The Migration Strategy, Diet & Foraging Ecology of a Small Seabird in a Changing Environment

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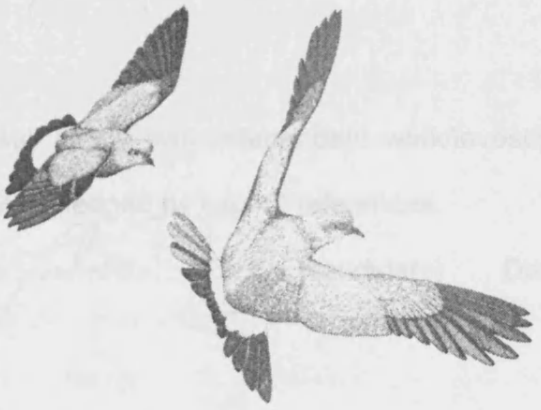

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Thesis Summary: This thesis examines the migration strategy, diet and foraging ecology of the smallest Atlantic seabird, the European Storm Petrel *Hydrobates pelagicus*. Evidence was found for sex-specific migration behaviour, opportunistic feeding (including on prey of inshore and even terrestrial origin), temporal variation in diet, and the strategic regulation of energy reserves in response to varying environmental conditions, as a buffer against starvation during migration. Molecular sexing from feather and faecal samples revealed an unexpectedly strong female bias in the sex ratio of Storm Petrels attracted to tape-lures of conspecific calls, during their northwards migration past the coast of SW Portugal. This bias was broadly consistent across seven years (mean ±SD = 85.5% female ±4.1%). The thesis describes the development and application of molecular techniques, in combination with stable isotope analysis, to study Storm Petrel diet by the detection of prey DNA from faecal samples. The major category of prey detected was fish (chiefly European Sardines *Sardina pilchardus*). Other components of the diet were other pelagic and demersal fish species, Cephalopoda (primarily cuttlefish *Sepia* spp.), Amphipoda, Isopoda and a range of terrestrial invertebrates, which were presumably scavenged from the sea surface by the Storm Petrels. Large between-year fluctuations in the level of body reserves carried by these birds were observed over the 21-year study period (1990-2010). The pattern of body mass variation followed a smooth oscillation, which was not an artefact of differences among years in the distribution of capture effort, body size or sex ratio changes. Local sea surface temperature (SST), net primary production (NPP) and European Sardine biomass were shown to be key factors associated with between-year changes in Storm Petrel body reserves. The direction of these associations suggests that Storm Petrels strategically regulate their body reserves to buffer against starvation in years of low food abundance.

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1.1 Overview

Seabirds are good indicators of changes in the marine environment, since their reproductive and foraging parameters reflect oceanographic changes, including climate-driven changes in ocean ecosystems. Anthropogenic climate change has major implications for the future of natural ecosystems as well as human societies (IPCC 2007). Understanding and predicting the diverse biological impacts of global climate change is therefore the central ecological challenge of our time, yet our ability to make such predictions is limited by the sheer complexity of ecosystems and of the interspecific interactions that they encompass. Emerging evidence of the impacts of climate change on ecosystems has generated great concern among scientists, policy makers and the wider public (McCarty 2001, Walther *et al.* 2002). Compelling examples of ecological change driven by the changing climate include case studies from marine ecosystems, involving commercially important species of fish and iconic seabirds.

One emblematic example is of climate driven changes in the recruitment of Atlantic Cod *Gadus morhua* in the North Sea. Variability in sea temperature affects cod survival mainly via their food supply; rising temperatures since the mid-1980s have modified plankton population cycles in a way that has reduced the food availability, survival and recruitment of young cod (e.g. Brander *et al.* 2001, Beaugrand *et al.* 2003, Sundby 2000, Ottersen & Loeng 2000). Similarly, there is a

well-documented relationship between sea temperature, sandeel *Ammodytes marinus* abundance, and the breeding success of seabird species also in the North Sea (Furness & Tasker 2000, Rindorf *et al.* 2000, Frederiksen *et al.* 2004a).

The study of trophic interactions within biological communities is crucial for a better understanding of the structure and function of ecosystems, as well as for predicting their response or resilience to climate change. Oceanic food webs have been described in detail as a result of concerns related to fisheries management as well as to climate change (e.g. Link 2002, Trites 2003, Dunne *et al.* 2004). However, most previous studies of trophic pathways in pelagic ecosystems have relied on methods for studying diet that have important limitations. Prior to the recent advent of biochemical approaches such as stable isotope and fatty-acid analysis (e.g. Williams *et al.* 2008), these methods primarily involved direct observations of foraging behaviour, or stomach-content analyses (e.g. Barrett *et al.* 2007, Monteiro *et al.* 1996). Combining such methods with new and complementary approaches has been shown to be highly beneficial (Trites 2003, Casper *et al.* 2007). Specifically, molecular techniques potentially provide a powerful new set of analytical tools for the study of foraging ecology and trophic relationships (e.g. Casper *et al.* 2007, Dunshea 2009, Lerner & Fleischer 2010). However, they have not yet been widely applied in ecological contexts in general, or in marine ecosystems in particular.

In this thesis, I investigate the foraging ecology and migration fuelling behaviour of a small pelagic seabird, the European Storm Petrel *Hydrobates pelagicus* (henceforth abbreviated to "Storm Petrel" where appropriate) and its behavioural responses to temporal changes in the marine environment. I developed and applied DNA-based methods to study Storm Petrel diet and foraging ecology, in

order to better understand the trophic mechanisms underlying the behavioural response of this species to the variable environment.

Evidence exists for the impacts of climate on the breeding parameters (Rindorf *et al.* 2000, Laaksonen *et al.* 2006), timing of migration (Miller-Rushing 2008, Smallegange *et al.* 2010), demography (Both *et al.* 2006, Sandvik *et al.* 2008), and adult survival (Grosbois & Thompson 2005, Sandvik *et al.* 2005) of different bird species. However, the present study is the first to invetesco's stigate in detail the connection between climate variation and the migration fuelling strategy of a seabird species. In this Introductory Chapter, I provide an overview of (i) climate change and its impacts on marine ecosystems, (ii) the study species, the European Storm Petrel, (iii) the range of methods available to investigate seabird diet and (iv) the methods for molecular analysis of diet and their development prior to the start of this research. I finish the Introduction with an outline of my studies that are presented in detail in the subsequent chapters of this thesis.

1.2 Marine Ecosystems: Their Importance and Conservation

For tens of thousands of years, people have had a close relationship with the oceans and their resources (Roberts 2009). Throughout history, these resources have provided a rich source of food (Roberts 2007), and are increasingly important for tourism, recreation, as a source of renewable forms of energy, and of various additives for foods or cosmetics. Thus, the diversity and productivity of marine ecosystems remains important to the survival and well-being of human societies.

Despite the importance and attractiveness of the marine environment to humans, its physical and biological oceanographic systems, processes, and changes are rather poorly understood when compared to the terrestrial environment. For

instance, unlike the land, the water column and wide ocean basins tend to be envisaged as fairly monotonous, uniform ecosystems but, in fact, there are many features that punctuate our oceans abruptly or gradually, dividing them into many different environments (Miller 2004, Kaiser *et al.* 2005). Furthermore, while the concepts of biomes and habitats are very well understood for the terrestrial environment, such patterns in the marine environment are often beyond our immediate perception. Only in the last few decades have technologies been applied (e.g. autonomous underwater vehicles with scientific instrumentation, or remote-sensors such as the NIMBUS satellite) to obtain a more detailed understanding of spatial and temporal patterns in the marine environment and the application of this understanding to the conservation of the oceans (Kaiser *et al.* 2005).

In contrast to terrestrial habitats, it is commonplace for marine habitats to be dominated (in terms of biomass) by animals rather than plants, and for the substratum to provide the main structure to the habitat (rather than plants providing the main structure, as in a forest). Only a small proportion of marine habitats have obvious dominant species, e.g. kelp forests (Laminariales), mussel beds (Bivalvia) and maerl beds (*Corallinaceae).* Many marine ecosystems are dominated by a few abundant mid-trophic species, usually pelagic schooling fish, with higher taxonomic diversity at lower and higher trophic levels (Rice 1995). These mid-trophic level fish (including the larval stages of all fish) typically feed on zooplankton (Hays *et al.* 2005) and are a key prey for predatory fish, marine mammals and seabirds.

In the marine environment, patchiness in topography, physical properties (temperature, salinity, and turbidity), biological production and biomass, exists at a wide range of spatial scales (cm to hundreds of km) and temporal scales (min to decades; Kaiser *et al.* 2005). Because the offspring of most marine species are small

(and most are pelagic), they may be more vulnerable to physical influences than terrestrial young, and thus experience wide fluctuations in survival and recruitment. Therefore, marine populations and communities often respond rapidly to (and hence are more temporally coupled with) changes in their physical environment (Steele 1985, 1998). This responsiveness is manifested over ecological time scales in dramatic changes in the composition of pelagic and benthic communities during community "regime shifts" over the order of one to several decades (e.g. Roemmich & McGowan 1995, Hayward 1997, Francis *et al.* 1998). Though such regime shifts are driven by atmospheric processes (such as the decade-scale climate oscillations described below), biotic responses to decadal regime shifts have been argued to be far more dramatic in marine systems compared to terrestrial systems (Steele 1998).

Marine ecosystems are exposed to a wide range of anthropogenic impacts, of which climate change and over-fishing are amongst those causing greatest concern (e.g. Beaugrand *et al.* 2002, Bhathal & Pauly 2007). The ecological impacts of over-fishing are intense and widespread. For example, more than 50% of the southeast Atlantic is either overexploited or depleted of its marine fisheries resources; the same is true of over 20% of the central east Atlantic and about 40% of the northeast Atlantic. Overall only 5%, 7%, 8% of the Mediterranean and Black Sea basin, southwest and northwest Atlantic, respectively, are still considered to be underexploited (Roberts 2007).

Despite abundant evidence of the overexploited and degraded state of most of the world's ocean ecosystems, the development of conservation plans for marine areas, including marine reserves, has proven to be a great challenge. Thus, the effects of local protection by marine reserves of ecological communities may be less predictable and, in the short term, more difficult to detect and validate both locally

and regionally than the effects of terrestrial nature reserves (but see Roberts 2007 for successful examples of marine protected areas). As a consequence, regardless of the growing interest by resource managers, policy makers, and academics in the potential for reserves in marine ecosystems (e.g. Carr *et al.* 2003, Thompson *et al.* 2008, Sen 2010), currently only 1% of the marine realm is protected within reserves, in contrast to over 12% in terrestrial systems (Groombridge & Jenkins 2002). This lack of protection from over-exploitation and degradation has obvious consequences for predators such as seabirds that rely on marine resources for their survival (Croxall 1992).

1.3 Climate Change

1.3.1 Climate Change and Oceanography

The world's oceans play a key role in shaping and regulating our climate and have a tremendous bearing on human future wellbeing in terms of their value for food production and a wide range of other ecosystem services, as well as their inherent biodiversity value (Kaiser *et al.* 2005). By absorbing, sequestering and releasing carbon, marine environments play a major role in the global carbon cycle and so directly influence the pace and extent of climate change (Takahashi 2004, Steinfeldt *et al.* 2009). One important ecosystem service provided by oceans over the historical period has been to buffer the climate against the anthropogenic increase in atmospheric carbon dioxide (CO_2). Despite this buffering, compelling evidence has accumulated for directional climate change that has diverse impacts on marine environments. Over the last century, global sea temperatures have increased, sea levels have begun to rise as a result of thermal expansion of sea water, while storms and waves have become more damaging (Kaiser *et al.* 2005, reviewed by Brierley &

Kingsford 2009). Furthermore, oceans around the world are becoming more acidic as a result of the increased concentration of CO_2 available to be absorbed at the sea surface (Kaiser *et al.* 2005, reviewed by Brierley & Kingsford 2009). Such changes are predicted to be amplified as atmospheric CO_2 continues to rise, but there is considerable uncertainty over the future extent and timing of these future impacts at global, regional or local scales (Watkinson *et al.* 2004). Similarly, predicting the frequency and timing of extreme events, such as severe weather, is important for predicting the response of ecological communities to a changing climate (Sutherland 2004), but changes in the occurrence of extreme events are notoriously difficult to predict.

The Atlantic is, after the Pacific, the world's second largest ocean, extending into both the Arctic and Antarctic. The North Atlantic region has an important climatic feature exerting a dominant influence over its marine system: the North Atlantic Oscillation (NAO), a decade-scale oscillation in latitudinal atmospheric pressure gradients across the North Atlantic (Stenseth *et al.* 2004), similar in nature to Arctic Oscillation (AO) in the polar region and the El Niño Southern Oscillation (ENSO) and Pacific Decadal Oscillation (PDO) in the Pacific Ocean. A high NAO index increases the degree of westerly winds, and consequently milder temperatures, over northern Europe. A low NAO index is usually associated with weaker westerly winds, allowing colder northerly winds to dominate over northern Europe (Stenseth *et al.* 2004).

Although the NAO is a natural mode of variability of the atmosphere, stratospheric and surface processes (including anthropogenic processes) may also influence its phase and amplitude (Ottersen *et al.* 2004). For example, it has been found that oceanic processes such as long-term changes in sea surface temperatures

can also have an important influence on the NAO, which in itself has a feedback impact on sea surface temperatures. However, there are different regional as well as seasonal patterns for this relationship and the mechanisms involved in it are poorly understood (Sutton *et al.* 2000, Edwards *et al.* 2001, Sutton & Hodson 2003).

The ecological effects of these cyclic decade-scale climate changes are of considerable interest, as they represent repeated "natural experiments", from which the possible consequences of longer term anthropogenic, directional changes may be inferred. In addition, the effects of anthropogenic climate change may themselves be compounded or mitigated in the shorter term by the effects of climatic cycles such as ENSO or the NAO.

The ecological effects of the NAO are widely reported from marine, freshwater and terrestrial ecosystems (Stenseth *et al.* 2004). Effects of the NAO on the organisms across a range of trophic levels from phytoplankton to predators, suggest that the NAO may also influence the dynamics of seabird populations, through variability in their food supply (e.g. Poloczanska *et al.* 2004, Bustnes *et al.* 2009). Indeed, recent studies have already shown associations between the NAO and different aspects of seabird ecology (reviewed by Durant *et al.* 2004) such as the likelihood of breeding (Thompson & Ollason 2001), timing of breeding (e.g. Frederiksen *et al.* 2004b), reproductive success (Thompson & Ollason 2001) and adult survival (Sandvik *et al.* 2005, Votier *et al.* 2005). However, the trophic relationships and behavioural mechanisms that may mediate such ecological associations remain largely unknown (Stenseth *et al.* 2004, Møller *et al.* 2004a). Moreover, to date, very little is known about the impacts of climate change on seabirds outside the breeding season or away from their breeding colonies.

1.3.2 Biological Impacts of Climate Change

The ways in which climatic variation influences behaviour, physiology and life history has long been a central theme of research in animal ecology (e.g. White 1789, Andrewartha & Birch 1954, Elkins 1983). In the context of anthropogenic climate change, this subject has gained an extra relevance and importance, providing a strong impetus and focus for new research on this topic (Møller *et al.* 2004a, McCarty 2001). Although animals must have been responding to natural variation in climate throughout their evolutionary history, great uncertainty remains as to how well most species may be able to respond (through behavioural plasticity or evolutionary adaptation) to the predicted rapid and substantial future changes in climate.

Studying the response of marine ecosystems to climate change is essential as we attempt to develop sustainable management of our living marine resources (Stenseth *et al.* 2004). Ecological responses to climate fluctuations are reflected in the productivity of marine ecosystems, from phytoplankton and the zooplankton communities that they sustain, to the dynamics of fish populations (Cushing 1990) and top predators such as seabirds (Ballance *et al.* 2007).

Climate-driven fluctuations in plankton populations can result in long-term changes in fish recruitment (Beaugrand *et al.* 2003). Recent studies have found that, across much of the world's oceans, recent warmer surface temperatures have been associated with lower oceanic productivity and standing biomass. For example, in the NASA's Sea-viewing Wide Field-of-view Sensor (SeaWiFS) time series, global chlorophyll and productivity increased sharply during 1997–98 as temperatures fell, and then declined gradually to 2005 as temperatures increased (Behrenfeld *et al.* 2006).

These observed reductions in ocean productivity during the recent post-1999 warming period provide insights into how future climate change might alter marine food webs, but these ocean-scale patterns are often far more complex at a regional or local scale. For example, a clearly discernable climate–plankton link is found primarily in the tropics and mid-latitudes, where there is limited vertical mixing of nutrients within the water column. At higher latitudes, productivity is often light-limited because more intense vertical mixing carries nutrients hundreds of metres down from the surface waters, into the deeper waters where sunlight does not penetrate. In these high-latitude regions, future warming and a greater influx of fresh water, mostly from increased precipitation and melting sea ice, is likely to contribute to reduced mixing that may actually increase productivity (Doney 2006). Climate-driven changes in sea surface temperature can therefore cause local primary production to either increase or decrease, depending on the nature of the controls on productivity at different spatial scales (Behrenfeld *et al.* 2006).

Regardless of the uncertainty over the magnitude and the timing of forthcoming climate changes, it is possible to predict qualitatively that the anticipated changes are likely to produce a wide range of major ecological changes, including regional changes in marine productivity (as outlined above), changes in the phenology and physiology of organisms, range shifts, changes in disease transmission, shifts in the structure of communities and ecosystems, species extinctions and consequent degradation of biodiversity (IPCC 2007). Of the types of change listed above, perhaps the most difficult to predict are changes at the community level, because of the frequently non-linear nature of species interactions. Migratory species, such as many seabirds, add extra complexity, since they can be affected by changes in climate across their whole distribution range, including

entirely different ecosystems at their breeding and non-breeding grounds, and at foraging sites along the migratory route.

1.3.3 Seabirds as Sentinels of Environmental Change

Both climate change and over-exploitation of resources by humans potentially exert strong effects on marine ecosystems. The manner in which the structure and function of each ecosystem is regulated will determine how both climate change and fisheries will affect productivity at different trophic levels (Frederiksen *et al.* 2006). Three general mechanisms might control the structure and function of the different ecosystems: strong bottom-up control, strong top-down control or weak trophic links (Cury *et al.* 2001).

Top-down effects imply control through predation, including fisheries, while bottom-up effects imply control through food abundance, often thought to be driven by climate or nutrient load. When bottom-up control is dominant, seabird populations are unlikely to be regulated through density-dependent prey depletion, because prey abundance will be controlled by production at lower trophic levels (Frederiksen *et al.* 2006). Instead, their foraging success, breeding productivity and ultimately population size are likely to track spatial and temporal variation in prey abundance (e.g. Frederiksen *et al.* 2005), although interference effects among seabirds and/or disturbance of their prey may still lead to density-dependent reductions in prey availability around large seabird colonies (Lewis *et al.* 2001). Nevertheless, when bottom-up effects are predominant, seabirds can be reliable, and often financially cost-effective, indicators of marine physical environmental conditions and biological productivity (Montevecchi 1993, Montevecchi & Myers 1996), if long-term monitoring is available (McGowan 1990).

Many seabirds are highly mobile and undertake long migration journeys. Therefore, it is likely that the same species could be impacted by changes in widely separated areas of the globe. For a more comprehensive understanding of these complex interactions it is important to study the ecology of seabird species throughout their life cycle, as well as the trophic levels on which they forage (Stenseth *et al.* 2004).

The foraging niche of most seabirds places them near the top of the food chain and the response of such species to climate change can be used as an integrative index of the effect of climate on the whole food web that sustains them (Stenseth *et al.* 2004). However, seabirds may take prey from various trophic levels, so that their relationship with climate may be highly complex, involving a large number of physical and biological processes. Most studies of the effect of climate on seabirds have focused on population-level effects, such as breeding performance and population change (e.g. Abraham & Sydeman 2004, Crick 2004, Both *et al.* 2006, Bustnes *et al.* 2009), but few studies have directly assessed the relationship between climate and behavioural change in seabirds in general, and north Atlantic seabirds in particular (Durant *et al.* 2004). Seabird behaviour may be directly affected by habitat features that differ with water mass (and may affect, for instance, thermoregulation), or they may respond to the availability of their prey, which may change with water mass, current systems, or other oceanographic features (Ballance 2001).

1.4 Seabirds

1.4.1 A Seabird Case Study: The European Storm Petrel

Seabirds are represented by only four orders and in the Northern Hemisphere only three of those are found: the Charadriiformes, Pelecaniformes and Procellariiformes.

This study is focused on a species within the Procellariiformes: the European Storm Petrel. "Storm petrel" is the common designation for members of the family Hydrobatidae, characterised by being the smallest of the seabirds, with generally dark plumage, relatively short-wings, square or slightly forked tails and long weak legs. Probably due to their vulnerability to predators on land, storm petrels are generally nocturnal at the breeding colony and nest in burrows or crevices (Brooke 2004). Like most of the Procellariformes, storm petrels are long-lived species that tend to delay their breeding until they are at least two or three years old, breeding colonially on remote islands or areas of difficult access and laying a single egg each breeding season (Brooke 2004).

The Atlantic subspecies of the European Storm Petrel is the smallest Atlantic seabird (weighing on average ~26g) and a long distance migrant: These birds breed in NW Europe, from the west coast of Spain to Iceland and northern Norway, but spend the winter in south Atlantic waters (Mainhood 1976, Cramp & Simmons 1977). About 90% of the known breeding population is concentrated in the Faroe Islands, United Kingdom, Ireland and Iceland, with smaller colonies in France, Norway and Spain (Cramp & Simmons 1977, Tucker & Heath 1994). There is also a breeding population in the Mediterranean area (Greece, Italy and Malta), described as a different subspecies (*H. pelagicus melitensis*, Cramp & Simmons 1977, Bretagnolle 1998, Cagnon *et al.* 2004). Contrary to the Atlantic populations, the birds breeding in the Mediterranean are believed not to be long-distance migrants (Cramp & Simmons 1977) and it has until very recently remained uncertain whether they ever enter the Atlantic (Hashmi & Fliege 1994, Brooke 2004, Robb & Mullarney 2008). Indeed, a very recent analysis using genetic screening indicates that very few Mediterranean Storm Petrels leave the Mediterranean via the Straits of

Gibraltar, with less than 1% of birds caught in Portuguese waters in early summer originating from Mediterranean breeding colonies (R.A. King, R. Medeiros *et al.*, unpublished data).

Despite some evidence for population decline, the estimated population size for the European Storm Petrel is relatively large (1,3M - 1,5M birds, Tucker & Heath 1994, Birdlife International 2004) and the species is classified as being of "Least Concern" under the IUCN Red List Classification. The major threats to this species seem to be related to the accidental introduction of predators such as rats, at the breeding colonies (De Leon *et al.* 2006, Ruffino *et al.* 2009, Ratcliffe *et al.* 2010). In some areas, recent increases in numbers of avian predators of Storm Petrels at breeding sites appear to have increased the rate of predation (Cadiou 2003, Sanz-Aguilar *et al.* 2009). At sea, there may be some risk from eating contaminated food items, taking indigestible matter or suffer from oil spills (Azcona *et al.* 2006).

Due to their relatively high metabolic rate and high surface area/volume ratio, small seabirds are likely to be more sensitive and respond more rapidly to changes in climate than larger seabirds. Moreover, it has been suggested that long-distance migrants might be more vulnerable to the impacts of climate change than short-distance or non-migratory species. This is because long-distance migrants rely on suitable conditions at a large number of locations during their annual cycle, any of which may be adversely affected by climate change. Furthermore, the cues they use to time their departure from their wintering grounds (e.g. photoperiod) do not change in response to climate, and these birds may be unable to take advantage of the earlier arrival of spring on their breeding grounds (Both & Visser 2001, Coppack & Pullido 2004). Certainly, there is evidence from among land-birds that long-distance

migrants have not been able to respond as rapidly to climate change as short-distance migrants or residents (Rubolini *et al.* 2010).

Though bound to the land for reproduction, most Procellariiformes, including storm petrels, spend most of their life at sea where they may forage over distances of hundreds to thousands of kilometres in a matter of days (Warham 1990, 1996). Although many details of seabird reproductive biology have been successfully elucidated, for smaller species much of their life at sea remains a mystery owing to the logistical constraints of research away from the breeding colonies. For example, satellite transmitters are not yet small enough to be applied to members of the Hydrobatidae.

Storm Petrels are fairly easy to capture at colony sites both using mist-nets at night or by capture on the nest. Pioneer work on this species was done by Ronald Lockley on Skokholm Island, Wales (which still holds a significant proportion of breeding European Storm Petrels in Europe) from the early 1930s (Lockley 1983). Many early studies of the species focused on breeding biology (Hemery 1973), movements (Mainwood 1976), predation (Love 1976), vocalizations (Hall-Craggs & Sellar 1976), physiology (Warham *et al.* 1976), and parasite loads (Bakke & Barus 1976). More recently, the focus has been on vocalization and its application for censusing (Slater 1991, Ratcliffe *et al.* 1998, Insley *et al.* 2002), on olfaction (Minguéz 1997, Léon, Mingués & Belliure 2003, Nevitt 2008), metabolism and breeding strategy (Bolton 1995a,b, 1996, Minguéz 1996, 1998) and demographics (Okill & Bolton 2005, Zuberogoitia *et al.* 2007, Cadiou *et al.* 2009, Sanz-Aguilar *et al.* 2009).

The great majority of studies on this species have focused on the breeding period, when Storm Petrels are frequently on land. In contrast, Storm Petrels at sea

are not easily accessible. A number of studies focused on the behaviour of European Storm Petrels at sea (Martinez-Abrain *et al.* 2002, Valeiras 2003, Poot 2008, Flood *et al.* 2009) but the information that can be derived from these studies is limited. The small size of Storm Petrels constrains the use of long-distance transmitters and remote-sensing technologies to study their movements, and samples such as feathers, vomit or faeces cannot be collected unless the birds themselves are captured. Procellariiformes are known to have a good olfactory sensitivity (Léon, Mingués & Belliure 2003, Bonadonna *et al.* 2004, Bonadonna *et al.* 2006, Nevitt 2008), so it is relatively simple to attract storm petrels close to a boat, at a considerable distance from the coast, using a "chum" of mashed fish. Attempts have been made to capture the birds attracted to such food-bait at sea, but the cost and effort required is high for limited number of successful captures that result (Brooke 2004, this study).

A different approach, developed by scientists collaborating with A Rocha, an environmental NGO in the south of Portugal, has proven to be efficient for capturing storm petrels away from their breeding colonies. Since 1990, large numbers of storm petrels have been caught in mist-nets every year in the south west coast of Portugal, many miles away from any known breeding colony, by attracting them to the coast at night using tape-lures (Harris, Fowler & Okill 1993). My research is partly based on the data collected in this way before and during my PhD.

1.4.2 Seabird Diet and Foraging Ecology

The issue of how seabirds locate their prey in the immense ocean is far from completely understood. In continental shelf systems, currents impinge upon topographically fixed features, such as reefs or seamounts, creating physical gradients predictable in space and time, at which seabirds can congregate to find

aggregations of food. In the open ocean, where currents and dynamic processes are less pronounced, locations of aggregations can be much less predictable, and this has important consequences for the adaptations necessary for seabirds to locate and exploit their prey. Under these circumstances, prey behaviour is likely to be a primary mechanism responsible for seabird aggregation, if seabirds are able to predict the behaviour of their prey and consequently its spatial and temporal distribution. In this context, visual cues from the activity of other birds and cetaceans are also good ways of finding prey (Nevitt *et al.* 2004).

The Procellariformes also rely on their highly developed sense of smell to locate their prey (Bang 1966, Wenzel & Meisami 1987, reviewed by Nevitt 2008). Studies have shown that one of the olfactory cues used by these birds is the dimethyl sulphide, a substance released by the phytoplankton while being grazed by zooplankton (Dacey & Wakeham 1986). Olfaction is more relevant for finding prey at large spatial scales, in order for the birds to orientate towards areas where phytoplankton accumulates and where animal prey is therefore likely to be abundant. Larger and more aggressive species, such as albatrosses, are better adapted to exploit a combination of visual and olfactory cues to exploit large patches of high prey density, while smaller species, such as storm petrels, rely more exclusively on the sense of smell and are adapted to forage opportunistically on small or less concentrated prey patches (Nevitt *et al.* 2004, Nevitt & Bonadonna 2005).

At the breeding grounds, seabirds are more restricted in terms of foraging habitats and subject to higher inter- and intra-specific competition. The breeding season, is therefore likely to be a period when seabirds are particularly sensitive to changes in the availability and distribution of their prey (Ricklefs 1987, Weimerskirch 1998). Accordingly, dietary studies on seabird colonies have

investigated links between climate or fisheries and seabird demography, recruitment or productivity (e.g. Sydeman *et al.* 2001, Carscadden 2002, Abraham & Sydeman 2004). However, it is very likely that the birds adapt their foraging strategies to the requirements of breeding, and that their diet can be strongly restricted by all the constraints that are inherent to the breeding process (e.g. the trade off between self-feeding and chick provisioning, Ydenberg *et al.* 1994). Despite the importance of breeding success in population regulation, focusing dietary studies on the relatively short breeding period limits understanding of the overall constraints on populations, particularly for long-lived birds such as Procellariiformes. These birds may delay the time of their first breeding attempt until they are over four or five years old and spend most of their lives at sea feeding in the open ocean, far from the breeding colonies. Nevertheless, almost all methods and studies on seabird diet refer to this period when birds are on or close to land, mainly because of the obvious logistic difficulties of accessing the birds at sea. Thus, because no satisfactory method of studying the diet of seabirds at sea has yet been found there is an almost total lack of knowledge on what these birds eat when they are not breeding, including when they are immature and therefore not yet attending colonies (Barrett *et al.* 2007). Furthermore, even at the colonies, dietary studies of seabirds face various limitations, as outlined below.

Commonly, studies on seabird diet (and the diets of most animals in general) have been based on visual identification of prey remains in stomach sampling (e.g. Neves *et al.* 2006a) or in faeces or pellets (e.g. Naves & Vooren 2006, Neves *et al.* 2006b), direct observations of feeding behaviour (e.g. Sydeman *et al.* 2001, Paiva *et al.* 2006a,b) or, more recently, biochemical methods (e.g. Quillfeldt *et al.* 2005, Williams *et al.* 2008).

Sampling of stomach contents can be carried out by dissection of dead birds, or by stomach flushing or spontaneous regurgitations from live birds. Dead birds can be hard to obtain, and samples from birds that have died from natural causes may anyway be unrepresentative of the diet of healthy, living birds. While killing birds to examine stomach contents was acceptable up to around 20 years ago (Duffy 1986), it is not an option that many modern ecologists would be willing to consider, or that most ethical committees would approve.

