($P < 0.05$) in the LSP.

A combined total of 41 polymorphic loci were then typed in 24 unrelated individuals (14 male and 10 female) belonging to the Gull Island population and displayed between 2 and 22 alleles (Table 1). Four loci displayed a genotype pattern consistent with linkage to the Z chromosome (Table 1) being homozygous (hemizygous) in all females (ZW) but heterozygous or homozygous in males (ZZ), in agreement with their Z chromosome location assignment (Fig. 1). *Ole16* was assigned to the Z chromosome based on sequence homologue but displayed heterozygous in females suggesting it was autosomal in the LSP. A Fisher's Exact test comparing numbers of male and female homozoygotes confirmed that 3 were Z-linked (*Ol10-39, Ole12, Ole26*; P-values < 0.001) but due to low heterozygozity in males, *Ole20* was not significant and therefore should be viewed with caution.

Observed and expected heterozygosities, and predicted null allele frequencies were calculated using CERVUS v3.0.3 (Kalinowski et al. 2007). Tests for departures from Hardy-Weinberg equilibrium (HWE) and assessment of linkage disequlibrium were conducted in GENEPOP v3.4 (Rousset 2008). Only males were used when Z-linked loci were analysed. Prior to a sequential Bonferroni correction, 6 loci deviated from HWE ($P < 0.001$; Table 1). After correction only 2 loci ($P < 0.001$; *Ole02 and TG05-053*) deviated. Eleven loci displayed a high estimated null allele frequency (above 0.10) including 9 loci that did not deviate from HWE (Table 1). When typed in 24 individuals, locus *Ole21* showed high levels of polymorphism (>20 alleles) and 6 loci included 1 bp allele size increments (Table 1). The combined first parent non-exclusion probability for the 37 autosomal polymorphic markers is <0.0001.

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