Stomach contents can be obtained from captured birds by a process called stomach flushing. This involves inserting a latex tube deep into the bird's oesophagus and pumping salt water through the tube, causing the bird to vomit (Montalti & Ruben-Coria 1993, Neves *et al.* 2006a). A major disadvantage of this approach is that it is highly invasive (potentially causing mortality), and is becoming less acceptable at a time when most scientists are trying more and more to adopt non-invasive or even remote techniques for animal sampling (e.g. Waits & Paetkau 2005). Moreover, it is more successfully applicable in larger seabird species.

Captured seabirds sometimes spontaneously regurgitate partially-digested food during handling (i.e. without the stomach-flushing method being applied by researchers). However, these spontaneous regurgitations are only common among birds captured at the colonies of species that routinely regurgitate food to offspring. Furthermore, when at the colony it is hard to differentiate whether a regurgitated meal was meant to be digested by the adult or to be provided to the chicks. This issue may be important if the diets of parents and offspring differ, as suggested by the results of the few studies on this subject in seabirds (e.g. Davoren & Burger 1999, Wilson *et al.* 2004)

The analysis of faeces or pellets of undigested hard parts is non-invasive, but consists generally of the visual identification of prey remains and a major limitation of the method arises from biased recovery of the remains due to differential digestion and the difficulties of identifying well-digested prey in the sample (e.g. Seefelt & Gillingham 2006, Tollit *et al.* 2007). Besides, not all taxa, including storm petrels, regularly produce pellets and for those that do, finding pellets is for practical reasons, once more restricted to the breeding colonies.

Direct observations of foraging behaviour and prey choice have the advantage of enabling the study of seabird diet directly at sea and, for many species, also at the colonies (primarily those that do not breed underground and carry the entire prey in their bill, to deliver it to their nestlings). However, observations are very time consuming and it is often difficult to accurately identify what the birds are catching, due to the distance and brevity of most of such observations. These observational studies are often anecdotal in nature, and have been most useful in highlighting unusual or previously unknown trophic links or to give some indication of where the birds feed rather than to provide detailed data on the composition of their diet (e.g. Bocher *et al.* 2000).

More recently, analysis of mercury burdens (Monteiro *et al.* 1995), stable isotope ratios (Kelly 2000) or fatty acid signatures (Williams & Buck 2010) have been widely used to infer information about the diet of seabirds. Such biochemical methods have the great advantage of being relatively non-invasive and providing data about diet composition over long time scales, implying that information about the diet during the non-breeding period can be obtained. Nevertheless, these techniques have their own limitations, including that they provide information only on the overall trophic level or broad geographical regions in which birds have been

foraging, from which can be inferred only broad dietary shifts or changes in foraging location (e.g. Monteiro *et al.* 1995, Quillfeldt *et al.* 2005).

Molecular techniques have also been recently developed to study the diet of predators by detecting prey DNA in their guts, regurgitations or faeces (reviewed by Symondson 2002, King *et al.* 2008). These molecular techniques have not yet been extensively explored for birds but are a promising tool to improve the study of multiple trophic links, including in marine ecosystems (e.g. Jarman *et al.* 2002, Blankenship & Yayanos 2005) and the seabirds that rely on them (Deagle *et al.* 2007). This research will take advantage of these new techniques and I will develop their application in the Chapter 3.

Studies on the feeding ecology of Procellariiformes have been mostly directed to the families Diomedeidae (albatrosses, e.g. Pinaud & Weimerskirch 2002, Thompson *et al.* 2000) and Procellariidae (fulmars, shearwaters and other petrels; e.g. Hilton *et al.* 1998, Gray & Hamer 2001, Weimerskirch 1998). Hence, very few studies are available on the Pelecanoididae (diving petrels; Brooke 2004), the most distinct group of petrels with only four species, restricted to the southern hemisphere. These are generally the least studied of all the petrels (Brooke 2004). On the contrary, there is a wide literature available on the Hydrobatidae (storm petrels) but relatively few studies have so far focused on their foraging ecology. This is not surprising since the birds' small size limits or at least complicates the range of techniques currently available for this type of study.

Many seabird species, including various species of storm petrels, take prey from the surface layer, within a half meter of the sea surface (Bried 1996, Flood *et al.* 2009). It is broadly known from direct observations that storm petrels feed either solitarily or in small groups, by dipping, hovering or pattering on the sea surface,

sometimes following ships or cetaceans, and may aggregate where food is concentrated, for example along hydrological fronts (Webb *et al.* 1990). Some species of storm petrels have been observed to occasionally dive beneath the sea surface for food (e.g. Prince & Morgan 1987, Warham 1990) and a recent study using depth gauges has shown that this is a typical foraging behaviour for the Madeiran Storm Petrel* *Oceanodroma castro* in the Azores, although to very shallow depths (less than 1 m) and not for extended periods (Bried 2005). It is possible that this is a common behaviour also for other storm petrel species, including the European Storm Petrel (Flood *et al.* 2009, pers. obs.).

Cramp & Simmons (1977) reviewed the available information on the diet of European Storm Petrels, which can be summarised as follows. From a total of five birds from northern Europe, cephalopod remains were present in all five birds, with one containing the remains of small fish together with aphid wings. European Storm Petrels may sometimes feed on whale carcasses, and offal and kitchen scraps from fishing boats. In studies of breeding Storm Petrels in Wales, UK, Davis (1957) found that the nestlings are fed regurgitated pre-digested grey pulp and Scott (1970) found that chick diet is mainly composed of small Atlantic Herrings *Clupea harengus* and Sprat *Sprattus sprattus,* with crustaceans provided infrequently.

The most detailed study on the diet of the European Storm Petrel was carried out by D'Elbée & Hémery (1998), on the spontaneous regurgitations of adult birds caught at a colony in NW France (these birds were presumably about to deliver the regurgitated prey to their chicks). In this study, each individual regurgitate sample contained on average only 3.6 identifiable organisms and very few samples contained more than two fish items. Taxa identified visually in these regurgitates include coelenterates, nematodes, chaetognaths, copepoda, isopods, ostracods, *Cypris*

* The denomination Madeiran Storm Petrel, *Oceanodroma castro*, has recently been attributed only to those birds that breed during the winter period. Those breeding during the summer were classified as a different species – Monteiro's storm petrel *O. monteiroi* (Bolton et al. 2008).

larvae (Cirripedia), decapod larvae, euphausiids, insects, fish larvae and plant seeds. Zooplankton represented 52% of the number of identifiable prey items eaten, but in terms of biomass fish were the most important, belonging to four different families: Gadidae, Gobiidae, Myctophidae and Ammodytidae. One species of gadid fish, Poor Cod *Trisopterus minutus,* was the most common species present in the regurgitations (11% of the total identifiable taxa) but the Gobiidae (mainly *Pomatoschistus* spp. and *Aphia minuta*) were the most common prey, found in the highest number of samples. Intertidal nocturnally-active isopods belonging to two different species (*Eurydice pulchra* and *E. affinis*) were also found to be an important food resource by number. The regular occurrence of these intertidal isopods suggests that, besides foraging offshore, Storm Petrels must regularly exploit the intertidal zone. There was previous evidence of inshore foraging at night (Maguire 1980) close to the breeding grounds. Thomas *et al.* (2006) reported similar behaviour far away from any known colonies, in southern Portugal. Some reports also suggest occasional diurnal inshore feeding (reviewed by D'Elbée & Hémery 1998).

Thus, the few studies to have looked in any detail at European Storm Petrel diet were of food delivered to nestlings by breeding birds (e.g. Bolton 1995a,b, D'Elbée & Hémery 1998), but there are no data on the diet of adult birds during migration. Even during reproduction, several studies of other seabird species show that the food provided to chicks does not necessarily reflect the diet of the adults at that same period; the adult diet consists of prey items in different proportions to the diet of chicks, and the adult diet is generally less diverse (Baird 1991, Ramos *et al.* 1998, Shealer 1998).

Such a scarcity of information on feeding ecology and diet is common to most storm petrels. The Leach's Storm Petrel *Oceanodroma leucorhoa* is one of the best

studied species in terms of feeding ecology and diet, and yet the literature available on the subject is nevertheless relatively scarce (some examples are Ricklefs *et al.* 1987, Vermeer & Devito 1988, Pitman & Ballance 1990, Hedd & Montevecchi 2006). A number of publications can also be found on the feeding ecology of the Wilson's Storm Petrel *Oceanites oceanicus* (e.g Obst & Nagy 1993, Quillfeldt 2001, 2002, Quillfeldt *et al.* 2005, Gladbach *et al.* 2007) and the Madeiran/Monteiro's Storm Petrel (Harris 1969, Prince & Morgan 1987, Monteiro *et al.* 1995, 1996). Very few studies on the feeding ecology and diet of other species of storm petrels are available (summarised by Brooke 2004). The development of a feasible and reliable method to study the diet of non-breeding as well as breeding storm petrels is therefore required. Such a method will greatly facilitate the study and conservation of these remarkable birds and will promote the applicability of similar techniques to other seabird and terrestrial species throughout their annual cycles.

1.4.3 Molecular Biology as a Tool to Study Seabirds

Over the last few decades, an increasing number of studies in the behavioural ecology and population biology of species belonging to great range of taxa, have been based on molecular techniques (e.g. Parker *et al.* 1998, Freeland 2005). Seabirds have not been an exception and molecular studies have greatly improved our understanding of several aspects of their ecology, such as mate fidelity (e.g. Swatschek *et al.* 1994, Mauck *et al.* 1995, Huyvaert *et al* 2006), kinship relationships (e.g. Nielsen *et al.* 2006), social behaviour (e.g. Hughes 1998) and population dynamics (e.g. Milot *et al.* 2008).

Many molecular studies on the phylogeny of seabirds have also been recently published (e.g. Nunn & Stanley 1998, Bretagnolle *et al.* 1998, Nunn *et al.* 1996,

Kennedy & Page 2002, Austin *et al.*, 2004, Gomez-Diaz *et al.* 2006) including phylogenies for the storm petrels. For example, Cagnon *et al.* (2004) have shown a phylogeographic differentiation of European Storm Petrels, confirming the distinction of two subspecies of *H. pelagicus,* namely: *H. p. melitensis* for birds that breed within the Mediterranean basin and *H. p. pelagicus* for birds that breed in the north-east Atlantic.

More recently, DNA based techniques have been applied to the dietary study of predators and subsequently to the trophic links within the food webs that they are part of. This relies on identifying DNA sequences unique to particular prey taxa in diet samples from the predators (obtained from their guts, regurgitations or faeces; reviewed by Symondson 2002). Prey DNA can be identified from even well-digested, amorphous remains in these samples (e.g. Jarman *et al.* 2002, Kvitrud *et al.* 2005, Parsons *et al.* 2005), but these studies depend upon appropriate primers that amplify target prey DNA from the samples. Primers can only be designed appropriately if the DNA sequences for a good range of species are available. Conveniently, a comprehensive database of animal DNA sequences from the mitochondrial Cytochrome Oxidase subunit I gene (COI) is being developed (Hebert *et al.* 2003a) and can be directly applied to identify prey DNA isolated in diet studies that used general primers to target the COI gene. This has been referred to as "DNA barcoding", by analogy with the bar codes used to identify manufactured goods, and is available in public databases such as GenBank and the Barcode of Life Data systems (BOLD). The COI gene was considered the most appropriate target gene for DNA barcoding because it is evolutionarily conserved enough to be amplified with broad-range primers, yet divergent enough to allow species discrimination for the great majority of taxa (Hebert *et al.* 2003b).

The CO1 barcodes by themselves distinguish about 98 percent of species recognized through previous taxonomic studies (Stoeckle & Hebert 2008), but recently diverged species and species that have arisen through hybridization may not be resolved by COI sequencing. Similarly, plants have too little mitochondrial sequence diversity (probably due to hybridization and introgression), such that the COI is not a suitable gene to distinguish them. To overcome these problems, investigations are being carried out to find other genes that could be included into the barcoding database (Hollingsworth *et al.* 2009). The most common primers used so far for the barcoding of invertebrate species target the region of the CO1 amplified by primers designed by Folmer *et al.* (1994). These primers amplify a region of approximately 700 bp. This is too large to be used in dietary analysis because digestion rapidly degrades long sequences of DNA into shorter sequences. Therefore, an amplified region of 300bp is usually the maximum size used for studying DNA in diet samples. Ideally, primers used in dietary studies would amplify a smaller region within that amplified by the Folmer primers (Folmer *et al.* 1994), in order to maximise the chances of finding matches in the databases. However, it is not always achievable to design taxon-specific primers within this region for the taxa of interest and increasing the diversity of amplified regions and genes available in the online databases will be very beneficial for the specificity of taxonomic identification that will be possible in future dietary studies.

The use of "universal" primers (i.e. primers which bind with DNA from any taxon) provides an alternative analytical approach when sequences of potential prey taxa are not available, or to find unexpected components of the diet. This approach involves amplifying the sequences bound to the universal primer, sequencing the amplified sequences, and comparing these sequences with those of databases such as

Genbank and BOLD. However, universal primers may fail to amplify all target sequences in these situations because the early round of the PCR is dominated by the more common sequences and rarer sequences may fail to be selected. Even if the target sequence is amplified, it then needs to be isolated from the pool of all amplified sequences. This generally involves cloning the PCR product and sequencing a number of clones proportional to the diversity of sequences in the library (Jarman *et al.* 2004, Deagle *et al.* 2007, Lerner & Fleischer 2010). The development of "Next-Generation" DNA Sequencing techniques, capable of producing thousands or millions of sequences at once and lowering the cost of each DNA sequence beyond what is possible with standard dye-terminator methods, greatly overcomes this problem and enhances the use of molecular techniques in the study of trophic interactions (Deagle *et al.* 2009). However, at the moment, the overall financial cost of applying such techniques is still considerable.

In addition to the potential lack of appropriate taxon-specific primers, some limitations of DNA-based methods to study predator diets can be (i) short or variable post-ingestion detection periods, (ii) secondary predation resulting in detection of DNA from the prey's own gut, and (iii) cross-amplification by the primers of the predator's DNA (King *et al.* 2008). Various predator taxa can differ markedly in their DNA digestion rates (e.g. Chen *et al.* 2000) and often this problem is overcome by performing feeding trials to quantify prey DNA detection periods. This involves keeping captive specimens of the predator and feeding it with known prey under controlled conditions to investigate the detection period of different prey types (e.g. King *et al.* 2010). However, this method may be unfeasible when dealing with species that cannot readily be kept in captivity, such as adult seabirds, in which case we can only refer to the available literature on their digestive physiology, or the

results need to be interpreted with caution. If the digestion rate of a certain species is very high, then the DNA detection periods for its prey will generally be short and effort needs to be put into obtaining samples that are as fresh as possible.

Secondary predation can lead to incorrect conclusions because the DNA-based techniques cannot distinguish what a secondary predator has eaten from what its prey (a primary predator) has eaten prior to itself being predated. This issue can be particularly serious for those studies using gut contents or regurgitation samples, rather than faecal samples (as the secondary prey is likely to have been thoroughly digested by the time it reaches the faeces of a secondary predator). Even so, Sheppard *et al.* (2005), working on beetle diet, used an empirical approach to evaluate the potential bias of secondary predation on DNA-based techniques and showed experimentally that secondary predation is only a problem when the primary predator had consumed its prey immediately before being consumed by the secondary predator. A similar issue to secondary predation is the accidental ingestion of non-prey organisms by marine predators, since large numbers of small planktonic organisms may be ingested in sea water together with the intended prey.

Despite these limitations, DNA based studies have the major advantage of identifying components of the diet that are not apparent through physical examination. They allow us to study the diet of vertebrate animals in a non-invasive way through analysis of their regurgitates and faeces. As these DNA based techniques become more widely applied to study the diet of wild animals, more DNA sequences, from different genes, will become available providing an enormous range of opportunities for research into diet and foraging ecology.

Nevertheless, in comparison with other sampling methods, few studies have yet applied molecular techniques to study the diet of animals (less than 100 in total,

most of which have been published since 2005). Of these, nearly 80% have focused on invertebrates, for example in soil food webs (e.g. Harwood & Obrycki 2005, Read *et al.* 2006, Cassel-Lundhagen *et al.* 2009, King *et al.* 2010). Within the marine invertebrates, molecular studies have been published on the diet of copepods (e.g. Nejstgaard *et al.* 2003, Nejstgaard *et al.* 2008), mysids (Gorokhova 2006, Gorokhova & Lehtiniemi 2007), amphipods (Blankenship & Yayanos 2005), euphausiids (Passmore *et al.* 2006, Vestheim *et al.* 2008, Tobe *et al.* 2010), lobster *Jasus edwardsii* (Redd *et al.* 2008), brown shrimp *Crangon crangon* and shore crab *Carcinus maenas* (Albaina *et al.* 2010) as well as giant squid *Architeuthis* sp. (Deagle *et al.* 2005a). Most commonly in studies on invertebrate diets, DNA is extracted from the gut of the predator after killing it, but some studies have also extracted DNA from faeces (Nejstgaard *et al.* 2003, Redd *et al.* 2008). When killing the predator is not an option, such as in most vertebrates, faeces or regurgitations are the only way of assessing the diet of predators using DNA-based methods.

Most of the vertebrate literature on faecal analysis has the aim of extracting DNA from the predator for genotyping (e.g. Goossens *et al.* 2006, Gillett *et al.* 2008), rather than extracting the DNA of the prey. Many studies have focused on showing the feasibility of using faecal samples for remote sampling of vertebrate populations and this has become common practice over the last decade (e.g. Jalil *et al.* 2008, Fernandes *et al.* 2008). Molecular methods have been used to study the diet of terrestrial mammals using faecal samples, including studies of western Gorillas *Gorilla gorilla* and Black and White Colobus Monkeys *Colobus guereza* (Bradley *et al.* 2007) and even extinct species such as the Ground Sloths *Nothrotheriops shastensis* (Poinar *et al.* 1998). Several studies have used molecular methods to study the diet of marine mammals: Steller's Sea Lions *Eumetopias jubatus* (captive;

Deagle *et al.* 2005b), seals *Arctocephalus* sp. (Parsons *et al.* 2005, Kvitrud *et al.* 2005, Casper *et al.* 2007, Matejusova *et al.* 2008, Deagle *et al.* 2009), the Pygmy Blue Whale *Balaenoptera musculus brevicauda* (Jarman *et al.* 2002) and bottlenose dolphins *Tursiops truncates* (Dunshea 2009). A few molecular studies have also been published on the diet of fish (Rosel *et al.* 2002, Jarman & Wilson 2004, Smith *et al.* 2005, Corse *et al.* 2010). The first study to apply molecular methods to study the diet of bird species was by Sutherland (2000), who successfully amplified and distinguished DNA from different species of Leptidoptera in the faecal samples of two species of tit *Parus* sp. Sutherland's (2000) study was a PhD project at Oxford University, UK, and some of this work was repeated a year later by Casement (2001) in an unpublished report by the same University. The method was then tried very briefly in a seabird species, the Adelie Penguin *Pygoscelis adeliae* by Jarman *et al.* (2002). In 2006, Nystrom *et al.* published a study on the diet of Gyrfalcon *Falco rusticolus* where DNA analysis was used to identify two species of potential prey from remains collected at the nest sites. However, Deagle *et al.* (2007) presented the first detailed investigation of a bird's diet using a molecular approach, focusing specifically on a seabird species, the Macaroni Penguin *Eudyptes chrysolophus.*

Overall, with a few exceptions (Sutherland 2000, Deagle *et al.* 2007, 2010), nearly all the literature applying molecular techniques to the study of animal diet is still either preliminary (e.g. Harper *et al.* 2006, Nejstgaard *et al.* 2008) or aims to evaluate the importance of a single prey in the diet of a certain predator (e.g. Jarman *et al.* 2002), rather than investigating the diversity of trophic interactions.

1.5 Thesis Outline

The overall aim of this PhD project was to combine a range of datasets and analytical approaches to understand how environmental variation is affecting the diet, foraging ecology and migration fuelling strategy of the European Storm Petrel. This species was chosen as a case study for its small size (and hence its anticipated sensitivity to environmental change), extreme migration strategy and ease of capture during active migration at sites remote from the breeding colonies. To my knowledge, this is the first study of temporal variation of diet and fuelling strategy in a migrating seabird. The Data chapters (Chapter 2 – Chapter 4) were written as self-contained papers.

Chapter 2 reviews what is currently known about Storm Petrel migration strategy and identifies a dramatically female-biased sex ratio during migration past my Portuguese study site. The possible origins of this sex ratio biased are discussed; furthermore, the bias is important to take into account (both qualitatively and statistically where necessary) in subsequent parts of the thesis.

Chapter 3 describes the development and application of molecular scatology methods, supported by stable isotope analysis, for a detailed investigation of the diet of migrating Storm Petrels.

Having identified possible key prey species in Chapter 3, Chapter 4 addresses the migration fuelling strategy of Storm Petrels and identifies large inter-annual variations in the level of fuel reserves carried by birds migration past SW Portugal. Causal mechanisms underlying these variations are then investigated, linking climate-driven changes in physical oceanographic conditions to cascading changes across trophic levels in the marine food web; from primary productivity to Storm Petrels.

Chapter 2

Molecular Sexing Reveals a Strongly Female-Biased Sex Ratio among Migrating European Storm Petrels

2.1 Abstract

Molecular sexing revealed an unexpectedly strong female bias in the sex ratio of pre-breeding European Storm Petrels *Hydrobates pelagicus*, attracted to tape-lures during their northwards migration past SW Portugal. This was consistent across seven years, ranging from 80.8% to 89.7% female (mean annual sex ratio ±SD = 85.5% female ±4.1%). The sex ratio did not differ significantly from unity (i.e. 50% female) among (i) chicks at a breeding colony in NW France, (ii) adults found dead on beaches in southern Portugal, (iii) breeding birds attending nest burrows in Scotland, captured by hand, and (iv) adults captured near a breeding colony in Scotland using the same sound recordings as used in Portugal, indicating that females are not inherently more strongly attracted to tape lures than males. A morphological discriminant function failed to provide a good separation of the sexes, despite males being significantly smaller than the females in terms of wing length, body mass and one aspect of bill morphology. There was no sex difference in the seasonal or nocturnal timing of migration past Portugal, but there was a significant tendency for birds to be caught in sex-specific aggregations. The preponderance of females captured in Portugal suggests that the sexes may differ in migration route or in their prospecting behaviour (susceptibility to tape-lures) far away from the original breeding colonies.

2.2 Introduction

Many species of bird exhibit marked differences between the sexes in aspects of their behaviour, including their foraging behaviour and migration strategies (e.g. Cramp & Simmons 1977, Cristol *et al.* 1999, Nebel 2007). Sex-specific foraging behaviour amongst birds is believed to be related either to social dominance and competitive exclusion (usual when one sex is larger than the other) or from niche specialization (related to differences in morphology or reproductive role; Marra 2000, Bearhop 2006, Phillips *et al.* 2004). These differences in foraging behaviour can potentially lead to differences in migration strategies, with males and females migrating at different times, travelling by different migration routes, or travelling to/from different wintering grounds (Cristol *et al.* 1999). Identifying and investigating sex-differences in migration behaviour is important for our understanding of species' ecology and conservation, but for monomorphic species such studies are hampered by the difficulty of identifying the sex of individuals, particularly outside the breeding season. Previous studies have attempted to address this problem by using morphometric methods such as discriminant function analysis, but such methods are by definition difficult to apply to monomorphic species, and often only a small proportion of individuals can be sexed with confidence (Brooke 2004, O'Dwyer *et al.* 2006, Warham 1996). As a result, there is a lack of information for monomorphic species on sex-differences in behaviour in general, and on migration strategies in particular. Only a few studies have addressed sex-specific differences in seabird behaviour outside the breeding season; these studies were based on stable isotope signatures among various Procellariid species (e.g. Hedd & Montevecchi 2006, Phillips *et al.* 2009).

The need to study species throughout their life cycle has been increasingly emphasised as more studies demonstrate the importance of carry-over effects of non-breeding processes into breeding productivity and population dynamics (e.g., Lindstrom 1999, Norris & Taylor 2006, Reudink *et al.* 2009). Molecular sexing methods now allow accurate sexing of individuals of even highly monomorphic species outside the breeding season (e.g., Bertellotti *et al.* 2002, Russello & Amato 2001), and in this study we apply molecular diagnostics to study differential migration patterns in a monomorphic migratory seabird, the European Storm Petrel *Hydrobates pelagicus*.

Storm petrels (family Hydrobatidae) are small but long lived pelagic seabirds, with delayed reproductive maturation. Pair bonds tend to last for many years. Females lay one large egg per year which both adults incubate. Both adults also feed the chick for about two months, until shortly before the chick is ready to fledge (Brooke 2004).

The European Storm Petrel (henceforth abbreviated to "Storm Petrel" where appropriate) is the smallest Atlantic seabird (~26 g), and birds of the Atlantic population are long-distance migrants between the breeding colonies in the north-east Atlantic and their wintering areas in the south Atlantic and Indian oceans, off southern Africa (Wernham *et al.* 2002). Like other Hydrobatidae, Storm Petrels normally come inshore only at night (Thomas *et al.* 2006), and pre-breeding birds can readily be attracted into mist-nets using nocturnal playbacks of sound recordings of conspecific nesting calls. These "tape-lures" are effective for catching Storm Petrels during their summer northwards migration, even at locations in SW Iberia, far from the nearest known colonies (Harris *et al.* 1993, Wernham *et al.* 2002). Most of the birds caught with this method are aged 2-4 years, returning northwards in the

years before their first breeding attempts in order to prospect for mates and breeding sites (Bolton & Thomas 2001, Wernham *et al.* 2002, Okill & Bolton 2005). Storm Petrels are usually absent from the Atlantic colony sites before the age of two and they usually only start breeding at the age of four or five (Okill & Bolton 2005). Little is known about what they do during the period before they begin returning to the colonies, but they are thought to remain in their wintering grounds at least during their first year (Bolton & Thomas 2001).

Breeding Storm Petrels are usually not attracted to playbacks of nesting calls since they tend to keep the same mate and nest site between years and they therefore cease to prospect for these once they are acquired. Breeding-age birds can still be caught in mist-nets without the need for tape-lures, but only at the colonies when they attend their nests. Nest sites are relatively easy to find, and both adults and chicks can be caught by hand in the nest. Therefore, as with other seabirds, much of what is known about Storm Petrels is derived from studies at or near the breeding colonies, where they are accessible to researchers. Like other storm petrels, European Storm Petrels are sexually monomorphic in terms of plumage features (Brooke 2004); breeding birds can (sometimes) be sexed on the basis of cloacal morphology or breeding behaviour (Scott 1970, Copestake *et al.* 1988), or discriminant function analysis can be used to predict the sex of individuals on the basis of biometric measurements (e.g. James 1983). As a result, little is known about sex-differences in the behaviour and ecology of Storm Petrels, such as dietary preferences (see Chapter 3), foraging and fuelling strategies (see Chapter 4), migration routes and natal site-fidelity. This lack of knowledge is most marked for the long period when birds are away from the breeding colonies, because of the difficulties involved with observing, catching and sexing the birds during the non-breeding season. Previous studies have

tested for differences between the sexes in the foraging behaviour of storm petrels (Stewart *et al.* 1999, Cherel *et al.* 2005, Hedd & Montevecchi 2006, Phillips *et al.* 2009, Gladbach 2009), but none of these involved the European Storm Petrel. Only one study (Gladbach 2009) found such a difference; in the chick provisioning strategies used by male and female Wilson's Storm Petrels *Oceanites oceanicus*; this sex-difference was only apparent in years of food shortage.

Molecular sexing techniques now enable tape-lured migrating Storm Petrels to be accurately sexed for the first time, providing novel insights into the behaviour and ecology of this pelagic seabird away from the breeding colonies. Instead of the X and Y chromosomes found in mammals, birds possess Z and W sex chromosomes, with males being homogametic (ZZ) and females being the heterogametic (ZW) sex. Griffiths *et al.* (1998), Kahn *et al.* (1998) and Fridolfsson & Ellegren (1999), published combinations of primers that allow the sex of individuals to be determined in most species of birds, using a simple PCR reaction based on size differences of the introns present in both the CHD1-W and CHD1-Z genes (the W- or Z-linked genes coding for the chromodomain-helicase-DNA-binding protein), which are found in most extant non-ratite birds. Several authors have now reported the use of this technique to sex fledgling and adult birds, mostly in captive-breeding projects (e.g., Bertault *et al.* 1999, Russello & Amato 2001) but also in the field (e.g., Hörnfeldt *et al.* 2000, Bertellotti *et al.* 2002, Nogueira *et al.* 2008).

The majority of molecular sexing studies have used DNA extracted from blood samples obtained relatively invasively (Bensch *et al.* 1999, Ewen *et al.* 2001, Genovart *et al.* 2003). However, molecular sexing can also be achieved much less invasively using DNA obtained from a single feather (Jensen *et al.* 2003, Harvey *et al.* 2006, Costantini *et al.* 2008) or a faecal sample (Waits & Paetkau 2005). Feathers

are becoming more widely used for molecular sexing of birds (Harvey *et al.* 2006), with the use of faecal DNA samples mainly used in mammals (e.g., Yamauchi *et al.* 2000, Bradley *et al.* 2001, Vidya & Kumar 2003). Despite a number of recent studies reporting successful DNA extraction from bird faeces, to our knowledge only four publications report molecular sexing by this means (Robertson *et al.* 1999, Segelbacher & Steinbrück 2001, Regnaut *et al.* 2006, Mäki-Petäys *et al.* 2007), but without presenting the details of the results obtained or methods used. This is possibly due to the greater challenge of amplifying nuclear DNA from faecal samples in comparison with mitochondrial DNA (Segelbacher 2002).

Using molecular sexing from feathers and faeces, the aims of the present study are: (i) To investigate the sex ratio of European Storm Petrels tape-lured to mist nets in Portugal over seven years, during the northwards migration of pre-breeders towards the Atlantic breeding colonies; (ii) To investigate if the sex ratios observed in Portugal are consistent with those in other parts of the annual cycle; (iii) To use the molecular sexing data generated to test for sex differences in aspects of migration behaviour of the species. The data on the sexes of the individual Storm Petrels in this dataset will also be used to examine sex differences in diet (Chapter 3) and migration fuelling (Chapter 4) in subsequent Chapters of this thesis.

2.3 Methods

2.3.1 Fieldwork

Storm Petrels were caught in mist-nets at the base of a sea-cliff on the south west coast of Portugal (37° 04' N, 8° 47' W, Figure 2.1), using tape lures of the calls that the males perform from their nest sites (usually referred to as the 'Purr' call; Cramp & Simmons 1977, Robb & Mullarney 2008). Tape-luring took place from dusk

(2100 GMT) to dawn (0400), within the period mid-May to late June, in all years from 2003-2009. This sampling period spans the main period during which migrating storm petrels can be attracted to tape lures in Portugal (Harris 1993). European Storm Petrels sampled using tape lures at this Portuguese field site have been found (using a combination of ringing data and molecular screening) to be comprised almost entirely of birds originating from the Atlantic population, with a very small number of vagrants (<1%) from the Mediterranean population (Robb & Mullarney 2008, Andrew King unpublished data).

Two sound recordings of Storm Petrel "purr calls" (James 1983, 1984) were used as tape-lures: (i) a recording obtained from the British Trust for Ornithology during the 1990s and (ii) track 11 of disc 1 in the CD collection by Roche (1997). The recording-locations of both of these recordings were unknown. These tracks were played on Technika MP Series MP3 players coupled to a Martley Megaphone 600 at a sound pressure level of approx. 70 dB, and were clearly audible at a distance of approx. 400 m offshore (personal observations). Males respond more strongly than females to playbacks of these purr calls in terms of calling in reply to the playbacks from inside the nest burrows (James 1984), but previous studies using tape-lures of purr calls to mist-net Storm Petrels in or near breeding colonies have found that there is no apparent sex bias in the birds attracted (see Table 2.IV).

Each captured individual was ringed and its age determined (as first-year or older than first-year, based on the abrasion and shape of the primary flight feathers; Bolton & Thomas 2001). Biometric measures were taken of body mass, wing length, tarsus length (from the depression in the angle of the intertarsal joint to the base of the last complete scale before the toes diverge), culmen length (from the tip of the bill to the feathering at the base of the bill), "bill depth 1" (from the bottom of the

mandible to the top of the nostril tube, taken at the depression mid-way along the tube), "bill depth 2" (from the bottom of the mandible to the top of the maxilla taken just anterior to the nostril), head-plus-bill length (from the tip of the bill to the back of the skull) and rump width (the anterior-posterior width of the exposed white feathering of the rump-patch). Wing length and body mass were the only measures taken during all seven years; the other measures were recorded only from 2006-2009 with the exception of head-plus-bill (recorded from 2006-2008) and rump width (only recorded in 2009). Between one and four breast feathers (most commonly two) were collected from each bird for molecular sexing, and kept in a paper envelope at ambient temperature. All the birds were processed at the site where they were caught, and were released shortly after capture.

We also acquired equivalent samples from Storm Petrel breeding locations in the NE Atlantic (Figure 2.1) - in July 2005, breeding birds attending nest burrows during daytime on Sanda Island, Scotland (55° 16′ N, 5° 34′ W), were captured by hand; In August 2006, tape-luring was carried out close to a small breeding colony on Ailsa Craig, Scotland (55° 15′ N, 5° 6′ W), using the same procedures as those used in Portugal, including using exactly the same sound recordings to attract European Storm Petrels into mist nets. At both of these sites, one breast feather was collected from each bird for molecular sexing, and kept in a paper envelope at room temperature. Fa ecal samples were collected from chicks at colonies in Brittany, France (48° 23′ N, 4° 57′ W, Figure 2.1) during the 2005-06 breeding seasons and stored in 80% ethanol.

In addition, European Storm Petrels found dead on beaches in southern Portugal (37°07'N 08°36' W) following severe storms in January 1996, were collected for anatomical sexing. On dissection, females were identified by the

presence of the single ovary on the left side, and males by the presence of a testicle on each side. Unfortunately these corpses subsequently became decomposed and molecular sexing could not be tested on them for this study.

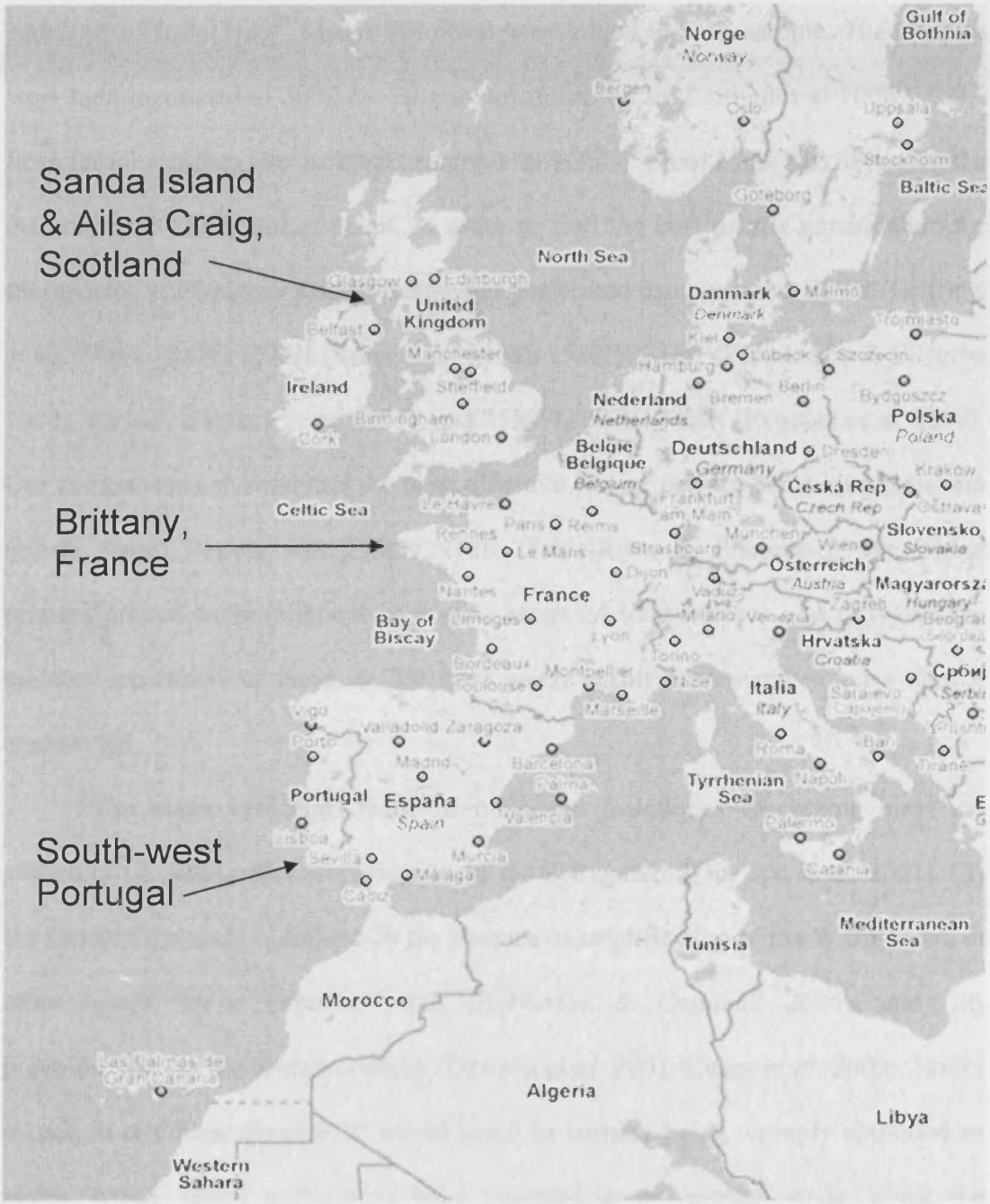


Figure 2.1 Location of the study sites used in the present study to sample European Storm Petrels in migration (Portugal), at the breeding colonies (adults - Sanda Island and chicks - Brittany) and near a breeding colony (Ailsa Craig).

2.3.2 Molecular Sexing

DNA from feathers was isolated using an adaptation of the Chelex extraction method (Walsh *et al.* 1991). The barbs towards the base of each feather were removed and approximately 5mm of the calamus of the feather was cut off. 50 µl of distilled H_2O and 20µl of InstaGene™ Matrix (BioRad) were added to each sample. The samples were then incubated at 50°C for 30 minutes, followed by 8 minutes at 100°C. DNA from faecal samples was isolated using the QIAGEN® Stool Mini Kit, following the manufacturer's standard protocol. In order to find the best primer combination for this species, preliminary primer testing was performed using primers P8/P2 (Griffiths *et al.* 1998), 1237L/1272H (Kahn *et al.* 1998), 2550F/2718R (Fridolfsson & Ellegren 1999), P8/M5 (Bantock *et al.* 2007), and 2550F/TuWR/ TuZR (Regnaut *et al.* 2006). Our comparisons showed that the most effective primer pair for separating male and female Storm Petrels was 2550F/2718R (Fridolfsson & Ellegren 1999). These primers proved to be efficient at a wide range of temperatures and provided the greatest separation of bands (~200 base pairs), easily differentiated on a simple agarose gel.

The major criticisms made of molecular techniques for sexing birds are related to (i) preferential amplification of the Z fragment (Dawson *et al.* 2001), (ii) the fact that the male is defined by the absence of amplification of the W fragment, in other words, by a negative result (Robertson & Gemmell 2006), and (iii) polymorphism in the Z chromosome (Dawson *et al.* 2001, Casey *et al.* 2009). Errors related to criticisms (i) and (ii) would result in females being wrongly classified as males, which seems unlikely to have occurred in the present study, given the direction of the sex-ratio bias in our main results. Primers 2550F/2718R have other advantages that minimise such potential sexing errors (Dawson *et al.* 2001, Casey *et*

al. 2009). Shizuka & Lyon (2008) developed a new W-specific primer (GWR2) to be used in combination with 1237L/1272H. This approach is very promising but it could not be tested in the present study because it was published after this research had been completed.

All PCRs included two positive controls to test for the success of the amplification and two negative controls, prepared with distilled water, to test for possible contamination. A gradient PCR was first performed in order to optimise the annealing temperature. One feather extraction and two faecal extractions were used for each temperature gradient PCR. These PCR reactions were performed on a BioRad PTC-225 DNA Engine® Peltier Thermal Cycle PCR machine (45°C to 60°C). The optimum annealing temperatures, obtained from these gradient PCRs, were 50°C for the feather samples and 47.5°C for faecal samples. Thirty individuals (15 males and 15 females) were selected at random to be sexed using both feathers and faeces, to compare the results obtained with the two types of samples and check for their consistency. Each male result was always checked at least three times and about 25% of all female results were checked at least twice.

Amplifications from feather extractions were made with a standard PCR, carried out in accordance with Fridolfsson & Ellegren (1999), using 1 µl of DNA template (~10 ng/ul). Those from faecal extractions were performed using a Multiplex kit, carried out in 20 µl reactions containing 1x of QIAGEN® Multiplex PCR Master Mix, 0.2 µM of each primer and 3 µl of DNA template (~3 ng/ul). The thermal conditions were 95°C for 15 min, 35 cycles of 95°C for 1 min, annealing temperature for 1 min 30 s, 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. All reactions were carried out using an Applied Biosystems GeneAmp® PCR

System 9700 PCR machine. Samples were run on 2% weight/volume agarose gels stained with ethidium bromide, unless specified otherwise.

2.3.3 Statistical Analysis

Chi-square tests were used to test for deviation from the expected 50:50 sex ratio, except for cases in which one or more expected values were less than five, in which case Fisher's exact test was used. *t*-tests were used to compare morphometric measurements between sexes and a discriminant function analysis was used to examine whether birds could be reliably sexed on the basis of morphometric measurements. Most of the analyses were carried out in SPSS v15.0; exceptions were the Fisher's exact tests, which were computed at www.langsrud.com/fisher.htm, and binomial confidence intervals, which were calculated using a Bayesian calculator available at: www.causascientia.org/math_stat/ProportionCI.html. Significance thresholds were set at $P = 0.05$. Note that the *P*-values presented in our tables are not corrected for multiple comparisons (see e.g., Perneger 1998, Moran 2003).

A runs test was performed in Rv2.6.7, to test the hypothesis that the European Storm Petrels captured using tape-lures in Portugal were captured in sex-specific groups. Given that unequal numbers of males and females were captured, we used the simulation-based method for a "biased coin" runs test presented by Crawley (2007) to test whether the observed number of runs of consecutive same-sex individuals was significantly different from the number of such runs expected if individuals of the two sexes occurred in a random sequence.

2.4 Results

2.4.1 Sex Ratios of Adult European Storm Petrels

A strongly female biased sex ratio (mean ± SE = 85.0% female ± 1.39%) was found in the sample of birds tape-lured in Portugal in all seven years (Table 2.I) with no significant differences in sex ratio among years (χ^2 = 11.794, *d.f.* = 6, *P* = 0.07) and no significant trend in sex ratio over the seven years (Pearson's *r* = 0.062, *n* = 7 years, *P* = 0.895). The vast majority of the birds caught were at least two years old, with only 0.01% of either undetermined age, or definitely in their first year (cf. Bolton & Thomas 2001). Among the birds from Portugal that were sexed, many carried rings from other countries, or were later recaptured in other countries; a female-biased sex-ratio was also found in these birds regardless of the country where they were previously ringed or subsequently recaptured (Table 2.II).

A total of 18 dead Storm Petrels were recovered from beaches in Portugal in 1996. Anatomical sexing revealed this sample to be comprised of 12 males and only six females, but this apparent male-bias was not significantly different from 50% female (Table 2.I).

Adult Storm Petrels tape-lured in Scotland, close to their breeding grounds, using the same sound recordings as used in Portugal, also showed a sex ratio that was not significantly different from 50% female (Table 2.I), suggesting that the sex bias in Portugal was not simply an artefact of the use of tape lures. Although this sex ratio is estimated from a relatively small sample of 30 birds, we found that 100 random sub-samples of 30 birds from the much larger Portuguese sample gave a mean sex ratio (± SE) of 84.7% (± 0.80), with only 4% of these sub-samples giving a female bias smaller than 64%, which was the upper 95% confidence interval of the sample tape-lured in Scotland. Thus, the apparent difference in sex ratio between birds tape-

lured in Portugal and Scotland does not appear to be an artefact of small sample size of the Scottish sample.

Breeding birds caught at their nest sites on Sanda Island in Scotland during the incubation period also showed a sex ratio that was not significantly different from 50% female (Table 2.I). This was expected given that both sexes incubate eggs equally (Cramp & Simmons 1977). In the absence of birds of known sex to validate the molecular sexing, this is a useful confirmation of the reliability of the molecular method.

2.4.2 Sex Ratio among European Storm Petrel Chicks

From the chicks examined at the breeding colony in France, nine faecal samples were collected in 2005 and 29 in 2006. In 2005, four chicks were found to be female and three were male (two samples could not be sexed); in 2006, 12 chicks were found to be female and 10 were male (seven could not be sexed). Data from both years were pooled to allow for statistical analysis. This indicated that the observed primary sex ratio of sexable chicks at this breeding colony did not deviate significantly from 50% female (Table 2.I).

2.4.3 Sex Differences in Biometrics and Behaviour of European Storm Petrels Tape-Lured in Portugal

On average, male Storm Petrels had significantly lower body mass, shorter wings and deeper bills (in terms of the measurement of bill depth 2) than females. However, there were no significant differences between the sexes in measurements of tarsus, culmen, bill depth 1, head-and-bill, or rump (Table 2.III). The best discriminant function, based on structural biometrics and using a randomly selected

subset of females to equal the sample size of the males, included two variables: the ratio of bill depth 2 to culmen and the ratio of wing length to tarsus. The resulting function is as follows:

Discriminant score = -0.654 * billdepth2 / culmen + 0.810 * wing / tarsus

(Wilks' Lambda = 0.900, χ^2= 9.396, P = 0.009).

This discriminant function correctly classified 63% of the individuals (n = 92) sexed with the molecular techniques, 62.2% of 45 males and 63.8% of 47 females. This is not a very useful level of discrimination in Portugal, since we could obtain a higher proportion of birds correctly sexed (~85%) by simply assuming they were all female.

Over the 1.5 months of the annual study period, there was no significant seasonal difference in when males and females were captured (mean difference = males 0.21 days before females, 95% CI limits -1.29 to +1.7 days, t-test = 0.280, $d.f.$ = 939, P = 0.781). Similarly, there was no significant difference in the time of night at which males and females were captured (mean difference = males 13 minutes before females, 95% CI limits = -8 minutes, to +33 minutes, t-test = 1.22, $d.f.$ = 939, P = 0.223). A runs test with unequal sample sizes showed that there were slightly, but significantly, fewer "runs" of consecutive catches of birds of the same sex (181 runs), than expected from random sequences of males and females, using the observed sample sizes for each sex (P < 0.01, 99% CI limits for expected number of runs = 184-219 runs). This result indicates that the observed same-sex runs were slightly, but significantly, longer than expected; hence there was a tendency for Storm Petrels to occur in sex-specific groups at our tape-lures in Portugal.

Table 2.I Sex ratios of European Storm Petrel adults and chicks in different locations and years. All samples were sexed using DNA extracted from feathers, except for the storm-killed birds in Portugal (sexed by dissection) and the chicks sampled in France (sexed using DNA extracted from faeces - see Methods).

Year	Female	Male	Total	Sex ratio (% female)	95% CI limits (% female)	$\chi 2$ test for deviation from unity (1:1), *d.f.* = 1
Tape-lured birds, Portugal						
2003	83	12	95	87.4	79.2-92.6	$\chi^2 = 53.1, P < 0.001$
2004	81	17	98	82.7	73.9-88.9	$\chi^2 = 41.8, P < 0.001$
2005	122	16	138	88.4	82.0-92.7	$\chi^2 = 81.4, P < 0.001$
2006	105	25	130	80.8	73.1-86.6	$\chi^2 = 49.2, P < 0.001$
2007	93	11	104	89.4	82.0-94.0	$\chi^2 = 65.6, P < 0.001$
2008	90	22	112	80.4	72.0-86.6	$\chi^2 = 41.3, P < 0.001$
2009	236	27	263	89.7	85.5-92.8	$\chi^2 = 166.1, P < 0.001$
All years combined	810	130	940	86.2	83.8-88.2	$\chi^2 = 491.9, P < 0.001$
Storm-killed birds, Portugal (1996)	6	12	18	33.3%	16.3-56.6%	$\chi^2 = 6.096, P = 0.297$
Tape-lured birds, Scotland (2006)	14	16	30	46.7%	30.2-64.0%	$\chi^2 = 0.133, P = 0.715$
Hand-caught birds, Scotland (2005)	15	17	32	46.9%	30.8-63.6%	$\chi^2 = 0.125, P = 0.724$
Chicks, France (2005 + 2006)	17	12	29	58.6%	40.6-74.5%	$\chi^2 = 0.862, P = 0.353$

Table 2.II Sex ratio of European Storm Petrels controlled in different countries or re-trapped in Portugal.

Location	Males	Females	Sex ratio (% female)	Fisher's Exact test for deviation from unity
Iceland, Norway & Denmark	1	16	94.1	$P = 0.007$
UK & Ireland	13	56	81.2	$P < 0.001$
France, Spain & Italy	3	15	83.3	$P = 0.07$
Same-year re-traps in Portugal	0	5	100	$P = 0.17$

Table 2.III Mean body measurements (mm) and body mass (g) for European Storm Petrels caught in Portugal among 1989-2008 (± SE).

Sex	Tarsus	Bill depth 1	Bill depth 2	Culmen	Head & Bill	Wing	Rump	Body Mass
Male	22.6 ± 0.78 (n = 81)	4.6 ± 0.35 (n = 52)	3.8 ± 0.31 (n = 52)	11.7 ± 0.53 (n = 71)	31.9 ± 0.77 (n = 53)	122.8 ± 2.80 (n = 130)	14.8 ± 2.23 (n = 27)	26.0 ± 2.05 (n = 129)
Female	22.5 ± 0.71 (n = 473)	4.5 ± 0.26 (n = 343)	3.7 ± 0.22 (n = 343)	11.8 ± 0.76 (n = 432)	31.8 ± 0.65 (n = 239)	123.8 ± 2.55 (n = 806)	14.9 ± 2.18 (n = 234)	26.4 ± 2.30 (n = 805)
***t*-test**	t =1.57 df = 552 P = 0.118	t = 0.51 df =393 P = 0.132	t = 2.10 df =58.5 **P = 0.040**	t =1.21 df = 501 P = 0.225	t = 0.79 df = 290 P = 0.428	t = 4.00 df = 934 **$P < 0.001$**	t = 0.26 df =259 P = 0.795	t = 2.04 df = 932 **P = 0.042**

Table 2.IV Previously published sex ratio data for European Storm Petrels. Sex ratios of birds caught at or near colonies are close to unity, regardless of whether they are captured using "purr call" tape-lures, or not.

Location	Year	Reference	At a colony?	Capture method	Tape lure used?	Sexing method	Males	Females	Sex ratio (% female)	Sig. different from unity?*
Throughout marine range	Prior 1977	Cramp and Simmons 1977	At colonies & at sea	Various	Mainly no	Museum skins dissection	20	25	56	No
Skomer, Wales	1981 & 1982	James 1984	Yes	Breeders taken on nest	No	Cloacal inspection	43	39	48	No
Skomer, Wales	1982	James 1983	Yes	Mist nets	Yes	Discriminant analysis (wing + tail)	31	26	46	No
Skomer, Wales	1982	James 1983	Yes	Mist nets	No	Discriminant analysis (wing + tail)	23	20	47	No
Skomer, Wales	1982	James 1983	Yes	Breeders taken on nest	No	Cloacal inspection	26	20	43	No
St. Kilda, Scotland	1983	R.W. Furness *In* Fowler *et al.* 1986	Yes "loose colony"	Mist nets	No	Dissection	11	10	48	No
Yell, Shetland	1983 & 1984	Fowler *et al.* 1986	No (but colony on same island)	Mist nets	Yes	Laparoscopy	21	28	57	No

2.4.4 Consistency of Sexing from Feathers and Faecal Samples

Overall, the proportion of feather samples that gave a result was 94% while that from faecal samples was 29.3%. When sexed from faecal samples, birds previously identified as female from the feathers often amplify only one of the two fragments, Z or W. When the W-fragment (female specific) is evident, birds can still be sexed with confidence. However, when only the Z-fragment (shared by males and females) is visible, females will be misidentified as males. Accordingly, 100% of birds sexed as male from feathers were also sexed as male from faeces, but 43% of females sexed from feathers were initially sexed as male from faeces. This proportion dropped to 14% after repeating each male result three times. Correcting the number of chicks that were potentially sexed incorrectly due to this type of error would still result in a non-significant sex bias ($\chi^2 = 2.793$, d.f. = 1, $P = 0.095$). For those birds sexed from feathers, less than 3% of the initial male results were found to be females after the three repeats and none of the initial female results appeared as males in subsequent testing.

2.5 Discussion

The molecular sexing analysis revealed a very strongly female-biased sex ratio among Storm Petrels sampled during their northwards migration past the Portuguese coast, several hundred kilometres from the nearest known breeding colonies. This sex ratio bias was broadly consistent over the seven years examined (varying between 81 and 90%), indicating that it is a stable feature of the birds available for capture using tape lures at this location (comprised almost entirely of wandering pre-breeders from the Atlantic population). To our knowledge, this is the first time that such a result is reported for a monomorphic seabird during migration.

The highly female-biased sex ratio that we observed among tape-lured birds in Portugal is strikingly and consistently different from the approximately 1:1 sex ratio found among European Storm Petrels of a variety of age classes sampled using a variety of techniques and sexing methods, at or near the NE Atlantic breeding colonies (Table 2.IV). We also found no evidence for any difference in geographical origin of the two sexes in our Portuguese sample (Table 2.II). This suggests that gender is more important than origin (and thus e.g., travel distance) in determining the migratory behaviour of these birds.

The strong female bias observed amongst the sample of birds caught in Portugal could be due to (1) a real sex-ratio bias in the population; (2) females being strongly attracted to the tape-lure, or (3) females being more likely to encounter the tape-lure (e.g., due to a sex difference in the timing or route of the migration journey). None of the above explanations are mutually exclusive, but we discuss them separately below.

For a sex ratio bias in a population to persist, a consistent bias in the primary sex ratio (amongst eggs/chicks) and/or a sex-specific mortality rate after fledging must be present. The primary sex ratio may be biased in some taxa, including some bird species (Mayr 1939, Sheldon 1998, Donald 2007). However, these are exceptional examples and most bird populations, especially in monogamous species, exhibit approximately 1:1 primary sex ratios (reviewed by Ellegren & Sheldon 1997). There was no bias in the primary sex ratio among the chicks hatched by Storm Petrels breeding at a colony in NW France, suggesting that this is not the explanation for any sex-ratio bias in the adult population.

A female-biased adult sex-ratio could arise from an unbiased primary sex ratio if males suffer greater mortality than females. In contrast to mammals, greater

male mortality is very uncommon among birds (reviewed by Donald 2007). The sex-ratio in the sample of European Storm Petrels killed during winter storms off the Portuguese coast did not differ significantly from unity (though we note that more males than females were killed; see Table 2.I). In one species of petrel (a diving petrel *Pelecanoides urinatrix*) a significant male biased mortality has been found among storm-killed individuals (Norman & Brown 1987). However, even if male Storm Petrels are more likely to be killed by storms, this may not be sufficient to give rise to a female-biased sex ratio, since other causes of death might be of greater importance in determining the relative numbers of surviving males and females. A total of 45 museum skins of Storm Petrels from throughout the species' range and annual cycle also show an unbiased sex ratio (Table 2.IV) and no sex ratio biases were found in any of the previous studies summarized in Table 2.IV. Furthermore, in the present study, the sex ratios among live birds tape-lured near a breeding colony in Scotland and among live birds captured without tape-lures at nest sites in Scotland were also unbiased. There is therefore little support for the hypothesis that there is an underlying bias in the sex ratio of the population as a whole.

The second hypothesis accounting for the female-biased sex ratio observed in Portugal is a sex bias in the attraction to the tape lures. It is possible that female Storm Petrels are inherently more attracted to tape lures of conspecific calls than are males, but this is not consistent with the finding that use of the same tape lures near a breeding colony resulted in an unbiased sex ratio. Similarly, the two studies presented in Table 2.IV on sex ratios of Storm Petrels caught either at- or close to- a breeding colony with tape lures show no sex ratio bias. James (1984) found that male Storm Petrels in nesting burrows responded more strongly than females to playbacks of "Purr" calls, which might be expected to result in a male bias among birds

attracted to tape-lures. However, no significant male bias was detected in any of the samples of tape-lured birds.

A differential attraction to the tape lures in Portugal could arise from a sex bias in the seasonality of prospecting behaviour, or in the distance from the natal colony at which prospecting may occur. For example, a biased sex ratio among pre-breeding Storm Petrels captured in Portugal could arise if males and females are differentially attracted to the tapes at different times in the season (e.g., males being more attracted earlier in the season) or at different locations (e.g., females being more attracted further south). The former could arise if males need to find their burrows earlier in the breeding season (Kokko *et al.* 2006), to which they subsequently attract a female, while the latter could occur if males exhibit stronger natal site fidelity, meaning that females may be more likely to disperse between breeding colonies, and so be more willing to investigate breeding locations in Portugal, well outside their main breeding range. No research has apparently yet investigated these possibilities, but among the sample of wandering pre-breeders in the present study, though there was temporal aggregation by sex over short timescales, there was no evidence of temporal segregation of males and females over the timescale of the migration season within the sampling period.

The third hypothesis accounting for the female-biased sex ratio observed in Portugal is that a sex difference in migration strategy leads to more females than males being present in Portuguese coastal waters during the May-June study period. There are several potential underlying mechanisms. Females could begin to wander north at a younger age than males, meaning more prospecting females than males reaching Portuguese waters. Pre-breeding Storm Petrels tape lured in the UK have an unbiased sex ratio (Tables 2.1 and 2.4), but the observed female bias in Portugal

could arise if younger females reach as far north as SW Portugal but do not wander all the way north to the breeding colonies.

Other possible mechanisms are a sex difference in the diurnal or seasonal timing of migration. Diurnal differences could arise since, at sea, European Storm Petrels are active both by day and by night (pers. obs.). Possibly, sex-differences in the diurnal/nocturnal pattern of migration along the Portuguese coast could make females more likely to come within hearing range the nocturnal tape-lures. However, there was no difference between males and females in the time of night at which they were captured. Seasonal differences could be related to sex-differences in the time of arrival at the colonies. In many migrant species, the breeding males are the first to arrive back on the breeding grounds (protandry), to set up territories or secure a mate (Rubolini *et al.* 2004, Smith & Moore 2005, Catry *et al.* 2005). No difference between males and females in capture date in Portugal was found, indicating that if males really are migrating at a different season than females, then this male migration must take place outside the study period of late May-June.

Finally, the sexes could have different migration routes. A different migration route could be a consequence (or the cause) of a difference in foraging strategy between the sexes. For example, due to differential nutritional demands, females may be more likely than males to exploit areas of high productivity (due to upwelling) close to the African and Portuguese Atlantic coasts (Stenseth *et al.* 2004), whereas males may migrate further offshore, along a more direct route between the wintering and breeding areas. This possibility could be tested by investigating the sex ratio of birds caught from boats further offshore than the range of our land-based tape-lures. This has been piloted in the present study, but has proven to be extremely difficult and to date only four birds have been caught, of which only one was a male.

Of the above hypotheses, it seems most likely that sex-differences in natal site fidelity (Hypothesis 2) or migration strategies (Hypothesis 3) account for the strong sex-ratio bias among Storm Petrels migrating past Portugal, but the underlying mechanisms remain unclear. Future work could further test these hypotheses through studies of the genetic structure of different breeding populations, and by capturing birds at different times of year and at additional locations off the Portuguese coast, further north and south along the migration route, and in the wintering grounds. Nevertheless, recognising these patterns is a first important step to investigate potential mechanisms and incorporate such information into conservation strategies, such as the implementation of marine protected areas that are now under consideration in many parts of the world. For example, by combining molecular sexing information with molecular identification of prey DNA in storm petrel faeces, it becomes possible to test for sex-differences in diet (Chapter 3), and migration fuelling strategies (Chapter 4). These findings show the importance of considering sex specific behaviour in interpreting ecological data.

Chapter 3

Investigating the Diet of Migrating European Storm Petrels Using Molecular Tools

3.1 Abstract

The diet of storm petrels (Hydrobatidae) is largely unknown, particularly outside the breeding season, due to the lack of a reliable non-invasive method to study it in detail. The present study describes the development and application of molecular techniques to study the diet of the European Storm Petrel, in combination with stable isotope analysis. This was achieved by the detection of prey DNA from faecal samples collected from Storm Petrels during their northwards migration past the coast of SW Portugal between 2006 and 2009. The diet of nestling Storm Petrels from a breeding colony in Brittany, NW France, was also studied in 2005 and 2006, for comparison with the migrating birds. Two complementary molecular approaches were used: 1) using taxon-specific primers to screen for the presence / absence of particular prey categories in individual faecal samples; and 2) amplifying prey DNA from a pool of samples using general primers, then using cloning and sequencing of the amplified sequences to identify the taxa present in the diet in each year. The major category of prey detected was fish (chiefly European Sardines *Sardina pilchardus*). Other components of the diet were Cephalopoda (primarily cuttlefish *Sepia* spp.), Amphipoda, Isopoda and a range of terrestrial invertebrates (primarily Lepidoptera, Hymenoptera and other insects), which were presumably scavenged from the sea surface by the Storm Petrels. Many prey taxa could be identified to species level using the cloning and sequencing approach, including deep-water

species that may have been made available to foraging Storm Petrels by the fishing industry. Individual migrating Storm Petrels typically had DNA of one or two different prey categories in their faecal samples, with few birds having no amplifiable DNA, or DNA of three or more different prey categories. Fish appeared more frequently in the diet of migrating birds than appeared in the diet of nestlings at the breeding colony. Furthermore, diet composition appeared to vary among years, and the migrating birds appeared to rely more on fish in 2009 than in the preceding three years. These results indicate that Storm Petrels may be opportunistic foragers, possibly varying their diet according to the changing availability of different prey, including scavenged material.

3.2 Introduction

Investigating an organism's diet is of primary importance for understanding its ecological requirements and its functional role in the ecosystems that it inhabits. However, most methods currently available for the study of diet in wild animals are either invasive, or have important limitations in the information that they can provide, or both (see Chapter 1 and below). Studying the diet of small, elusive, highly mobile animals such as the pelagic storm petrels (Hydrobatidae) is particularly challenging, with no satisfactory single method or combination of methods currently available. As a result, the diet and foraging ecology of such taxa is often poorly understood. There is therefore a major requirement for developing a widely applicable, non-invasive and objective method for the study of animal diet in the wild (Barrett *et al.* 2007). Molecular analysis of prey DNA in the faeces of foragers potentially fulfils this requirement, and this Chapter describes the application of two complementary molecular approaches for the study of the diet of

the European Storm Petrel *Hydrobates pelagicus*; (i) screening for presence / absence of different prey taxa with taxon-specific primers and (ii) cloning and sequencing of prey DNA, amplified using general primers. The advantages and limitation of these molecular approaches are discussed in contrast to, and in combination with, other methods for investigating diet in seabirds, primarily stable isotope analysis.

3.2.1 Current Methods for Investigating Avian Diet; Advantages and Limitations

(i) Observations of Foraging Behaviour

One of the most direct and basic approaches for studying diet and foraging ecology is simply to observe foraging animals and to visually identify food items as they are eaten or as they are being carried to feed offspring. This can be successfully applied to large animals eating large and conspicuous food items (e.g. a Peregrine Falcon *Falco peregrinus* capturing a Feral Pigeon *Columba livia* in flight) and it has been used in some seabird species such as puffins *Fratercula* spp. and terns *Sterna* spp. delivering food to their chicks (e.g. Paiva *et al.* 2006a,b). However, this approach is difficult or impossible to apply when the foragers are small, difficult to approach, or eating a mixture of small or indistinct food items (e.g. swifts *Apus* spp. eating flying insects at high altitudes, or storm petrels (Hydrobatidae) taking small food items from the moving sea surface). Even if the individual food items can be discerned, specific identification is often impossible or biased towards larger and more easily identified prey.

(ii) Examining the Contents of the Digestive Tract

The normal practice in many early studies of animal diet was to kill a sample of the animals and examine their stomach contents (e.g. much of the dietary information summarised in Cramp & Simmons 1977). However, for ethical reasons this is increasingly unacceptable for many vertebrate taxa (Cuthill 1991, Broom & Johnson 1993). Alternatively, the stomach contents of animals that are found dead can be investigated, but the diet of animals that have died from natural causes may not be typical of healthy living individuals and the sample size is tipically small.

A commonly used, non-fatal method for sampling stomach contents, widely applied to large seabirds, is stomach flushing (also known as lavage). This involves inserting a tube down the oesophagus of captured individual, and using a saline solution injected into the stomach/crop to flush the stomach contents out through the subject's mouth. This procedure is generally considered to be invasive, even for relatively large species, and it can be risky to apply it to small species such as storm petrels (pers. obs.).

Food delivered to nestlings can be sampled from the very top of the digestive tract by applying temporary neck ligatures, which prevents the food put into the nestlings' mouths by their parents from being swallowed. The food can then be scooped out from the mouth by the researcher and the ligature removed (e.g. Douglas *et al.* 2008). This is a very direct but invasive method for sampling chick diet, but cannot be used to study adult diet (which may differ from the diet provided to the chicks), or to study diet outside the nestling phase of the breeding season.

Prey remains may be made available to researchers by the animals themselves, through the natural regurgitation of pellets (i.e. a compact ball of undigested hard parts of prey, regurgitated by many bird species) or the defensive

regurgitations of undigested or semi-digested meals by taxa such as Procellariiformes and herons *Ardeidae* when captured or closely approached (e.g. Gilbert *et al.* 2003). Such samples potentially provide less invasive means (or non-invasive means, in the case of pellets) to study stomach contents.

Whatever the methods by which they are obtained, an important limitation of the visual identification of prey in samples of stomach contents is that digestion may already have begun to degrade the food items, so that taxa may become impossible to identify. Furthermore, different prey taxa may differ markedly in the rate at which they become unidentifiable; for example, soft-bodied taxa will generally be rapidly degraded, whereas certain hard parts (e.g. fish otoliths) may remain intact through the digestive tract. Such differences in digestibility and identifiability make it difficult to interpret direct comparisons of the contribution of different food types to the diet. Furthermore, skills are needed for the identification of these hard parts.

(iii) Stable Isotope Analysis

Many chemical elements have two or more stable (i.e. non-decaying) atomic forms, known as stable isotopes, which differ in the number of neutrons that the nucleus of the atom contains. For example, carbon has two stable isotopes ^{12}C (more common, containing 6 neutrons) and ^{13}C (less common, containing 7 neutrons). Nitrogen similarly has two stable isotopes, ^{14}N (more common) and ^{15}N (less common). The differences in the nuclear masses of the atoms can result in small but consistent differences in the ways in which the heavy and light isotopes of an element are affected by physical processes, and hence systematic spatial and temporal variation in the ratio of different isotopes across the environment.

On a molecular scale, heavier molecules have a lower diffusion velocity so, for example, they diffuse out of cells more slowly than lighter molecules. Furthermore, the collision frequency with other molecules (the primary condition for chemical reactions) is lower for heavier molecules; this is one of the reasons why lighter molecules tend to react faster. At the scale of a living organism, the net effect of these processes is fractionation; in effect, the selective metabolic loss of the lighter isotopes (^{12}C and ^{14}N) from living tissue. Fractionation of isotopes of different molecular weights occurs progressively as elements pass between the different trophic levels of a food chain, resulting in systematic variation in the ratio of different isotopes across trophic levels in any particular habitat. Measuring the isotopic delta-value (δ; the ratio of the heavier isotope to the lighter isotope, expressed as parts per thousand, ‰) in tissues taken from foragers can therefore be used to infer information about the trophic level(s) or location(s) at which a forager has predominantly fed.

In marine food webs, fractionation generally results in an enrichment in $\delta^{15}N$ of approximately 3.0 to 5.0‰ and in $\delta^{13}C$ of 0.8‰ per trophic level (Minagawa & Wada 1984, Owens 1987, Michener & Shell 1994), though these enrichment values can themselves depend on the specific type of tissue sampled (Hobson & Clark 1992). Enrichment rates measured in the transfer of isotopes into bird feathers have been in the order of 0.8 to 0.9‰ for $\delta^{13}C$ and 3.1 to 3.3‰ for $\delta^{15}N$ compared to the equivalent $\delta^{13}C$ and $\delta^{15}N$ values in the diet components (Hobson 1995).

Stable isotopes of different elements differ in the extent to which they are fractionated in different contexts, and hence in their suitability for addressing different types of questions about foraging ecology. For example, the different stable isotopes of nitrogen vary primarily across trophic levels (rather than between

habitats). Therefore, nitrogen isotope ratios are commonly used to determine the trophic level at which foragers primarily feed.

In contrast, the relative abundance of the different stable carbon isotopes varies primarily across habitats (rather than across trophic levels). For example, marine and terrestrial communities differ in their $\delta^{13}C$ signatures, so that $\delta^{13}C$ signatures in the tissues of foragers may reflect their foraging habitat. Therefore, carbon isotope ratios are commonly used to assess foraging location, such as whether animals are foraging primarily from terrestrial versus marine, inshore versus offshore and pelagic versus benthic food webs. However, such inferences rely not only on prior knowledge of the carbon isotope ratios in each habitat, but also of how these underlying differences may vary geographically across the globe.

Stable isotopes analyses have been widely used to study the nutrition of a range of species from copepods to humans (Hobson *et al.* 2002, Tykot 2004), and have been increasingly applied to the study of the foraging ecology of birds (e.g. Bearhop *et al.* 2006, Bond & Jones 2009, Weiss *et al.* 2009). This approach offers many advantages compared to the more traditional methods described above, namely being less invasive or even non invasive (depending on the tissue sampled), not biased towards less digestible material, and allowing for the study of animal diet over a range of time-scales. The time-scale over which stable isotope analysis can reveal a forager's diet depends on the rate of molecular "turnover" in the tissue sampled. Metabolically inert tissues such as fully grown feathers can be used to infer diet at the time that these were grown (up to 1 year ago in most birds), whereas actively growing feathers can be used to infer recent diet (generally over less than 1 month). Different fractions of blood samples differ in their molecular turnover, so that red blood corpuscles and blood plasma can provide information about diet over recent

weeks and days, respectively. This is particularly relevant in the study of birds; for example, fully grown feathers can often be used to infer the diet of migratory birds on their wintering grounds (Bearhop *et al.* 2004, Wiley *et al.* 2010).

Stable isotopes have been used to study the diet of many species of Procellariiformes from albatrosses and shearwaters (Bugoni *et al.* 2010, Paiva *et al.* 2010, Wiley *et al.* 2010) to smaller petrels (Hedd & Montevecchi 2006). Despite their widespread application, stable isotopes analyses have important limitations in that knowing the isotopic signatures of the prey is essential to interpret the data and, more importantly, in the lack of taxonomic detail on the information obtained. New analytical developments such as isotope mixing-models (e.g. Bugoni *et al.* 2010) are allowing a degree of quantitative discrimination of the prey consumed, but these methods are still best applicable to species feeding on a limited range of prey (two or three taxa) of known isotopic signatures.

(iv) Fatty Acid Analysis

This method is based on the overall premise that fatty acid composition tends to vary more among species than within species, and that long chained fatty acids (>14 carbon units) pass to the predator from the prey with relatively little degradation and are stored in the predators' adipose tissue, which can be sampled using biopsies (Williams & Buck 2010). By comparison of the predator's fatty acid profile with those of potential prey taxa,, some detail of diet composition can be assessed, rather than just the trophic level and/or foraging location obtained with the stable isotopes approach described above. However, fatty acid analysis still does not allow species-level identification of dietary components. Further limitations of this technique are (i) that a fatty acid database of all possible prey is needed to interpret predator fatty

acid signatures accurately, and (ii) because most predators feed on more than one prey type, the interpretation of the fatty acid signatures is not straightforward. Possible errors of measurement or interpretation of fatty acid signatures can occur, related to the predator's intrinsic rates of fatty acid production and metabolism, variability of fatty acid signatures between individuals of the same prey species and the need to calibrate the metabolic shifts in fatty acid signatures between the forager and its food. This approach has been applied relatively frequently in the marine environment (e.g. Iverson 2009, Hanson *et al.* 2010, Young *et al.* 2010, Skoglund 2010), including in the study of seabird diet (e.g. Williams *et al.* 2008, Williams & Buck, 2010, Kakela *et al.* 2010). Although better than stomach flushing in terms of animal welfare, the biopsy procedure is still relatively invasive, especially if compared to the simple collection of faecal samples used in the molecular approaches described below.

(v) Identification of Prey DNA from Predator Faeces

The biochemical methods described above, using "intrinsic markers" (stable isotopes, fatty acids), have improved the knowledge of the foraging ecology of many species of animals (e.g. Hobson *et al.* 2002, Caut *et al.* 2008, Mancina & Herrera 2010). However these methods are all still limited when used in isolation, particularly in that they do not provide species-specific prey identification. Molecular (i.e. PCR-based) methods, involving extraction and analysis of prey DNA from the digestive tract of foragers, have the clear advantages of being non invasive and providing very detailed information on diet composition, potentially to the species level (Symondson 2002, King *et al.* 2008). Prey DNA becomes progressively degraded by digestion and therefore, these studies typically use primers that target

relatively short DNA fragments from mitochondrial genes. Mitochondrial DNA is more abundant in animal cells than nuclear DNA and it is therefore more likely that intact fragments of the relevant genes are available to be detected.

DNA-based studies require reference DNA sequences of the prey consumed and the existence (or design) of relevant primers for isolating prey DNA. Such primers would ideally amplify all the potential prey without amplifying the DNA of the predator. This is difficult to achieve, however, as primers general enough to amplify DNA from a range of prey species will almost inevitably amplify the predator's own DNA. Solutions to this problem have been developed, including blocking the amplification of predator DNA (Dunshea 2009), but there are still limitations as it is impossible to guarantee that prey DNA is not being blocked, especially if the predator's diet includes species closely related to the predator itself.

An alternative molecular approach is to use taxon-specific PCR primers to screen samples for the presence or absence of particular prey taxa of interest. It is difficult to be certain that the primers used are amplifying all the species within the targeted group, or how specific they are to that group; nevertheless, a good level of group specificity can be achieved relatively easily. Moreover, as more sequences become available for potential prey species, and more taxon-specific primers are developed and tested, this approach will become even more powerful and easily applicable.

The only previous studies to apply molecular techniques to investigate diet of a seabird species were published recently by Deagle *et al.* (2007) and Deagle *et al.* (2010). The first focused on adult Macaroni Penguins *Eudyptes chrysolophus* attending a colony. Deagle *et al.* (2007) used the two different molecular approaches described above, and compared the results with those from stomach content analysis.

This was however a preliminary study with a relatively small sample size in a single breeding season. The second study was on Little Penguins *Eudyptula minor*, using pyrosequencing to scale-up the number of prey DNA sequences that could be obtained (Deagle *et al.* 2010). Each of these studies described the diet of the birds from just one colony in a single breeding season.

In the present study, a DNA-based method is used to analyse the diet of pre-breeding (mainly 2-4 year old) European Storm Petrels, captured over four years (2006-2009) during migration past the coast of Portugal, from their wintering grounds in the south Atlantic, *en route* to their future breeding sites along the NE Altantic seaboard, between the north coast of Spain and Iceland/Norway (Bolton & Thomas 2001). The diet of nestling Storm Petrels from a breeding colony in Brittany, NW France, was also studied to some extent in 2005 and 2006, for comparison with the migrating birds.

European Storm Petrels (henceforth "Storm Petrels") are amongst the smallest of the seabirds (~26 g) and due to their small size, their movements at sea cannot be studied through any currently available remote-tracking equipment. Studies on their foraging ecology and diet have been limited to behavioural observations at sea (reviewed in Cramp & Simmons 1977, Poot 2008), early description of stomach contents of dead birds (reviewed in Cramp & Simmons 1977), and a small number of studies at breeding colonies (Davis 1957, Scott 1970, D'Elbée & Hémery 1997). Molecular techniques have been tested in a wide range of predator species, but with greatest focus on invertebrate species (e.g. Symondson 2002, King *et al.* 2008). The present study is, to my knowledge, the first detailed investigation of the diet of a seabird species during migration, and one of the few to apply molecular techniques to investigate the diet of seabirds.

In the present study, the same two molecular approaches used by Deagle *et al.* (2007) are applied, combined with stable isotope analysis, to investigate Storm Petrel diet. The first molecular approach was to determine the presence/absence of DNA from relevant prey taxa for each faecal sample. This was achieved by performing PCR tests using primers that specifically amplify DNA from certain relevant prey groups. This screening approach was used on faecal samples from migrating adult Storm Petrels caught in the south of Portugal and on faecal samples from nestlings at a Storm Petrel breeding colony in Brittany, France. This approach gives the proportion of individuals that consumed certain prey groups and requires some prior knowledge of taxa likely to appear in the predator's diet.

The second molecular approach, used only for the migrating Storm Petrels, involves amplifying DNA from general prey groups (fish and non-fish) followed by cloning and sequencing the DNA to separate and identify individual prey sequences. This approach provides a list of DNA sequences of prey species consumed by the predator. These sequences can be identified by comparison against sequences of known species. In theory, the number of sequences obtained for each prey species should reflect the relative contribution by mass of each prey taxon to the diet of the predator (based on the assumption that larger prey items contain greater amounts of DNA than smaller prey). However, prey species may differ in the number of mitochondria that their cells contain, and therefore some prey species will contribute more DNA per unit mass consumed than others, to the total amount of mitochondrial DNA present in the faecal sample. Furthermore, primers might not be equally sensitive to each prey species and preferably amplify some species over others.

To supplement and validate the information obtained from the molecular analyses, for a subset of the years studied using the molecular approach (2008 and

2009), stable isotope analysis of growing feathers was used to infer the overall trophic levels and foraging location (i.e. coastal/offshore) of migrating Storm Petrels. The relative merits of the molecular and stable isotope methods, and the degree to which they are complementary, will be discussed.

3.3 Methods

3.3.1 Collection of Samples from European Storm Petrels

Faecal samples from Storm Petrel chicks from a colony in Brittany, NW France (48° 23′ N, 4° 57′ W) were collected from inside the nests during the 2005 and 2006 breeding seasons, by a collaborator (B. Cadiou). These samples were collected onto filter paper and stored in 80% ethanol.

Migrating Storm Petrels were captured in mist nets during their northwards journey past the south-west coast of Portugal (37° 04' N, 8° 47' W). Birds were attracted to shore using tape lures of storm petrel male song, played at night between dusk (approx. 22:00 and dawn approx. 05:00 GMT). Captured birds were ringed and weighed and biometric measurements were taken (see Chapter 2 for full details of these procedures). One to five breast feathers were taken for molecular sexing (see Chapter 2 for details). Faecal and vomit samples were collected over four field seasons in late spring (late May-mid June) between 2006 and 2009.

Throughout the capture and handling process, and while the birds were preparing to fly off following release, the birds were observed closely in order to collect any faeces or vomit that they produced. Birds were released onto flat rocks, a few metres away from the ringing area and most faecal samples were collected from this substrate after the bird had flown. More rarely, faecal samples were obtained during the ringing process, from the bird bag or other surfaces. In contrast, vomit

samples were all obtained either when the bird was in the net or during the ringing process. The samples were collected into 2ml Eppendorf tubes using a paper disc or cotton bud and preserved with 80% ethanol. Each sample was labelled with the ring number of the individual bird that produced it, and subsequently stored at -20°C until the DNA was extracted in the laboratory. In 2007, samples were frozen directly in the field without ethanol. Before collecting each sample, the sampling equipment and the sampling surface were sterilized by flaming.

In 2008 and 2009, captured birds were inspected for any growing body feathers and these were collected for stable isotope analysis. Stable isotope values integrate diet during the period of feather growth. The time taken for a body feather to grow is not known for European Storm Petrels, but this species replaces all eleven of its primary flight feathers over a period of approximately 7 months (Scott 1970). Since body feathers are much smaller than primaries, it was assumed that growing feathers integrate information about diet of storm petrels over a period of one to two months.

3.3.2 Reference DNA Sequences

In order to increase the chances of identifying prey DNA sequences obtained from the faecal samples of Storm Petrels, a reference collection was built, of sequences from potential prey caught near the study site in Portugal. Samples of fresh potential prey were collected by sweeping the sea surface with nets from the coast and further offshore (up to 12km) from a boat, both during the night and during the day. These samples consisted mainly of invertebrates later identified by experts in the different taxonomic groups encountered (pers. comm.). Samples of fresh-caught local fish

species were obtained from a market in the fishing port of Lagos, 10km from the study site in SW Portugal.

DNA from these fresh invertebrates and fish was extracted with the Chelex method (Walsh *et al.* 1991). Extractions of fish DNA were made from the liver, cut into small pieces and dried at 45°C for 45 min. For each fish species, 30 mg of dried liver was used per extraction in 150 µl of water and 60 µl of Instagene Matrix. One or two specimens of each invertebrate species were used per extraction. These invertebrate samples were put into a 2 ml Eppendorf tube, in 50 µl of water to which 20 µl of Instagene Matrix (Invitrogen) was added. Samples were mixed by vortex and incubated for 1h at 50°C, followed by 8 min incubated at 100°C.

Two different primer pairs were used to amplify DNA from the fish or the invertebrate species: one primer pair designed to amplify Osteichthyes (bony fish) DNA, and one initially designed to amplify DNA from a wide range of invertebrate species, but that was subsequently found also to amplify some vertebrate species and thus will be referred to as "non-fish" (Table 3.I). Amplifications were performed using the Multiplex PCR Kit (Qiagen) in 25 µl reactions containing 1x Multiplex PCR Master Mix, 0.2 µM of each primer and 0.1 mg/ml of BSA (New England Biolabs). The template was 1 µl of the DNA extract. Thermal cycling conditions were as follows: 95°C for 15 min, 35 cycles (94°C for 30 s followed by 56°C (for FishF1/R1) or 46°C (for CI-J-2183/CI-N-2353) for 90 s followed by 72°C for 90 s), concluding with 72°C for 10 min. A minimum of three negative controls (the extraction control, plus at least two distilled water blanks) were included in each set of PCR amplifications. PCR products were separated by electrophoresis in 1.5% agarose gels and visualised by staining with ethidium bromide, visualised by transillumination with UV light.

Prior to sequencing, PCR products were purified by filtration using the Qiagen Qiaquick cleaning kit according to the manufacturer's protocol. Samples were sequenced in an Applied Biosystems 3130xl Genetic Analyzer using a 50 cm capillary array with POP-7 polymer.

3.3.3 DNA Extractions from Faecal and Vomit Samples

DNA from storm petrel faecal and vomit samples was extracted using the QIAamp DNA Stool Mini Kit (Qiagen), following the manufacturer's standard protocol. Depending on the size of the faecal sample (i.e. number of cotton buds or amount of paper; total sample volume per extraction was approximately 0.5ml), one or more extractions (up to four) were performed so that DNA was extracted from the whole sample. For samples from which more than one extraction was performed, often the final extraction was done from the ethanol in which the sample had been immersed.

Vomit samples were typically larger in volume than the faecal samples, and three different approaches were used for these as appropriate: a) the sample was homogenized by vortexing and DNA was extracted from a subsample; b) the sample was centrifuged, the top lipid layer was removed and DNA was extracted from the bottom layer; c) the DNA was extracted from solid parts of the sample (e.g. lumps of fish, parts of invertebrates). For the latter approach, hard parts were separated and placed into fresh ethanol at least two days before extracting. To extract the DNA from vomit samples, both the Qiagen DNeasy Blood and the Qiagen Tissue Kit and the QIAamp DNA Stool Mini Kit were tried. One blank extraction, using only water, was included in each batch of 24 extractions to test for any cross-over contamination.

3.3.4 Testing for Presence / Absence of Prey DNA

In order to first test for the success of the extraction process, DNA extracts were screened using primers specific to storm petrel DNA, which amplify a region of approximately 200 bp from the mitochondrial Cytochrome B gene. These primers were designed from sequences in GenBank (Cagnon *et al.* 2004) using the software Amplicon (Jarman 2004). Any samples giving a negative result were tested a second time, to confirm that they were indeed negative. Successful extracts were then screened for prey DNA. Solid parts of prey in vomit samples were identified as either fish or invertebrate and tested directly with the relevant primers (FishF1/R1 or CI-J-2183/CI-N-2353) rather than with storm petrel primers.

Successful extracts were screened with the two more general sets of primers, targeting fish (FishF1/R1) and non-fish prey (CI-J-2183/CI-N-2353), also used for the reference collection (described above). For the migrating birds caught in the south of Portugal but not for the samples from Brittany, representative faecal samples containing fish DNA were subsequently screened for the specific sequences of clupeiformes. This order is represented in the seas off SW Portugal by the highly abundant European Sardine *Sardina pilchardus* (henceforth "Sardine") and by five other species: *Engraulis encrasicolus*, *Alosa fallax, A. losa, Sardinella aurita* and *Sprattus sprattus*; Borges 2007). For those faecal samples containing non-fish DNA, the presence/absence of four particular invertebrate prey taxa was determined with separate PCR assays using group-specific primers testing for: i) amphipods, ii) isopods, iii) Mysidacea, and iv) cephalopods (Table 3.I). These taxa were chosen to be screened for, as cephalopods (cuttlefish) and isopods have previously been described as part of the Storm Petrel's diet (D'Elbé & Hémery 1998, Thomas *et al.* 2006); amphipods (sandhoppers) and mysidacea (opossum shrimps) were the most

abundant taxa obtained from net sampling in the field (decapods were also abundant, but no specific primers were already available or could be successfully designed for these taxa), and Sardine is the most abundant fish in the area (FAO 2004).

Table 3.I Primers used for predator and prey DNA screening from faecal samples of European Storm Petrels.

Target	Primer name	Sequence 5'-3'	Product size	Annealing temp.	Reference
Storm Petrel mitochondrial Cytochrome b	PetrelF1 PetrelR1	TCATCAGTCGCACACACATGC CAGTTGCTATGAGGGTGAGTA	200	58°C	This study
Osteichthyes mitochondrial 12S	FishF1 FishR1	CGGTAAAACTCGTGCC CCGCCAAGTCCTTTGGG	300	56°C	Jarman unpubl.
non-fish species mitochondrial COI	CI-J-2183 CI-N-2353	CAACATTTATTTTGATTTTTTGG GCTCGTGTATCAACGTCTATWCC	216	46°C	Simon *et al.* 1994; Simon *et al.* 2006
Clupeiformes mitochondrial Cytochrome b	C-CB285dF C-CB431R	CGCCCACATTGGNCGAGG GTGGCCCCTCAGAAGGACATTTGGCC	147	61°C	Jérôme *et al.* 2003
Isopoda nuclear 18S rDNA	IsopodNSSf1 IsopodNSSr1	TCATGATTYATGGGATGT AAGACCTCAGCGCTCGGC	201-278	57°C	Jarman *et al.* 2006
Amphipoda nuclear 18S rDNA	AmphNSSf1 AmphNSSr1	CTGCGGTTAAAAGGCTCGTAGTTGAA ACTGCTTTRAGCACTCTGATTTAC	204-375	58°C	Jarman *et al.* 2006
Mysidacea mitochondrial COI	MysF1 MysR2	TTCCTTGAGCGTGCTGGTTC GAGGAAAGGCCATATCAGGC	194	47°C	Swan & King, unpubl.
Cephalopoda nuclear 28S rDNA	Squid28SF Squid28SR	CGCCGAATCCCGTCGCMAGTAAAMGG CTTC CCAAGCAACCCGACTCTCGGATCGAA	180	60°C	Deagle *et al.* 2005

Amplifications were performed separately for each primer pair, using the Multiplex PCR Kit (Qiagen) in 20 µl reactions containing 1x Multiplex PCR Master Mix, 0.2 µM of each primer and 0.1 mg/ml of BSA (New England Biolabs). The template was 2 µl of the DNA extract. Thermal cycling conditions were as follows:

95°C for 15 min, 35 cycles (94°C for 30 s followed by the primer specific annealing temperature for 90 s followed by 72°C for 90 s), concluding with 72°C for 10 min. A minimum of three negative controls (the extraction control, plus at least two distilled water blanks) were included in each set of PCR amplifications. PCR products were separated by electrophoresis in 1.5% agarose gels and visualised by staining with ethidium bromide, visualised by transillumination with UV light.

3.3.5 Cloning and Sequencing Prey DNA

Subsets of the faecal samples that contained prey from 2006-2009, were used to make the clone libraries. One clone library was produced for each of the two years, for each prey type (i.e. fish - FishF1/R1 or non-fish – CI-J-2183/CI-N-2353). The DNA concentration of the PCR products was measured using Picogreen and the samples were pooled according to their concentration. The number of samples pooled per treatment (year/prey type) varied between 6 and 19. Products were cloned using the TOPO TA cloning system (Invitrogen) following the manufacturer's protocol. Colonies containing the recombinant clones were cultured in LB broth and plasmid DNA was amplified with M13 primers in 25 µl reactions containing 1 µl of culture medium, 1x of buffer, 0.1 mM of dNTPs (Invitrogen), 1 mM of Mg^{-2}, 0.5 µM of each and 0.4 U of Taq (Invitrogen). Thermal cycling conditions were as follows: 94°C for 3 min, 35 cycles (94°C for 20 s followed by 60°C for 20 s followed by 72°C for 90 s), concluding with 72°C for 10 min.

The PCR products were sequenced in an Applied Biosystems 3130xl Genetic Analyzer using a 50 cm capillary array with POP-7 polymer. Due to unexpected low efficiency of the cloning reactions, the number of sequences retrieved per treatment (year/prey type) was relatively small and uneven across the years. All the species /

taxa identified from the clone library were confirmed to exist in the study region from published literature and/or from the reference sequences from locally-caught samples.

3.3.6 Primer Optimisation

All the primers used were initially optimised to the needs of the study. Gradient PCRs (45° to 60°) were performed for each primer pair, in order to optimise the annealing temperatures of the target taxon. The highest temperature at which the primers were still amplifying target DNA was selected and a range of cross-reactivity tests were performed to verify the specificity of the primers. This way, the primers were optimised in a way that it would be more likely to underestimate the presence of a prey type (the primers failing to amplify all the species of that prey type) than to overestimate it (the primers amplifying non-target prey types). Each primer pair was tested against European Storm Petrel, 13 species of fish, 15 species of Amphipoda, three species of Decapoda, six species of Isopoda, two species of Mysidacea, one species of Tanaidacea, Gastropoda, Copepoda, Cumacea, Cephalopoda and Annelida (Table 3.II). None of the primers used amplified DNA from European Storm Petrel.

Fish primers (FishF1/R1) designed for bony fish (Osteichthyes) were very robust and amplified all target fish species tested. As expected, they did not amplify ray (a cartilaginous fish of the superorder Batoidea). The only non-target species that these primers amplified was an isopod. Other individuals of the same species and other isopod species were not amplified with these primers and therefore, it was assumed that the amplification was due to the gut contents of the isopod, which presumably had recently eaten fish flesh (which is likely, considering the foraging behaviour of isopods; Thomas *et al.* 2006).

Table 3.II List of taxa used for specificity tests and cross-reactivity tests of the primers used in this study.

Phylum	Class	Order	Family	Species
Annelida	Oligochaeta	Haplotaxida	Lumbricidae	Unknown
Arthropoda	Maxillopoda	Copepod	Unknown	Unknown
	Malacostraca	Cumacea	Unknown	Unknown
		Amphipoda	Melitidae	*Melita hergensis*
			Dexaminidae	*Atylus swammerdami*
				Dexamine spiniventris
			Hyalidae	*Hyale schmidtii*
			Talitridae	*Talitrus saltator*
			Gammaridae	*Echinogammarus planicrurus*
			Ampithoidae	*Ampithoe helleri*
			Ischyroceridae	*Jassa falcate*
				Jassa ocia
				Jassa marmorata
				Jassa pusilla
			Eusiridae	*Apherusa jurinei*
				Apherusa mediterranea
			Podoceridae	*Podocerus variegates*
			Oedicerotidae	*Pontocrates arenarius*
		Tanaidacea	Tanaidae	*Tanais dulongii*
		Isopoda	Gnathiidae	*Paragnathia formica*
			Cirolanidae	*Eurydice pulchra*
				Eurydice spinima
				Eurydice naylory
			Sphaeromatidae	*Sphaeroma* sp.
				Dynamene sp.
		Mysidacea	Mysidae	*Gastrosaccus roscoffensis*
			Gardonae	*Siriella gracilipes*
		Decapoda		Unknown
			Grapsidae	*Pachygrapsus marmoratus*
			Portunidae	*Polybius henslowii*
Mollusca	Gastropoda	Unknown	Unknown	Unknown
	Cephalopoda	Teuthida	Unknown	Unknown
Chordata	Chondrichthyes	Batoidea	Unknown	Unknown
	Osteichthyes	Clupeiformes	Clupeidae	*Sardina pilchardus*
			Engraulidae	*Engraulis encrasicolus*
		Gadiformes	Phycidae	*Phycis* sp.
			Merlucciidae	*Merluccius* sp.
		Perciformes	Moronidae	*Dicentrarchus* sp.
			Carangidae	*Trachurus* sp.
			Sparidae	*Pagellus* sp.
			Sparidae	*Pagrus*
			Mullidae	*Mullus* sp.
			Scombridae	*Scomber* sp.
		Pleuronectiformes	Soleidae	Unknown
		Salmoniformes	Salmonidae	Unknown

As desired, the non-fish primers (CI-J-2183/CI-N-2353) amplified all the invertebrate species tested but they also, occasionally, amplified DNA from six of the fish species. This did not seem to a major problem since the amplification signal was weak (faint DNA bands in the agarose gels), not consistent among assays (it did not always amplify any of the fish species in different PCRs), meaning that in the context of the faecal samples the likelihood of amplification of fish sequences was much lower than for non-fish sequences. Nevertheless, this was taken into account when interpreting the results and all the results and statistics presented were also repeated using only the subset of samples that were certain to contain non-fish DNA (either because they did not test positive using the fish primers or they tested positive for one or more of the invertebrate groups; Amphipoda, Isopoda, Mysidacea or Cephalopoda), to make sure that the nature of the results did not change.

Primers specific to the order Clupeiformes (C-CB285dF/C-CB431R) were optimised with the aim to reduce the number of species amplified so that they could give an indication of Sardine consumption only. After optimisation, these primers amplified DNA from *Sardina pilchardus* but not from *Engraulis encrasicolus*. It was not possible to test the other four potential species of Clupeiformes since there were no reference sequences available for those species. However, according to Jérôme *et al.* (2003), these primers were not very sensitive to *Sprattus sprattus,* even at lower temperatures than used in the present study, so it can be predicted that this species' DNA was also not amplified from the Storm Petrel faecal samples. Amongst the species tested for cross-reactivity, these primers amplified DNA from two non-target fish species, both Perciformes: *Mullus* sp. and Chub Mackerel *Scomber japonicus*. The latter had only a weak amplification signal.

The primers specific to the class Cephalopoda (Squid28SF/R) were not tested in a range of cephalopod species in this study but these have been successfully used in previous studies, amplifying a good range of species (Deagle *et al.* 2005, 2007). These primers were very consistent throughout all the different PCR assays. Amphipod primers (AmphNSSf1/r1) failed to amplify all the amphipod species tested due to the need to increase the annealing temperature in order to increase their specificity to the group. Although amphipod consumption is likely to be slightly underestimated, these primers still consistently amplified a good range of species. Isopoda and Mysidacea are likely to be most underestimated groups in the Storm Petrel diet since the primers designed for these taxa (IsopodNSSf1/r1 and MysF1/R1, respectively) were not shown to be very reliable, since they failed to consistently amplify a range of species within the target groups.

3.3.7 Stable Isotope Analysis

The sampled body feathers were washed vigorously in triple baths of 0.25 N sodium hydroxide solution, alternated with triple baths of deionized water, in order to remove adherent external contamination as well as any external lipid layer resulting from the bird's preening oil. Each bath lasted 5 min, and an ultrasound system was used, to increase the efficiency of cleaning. Feathers were then dried in an oven for 24 h at 50°C and cut into small fragments for isotopic analysis. Stable carbon and nitrogen isotope assays were carried out on 0.35 ± 0.05 mg subsamples loaded into tin cups. Isotopic ratios were determined by continuous-flow isotope-ratio mass spectrometry (CF- IRMS). Results are presented conventionally as δ values (‰) relative to Pee Dee Belemnite (PDB) for δ13C, and atmospheric nitrogen (N_2) for δ15N.

3.3.8 Data Analysis

Chromatograms of sequences obtained from the clone libraries were examined by eye to check base calling using Sequencher DNA Software. To identify these sequences they were analysed with two complementary approaches:

i) Sequences were compared with sequences in GenBank, using the BLAST software, and in the Barcode of Life Data systems (BOLD) v2.5; Judgements on the level of identification (species, genus, family, order, class, phylum, etc.) were made using the degree of match (% similarity) from both databases and the Maximum Score given in GenBank. The thresholds used were created based on the range of results obtained (e.g. by comparing all the values against one another) and the knowledge of the fauna present in the study area. The scores obtained from sequences of known species of potential prey collected in the area were also used as reference. The criteria for the thresholds varied between fish and non-fish data since sequences for the DNA region used for fish were more abundant in GenBank and the fragment size was bigger, meaning that fish sequences overall had much higher scores than non-fish sequences;

ii) Sequences were aligned, together with sequences from reference species collected at the field site, and grouped into clusters using a Neighbour-Joining phylogenetic analysis (Saitou & Nei 1987), conducted in MEGA4 (Tamura *et al.* 2007). Cloned sequences were clustered together first and sequences of known species were added one at a time. Only those sequences from known species that helped the classification of the cloned sequences stayed in the analysis.

Logistic regression analyses were used to test for differences in the likelihood of presence or absence of prey taxa between sexes and among years, along the season (i.e. days from May 1st) and according to their body mass. Chi-square contingency

tests or Fisher's exact tests (2-tailed) were used where appropriate, to compare frequencies of different prey taxa among years.

3.4 Results

Table 3.III summarizes the number of birds caught and the numbers of different types of samples (i.e. faeces, vomit or growing feathers) obtained in each year and location. The number of samples obtained depended on the total number of birds caught and the proportion of birds that yielded a sample. Overall, more than 10% of the birds caught produced faecal samples (range 9-14% per year, Table 3.III), whereas the number of vomit samples obtained was comparatively lower in all years, with only 3% of birds producing vomit samples overall (range 2-7% per year, Table 3.III).

Table 3.III Summary of faecal, vomit and feather samples obtained from European Storm Petrel nestlings at a breeding colony in NW France, and from migrating European Storm Petrels in SW Portugal.

Location / Year	Birds caught	No. of faecal samples (% of birds caught)	No. of vomit samples (% of birds caught)	No. of growing feathers
Portugal 2006	136	19 (14%)	10 (7%)	-
Portugal 2007	520	49 (9%)	14 (3%)	-
Portugal 2008	639	82 (13%)	7 (2%)	15
Portugal 2009	370	40 (11%)	25 (7%)	29
Portugal total (2006-2009)	1,665	190 (11%)	56 (3%)	
France 2005	-	12	9	-
France 2006	-	29	29	-
France total (2005-6)		41	38	

Not only were faeces more frequently obtained than vomit samples, but faeces were much more likely to yield DNA; the overall proportion of faecal samples from which DNA was successfully amplified was 88.3%. However, none of the vomit samples produced amplified DNA, except for the few samples where extractions could be attempted from solid items found within the liquid vomit.

3.4.1 Presence / Absence of Prey DNA in Faecal Samples

(i) Prey DNA in the Faeces of European Storm Petrel Nestlings

At the breeding colony in NW France, in both 2005 and 2006 the proportion of nestlings whose faeces contained DNA from non-fish prey was higher than the proportion of chicks whose faeces contained DNA from fish (Figure 3.1). This difference in the proportion of the two prey categories was significant in 2006 (Fisher's exact test, $P = 0.001$) but not in 2005 (Fisher's exact test, $P = 0.294$). Nevertheless, the proportion of fish and non-fish prey was very similar in the two years studied (Fisher's exact tests: Fish, $P = 0.427$; Non-fish, $P = 0.678$). The proportion of faecal samples that contained amplifiable bird DNA but from which no prey DNA could be amplified (possibly indicating a period of fasting), was not significantly different between 2005 (11%) and 2006 (3.4%) (Fisher's exact test, $P = 0.422$).

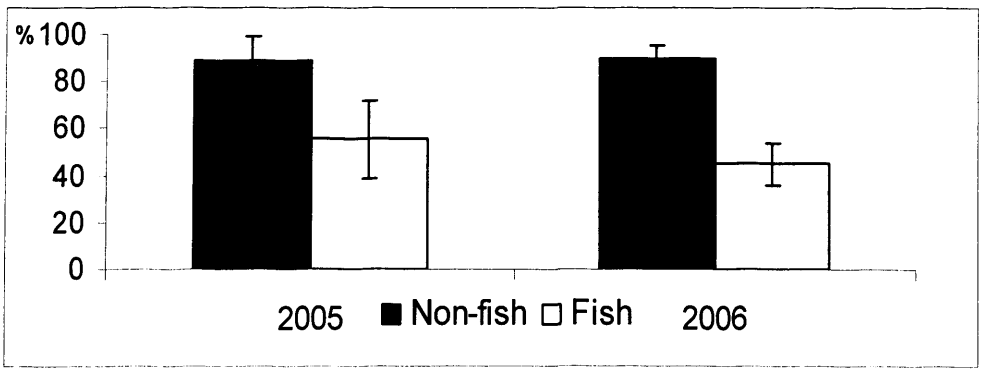


Figure 3.1 Proportion of European Storm Petrel chicks sampled in NW France, which tested positive for fish or non-fish DNA in 2005 and 2006.

(ii) Prey DNA in the Faeces of Migrating European Storm Petrels

Amongst the migrating Storm Petrels caught in the south of Portugal, across the four years, faecal samples from individual Storm Petrels typically tested positive for either one or two different prey "types" (i.e. prey DNA from the following categories tested: fish, Cephalopoda, Amphipoda, Isopoda, Mysidacea or other non-fish prey), with few birds having no amplifiable prey DNA or DNA from more than two prey types (Figure 3.2).

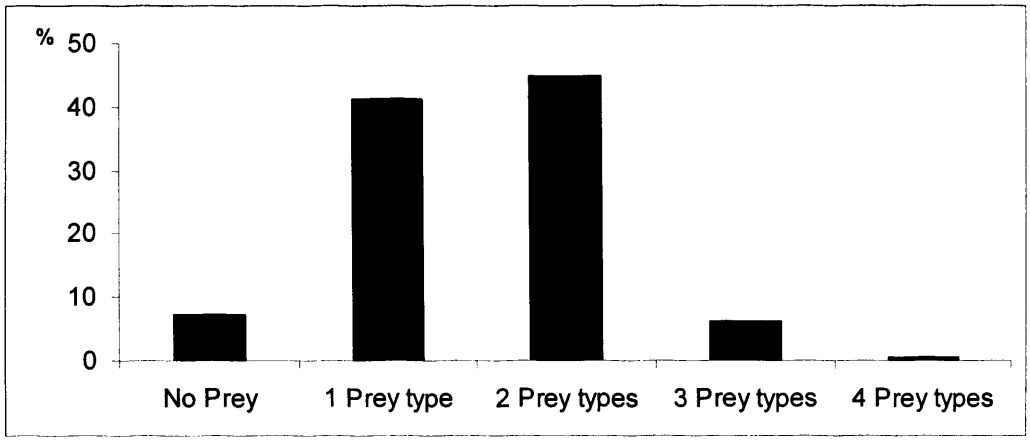


Figure 3.2 Number of prey "types" (categories: fish, Cephalopoda, Amphipoda, Isopoda, Mysidacea, or other non-fish prey) detected in the faeces of individual European Storm Petrels sampled in SW Portugal among 2006 and 2009.

Neither season, sex nor body mass were significant predictors of the presence / absence of either fish or non-fish prey DNA in the faeces; Logistic regression: all *Wald* values < 1.268, *d.f.* = 1, all *P* values > 0.260. The proportion of birds found to have consumed fish was not significantly different among years (Logistic Regression: *Wald* = 3.611, *d.f.* = 3, *P* = 0.317) but there was a significant difference among years in the proportion of birds eating non-fish prey (Logistic Regression: *Wald* = 9.198, *d.f.* = 3, *P* = 0.027; Figure 3.3). Although direct comparisons between the presence / absence of prey taxa detected using different primers cannot be made

due to potential differences in the sensitivity of the primers, it is worth noting that, contrary to all other years, in 2009 there was a significant increase in the relative frequency of birds testing positive for fish compared to non-fish (for 2009, $\chi^2 = 8.250$, $d.f. = 1$, $P = 0.002$; for 2006-2008, all P values > 0.616; Figure 3.3).

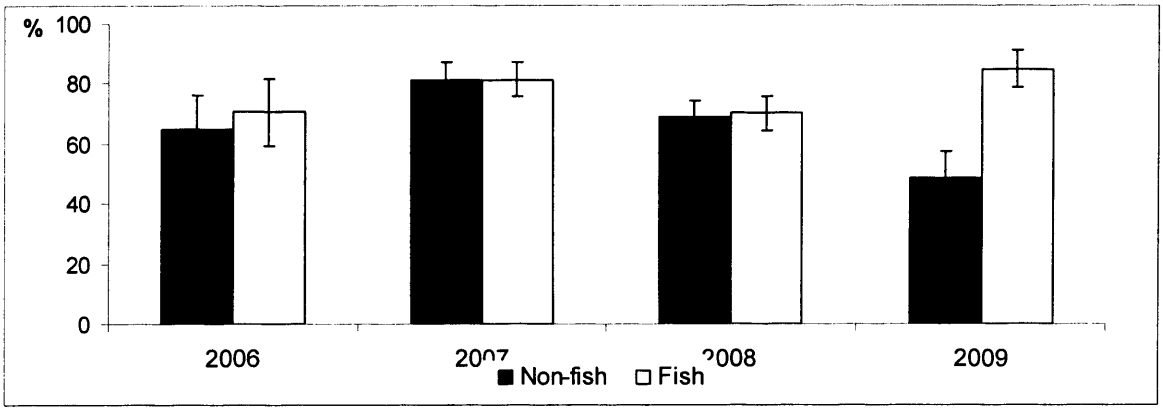


Figure 3.3 Proportion of European Storm Petrels sampled in SW Portugal among 2006 and 2009 which tested positive for fish and non-fish prey in each year.

The shift in prey taxa detected in 2009 is mainly due to an increase in the proportion of birds which ate only fish (Logistic Regression: *Wald* = 9.275, *d.f.* = 3, $P = 0.026$; Figure 3.4), despite the proportion of birds eating only non-fish or both prey types remaining similar among years (Logistic Regression: both *Wald* values < 5.496, *d.f.* = 3, both P values > 0.139, Figure 3.4). The proportion of birds eating only fish in 2009 was significantly different from that in each other year (except 2006 when the difference was marginally non-significant, $P = 0.061$), suggesting that a higher proportion of birds were specialising more on fish as their main prey in 2009. Very few faecal samples had no amplifiable prey DNA (<20% of samples in each year, Figure 3.4) and the proportion of samples yielding no prey DNA did not vary significantly among years (Logistic Regression: *Wald* = 3.161, *d.f.* = 3, $P = 0.367$).

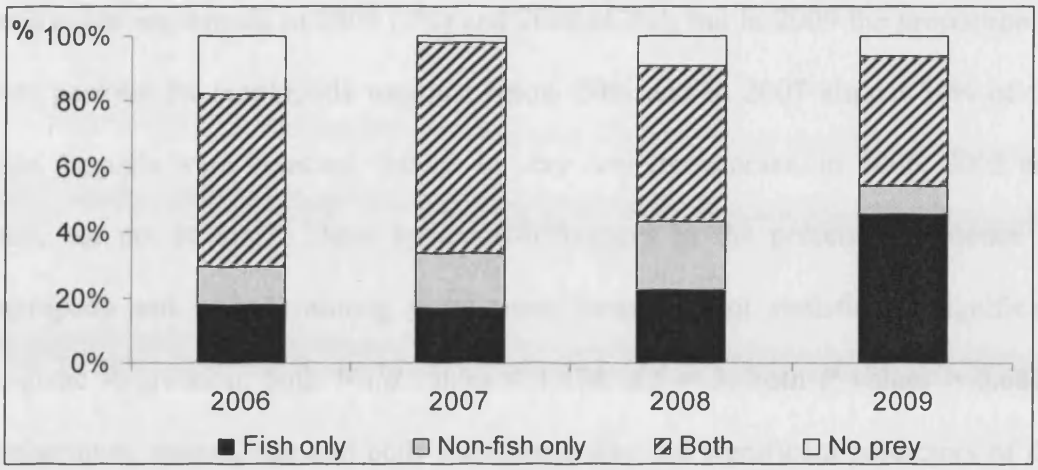


Figure 3.4 Proportion of European Storm Petrels sampled in SW Portugal among 2006 and 2009 which tested positive in each year for only non-fish DNA, only fish DNA, both fish and non-fish DNA, or yielded no prey DNA.

A large proportion of the Storm Petrels that had consumed fish were subsequently shown (by screening with clupeiform-specific primers) to have consumed clupeiform fish (most likely to be European Sardine) in each year: 46% in 2006, 73% in 2007, 57% in 2008 and 64% in 2009. There was no significant variation among years in the proportion of birds testing positive for clupeiform DNA (Logistic Regression: *Wald* = 1.350, *d.f.* = 3, *P* = 0.717). The presence / absence of these prey was also not related to date, sex or body mass; Logistic regression: all *Wald* values < 1.750, *d.f.* = 1, all *P* values > 0.186.

Amongst the non-fish taxa tested using more taxon-specific primers (Figure 3.4), cephalopod DNA was abundant in all four years, particularly in 2009. Differences among years in the relative frequency of cephalopods were however, not statistically significant (Logistic Regression: *Wald* = 2.661, *d.f.* = 3, *P* = 0.447). No birds were found to have eaten Mysidacea in any of the years studied, despite these crustaceans being highly abundant on the shoreline of the capture site during the migration season (Thomas *et al.* 2006, pers. obs.). A small proportion of birds tested

positive for amphipods in 2006 (9%) and 2008 (13%), but in 2009 the proportion of birds positive for amphipods was just below 20% and in 2007 almost 30% of the birds. Isopods were detected, though in very low frequencies, in 2006, 2008 and 2009, but not in 2007. These apparent differences in the presence / absence of amphipods and isopods among years were, however, not statistically significant (Logistic Regression: both *Wald* values < 1.478, *d.f.* = 3, both *P* values > 0.687). Furthermore, season, sex and body mass were also not significant predictors of the presence / absence of amphipods or isopods; Logistic Regression: all *Wald* values < 2.020, *d.f.* = 1, all *P* values > 0.155.

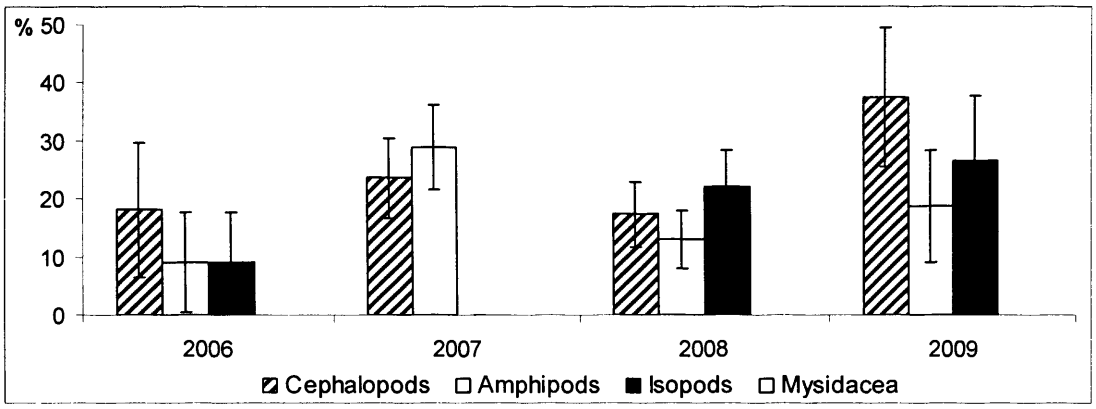


Figure 3.5 Proportion of European Storm Petrels sampled in SW Portugal among 2006 and 2009 which tested positive for different invertebrate taxa, using taxon-specific primers.

3.4.2 Cloning and Sequencing of Prey DNA from the Faeces of Migrating European Storm Petrels

Across the four years, a high proportion of sequences (43%) obtained through cloning and sequencing non-fish DNA from Storm Petrel faecal samples were identified as fungi by comparison with sequences in GenBank. Although the species matches were not very high (mean match = 82%), it is entirely possible that these sequences are indeed from fungi. Since these do not represent prey taxa, they could

either represent unintentional ingestion of fungal material with animal prey, mycological components of the gut flora, or contamination of the faecal samples with fungi after they were collected from the field site or in the lab. Therefore, these fungal sequences were not considered for further analysis.

Across the four years, a total of 170 prey DNA sequences were obtained. Tables 3.IV and 3.V present, respectively, the results for the identification of sequences for fish and non-fish prey DNA in the four years of study. For the fish prey it was possible to obtain species or genus level identification for a good proportion (70%) of sequences. However, in general the specific identification of sequences from the non-fish primers was poor and many prey sequences could only be identified with certainty to the phylum level. The phylogenetic analysis was therefore performed for the non-fish prey as a complementary approach to improve the identification of non-fish sequences. The sequences from Dolphin *Delphinus* sp. listed in Table 3.V were not included in this analysis, since these were the only mammal species detected.

This analysis provided another means of visualising the taxonomic distribution of sequences from non-fish diet components among years, and improved some of the identification of sequences from these primers (Figure 3.6). For example, a cluster of sequences from 2008 that were mostly identified in GenBank as possible arthropods, grouped with a decapod species. Some sequences, also identified only to the phylum in GenBank, clustered with isopods of the genus *Eurydice*. Another putative arthropod sequence clustered with an amphipod species. Furthermore, sequences from 2009 also identified only to the phylum Arthropoda clustered with one sequence from 2007 classified to the class (Insecta), suggesting that the former were also likely to be insects.

Figure 3.7 presents the relative abundance of each identified taxon for each year, combining information from the GenBank identification and the phylogenetic analysis. Decisions on the level of identification of the sequences were made based on the proportion of match and on maximum scores (Tables 3.IV and 3.V). Sequences found comprised prey from a total of 13 distinct taxa of fish and 12 distinct taxa of non-fish. European Sardine was the most common species identified in all the years but significant differences were found in the proportion of DNA sequences from this species among years (Fisher's exact test: $P = 0.036$). The order with higher representation is that of the Perciformes, which includes families such as: Scombridae, Carangidae, Gobiidae and Sparidae. The Perciformes is also the most represented order in the study area, in terms of number of species (Borges 2007). Demersal fish (Gadidae, Phycidae, Myctophidae, Peristediidae and Pleuronectiform) were identified in all years, except 2007 (though it is possible that the unknown sequences belong to a demersal species, as suggested by Table 3.IV). Assuming that the unknown sequences in 2007 belong to a demersal fish species, there is no significant difference in the proportion of demersal fish species among the years (Fisher's exact test; $P = 0.5271$). The only species of Peristediidae present in the study area is the African armoured searobin *Peristedion cataphractum*, therefore this must be the species consumed by the birds. The identification of shark (Carcharhiniform) DNA in 2008 indicates that the fish primers do amplify some Chondrichthyes species.

Table 3.IV Fish taxa identified in the faeces of European Storm Petrels using the cloning and sequencing approach. In 'bold' is the taxonomic detail determined by the Maximum Score and % Match.

Year (no. Samples pooled)	Species	Common name	Number of sequences	% Match GenBank	Max. Score	Family / Order
2006 (11)	***Sardina pilchardus***	European Sardine	9	100	564	Clupeidae Clupeiform
	Microgadus proximus[1]	Pacific Tomcod	2	93	440	**Gadidae** Gadiform
2007 (13)	***Sardina pilchardus***	European Sardine	14	100	564	Clupeidae Clupeiform
	Scomber japonicus	Chub Mackerel	8	100	553	Scombridae Perciform
	Trachurus *japonicus*[2]	Jack Mackerel	8	99	545	Carangidae Perciform
	Crystallogobius linearis	Cristal Goby	6	92	250	Gobiidae **Perciform**
	Opisthoproctus spp. **(unknown)**	Barreleye	4	82	259	Opisthoproctidae Argentiniform
2008 (6)	***Sardina pilchardus***	European Sardine	17	100	564	Clupeidae Clupeiform
	Scomber japonicus	Chub Mackerel	2	100	553	Scombridae Perciform
	Pagrus auriga	Redbanded seabream	2	94	459	**Sparidae** Perciform
	Mustelus manazo[2]	Smooth-hound	7	93	315	Triakidae **Carcharhiniform**
	Solea solea	Common sole	1	86	320	Soleidae **Pleuronectiform**
	Opisthoproctus spp. **(unknown)**	Barreleye	2	82	259	Opisthoproctidae Argentiniform
2009 (18)	***Sardina pilchardus***	European Sardine	17	100	564	Clupeidae Clupeiform
	Scomber japonicus	Chub Mackerel	4	100	553	Scombridae Perciform
	Scomber scombrus	Atlantic Mackerel	5	96	492	**Scombridae** Perciform
	Trachurus *japonicus*[2]	Jack Mackerel	2	99	545	Carangidae Perciform
	Satyrichthys amiscus[1]	Armored Gurnard	1	93	438	**Peristediidae** Scorpaeniform
	Phycis blennoides	Greater Forkbeard	5	99	337	**Phycidae** Gadiform
	Hygophum hygomii	Bermuda Lantern Fish	1	99	401	**Myctophidae** Myctophiform

[1] Genus does not exist in the study area. [2] Species does not exist in the study area but Genus does.

Table 3.V Non-fish taxa identified in the faeces of European Storm Petrels using the cloning and sequencing approach. In 'bold' is the taxonomic detail determined by the Maximum Score and % Match.

Year (no. samples pooled)	Species	Nr. of sequences	% Match GenBank	Max. Score	Order / Family	Phylum / Class
2006 (11)	*Neodiprion* spp. **(unknown)**	1	97	67.6	Hymenoptera Diprionidae	Arthropoda Insecta
	Actinote thalia	1	92	196	Leptidoptera Nymphalidae	Arthropoda **Insecta**
	Cicurina pampa / C. madla	10	90	185	Araneae Dictynidae	**Arthropoda** Arachnida
	Scathophaga stercoraria	1	99	307	**Diptera** Scathophagidae	Arthropoda Insecta
	Dolichopoda makrykapa	1	92	239	**Orthoptera** Rhaphidophoridae	Arthropoda Insecta
	Sepia officinalis	1	98	302	Sepiida **Sepiidae**	Mollusca Cephalopoda
	Demodex folliculorum	2	98	294	**Acarina**	Arthropoda Arachnida
	Unknown	1	-	-	-	-
2007 (6)	*Protocalliphora sialia*	1	86	202	Díptera Calliphoridae	Arthropoda **Insecta**
	Acrodipsas mortoni	1	86	189	Leptidoptera Lycaenidae	**Arthropoda** Insecta
	Charaxes marmax	1	92	244	**Leptidoptera** Nymphalidae	Arthropoda Insecta
	Euphausia superba	1	84	176	Euphausiacea Euphausiidae	**Arthropoda** Crustácea
2008 (11)	***Apis mellifera** iberica*	2	98	326	Hymenoptera Apidae	Arthropoda Insecta
	Aegla prado / A. denticulate	8	86	193	Decapoda Aeglidae	**Arthropoda** Crustácea
	Orconectes etnieri	2	82	106	Decapoda Cambaridae	**Arthropoda** Crustácea
	Lepetodrilus spp. **(unknown)**	1	80	91.6	Lepetodrilidae	Mollusca Gastropoda
	Arrhipis vassei	1	84	104	Coleoptera Eucnemidae	**Arthropoda** Insecta
	Napeogenes lycora	3	94	276	**Leptidoptera** Nymphalidae	Arthropoda Insecta
	***Delphinus** delphis*	3	98	320	Cetacea Delphinidae	Chordata Mammalia
	Unknown	3	-	-	-	-
2009 (11)	*Sepia officinalis*	2	98	302	Sepiida **Sepiidae**	Mollusca Cephalopoda
	-	3	82	121	*Curculionidae* Coleoptera	**Arthropoda** Insecta
	Myrmecocystus mexicanus	1	89	172	Hymenoptera Formicidae	**Arthropoda** Insecta
	Unknown	3	-	-	-	-

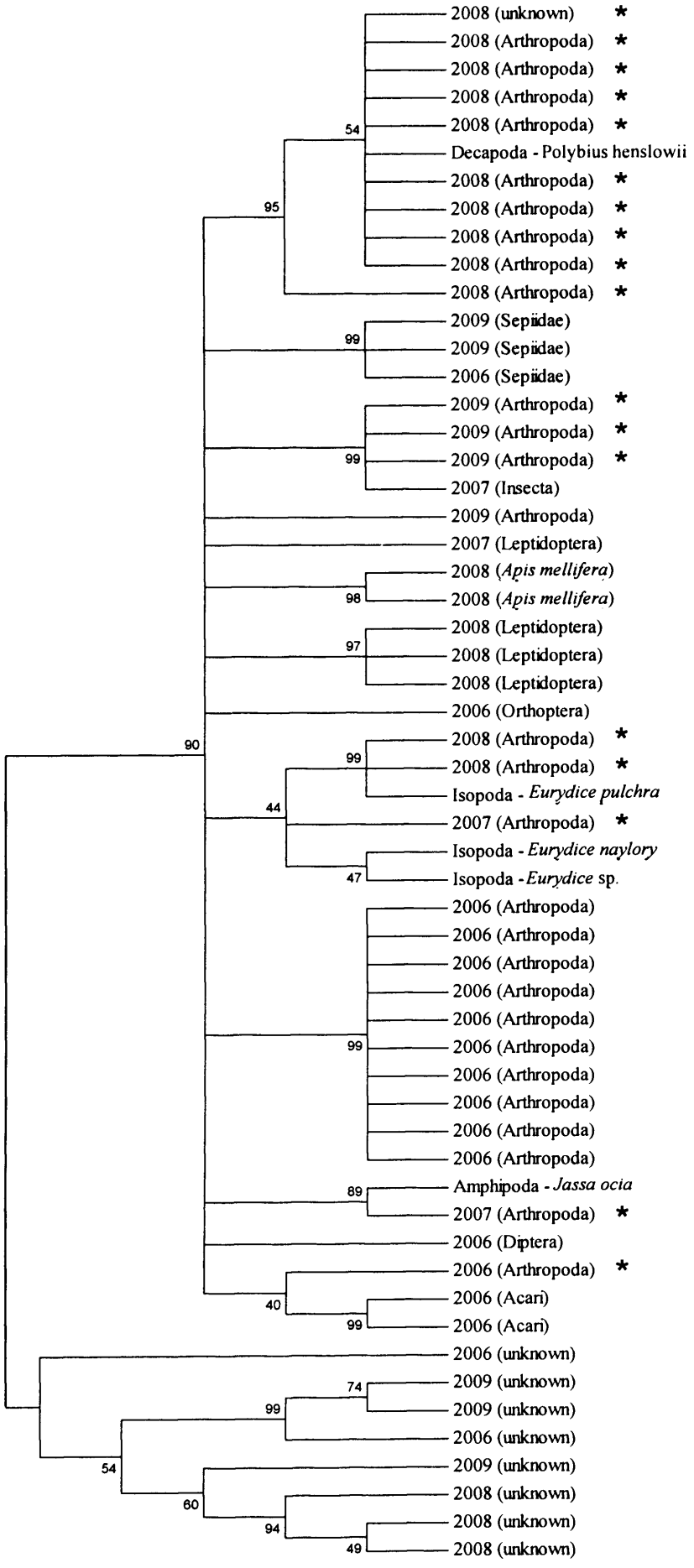


Figure 3.6 Phylogenetic tree of invertebrate prey DNA sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branch nodes. Branches corresponding to partitions reproduced in less than 40% bootstrap replicates are collapsed. Taxomonic names in brackets show sequence identifications from online databases (Genbank and BOLD), and * indicate those sequences for which identification was improved with the phylogenetic analysis.

The diversity of taxa among years can not be directly compared due to the different number of individuals pooled in each year and the differences in the number of sequences obtained (e.g. higher diversity of fish taxa found in 2009 is probably related to a higher number of individual faeces samples pooled), but it is notable that the composition of taxa seems to vary greatly among years, both for fish and non-fish prey (Figure 3.7; note that 2007 had a very small sample size for non-fish prey). In 2008 there was a high proportion of DNA sequences from Decapods but these were absent in other years. Sequences from terrestrial invertebrates (Leptidoptera, Hymenoptera and other insects) were present in all years but there was a significant difference in the proportion of these prey among years (Fisher's exact test: P = 0.003). Cuttlefish (Sepiidae sp.) were identified using the sequencing approach in 2006 and (apparently more abundantly) in 2009, although no significant difference was found in the prevalence of this prey among years (Fisher's exact test: $P = 0.119$).

Evidence for scavenging of food from large species was found in 2008 by the detection of Common Dolphin (*Delphinus delphis*) and hound shark (Triakidae: Carcharhiniformes) DNA. Though the latter are small sharks and some species produce eggs, even the eggs are probably too big for a Storm Petrel to consume, besides being protected by a hard, leather like, capsule (Flammang *et al.* 2007, Concha *et al.* 2010). Parasitic mites (Acarina) found in 2006 are likely to have been ingested by the birds during preening their feathers and therefore can not be considered as prey in the usual sense.

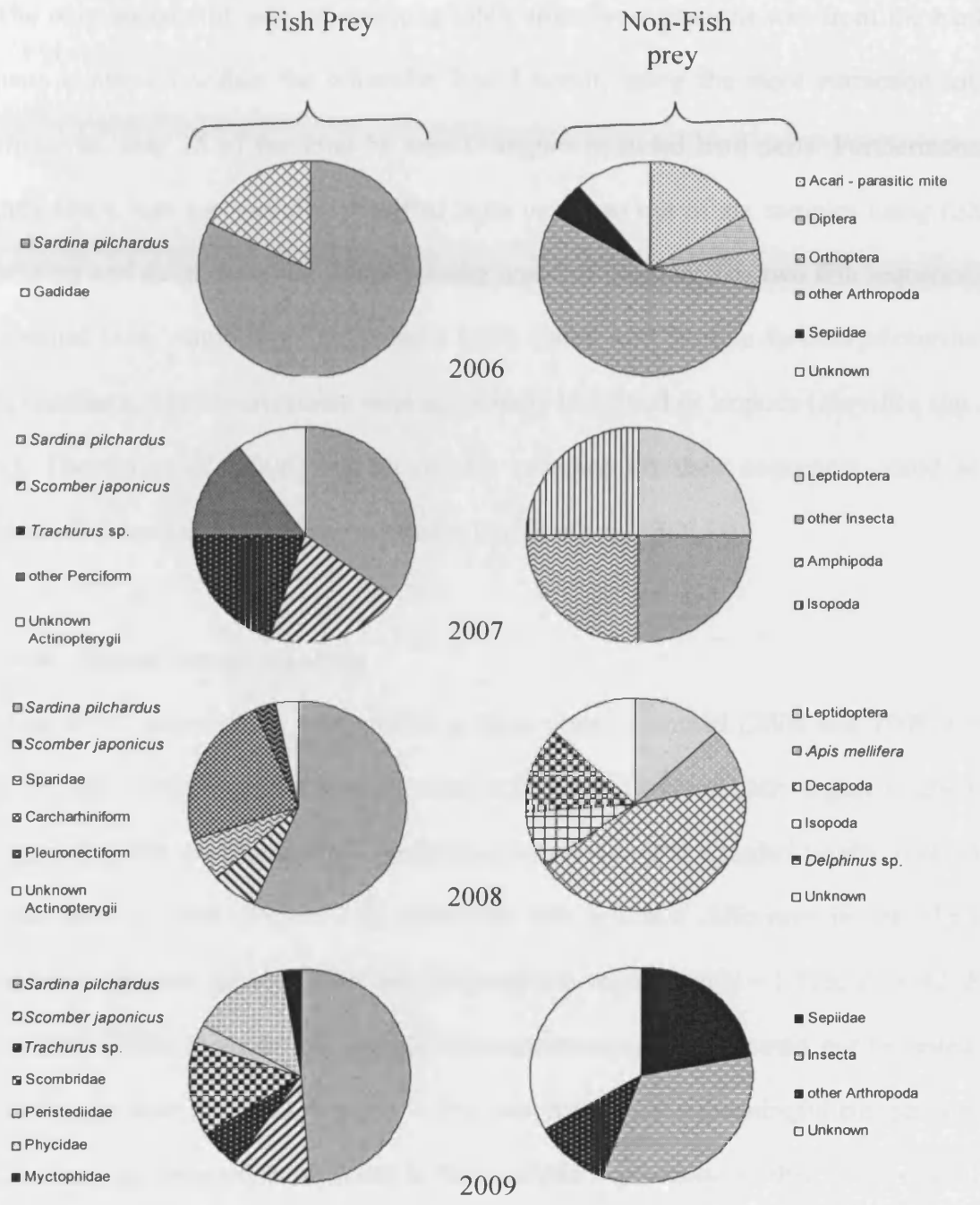


Figure 3.7 Pie charts showing proportions of various fish and non-fish prey DNA sequences obtained from the faecal samples of European Storm Petrels and identified on GenBank/BOLD.

3.4.3 Analysis of Prey DNA from Hard Parts in Vomit Samples

The only successful way of obtaining DNA from regurgitations was from the hard parts contained within the otherwise liquid vomit, using the stool extraction kit. However, only 18 of the total 56 vomit samples included hard parts. Furthermore, prey DNA was successfully amplified from only two out of six samples using fish primers and three out of 12 samples using non-fish primers. The two fish sequences obtained from vomit samples showed a 100% match with Sardine *Sardina pilchardus* in GenBank. The invertebrates were all visually identified as isopods (*Eurydice* spp., c.f. Thomas *et al.* 2006) but no reliable matches for their sequences could be obtained from online sequence databases (GenBank and BOLD).

3.4.4 Stable Isotope Analysis

Mean δ13C values were very similar in both years examined (2008 and 2009, t = 0.555, $d.f.$ = 41, P = 0.582), while mean δ15N values were slightly higher in 2009, suggesting that the Storm Petrels may have been feeding at a higher trophic level in 2009 than in 2008 (Figure 3.8). However, this apparent difference in the δ15N isotopic signature between 2008 and 2009 was non-significant (t = 1.716, $d.f.$ = 42, P = 0.094). Differences in the isotopic signatures between sexes could not be tested, since there were not enough males in the dataset to allow a meaningful comparison. However, the proportion of males in this analysis was similar to that in the overall study sample, so therefore differences between sexes should not be a confounding factor in the interpretation of the results.

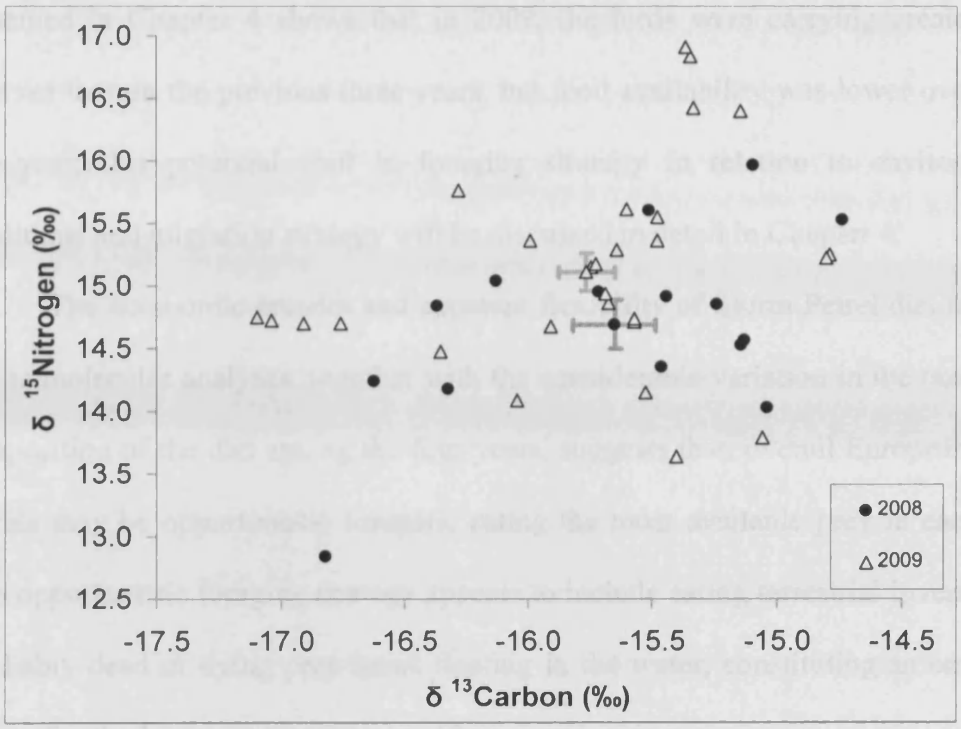


Figure 3.8. Isotopic ratios from growing feathers of European Storm Petrels, sampled during their northwards migration past SW Portugal in 2008 and 2009.

3.5 Discussion

3.5.1 Overview

This study is one of the first detailed studies of the diet of the European Storm Petrel, including temporal variation in diet, and the first to examine diet of any storm petrel species during migration. The molecular techniques used here allowed the identification of many prey items to the species and genus levels, some of which would not be likely to be identified with any other method.

This study has also revealed potential shifts in the foraging strategy of Storm Petrels, depending on the stage of the annual cycle (e.g. migrating adults appear to take fish more frequently than do birds foraging to feed nestlings), and among years (in particular, 2009 presented a range of differences in diet composition in relation to the preceding three years). The analysis of Storm Petrel body mass variations

presented in Chapter 4 shows that in 2009, the birds were carrying greater body reserves than in the previous three years, but food availability was lower overall for that year; this potential shift in foraging strategy in relation to environmental conditions and migration strategy will be discussed in detail in Chapter 4.

The taxonomic breadth and apparent flexibility of Storm Petrel diet revealed by the molecular analyses, together with the considerable variation in the taxonomic composition of the diet among the four years, suggests that, overall European Storm Petrels may be opportunistic foragers, eating the most available prey in each year. This opportunistic foraging strategy appears to include eating terrestrial invertebrates (probably dead or dying prey items floating in the water, constituting an easy food source for the birds) and scavenging (from corpses or even faeces) of other taxa that would otherwise be too large for Storm Petrels to consume (e.g. Common Dolphin and probably hound sharks).

Thus, Storm Petrels might respond to changes in their environment by changing their foraging strategy according to spatial or temporal changes in foraging conditions. The European Sardine was identified as a potentially very important component of the diet of migrating Storm Petrels across the four years, both in terms of the number of birds consuming it, and the biomass consumed. Further investigations of inter-annual variations in the abundance and biomass of Sardine populations off SW Portugal, in the context of Storm Petrel foraging ecology, are presented in Chapter 4.

The importance of prey from higher trophic levels, such as fish, particularly in 2009, was supported by the stable isotope analysis, as suggested by the relatively high $\delta^{15}N$ values (see e.g. Bearhop *et al.* 2006, Weiss *et al.* 2009, Paiva *et al.* 2010). A connection to the coastal/benthic zones was also indicated by the stable isotope

ratios, particularly by the relatively high values of $\delta^{13}C$ in the growing feathers of Storm Petrels compared with those of the pelagic-foraging Cory's Shearwaters *Calonectris diomedea* sampled approximately 100km further north of the study site, at the Berlengas Islands off the Portuguese coast (Paiva *et al.* 2010). This view of Storm Petrels as coastal foragers, rather than entirely pelagic foragers, is supported by previous observations of Storm Petrels occasionally regurgitating undigested isopods of species that are restricted to the intertidal zone (Thomas *et al.* 2006). Similarly, the identification of the demersal fish taxa (Gadidae, Phycidae, Myctophidae, Peristediidae and Pleuronectiform) in the molecular analysis of diet, supports the view that Storm Petrels may opportunistically take prey with a demersal origin, at least in some circumstances. Since Storm Petrels do not dive more than a few cm below the sea surface (Brooke 2004, Flood *et al.* 2009, pers. obs.), demersal prey was most likely to have been obtained from fisheries discards. Storm Petrels are not commonly seen following fishing boats during the day (perhaps in order to minimise the risk of predation by larger seabirds such as gulls *Larus* spp.). However, they may do so more frequently by night and they readily appear around small boats when a "chum" of mashed fish is placed into the water by day or night (pers. obs.).

The stable isotopes failed to detect significant differences in the foraging strategy of European Storm Petrels between 2008 and 2009 (note however the relatively small sample size). In a study of stable isotopes in Sardine and plankton in Galicia, NW Spain (Bode *et al.* 2004), young Sardines (< 18cm long) and older individuals (> 18cm) had mean $\delta^{15}N$ values of 10.5‰ and $\delta^{13}C$ of –17‰. Among plankton, N values were between 3‰ and 8‰, varying with size class; $\delta^{13}C$ values ranged from -18‰ to -22‰. Thus, both $\delta^{13}C$ and $\delta^{15}N$ values were considerably lower than found in the Storm Petrels in the present study, supporting the view that

Storm Petrels forage mainly at higher trophic levels prior to their arrival at the Portuguese coast. Combining stable isotopes analysis with molecular scatology in the study of animal species' diet can greatly enhance the outcome results provided by each of the methods separately.

3.5.2 Comparison and Integration of Results

The use of primers of varying taxonomic specificity to screen for the presence / absence of prey DNA has the advantages of giving a semi-quantitative measure of prey consumed, based on the proportion of birds that consumed each prey type. This approach is typically more economical than the cloning and sequencing approach in terms of financial cost of the analysis, and it is ideal to identify levels of predation on one or few key prey types (e.g. Sardines and cuttlefish *Sepia* sp. in the present study). However, this approach is highly dependent on the availability or design of primers of appropriate specificity, which need to be designed, optimised and tested before they can be applied. Furthermore, it is almost impossible to be certain about the primers characteristics (e.g. sensitivity to the different prey, specificity), since it is virtually unfeasible to test most primers against all potential non-target species within a marine system. For example, in this study, primers designed to amplify invertebrate species (CI-N-2535/CI-J-2183), were shown to also amplify vertebrate species such as dolphin and some fish. This was not a problem in this study, since the cross-amplification of fish by "non-fish specific" primers was minor (controlling for it did not change the nature of the results) and it allowed the detection of a broader range of non-fish prey. Similarly, primers designed for Clupeiformes (C-CB285dF/C-CB431R) amplified at least two species of perciform fish. While one of these, the perciform genus *Mullus* sp., did not seem to be a common prey for

migrating Storm Petrels, DNA of *Scomber japonicus* was found in relatively high proportions in most years. Although the Clupeiforme primers did not seem very sensitive to this species compared to Sardine, it is possible that amplification of *Scomber japonicus* might contribute to the high frequency of Clupeiformes found across the years. Nevertheless, based on the clone library results and considering the different specificity of the primers, Sardine DNA is likely to have made the major contribution to the high proportion of birds testing positive for clupeiform DNA in the present study. The Osteichthyes (bony fish) primers (FishF1/R1) also amplified unexpected prey DNA sequences from Chondrichthyes (cartilaginous fish), but again, in this case it was beneficial to the study in that additional fish taxa could be sequenced and identified. As more primers become designed and tested in future studies, the chances of finding reliable primers for dietary studies will be increased.

The presence / absence screening approach has also the limitation of requiring some prior knowledge or expectation of the potential prey. While fish are obvious prey to search for in pelagic species such as Storm Petrels, specific groups of fish or non-fish prey are harder to predict as being important unless previous studies have shown them to appear in the birds' diet. For example, in this study it was not expected to find such an apparently high contribution of terrestrial invertebrates in the birds' diet and, therefore, primers targeting Leptidoptera or other terrestrial invertebrates were not selected for the presence / absence approach.

Another limitation of these molecular methods is that the comparison of results obtained from different primer pairs might not be valid due to differences in the sensitivity of the different primers. Therefore, the relative abundance in the faecal samples of fish and and non-fish, or of the different invertebrates (Cephalopoda, Amphipoda, Isopoda, Mysidacea), can not be directly compared within each year.

This is also true for the cloning approach. Nevertheless, it is possible to establish comparisons on the patterns among years. Furthermore, the faecal samples from nestlings showed a reverse pattern, suggesting that the patterns obtained for the migrating adults are not just related to differential sensitivity of the fish and non-fish primers. Even assuming that the proportion of birds feeding on non-fish prey was underestimated, the proportion of birds consuming fish is high and a fish meal will, almost certainly, be more energy-rich on average than an non-fish meal of equivalent mass (Beukema 1997, Hilton *et al.* 1998, Paiva *et al.* 2006a, Hilton *et al.* 2000).

Information on the predator's digestive physiology is useful when applying molecular methods to dietary studies, since the rate at which a predator digests each prey taxon will affect the results obtained. Feeding trials with captive animals fed known prey can be performed in order to calibrate the results obtained from the wild (e.g. Deagle *et al.* 2005b, 2006, 2010). This has been done mainly with invertebrate species (e.g. Agustí *et al.* 2003, Harper *et al.* 2005, Juen & Traugott 2007) and captive mammals, such as Steller's Sea Lions *Eumetopias jubatus* (Deagle *et al.* 2005). Feeding trials can not be easily be performed on Storm Petrels though this have been done for other purposes in a range of seabird species, suggesting a digestion period for fish prey of about five hours (Hilton *et al.* 2000). However, trials on young Little Penguins showed that the DNA signal of fish prey items could be detected in the faeces of the penguins up to four days after feeding (Deagle *et al.* 2010). These detection periods can also vary depending on the sensitivity of the primers used. A feeding trial could potentially be performed on Storm Petrel nestlings at the colonies if these are fed items not provided by the adults. On this matter, different prey will have different retention times (i.e. period of time in which the prey is retained in the predators' guts) (Hilton *et al.* 1998, Hilton *et al.* 1999) For

instance, digestion rates of fish were found to be higher than those for squid which are themselves digested more rapidly than crustaceans (Wilson *et al.* 1985, Jackson & Ryan 1986, Jackson 1992). Different species of fish might also have different digestion times related to size or lipid content (Hilton *et al.* 1998). Furthermore, molecular methods have the limitation of not distinguishing between age classes of prey (e.g. eggs, larvae or fully developed individuals), making it harder to evaluate differences in digestion periods among different prey types.

In the context of the present study, such variations in primer sensitivity, specificity and detection periods of different prey taxa emphasise the need for caution in interpreting differences in the frequency with which different categories of prey are detected using different primer pairs. Nevertheless, such methods are potentially powerful tools for tracking changes in diet composition over space and time, when using the same primer combinations to examine diet composition in different contexts (e.g. among years and different stages of the annual cycle.

The cloning approach provided novel and detailed information on the type of prey that the birds feed on and on changes in the composition of prey among years. The specificity of taxonomic identifications possible using this approach frequently allows the presence of individual species in the diet to be confirmed, and sometimes identification is even possible to the level of subspecies (e.g. the Iberian subspecies of honeybee, *Apis mellifera iberica*, Table 3.IV).

As with the presence / absence screening approach discussed above, caution is required in interpreting some aspects of the cloning and sequencing results. For example, the frequency with which different sequences are detected will depend on primer sensitivity and specificity, as well as, potential differences in the number of mitochondria per unit mass in different prey taxa. These limitations could be

explored further in future work, for example by comparing the number of sequences that can be amplified per unit biomass, from samples of fresh prey (cf. Deagle *et al.* 2010).

The success of the cloning reactions in this study was unexpectedly low which resulted in a limited number of DNA sequences obtained for each year / prey type, particularly for non-fish prey in 2007. This increases the likelihood of bias towards certain prey types and makes the interpretation of the results more difficult. For example, the apparently high proportion of terrestrial invertebrates in 2007 (50%), is derived from only two DNA sequences and it is likely not to be representative of the real composition of the diet. In 2008, with a larger sample of sequences, this proportion is much lower.

Increasing the number of sequences obtained would allow a more reliable semi-quantitative interpretation of the results (Deagle 2010). Even with higher success rates of the cloning, the sequencing process is expensive and usually limits the number of sequences one can get. Next-generation sequencing (e.g. pyrosequencing) can overcome this issue by producing massive amounts of sequencing data, largely reducing the individual cost of each sequence (Deagle *et al.* 2009, Lerner & Fleischer 2010, Deagle *et al.* 2010). Although molecular methods are currently a relatively costly way to study diet (compared to visual identification, stable isotopes or fatty acids), this is largely compensated by the being non-invasive and having the potential to describe an animal's diet with extraordinary and unprecedented detail. The ongoing expansion of DNA online databases (GenBank and BOLD) will continue to enhance the power and applicability of molecular methods for the study of the diet of free-living animals.

Chapter 4

Climate-Driven Changes in the Strategic Regulation of Body Reserves by Migrating European Storm Petrels

4.1 Abstract

Understanding and predicting the impacts of climate change on ecosystems requires knowledge of the mechanisms, such as climate-driven changes in trophic relationships, underlying such changes. In this study, I report large and previously undescribed changes among years in the body reserves of European Storm Petrels *Hydrobates pelagicus*: small, surface-feeding oceanic seabirds, sampled over 21 years (1990-2010) during their northward migration past the coast of southern Portugal. These changes in the birds' body reserves are associated with local sea temperatures, marine primary productivity and the abundance of a major food source, the European Sardine *Sardina pilchardus*. European Storm Petrels were heavier during their summer migration in years when spring sea temperatures, summer primary productivity and Sardine abundance were lower. These relationships suggest that the large changes in body reserves among years were the result of strategic regulation of reserves as a buffer against starvation in response to changes in food availability. Local variables were more successful at accounting for among-year variance in body reserves than the ocean-basin scale North Atlantic Oscillation; the major index of climate variability across the North Atlantic. This suggests that birds were responding directly to climate-driven changes in local foraging conditions, which are themselves driven by larger-scale climate processes. This study shows that

body mass regulation behaviour can act as a sensitive bio-indicator of the effects across trophic levels of climate-driven environmental changes.

4.2 Introduction

Improving our understanding of the potential impacts of climate change on different ecosystems requires knowledge of the mechanisms underlying climate-driven ecological changes (Møller *et al.* 2004a). The impacts of climate change are manifested at a range of spatio-temporal scales (Miller 2004), from short-term changes in local phenomena (hours - days, 10s of m) such as air temperature, wind speed and direction, through mesoscale phenomena (weeks - months, 100m - 100km) such as upwelling intensity, salinity, sea surface temperature and currents, to long-term and large-scale phenomena (months - years, 100 - >1,000 km) including decadal climatic oscillations such as the North Atlantic Oscillation (NAO, Hurrell 1995, Hurrell & van Loon 1997) and the apparent ongoing anthropogenic increases in global surface temperatures (IPCC 2007). Local, short-term variations are likely to most proximately mediate the impacts of larger-scale and longer-term climate variations on animals (Møller *et al.* 2004a). However, phenomena such as the near simultaneous fluctuations of fish stocks in widely separated regions support the view that such ecological effects can also be driven by climate processes operating at a global scale (Schwartzlose *et al.* 1999).

Climate variability can affect animals directly through physiological mechanisms (e.g. metabolism, reproduction, mortality), as well as indirectly through affecting their biological environment (e.g. predators, prey, within-population interactions and disease; Ottersen *et al.* 2004). Climate-driven change in trophic relationships is considered to be one of the crucial mechanisms through which

climate will impact ecosystems (Werner *et al.* 2004, Durant *et al.* 2007, Miller-Rushing *et al.* 2010). Indeed, the response of seabirds to climate change appears to be a good integrative index of the cumulative effects of climate across the trophic levels below their position in the food chain (Durant *et al.* 2004, Piatt *et al.* 2007 - but see Gremillet & Charmantier 2010 for important limitations of such indices).

Although there has been an increasing number of studies on the relationship between birds, food supply and climate (e.g. Furness & Tasker 2000, Frederiksen *et al.* 2004, Kendall *et al.* 2004), direct behavioural responses of birds to climate are much less explored and have been identified as a priority for future studies (Møller *et al.* 2004b, Stenseth *et al.* 2004). In this Chapter, evidence is reported for an effect of climate-driven ecological changes on a behavioural survival strategy, the regulation of body reserves in a small migrating seabird – the European Storm Petrel *Hydrobates pelagicus*. Small seabirds are likely to be more responsive to changes in their environment (e.g. thermal conditions, food supply) than larger seabirds because of their higher metabolic rate and greater surface area / volume ratio, and therefore provide good case-studies to investigate impacts of climate. However, the smallest seabirds (storm petrels) tend to be harder to study (e.g. due to underground nesting, being nocturnal at the colonies, more sensitive to disturbance), particularly away from the breeding grounds (e.g. being harder to observe, too small for tracking devices).

European Storm Petrels (henceforth "Storm Petrel") are small (~26g), long-lived pelagic seabirds, which forage mainly in flight by picking small items of food from the water surface (Cramp & Simmons 1977). The species breeds along the Atlantic seaboard of Europe (the migratory subspecies *H. p. pelagicus*) and around the Mediterranean basin (the apparently non-migratory subspecies *H. p. melitensis*,

Robb & Mullarney 2008). Birds from the Atlantic breeding colonies migrate south to overwinter off the coasts of Western and Southern Africa (Wernham *et al* 2002). The present study investigated variation in the body reserves of migrating birds of the Atlantic population (i.e. *H. p. pelagicus*), sampled over a 21-year period, during their late spring / early summer northward return migrations past the coast of south-west Portugal. The birds sampled were presumably mainly female pre-breeders prospecting potential breeding sites (see Chapter 2).

The most likely causal link between climate and the migration fuel loads of Storm Petrels is food availability (Stenseth *et al.* 2004). Storm Petrels, like most Procellariiformes, store energy in the form of stomach oil (Place *et al.* 1989, Warham 1990, 1996) and probably also as subcutaneous fat reserves (Blem 1990). Such energy storage has been interpreted as an adaptation to a pelagic feeding environment in the context of reproduction (Lack 1968, Ashmole 1971), enabling the birds to buffer themselves against starvation during their incubation shifts, shared by both sexes, which lasts on average three days for the European Storm Petrel (Scott 1970, Bolton 1996). Lipid accumulation is also very important for the nestlings, which are able to survive for many days without being fed by their parents (Warham 1990, 1996). Although it is known that storm petrels carry stomach oil throughout their life cycle and not just during breeding (Jacob 1982), there is an almost total lack of knowledge on its function as fuel as part of the migration strategy. Similarly, very little is known about the diet of storm petrels during migration (see Chapter 4). Studies of European Storm Petrel diet come mainly from food provided to chicks at colonies (composed chiefly of zooplankton, small fish and cephalopods) (Cramp & Simmons 1977, D'Elbee & Hemery 1998) but there is evidence that this can be

markedly different from the diet of adults, even during the breeding season (Scott 1970).

The abundance of the taxa that comprise the diet of Storm Petrels may itself depend ultimately on the level of marine primary productivity, which can vary substantially from year to year, depending on climatic and oceanographic conditions. Previous work has shown that in the waters off the western seaboard of Iberia, net primary productivity (NPP) is driven primarily by oceanographic conditions, particularly sea surface temperatures (SST) and changes in wind direction, at the end of the winter (Relvas *et al.* 2007, Santos *et al.* 2007). However, the trophic links from primary productivity to the abundance and availability of food for seabirds are complex, with several other interrelated climatic and oceanographic variables contributing directly or indirectly to inter-annual variability in abundance and availability of prey taxa. These variables include surface air temperature, sea-level pressure, wind speed and direction, upwelling intensity and changes in ocean currents (e.g. Abraham & Sydeman 2004, Behrenfeld 2006, Hipfner 2009).

Warmer temperatures at the sea surface tend, in general, to decrease phytoplankton productivity, but there may be marked geographical variation in the relationship between SST and NPP. For example at a local scale, NPP is higher in areas of upwelling and fronts between water bodies of contrasting temperatures. At a larger spatial scale, NPP varies along gradients of light and nutrient availability such that contrasting climate controls on ocean productivity can cause primary production to vary either positively or negatively with SST in different locations (Behrenfeld 2006). Furthermore, depending on the composition and flexibility of seabird diet, climate-driven changes in sea temperatures may have varied effects on seabird foraging ecology and migration fuelling strategies (e.g. Kitaysky & Golubova 2000).

During breeding, zooplankton taxa (other than fish larvae) seem to comprise a major part of the diet of European Storm Petrels (Witherby *et al.* 1965, D'Elbée & Hémery 1997), despite fish having higher calorific density (Beukema 1997, Pérez 1994, Paiva *et al.* 2006a). The analysis of diet presented in Chapter 4 suggests that, during migration, Storm Petrels feed extensively on fish, of which the European Sardine (henceforth "Sardine") is an important component (see Chapter 2). The Sardine is the most abundant fish species present off the coast of Portugal (FAO 2004) and constitutes an energy-rich diet for the birds (Paiva *et al.* 2006b). The spawning season of Sardines in Iberian waters ranges from November to April, but along the southern coast of Iberia it occurs mainly in the spring (March - May; Ré *et al.* 1990, Santos *et al.* 2001). This means that by May - June, when Storm Petrels are migrating past this coast, the Sardines will be potentially available as prey for the birds mainly in the stages of eggs, larvae or early juvenile (size range: 11-60 mm; Santos *et al.* 2005).

In the current Chapter, inter-annual variability in the North Atlantic Oscillation (NAO) index, temporal patterns in SST and NPP, as well as data on local surveys of adult and juvenile Sardines, were used to investigate climate-driven changes in food availability for migrating European Storm Petrels and their consequent regulation of energy reserves during migration. To my knowledge this is the first study to investigate such links in a migrating seabird.

4.3 Methods

4.3.1 Study Area and Ecosystem Features

This study was conducted on the SW coast of Portugal (37° 04' N, 8° 47' W) in the temperate NE Atlantic region. The study area is located at the northern limit of the

North Atlantic Upwelling System, characterized as one of the world's major upwelling areas. In this area the upwelling is induced by the prevalence and steadiness of northerly winds between April and September, strengthened during the summer by a thermal low pressure centre typically located over the Iberian Peninsula. Associated with the upwelling there is typically a bloom of phytoplankton in April - May, which in turn triggers a bloom in zooplankton in May – June (Arístegui *et al.* 2004). Since phytoplankton blooms are strongly influenced by SST (Stenseth *et al.* 2004), SST in March - April and NPP in April - May could potentially be useful indices of inter-annual variability in the bottom-up control of the marine ecosystem in Portuguese waters.

The study area is generally highly productive and the focus of intensive commercial fisheries. The most commercially important fish species in this area is the European Sardine (37% of landings by mass in 2004), followed by Atlantic Mackerel *Scomber scombrus* (9%) and Horse Mackerel *Trachurus trachurus* (8%) (FAO 2004).

4.3.2 European Storm Petrels

Between 1990 and 2010, Storm Petrels were captured at the study site during their northward migration, between May 16th and August 17th (study periods varying to some extent between years; median dates ranged from 1st June to 29th June, with the great majority of individuals caught between May 20th and June 20th). Acoustic playbacks of the species' "purr" call (Cramp & Simmons 1977) were used to attract the birds into mist nets at night. Birds captured with this method are mainly immature birds (Fowler *et al.* 1982), migrating rapidly northwards (often >200km/day, Bolton & Thomas 1999) towards the Atlantic breeding colonies (Harris

et al. 1993). Only a very small proportion of the storm petrels caught are of the Mediterranean subspecies *H. p. melitensis* (<1%, R.A. King, R. Medeiros *et al.*, unpublished data) or are subsequently retrapped at the study site (<1%). There is a substantial female bias in the sex ratio of the sampled birds, though this bias is largely consistent between years (mean sex ratio ± SD = 85.5% female ± 4.1%, see Chapter 2). All birds captured were weighed (to 0.1g) and wing length (flattened, maximum chord in mm, Svensson 1992) was measured. Date (no. of days from May 1st) and time of capture (hours relative to midnight) was also recorded for each capture.

4.3.3 North Atlantic Oscillation (NAO)

The NAO is a cyclic oscillation in latitudinal pressure gradients across the North Atlantic, which captures a large amount of the inter-annual variation in climatic, oceanographic and ecological conditions across the North Atlantic basin (Hurrell 1995, Stenseth *et al.* 2004). The NAO can be quantified as an NAO index – the difference in atmospheric pressure between Iceland and Lisbon in Portugal (Hurrell 1995), Iceland and Gibraltar (Jones *et al.* 1997), or Iceland and the Azores (Walker 1924, Uppenbrink 1999). The latitudinal pressure gradient across the NE Atlantic is most pronounced during the winter months, and NAO index values for the winter period have been shown to be more strongly associated with oceanographic and ecological processes than values over the rest of the year (Hurrell 1995, Rogers 1997). In the present study, the winter (December-March) NAO index values between Portugal and Iceland were used. Since delayed NAO impacts on ecosystems can sometimes be stronger than more direct ones (Stenseth *et al.* 2004), one-year and two-year lagged NAO index values were also tested (i.e. the winter NAO index value

from 1 year and 2 years previously), henceforth referred to as NAO^{-1} and NAO^{-2} respectively. These data were obtained for the whole study period, from the Climate & Global Dynamics Division of the NCAR Earth System Laboratory, available to download from: http://www.cgd.ucar.edu/cas/jhurrell/indices.html (last accessed 01/06/2010).

4.3.4 Sea Surface Temperatures (SST)

Means values for sea surface temperatures from January to June were obtained for 1990-2010 from the British Atmospheric Data Centre, provided by the Hadley Centre at the UK Meteorological Office. These data are obtained from *in situ* sea surface observations and satellite derived estimates of temperatures at the sea surface. Data available includes monthly mean gridded, global SSTs from 1870 to present, downloadable from: http://badc.nerc.ac.uk/home/index.html (last accessed 01/08/2010).

The data are downloaded as grids, in which the grid spacing is 1° in both latitude and longitude. The sea area over which mean SST was calculated was defined by estimating the birds' flight range over the period during which birds could be adjusting their stored energy reserves. This was achieved by taking the observed variance in individual mass across the whole study period (5.21g) and calculating the period of time over which this degree of variation in body mass is likely to be generated, considering that a breeding European Storm Petrel gains weight at the rate of 1.6g/day whilst away from the nest foraging at sea (Bolton 1996; 5.21g / 1.6 g day^{-1} = 3.26 days). Using data from European Storm Petrels ringed in Portugal and subsequently recaught in different countries along the coasts of NW Europe, Bolton & Thomas (1999) estimated the average daily cross-country travel speed for

migrating individuals as 192.4 km/day. Based on this, the estimated foraging range to account for the observed variation in mass was 3.26 days * 192.4km/day = 626.5km. A sea area of 600km, just within this estimated flight range, between the bearings of South to SW from the capture location in SW Portugal (Appendix 4.I), was used to extract mean monthly SST values for each year of the 21 year study period, for use in the subsequent analyses.

4.3.5 Net Primary Productivity (NPP)

Net primary productivity is defined as the carbon produced by photosynthesis that is not immediately used by the plants (in terrestrial habitats) or phytoplankton (in marine habitats) to support their own maintenance requirements. Data on NPP were obtained for the years 1998 to 2007 from the Sea-viewing Wide Field-of-view Sensor (SeaWiFS) database at: http://www.science.oregonstate.edu/ocean.productivity/index.php (last accessed 01/08/2010).

Mean monthly NPP values were downloaded for the months January to June in each year. This database comprises estimations made using the standard Vertically Generalized Production Model (VGPM, Behrenfeld & Falkowski 1997). The VGPM is a model that estimates NPP from the upper-ocean chlorophyll concentration using a temperature-dependent description of chlorophyll-specific photosynthetic efficiency, given as milligrams of carbon fixed per day per unit volume (mg C / m^2 / day). The global data are downloaded in a 1080 x 2160 grid, in an equidistant cylindrical projection, in which the grid spacing is 1/6 of a degree in both latitude and longitude. As for the SST data described above, the monthly mean was obtained from the same 600km sea area, obtained by estimating the birds' flight range over the

period during which birds could be generating the variation observed in their stored energy reserves (Appendix 4.II).

4.3.6 Sardine Abundance and Biomass

Data on the abundance and biomass of European Sardines in waters off the Algarve region of southern Portugal were obtained from IPIMAR (Portuguese Research Institute for the Fisheries and the Sea) reports on sardine surveys, using acoustic survey methods together with standardised fishing data (Marques *et al.* 2005). Data were available on the following variables: (i) abundance of adult Sardines, (ii) abundance of juvenile Sardines, (iii) total biomass of adults and juveniles. Data on total Sardine biomass were available for surveys performed between 1995 and 2005, but data on the numbers of adults and juveniles are only available from 1995 until 2002. These surveys were usually carried out in March, but the timing of surveys varied to some extent across the years, between February and June (Marques *et al.* 2005).

4.3.7 Data Analysis

Data were analysed in SPSS v16 (SPSS Inc.) and R v2.10.1 (The R Foundation for Statistical Computing, 2009). An initial general linear model (GLM) was performed to investigate the changes in body mass among years, accounting for other non-environmental variables (i.e. date, time of night and sex) and controlling for wing length (as a measure for body size). The variable "date" was quantified as the number of days from May 1st in each year. The model tested for linear and non-linear (quadratic and cubic) effects of date and time. A stepwise approach was used, sequentially removing the least significant variables one at a time to reach the

minimum adequate model containing only significant parameters (Appendix 4.III). From this "baseline model", parameter values for each year were used to calculate the relative body mass for each year (i.e. body mass corrected statistically for wing length, date and time of night). These annual mean relative body mass values were used to analyse the direct relationships with the environmental variables using Pearson correlations ($n \leq 21$ years).

Further analysis involved using GLMs to test for the effect of different environmental variables that could account for the variation in body mass between years. Parameters from the baseline model described above (i.e. wing length, date (linear term), date^2 (quadratic term), and time of night) were all retained in all of the models testing for associations between body mass and the following environmental variables: NAO, SST, NPP and the abundance or biomass of Sardines. These environmental variables were included in models to test whether they could account for the observed variation in body mass among years and therefore year was not included in these models. A separate set of models was run for each class of environmental variables (i.e. SST and NPP from January to June, direct and lagged NAO effects, numbers of juvenile and adult Sardines and total biomass of adult and juvenile Sardines). The explanatory power and fit of each of these different models was compared within each class of environmental variable, using a range of statistics relating to individual variables (probability values, partial Eta^2 values, parameter estimates) and model parameters (model R^2 and model AIC values). The best individual predictors within each class of environmental variable were compared using the models' adjusted R^2 values. The non-linear (quadratic) effect of each environmental variable was also investigated and compared with the linear-only models using the model AIC values.

4.4 Results

A total of 5,258 individual European Storm Petrels was caught in the 21 years of study. Mean body mass ± SD for the whole study period was 26.31g ± 2.28g (range = 19.2g – 37.7g; 99% of individuals weighed 20.9g – 33.2g). Mean annual relative body mass (i.e. controlling for variation in wing length, date and time of capture) varied dramatically among years, over a range of 3.41g over the whole study period (equivalent to 13% of the mean body mass across all 21 years). Rather than varying erratically between successive years, the pattern of variation in body mass followed a clear trend (mean relative body mass in one year was positively correlated with that in the next year, r = +0.453, n = 20 contrasts, p = 0.045), although in the last three years (i.e. 2008-9, 2009-10) changes between years have been more dramatic than over the rest of the study period (Figure 4.1).

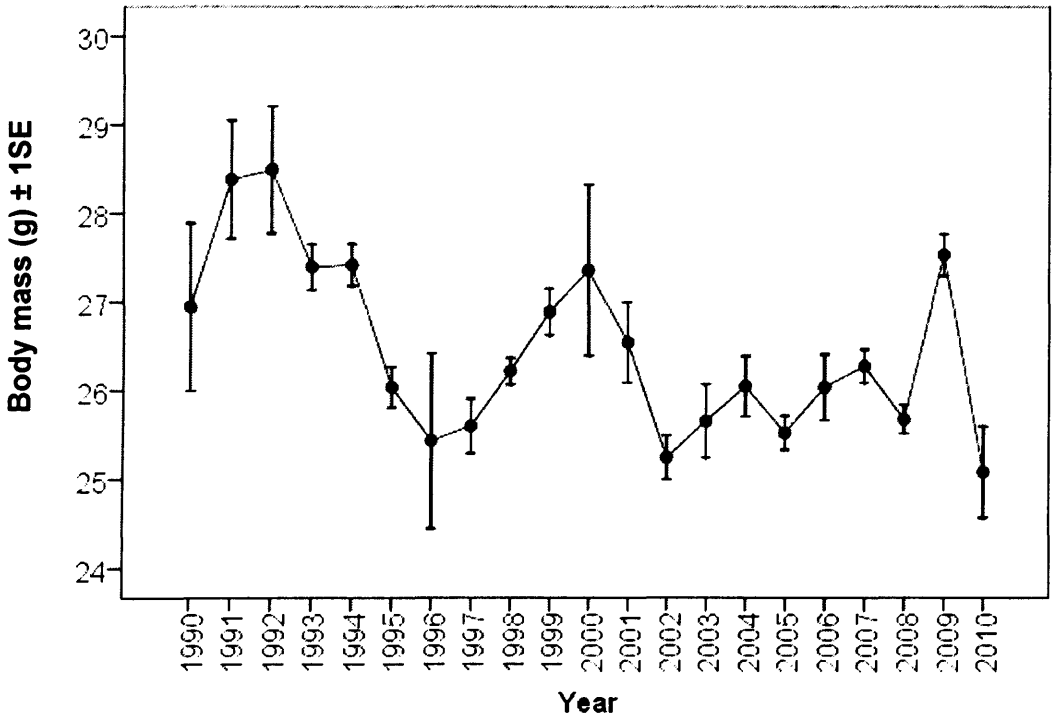


Figure 4.1 Changes in body mass (mean annual body mass ± 1SE) of European Storm Petrels captured in SW Portugal among 1990 and 2010. Numbers of birds sampled each year are presented in Table 4.I.

An initial GLM testing for significant variation in body mass according to sex, year, season and time of night, showed no significant differences in the European Storm Petrels' mean body mass between sexes (using the sex data for birds caught in 2003-9 see Chapter 2), but significant effects of year, time of night (increasing body mass over the course of the night) and date (decreasing body mass over the course of the migration season). The date effect is, furthermore, non-linear (the steepest decline in mass with date occurs at the start of the migration season). Therefore, to control statistically for these effects, the variables date, date^2, time of night and wing-length (as a measure of body size) were included in all subsequent GLMs testing for the effects of environmental conditions on changes in the birds' body mass among years.

According to the results of the GLMs, NAO^{-1}, SST-April, NPP-May and biomass of Sardines were the best individual predictors of Storm Petrel body mass within each class of environmental variable (Figure 4.2, Appendix 4.III.A). The direction of the association between the Storm Petrels' body mass and SST in each month from January to June was always negative, while that between body mass and NPP in each month changed from positive in February, to negative in May. NAO, NAO^{-1} and NAO^{-2} each had a positive association with the birds' body mass. Local numbers of juvenile and adult Sardines and total Sardine biomass were each negatively associated with the birds' body mass, though the partial Eta^2 values indicated that total Sardine biomass had the strongest relationship with the birds' body mass.

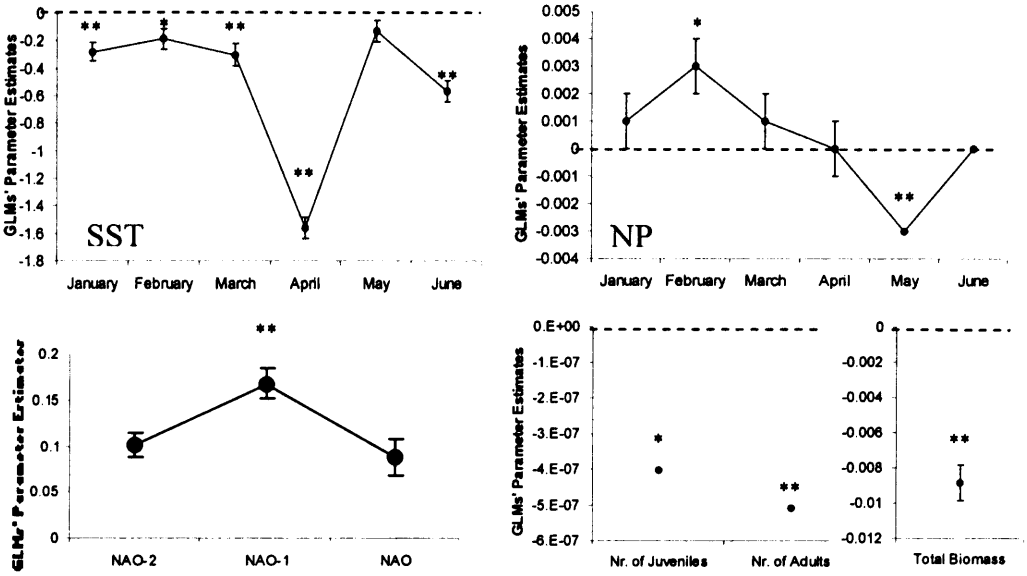


Figure 4.2 Parameter estimates (±SE) obtained from GLM analyses, for the association between European Storm Petrel body mass (dependent variable), and sea surface temperature (SST), net primary productivity (NPP), the North Atlantic Oscillation (NAO) or the abundance and biomass of Sardines (independent variables). The symbols indicate the significance of the association in the GLM; * indicates $p < 0.01$, ** indicates $p < 0.001$.

Table 4.I shows the annual mean values for the body mass of Storm Petrels over the study period, together with the most relevant environmental variables identified by the comparisons of GLM models shown in Appendix 4.III. According to the individual GLMs, SST-April is the variable that alone explains the highest proportion of variation in the birds' body mass (summarised in Table 4.II, full details in Appendix 4.III). Whilst quadratic relationships did result in a very minor improvement in adjusted R^2 values (Appendix 4.III), the relationships were broadly linear. In particular, there was no evidence to suggest that body mass peaked at intermediate values of any of the environmental variables, within the range of environmental variation observed in the present study (Figure 4.3). Since the

deviation from a linear relationship was extremely small, the linear relationships are reported below.

Table 4.I Inter-annual variation in the body mass of European Storm Petrels, together with equivalent data for environmental variables which GLM model comparisons identified as being most strongly associated with Storm Petrel body mass. Tests for temporal trends are also presented (Pearson correlations of each variable with year, n = no. of years). Mean relative body mass is the annual mean body mass corrected for individual variation in wing-length, date and time of night.

Year	NAO^{-1} Index (1-year lag)	SST April (°C)	NPP May (mg C / m^2 / day)	Sardine biomass (kt)	Mean Storm Petrel body mass (g)	Mean relative body mass (g)	No. of Storm Petrels caught
1990	5.08	17.7	-	-	26.9	26.6	7
1991	3.96	17.2	-	-	28.4	28.2	31
1992	1.03	17.2	-	-	28.5	28.2	52
1993	3.28	17.6	-	-	27.4	27.3	340
1994	2.67	17.3	-	-	27.4	27.1	483
1995	3.03	18.0	-	133	26.0	25.8	396
1996	3.96	18.3	-	106	25.4	25.1	19
1997	-3.78	18.9	-	96	25.6	25.3	180
1998	-0.17	18.3	714.12	65	26.2	26.0	786
1999	0.72	17.8	774.55	39	26.9	26.7	241
2000	1.7	17.8	618.21	59	27.4	27.2	28
2001	2.8	18.1	827.75	24	26.5	26.6	88
2002	-1.9	17.9	893.27	105	25.2	25.2	225
2003	0.76	18.1	888.04	60	25.7	25.6	112
2004	0.2	18.1	702.69	39	26.0	26.0	116
2005	-0.07	18.1	957.14	62	25.5	25.5	435
2006	0.13	18.4	784.88	-	26.0	25.9	136
2007	-1.09	18.2	806.45	-	26.3	26.2	519
2008	2.79	18.3	-	-	25.7	25.6	637
2009	2.1	17.5	-	-	27.5	27.5	367
2010	-0.41	18.7	-	-	25.1	25.1	60
Tests for directional change over the sampling period							
r =	-2.85	0.514	0.401	-0.593	-0.536	-0.464	n.a.
n =	21	21	10	11	21	21	(effort-
p =	0.211	0.017	0.251	0.055	0.012	0.034	dependent)

Table 4.II Summary of four different general linear models (GLMs) explaining variation in body mass (dependent variable) of European Storm Petrels captured in SW Portugal in May - June. In addition to one of the environmental variables shown in the table, all four models also contained: wing, date, date^2 and time of night. Full details of these and related models are given in Appendix 4.III.

Environmental variable in the GLM	Model R^2 (adjusted)	Partial Eta^2	Parameter values	F (d.f.)	P
NAO lag-1yr	0.060	0.019	+ 0.168	96.973 (1, 5045)	<0.001
SST-April	**0.111**	**0.072**	**- 1.561**	**390.547 (1, 5045)**	**<0.001**
NPP-May	0.065	0.017	- 0.003	45.001 (1, 2678)	<0.001
Total Sardine biomass	0.060	0.015	-0.009	40.689 (1, 2622)	<0.001

Figure 4.3 summarizes the direct correlations amongst the four classes of environmental variables (i.e. NAO, SST, NPP and Sardines), and between each of these and the European Storm Petrels' body mass. The direct relationships between mean annual relative body mass of the Storm Petrels and each of these parameters are shown in Figure 4.4.

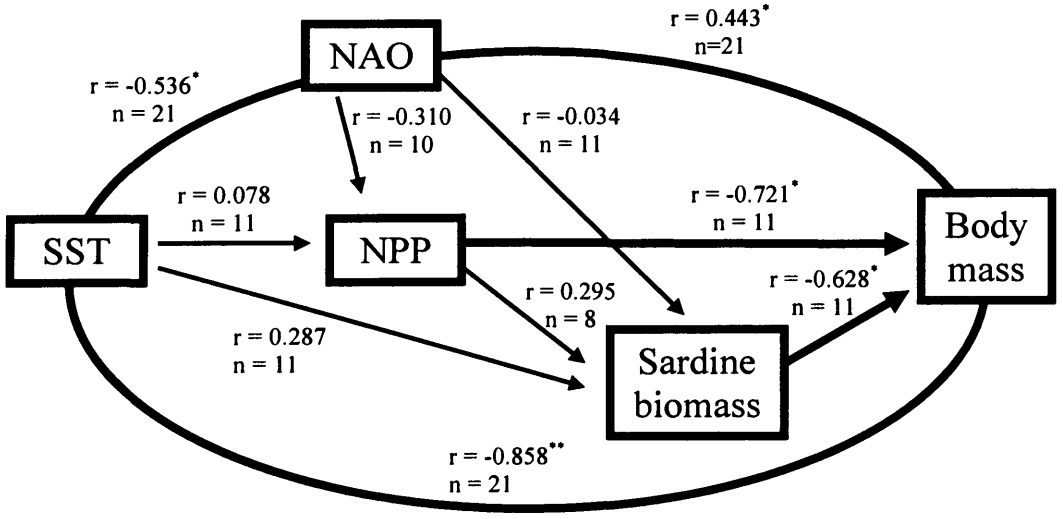


Figure 4.3 Direct correlations amongst environmental variables, prey abundance, and the mean annual relative body mass of European Storm Petrels. Sample size (n) is the number of years for which data was available for each correlation.

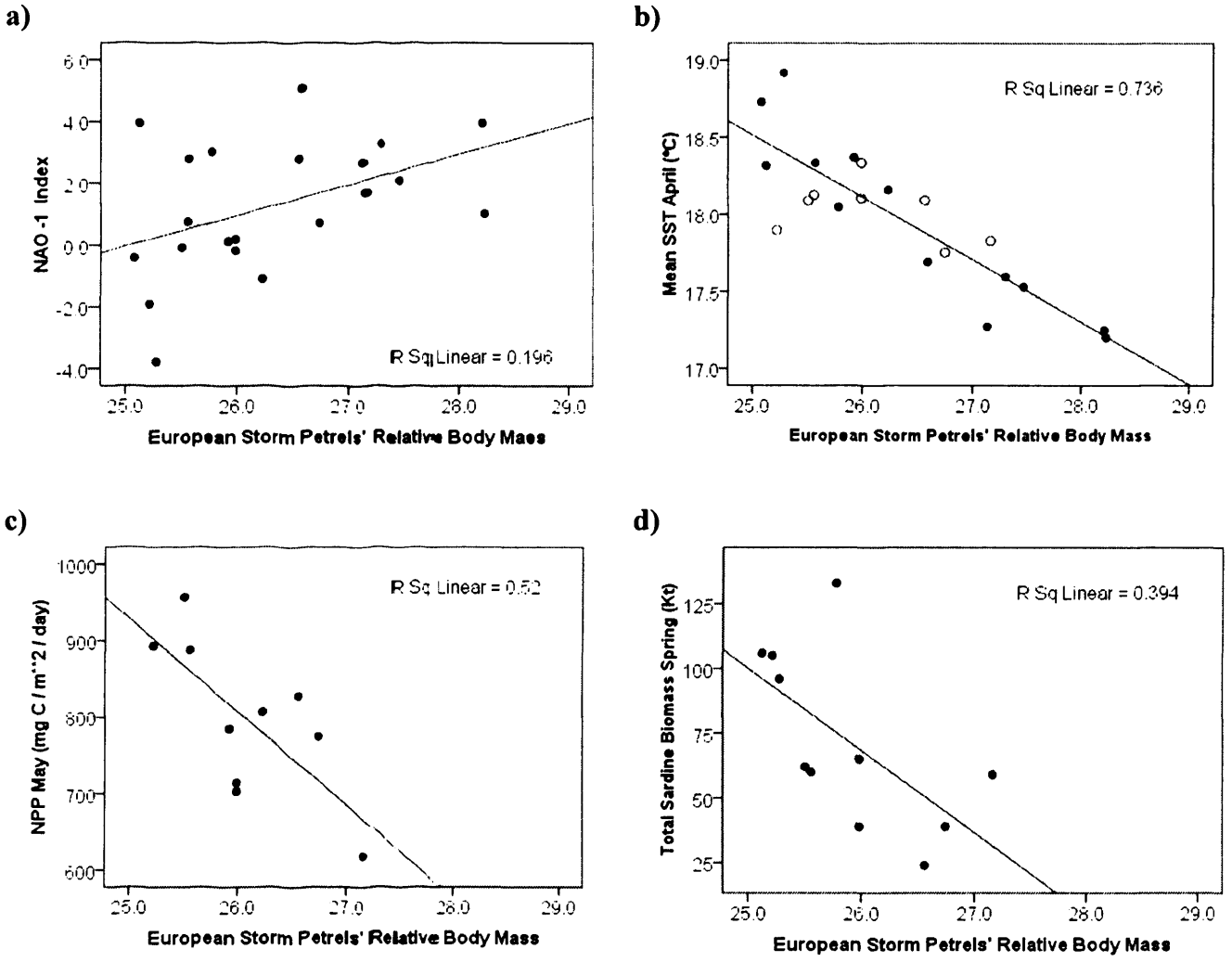


Figure 4.4 Relative body mass (corrected for wing size) of European Storm Petrels captured in SW Portugal in May/June, plotted against a) 1-year lagged NAO index (NAO^{-1}); b) mean Sea Surface Temperatures (SST) in April; c) mean Net Primary Productivity (NPP) in May; and d) Sardine biomass in Spring. Graphs a) and b) include data from 1990-2010; graph c) includes data from 1998-2007. The open circles in Figure 4.4b) represent those years (1998-2005) when data are also available for NPP and Sardine biomass.

Each of these parameters alone explained respectively 19.6% (NAO^{-1}), 73.6% (SST-April), 52% (NPP-May) and 39.4% (Sardine biomass) of the among-year variations in the birds' relative body mass (Figure 4.4). However, note that the years for which data are available vary among the different environmental variables, so these values are not necessarily directly comparable. Although SST-April again explains most of the variation in the birds' body mass, Figure 4.4a suggests that the period for which

there are NPP and Sardine data available (open dots) did not show such a large amplitude in SST, compared with the whole sampling period of 1990-2010.

4.5 Discussion

The large inter-annual variations in the body mass of European Storm Petrels remain apparent and highly significant when structural body size (i.e. wing length), time of night and seasonal variations are controlled for statistically. These inter-annual variations span more than 13% of the mean body mass across the study period, and therefore represent large fluctuations in the size of the birds' stored energy reserves (likely to be primarily subcutaneous fat and /
or stomach oil).

Storm Petrels appear to respond sensitively to inter-annual changes in their environment, namely to SST, NPP and Sardine availability. Although migrating Storm Petrels probably sample food availability over a wide area prior to capture in Portugal, the level of body reserves which these birds carry seems more sensitive to variations in local climate conditions (e.g. SST) than to large-scale climate variations (such as those captured by the NAO index). Other studies, focusing on variation in fish abundance and recruitment in Atlantic waters off the western Iberian coast have found a similar enhanced level of responsiveness to local conditions compared to the NAO (Ottersen *et al.* 2001, Guisande *et al.* 2004).

The time-lag in the strong association between SST in April and the birds' body reserves in the subsequent migration season (late May - late June) suggests that this relationship between sea temperatures and migration fuelling decisions is not a direct thermal effect (e.g. if birds were increasing subcutaneous fat for thermal insulation when the sea was colder), but may instead be mediated via an effect of sea

temperatures on the birds' food abundance. The direction of these relationships indicates that Storm Petrels carry lower body reserves in years when the sea is warmer and food is abundant overall, as suggested by the higher levels of primary productivity and of Sardine biomass in years when the birds have relatively low body mass. In other words, the body mass of Storm Petrels does not appear to be directly limited by food availability; rather, the birds appear to be using their body reserves as a strategic "buffer" against starvation in years when food availability is relatively low. However, the lack of a significant direct association between SST, NPP and Sardine biomass or abundance in the waters off SW Portugal (at least over the years for which such data are available) suggests that strong temperature regulation is not the main mechanism by which SST affects food abundance for migrating Storm Petrels, and that variation in SST and NPP is not the only mechanism driving changes in Sardine abundance. Therefore, changes in Storm Petrel body mass seem to integrate various levels of variability in their environment. This illustrates the challenge of predicting the impacts of climate change across trophic levels in complex ecosystems.

Although well reported for terrestrial birds in the context of overnight survival (Thomas & Cuthill 2002) and migration fuelling decisions (Bayly 2006), strategic adjustment of fuel reserves for migration during years of food scarcity has not previously been reported in pelagic seabirds. The level of body reserves carried by birds is always the outcome of a trade-off between the costs and benefits of carrying those reserves (Witter & Cuthill 1993) and these may depend on environmental conditions, particularly the availability of food. The benefits of carrying body reserves are perhaps obvious, as they act as "fuel" for long-distance migrations, such as that being undertaken by Storm Petrels as they pass the SW coast of Portugal in

early summer. This species forages largely by taking food from the sea surface in flight, by dipping briefly onto the water or by using their legs to "patter" across the sea surface, while using their bills to reach into the water to take food items (see Chapter 1). Therefore, since Storm Petrels can potentially migrate as they feed (i.e. by feeding in a particular direction), they may not need to migrate between discrete stopover sites as many terrestrial migrants do (e.g. Newton 2010, Bayly 2006, Wernham *et al.* 2002). However, the distribution of food across the ocean surface is not uniform (e.g. Miller 2004, Kaiser.*et al.* 2005), and body reserves may be important in avoiding starvation during migration between patches of food in years when food availability overall is low.

Balanced against these benefits can be important costs, namely: greater body mass, resulting in increased flight costs (higher energy expenditure), reduced flight speed and decreased maneuverability. For a species that forages using aerobatic flight to take food from the moving sea surface, maneuverability and agility are likely to be particularly important for efficient foraging during migration. Increased body mass may also cause a reduced ability to evade predators in flight (Cuthill *et al.* 2000). Although mortality at sea is estimated to be low for Storm Petrels (Cramp & Simmons 1977) and predation risk is not known as a major pressure for these birds, near the colonies the Storm Petrel is easily predated by bigger birds such as gulls *Larus* spp. and skuas *Stercorarius* spp. (Warham 1996). It is possible that this phenomenon has been underestimated when the birds are at sea. The trade-off between the benefits of storing energy reserves and the suite of mass-dependent costs will favour a reduction in body reserves in circumstances when starvation risk is low (Cuthill *et al.* 2000).

Despite the observed responsiveness of the body reserves of Storm Petrels to climate-driven changes in foraging conditions, there are ultimately limits to the extent of this response. Body reserves cannot fall below zero, or the bird will, by definition, starve to death. The lean body mass of European Storm Petrels is likey to be around 18-19g (though individuals blown inland by gales, which may have metabolised additional muscle tissue, weighed only 14.5 – 17g, Cramp & Simmons 1977). At the other extreme, body reserves cannot become so large that the bird cannot fly. Indeed, for a species relying on aerobatic flight, mass-dependent flight costs may effectively limit body reserves well before the maximum fuel load that can be carried in flight is reached. It is possible that some individual Storm Petrels in the present dataset may have reached such limits; the lightest bird captured in the present study was 19.2g and the heaviest was 37.7g, though 99% of birds captured fell within the range 20.9 – 33.2g.

The molecular and stable isotope analyses of diet described in detail in Chapter 3, indicates that fish in general, and the European Sardine in particular, make up a major part of the diet of European Storm Petrels during their migration past SW Portugal. Predictions of forthcoming climate change include mean SST increases of 2-3°C off SW Iberia by 2100 (IPCC 2007). Over the study period (1990-2010), upwelling intensity has decreased (Pérez *et al.* 2010) and sea surface temperatures have increased (Table 4.I). Fish populations are affected by these physical parameters, and studies suggest that the abundance of Sardines has been decreasing in the south of Portugal, particularly in recent years (Santos *et al.* 2001, Marques *et al.* 2005). Although varying according to the geographical location (Planque *et al.* 2007), Sardine spawning activity is temperature dependent, with preferences for spawning in the study area at 14-15°C and avoidance for temperatures below 12°C

and above 16°C (Stratoudakis *et al.* 2007). SST values in the present dataset vary between years as well as seasonally, but late-winter/spring SST values are generally above this range. In fact, the minimum temperature recorded across all months and years was 16.78°C (though these were monthly means and not instantaneous temperature values). Thus, an increase in SST within or above the current range will take conditions further from the optimal temperature range for Sardine spawning, accounting for the observed negative association between SST and spawning (Coombs *et al.* 2010). In contrast, higher temperatures are usually associated with increased growth rates in many marine organisms (e.g. Wiedenmann *et al.* 2008), including small pelagic fish such as the Sardine (Montevecchi & Myers 1997). However, this might also reduce the food availability of Storm Petrels at higher temperatures, since it reduces the period of time in which the young fish are more vulnerable to predation (larvae and young juveniles). Despite these relatively direct and simple temperature dependent effects, it is important to acknowledge that the impact of climate on fish stocks is highly variable, often indirect and complex (Stenseth *et al.* 2004). For example, there may be differing effects of SST variations on different life-history stages of different species of fish, and these may vary geographically as well as being modulated by a range of other environmental variables (e.g. upwelling intensity, wind conditions and offshore transport; Santos *et al.* 2001, Planque *et al.* 2007, Takasuka *et al.* 2008).

Thus, future increases in sea surface temperatures may have varied effects, depending on a species' feeding biology (e.g. planktivorous or piscivorous, Kitaysky & Golubova 2000). In addition to using their body reserves as an energetic buffer against starvation, birds may respond to climate change by strategically adapting their diet to the changed foraging conditions. The analysis of Storm Petrel diet

presented in Chapter 3 suggests that this might indeed be how Storm Petrels behave, by eating more fish and possibly, squid in years when overall productivity (in terms of NPP and hence zooplankton abundance) is low. Specifically, in 2009 when body mass was relatively high, unlike in the three previous years (2006-2008) when body mass was relatively low, there was a higher number of birds presenting fish in their diet (Chapter 3). This makes ecological sense, since in years of lower food availability the birds must increase their foraging effort in order to build up their body reserves. Focusing on more energetically efficient prey such as fish and squid (Adams *et al.* 1984, Beukema 1997, Pérez 1994, Paiva *et al.* 2006b) is presumably a more efficient way to do so. However, the ability to store reserves is in itself dependent on the availability of prey. If future changes in climate further reduce the abundance of such prey, the ability to build up a strategic buffer of body reserves in years of low food availability might ultimately be reduced, resulting in a shift in the nature of the relationships between environmental variables and the birds' body mass, from the strategic buffering described in this Chapter, to direct limitation of energy reserves.

Although the Sardine appears to be a key prey species for European Storm Petrels during migration (Chapter 4), changes in the availability of other prey types (e.g. other fish taxa, cephalopods, isopods, amphipods, decapods – see Chapter 4) are potentially more directly mediated by SST or NPP, and are therefore also important to consider. These are more difficult to investigate since long-term studies of such taxa in Iberian waters are scarce, incomplete or entirely lacking.

In the present study there is an apparent oscillation in the body reserves of Storm Petrels for the first 15 years (1990-2004) but this seems more erratic in more recent years (2005-2010). So far, the long-term trends in SST and NPP may be

favouring the birds, since their body mass has shown an overall decrease over the study period (though total Sardine biomass has shown a significant decrease over an even shorter period, Table 4.I), Regardless of the observed changes over the last 2 decades, the scale of the predicted increases in SST over the next 50-100 years may lead to severe disruption of ecosystem processes and functions, ultimately leading to decreases in food availability and direct food limitation among migrating Storm Petrels.

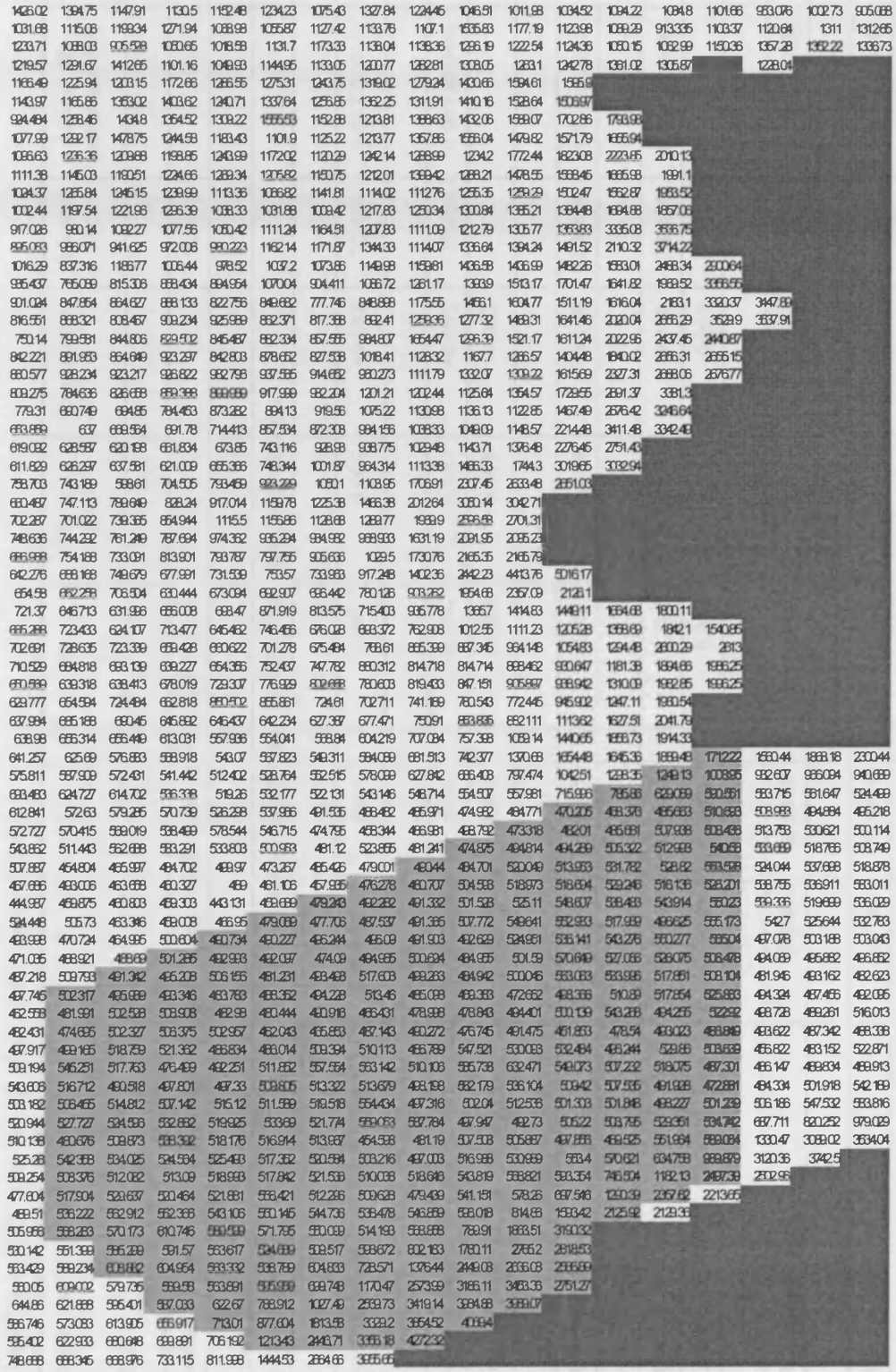
Changes across trophic levels in marine ecosystem dynamics are difficult to monitor directly, but understanding the mechanisms underlying such changes is vital if higher trophic level foragers such as Storm Petrels are to be used as monitors of the marine environment and bio-indicator of climate change, as has been advocated (Furness and Camphuysen 1997, Gremillet and Charmantier 2010, Kazama *et al.* 2010). Previous uses of birds as bio-indicators of climate change have focussed on breeding productivity, population dynamics, or phenology (Aebischer *et al.* 1992, Furness & Greenwood 1993, Crick *et al.* 1997, Dunn & Winkler 1999). The present study highlights the potential value of the body mass regulation behaviour of seabirds as a new and sensitive class of bio-indicator of climate change.

4.6 Appendices

Appendix 4.I. Example of database for SST around the Portuguese and African coast in April 1998. Dark shading represents land and light shading represents the sea area used to calculate the mean SST for each month.

14.47	14.42	14.38	14.41	14.48				
14.87	14.84	14.81	14.83	14.92	15.16			
15.39	15.38	15.32	15.29	15.31	15.16			
15.86	15.84	15.75	15.69	15.68				
16.25	16.21	16.10	16.06	16.11				
16.63	16.59	16.52	16.50	16.56	16.79			
16.97	16.96	17.00	16.98	16.91	16.88	16.91	16.79	16.62
17.30	17.30	17.39	17.38	17.26	17.16	17.06	16.94	16.62
17.60	17.58	17.64	17.64	17.56	17.44	17.33	17.10	
17.84	17.80	17.84	17.83	17.77	17.54	17.44		
18.02	17.95	17.98	17.98	17.94				
18.23	18.14	18.16	18.12	18.07				
18.44	18.38	18.40	18.33	18.12				
18.58	18.48	18.47	18.46					
18.72	18.48	18.43						
18.79								

Appendix 4.II. Example of database for NPP around the Portuguese and African coast in May 1998. Dark shading represents land and light shading represents the area used to calculate the mean NPP values for each month.



Appendix 4.III. Summary table of general linear models explaining variation in body mass (dependent variable) of European Storm Petrels captured in SW Portugal in May - June. In addition to the environmental variables shown in the table, all models also contained the following variables from the baseline model: wing length, date, date^2, time of night.

Models and variables	F	d.f.		P	Partial Eta^2	Parameter	Model AIC	Model R^2 (adjusted R^2)	Years
Baseline model (independent variables: **year**, wing length, date, date^2, time of night)							22,470	0.043	**1990-2010**
(a) Environmental variable: North Atlantic Oscillation (NAO) + wing length, date, date^2, time of night.									**1990-2010**
NAO current yr	19.704	1, 5045		<0.001	0.004	+0.088	22,450	0.047 (0.046)	
NAO current yr	15.745	1, 5044		<0.001	0.003	+0.093	22,450	0.047 (0.046)	
(NAO current yr)^2	0.165	1, 5044		0.685	<0.001	-0.003			
NAO lag 1-yr	**96.973**	**1, 5045**		**<0.001**	**0.019**	**+0.168**	**22,370**	**0.061 (0.060)**	
NAO lag 1-yr	**97.561**	**1, 5044**		**<0.001**	**0.019**	**+0.190**	**22,370**	**0.062 (0.061)**	
(NAO lag 1-yr)^2	**6.051**	**1, 5044**		**0.014**	**0.001**	**-0.020**			
NAO lag 2-yr	54.880	1, 5045		<0.001	0.011	+0.101	22,410	0.054 (0.053)	
NAO lag 2-yr	55.198	1, 5044		<0.001	0.011	+0.101	22,410	0.056 (0.054)	
(NAO lag 2-yr)^2	10.891	1, 5044		0.001	0.002	+0.019			
Continued overleaf									

Appendix 4.III., *continued.*

Models and variables	F	d.f.		P	Partial Eta²	Parameter	Model AIC	Model R² (adjusted R²)	Years
(b) Environmental variable: Sea Surface Temperature (SST) + wing length, date, date², time of night.									**1990-2010**
SST January	15.797	1, 5045		<0.001	0.003	-0.279	22,450	0.046 (0.045)	
SST January	10.643	1, 5044		0.001	0.002	+16.593	22,440	0.048 (0.047)	
(SST January)²	11.006	1, 5044		0.001	0.002	-0.469			
SST February	6.664	1, 5045		0.010	0.001	-0.187	22,460	0.044 (0.044)	
SST February	1.554	1, 5044		0.213	<0.001	-8.000	22,460	0.045 (0.044)	
(SST February)²	1.482	1, 5044		0.224	<0.001	+0.223			
SST March	14.644	1, 5045		<0.001	0.003	-0.300	22,450	0.046 (0.045)	
SST March	97.993	1, 5044		<0.001	0.019	-73.051	22,360	0.064 (0.063)	
(SST March)²	97.202	1, 5044		<0.001	0.019	+2.065			
SST April	**390.547**	**1, 5045**		**< 2.2e-16**	**0.072**	**-1.561**	**22,090**	**0.112 (0.111)**	
SST April	**27.395**	**1, 5044**		**<0.001**	**0.005**	**-28.788**	**22,070**	**0.116 (0.115)**	
(SST April)²	**24.510**	**1, 5044**		**<0.001**	**0.005**	**+0.757**			
SST May	2.4247	1, 5045		0.119	<0.001	-0.127	22,470	0.044 (0.043)	
SST May	61.151	1, 5044		<0.001	0.012	-62.466	22,410	0.055 (0.054)	
(SST May)²	60.909	1, 5044		<0.001	0.012	+1.635			
SST June	53.231	1, 5045		<0.001	0.010	-0.563	22,420	0.053 (0.052)	
SST June	31.025	1, 5044		<0.001	0.006	-47.310	22,390	0.059 (0.058)	
(SST June)²	30.294	1, 5044		<0.001	0.006	+1.129			

Appendix 4.III., *continued.*

Models and variables	F	d.f.		P	Partial Eta²	Parameter	Model AIC	Model R² (adjusted R²)	Years
(c) Environmental variable: Net Primary Productivity (NPP) + wing length, date, date², time of night.									**1998-2007**
NPP January	1.0302	1, 2678		0.310	<0.001	+0.001	11,610	0.051 (0.049)	
NPP January	18.179	1, 2677		<0.001	0.007	-0.286	11,590	0.058 (0.056)	
(NPP January)²	18.357	1, 2677		<0.001	0.007	<+0.001			
NPP February	10.965	1, 2678		<0.001	0.004	+0.003	11,600	0.055 (0.053)	
NPP February	12.877	1, 2677		<0.001	0.005	+0.047	11,590	0.059 (0.057)	
(NPP February)²	11.478	1, 2677		<0.001	0.004	-3.500e-1			
NPP March	1.1577	1, 2678		0.282	<0.001	+0.001	11,610	0.051 (0.049)	
NPP March	28.595	1, 2677		<0.001	0.011	+0.092	11,580	0.061 (0.059)	
(NPP March)²	28.257	1, 2677		<0.001	0.010	-5.819e-1			
NPP April	0.1577	1, 2678		0.691	<0.001	+2.424e-04	11,610	0.051 (0.049)	
NPP April	25.530	1, 2677		<0.001	0.009	+0.068	11,580	0.060 (0.058)	
(NPP April)²	25.400	1, 2677		<0.001	0.009	-5.885e-5			
NPP May	**45.001**	**1, 2678**		**<0.001**	**0.017**	**-0.003**	**11,560**	**0.066 (0.065)**	
NPP May	**5.554**	**1, 2677**		**<0.019**	**0.002**	**+0.021**	**11,560**	**0.069 (0.067)**	
(NPP May)²	**7.292**	**1, 2677**		**<0.007**	**0.003**	**-1.483e-5**			
NPP June	0.186	1, 2678		0.666	<0.001	+1.733e-04	11,610	0.051 (0.049)	
NPP June	21.633	1, 2677		<0.001	0.008	-0.048	11,590	0.059 (0.056)	
(NPP June)²	21.821	1, 2677		<0.001	0.008	+3.117e-5			

Appendix 4.III., *continued.*

Models and variables	F	d.f.		P	Partial Eta²	Parameter	Model AIC	Model R^2 (adjusted R^2)	Years
(d) Environmental variable: Sardine abundance or biomass + wing length, date, date², time of night.									
Juvenile Sardine abundance	6.868	1, 1958		0.009	0.003	-4.03e-07	8,597	0.040 (0.038)	1995-2002
Juvenile Sardine abundance	12.376	1, 1957		<0.001	0.006	+2.078e-6	8,580	0.049 (0.046)	
(Juvenile Sardine abundance)²	18.920	1, 1957		<0.001	0.010	-2.660e-12			
Adult Sardine abundance	51.816	1, 1958		<0.001	0.026	-5.10e-07	8,553	0.061 (0.059)	1995-2002
Adult Sardine abundance	40.393	1, 1957		<0.001	0.020	-1.960e-6	8,580	0.072 (0.070)	
(Adult Sardine abundance)²	23.228	1, 1957		<0.001	0.012	4.532e-13			
Juvenile + adult Sardine biomass	**40.689**	**1, 2622**		**<0.001**	**0.015**	**-8.854e-03**	**11,370**	**0.061 (0.060)**	**1995-2005**
Juvenile + adult Sardine biomass	**44.656**	**1, 2621**		**<0.001**	**0.017**	**-0.051**	**11,340**	**0.073 (0.070)**	
(Juvenile + adult Sardine biomass)²	**31.604**	**1, 2621**		**<0.001**	**0.012**	**<0.001**			

Chapter 5

General Discussion

5.1 Overview

The work presented in this thesis has provided novel insights into the migration strategy, diet and foraging ecology of the European Storm Petrel *Hydrobates pelagicus*, one of the world's smallest seabird species. Despite its small size, the European Storm Petrel (henceforth "Storm Petrel") is remarkably long-lived (longevity is regularly in excess of 20 years, with the oldest known individual exceeding 38 years, M. Bolton, pers. comm.). Storm Petrels are generally thought to spend most of their life on the open ocean, coming onshore only to breed. They breed on mainly small, rat-free, islands in the north Atlantic with colonies located from Norway and Iceland in the north of the breeding range, to the Canary Islands in the south (Cramp & Simmons 1977, Brooke 2004). When the nestlings fledge, they are assumed to undertake their first migration to the southern hemisphere and spend their first year in the south Atlantic, off the coast of South Africa (Cramp & Simmons 1977, Wernham *et al.* 2002). The birds sampled in this study in SW Portugal were pre-breeders (aged 2-5 years old; Bolton & Thomas 2001, Wernham *et al.* 2002) undertaking their northwards migration to prospect colonies for future breeding attempts. Over the course of the subsequent annual cycles, Storm Petrels complete this long-distance migration from the breeding colonies in the NE Atlantic, to wintering areas in the southern hemisphere (Wernham *et al.* 2002). The extreme nature of the Storm Petrel's biology makes it an excellent case-study for examining the impacts of climate variation on migration behaviour and foraging ecology, which is the central theme of this thesis.

A range of approaches was applied in this research, to describe and understand the mechanisms underlying the impacts of climate variability on such a diminutive and distinctive species. Evidence was found for sex-differences in migration behaviour, opportunistic (non-specialist) feeding, including prey of inshore and even terrestrial origin, temporal variation in diet, and the strategic regulation of energy reserves in response to varying environmental conditions, as a buffer against starvation during migration. This study is one of few to look in detail at the ecology of migrating non-breeding seabirds; a class of birds that is usually not easily accessible to researchers. This is also one of the few studies to look at the relationship between climate change and the behaviour of individual birds. Such behavioural responses to changing environments may be important mechanisms by which the effects of climate variation may manifest themselves at a population level, leading to the observed widespread changes in avian distribution, phenology, demographics, breeding success and population size (reviewed by Stenseth *et al.* 2004, Crick 2004).

These analyses have linked field biology, molecular ecology and the analysis of long-term datasets (bird-ringing, fisheries and remote-sensing datasets). Each of these approaches can potentially be developed much further than has been possible within the scope of a time-limited PhD project. However, this thesis illustrates the value of an integrated approach to studying seabird behaviour and ecology, by combining several normally distinct areas of research to obtain novel insights. This final Chapter reviews the progress achieved in each area and highlight priorities and opportunities for further developing this research.

5.2 Sex-Specific Migration Behaviour of Storm Petrels

Storm Petrels are sexually monomorphic, so very little has previously been known about sex differences in their migration behaviour. Molecular sexing methods now allow accurate and relatively non-invasive sexing of birds from routinely collected field samples (feathers and faeces). In Chapter 2, this method was refined and applied to Storm Petrels, revealing an unexpectedly strong female bias in the sex ratio of pre-breeding Storm Petrels attracted to tape-lures during their northwards migration past SW Portugal. This sex bias was remarkably consistent across seven years, ranging from 80.8% to 89.7% female (mean annual sex ratio ±SD = 85.5% female ±4.1%).

While the initial aim in sexing the birds was primarily to study differences between sexes in the birds' diet and body reserves (see below), the discovery of the strong female bias in the sampled population raised new questions about sex-specific migration behaviour. No definitive explanation for the sex bias is yet available, but hypotheses include a different distribution of the two sexes at sea during migration, sex-differences in the seasonal timing of migration, or differences in the willingness to explore potential colonies as far south as Southern Iberia. Testing these hypotheses could involve catching Storm Petrels at other times of year (e.g. in April and July, to test whether males are migrating much earlier or later than females) or in other locations (e.g. at sea off the Portuguese coast, to test whether males are migrating further off shore than females). Ultimately, remote-tracking devices may become small enough to be fitted to Storm Petrels of each sex, revealing much more detail about sex-differences in migration and behaviour at sea.

There was a slight tendency for male and female Storm Petrels to be captured in sex-specific aggregations, suggesting that there may be some segregation of the

sexes at sea. Although some comparisons could be made between the sexes in terms of diet (Chapter 3) and the regulation of body reserves (Chapter 4) between males and females, no substantial sex-differences in foraging ecology were found. However, the small sample size for males restricted the power of some of these analyses, and the continued use of molecular sexing to build up the sample size of male birds will soon allow more powerful comparisons of diet, foraging ecology and migration fuelling strategies between male and female Storm Petrels sampled during their migration past the Portuguese coast.

5.3 Molecular Investigations of Storm Petrel Diet and Foraging Ecology

A detailed understanding of an animal's diet is fundamentally important for understanding its ecological requirements (and hence its conservation needs), its functional role in an ecosystem, and its potential as a biological indicator of environmental change. For many organisms, such as small pelagic seabirds, there are major practical, logistical or ethical obstacles to studying diet in the field (Barrett *et al.* 2007). As a result, the diet of Storm Petrels is largely unknown, particularly outside the breeding season, due to the lack of an appropriate method to study it in detail. Stable isotope analysis and fatty acid analysis are increasingly widely applied to the study of avian diet, but these approaches are limited in the degree of taxonomic resolution that can be achieved, particularly for studying the diet of generalist foragers whose diet may be composed of a large number of prey taxa, originating from a wide range of different habitats and trophic levels (Bond & Jones 2009). The emerging field of molecular scatology (extraction and identification of DNA of food taxa from a forager's faeces) provides a potentially powerful toolkit for the non-invasive investigation of diet in free-living animals (Symondson 2002, King

et al. 2008, Lerner & Fleischer 2010). Chapter 3 describes the refinement and application of methods for molecular scatology in the context of Storm Petrel diet and foraging ecology.

Two complementary molecular approaches were used: 1) using taxon-specific primers to screen for the presence / absence of particular prey categories in individual faecal samples; and 2) amplifying prey DNA from a pool of samples using general primers, then using cloning and sequencing of the amplified sequences to identify the taxa present in the diet in each year. Each of these methods has its advantages and limitations, but together, particularly in combination with analysis of carbon and nitrogen stable isotope signatures from growing feathers, they can provide a comprehensive account of diet, from identification of individual prey taxa right down to the level of subspecies (cloning and sequencing), through semi-quantitative assessments of the occurrence of key prey taxa in the diet at a population level (screening with taxon-specific primers), to an overall assessment of the location and trophic level at which the storm petrels had fed over larger spatial and temporal scales, prior to their capture on the Portuguese coast.

This study identified European Sardine (*Sardina pilchardus*) as a major prey species eaten by Storm Petrels at this stage of their migration. This information was important in informing the parallel investigation of the strategic response of migrating Storm Petrels to fluctuations in their foraging environment, presented in Chapter 4. Other notable results from this part of the analysis included the regular occurrence of prey DNA from terrestrial invertebrates (which are likely to be blown out to sea and taken from the sea surface by the foraging petrels), as well as fish from deep in the water column (which would normally be inaccessible to foraging Storm Petrels, but may be brought to the sea surface by human fisheries. These results

provide abundant detail about the foraging ecology of Storm Petrels, and suggest that they are overall rather generalist foragers, exhibiting substantial variation in their diet among years, perhaps in response to changes in the availability of different prey taxa.

In addition to revealing in detail aspects of diet and foraging ecology of species that are otherwise difficult to study, molecular methods for the identification of prey DNA in predator gut contents are of great relevance to conservation organisations seeking to manage adequate food supplies for taxa of conservation concern. Future developments to enhance the value of this approach for ecologists and conservationists is the use of next-generation sequencing technologies, such as pyrosequencing, to scale up the capacity and hence greatly increase the level of detail and the quantitative analysis of dietary data that can be achieved.

5.4 Responses to Climate-Driven Changes in the Foraging Environment

Since the early 1990s, a vast number of studies have been published describing associations between climate variables and ecological changes. Bird studies have been prominent in this rapidly developing field, with many studies describing climate-linked changes in the timing of migration, timing of breeding, changes to breeding or wintering ranges, and population changes (reviewed in Møller *et al.* 2004a). However, most studies described observed patterns on birds' response to climate change but fail to provide the underlying mechanisms driving those patterns (Stenseth *et al.* 2004). Furthermore, most studies are restricted to the breeding period and breeding area, but changes in climate are likely to have different impacts at different stages of the birds' annual cycle (Møller *et al.* 2004b). Studies of bird migration in the context of climate change have typically been constrained to describe patterns in terms of migration timing, mainly timing of arrival at the

breeding areas (Møller *et al.* 2004a). In the present study, changes in the abundance of food supply, in particular, the abundance of an identified potential key prey species, the European Sardine, has been identified as one of the mechanisms driving the observed pattern of changes in European Storm Petrels' body mass during migration. The birds were heavier in years when the sea was colder and food abundance was lower, suggesting strategic foraging behaviour to increase the body reserves and buffering against starvation in years when food resources were less predictable, similar to the responses of small terrestrial birds to energetic stress over the winter or during the night (Cuthill and Houston 1997)

The migration ecology of the Storm Petrel represents an extreme case-study for examining the impacts of climate change on a migratory seabird. This species is the smallest of the Atlantic seabirds with an average body mass of only 26g, potentially making it particularly susceptible to climate-driven changes in the marine environment. The long-distance migration undertaken by these birds, spanning a large part of the western hemisphere, potentially makes Storm Petrels susceptible to environmental changes across the Atlantic latitudes from the breeding colonies in NW Europe, to the wintering grounds off southern Africa. Migratory species inhabit widely separated locations over the course of their annual cycle, and are therefore exposed to a range of different climatic patterns that can themselves have differential ecological impacts. Changes in climate, manifested as variations in sea surface temperatures, are not constant across the globe and, even the same climate patterns might have different ecological impacts in different areas of the globe. Behrenfeld *et al.* (2006) showed that between 1999 and 2004 all four combinations of changes in SST and NPP occurred in different areas of the globe (increasing SST with increasing NPP, increasing SST with decreasing NPP, decreasing SST with

increasing NPP and decreasing SST with decreasing NPP). During this period, there was an overall decrease in SST and increase in NPP for the south Atlantic and the inverse patterns for the North Atlantic (Behrenfeld *et al.* 2006). There is however a marine area off the Iberian coast where the observed changes between 1999 and 2004 were of an increase in both SST and NPP (Behrenfeld *et al.* 2006), which is in accordance with the data presented in Chapter 4.

Despite the behavioural flexibility of Storm Petrels in regulating their own body reserves according to environmental conditions during migration, during the breeding season the birds are limited to foraging in the proximity of the colony (particularly when feeding nestlings) which might contrain their ability to cope with such changes (Weimerskirch 1998, Quillfeldt 2001, Pinaud and Weimerskirch 2002). If the trend described in Behrenfeld *et al.* (2006) of increasing temperatures and decreasing marine productivity in the north Atlantic (where most of the Storm Petrel colonies are located) continues in the future, as suggested by many climatic models (IPCC 2007), negative impacts on breeding productivity and population size might become a serious problem for the European Storm Petrel.

5.5 Conclusions

The field of climate change biology has developed rapidly over the past 20 years, in which the biological impacts of climate change have become one of the central issues in the study of ecology as well as of great concern in society as a whole. Several authors (e.g. Møller *et al.* 2004b, Stenseth et al. 2004, Crick 2004) identified priority areas for research to address issues arising from the increasing volume of studies into how birds respond to changing climate. This PhD research has focused on investigating several of these priorities, using the European Storm Petrel as a major

case-study. Table 5.I summarises a number of key ways in which this thesis has addressed these priorities.

Typically, in addressing such problems, many new questions are raised, illustrating the complexity of the marine ecosystem, of the climate that drives ecological changes, and of the individual behavioural decisions, such as what to eat and how much to eat. These behavioural decisions constitute a key set of mechanisms by which animals may respond effectively to changing environments, potentially enabling them to track even rapid directional changes in ecosystems. Such behavioural plasticity may itself provide some of the phenotypic variation on which selection can act, in turn leading to micro-evolutionary change. Nevertheless, there may be limits to the extent that behavioural plasticity may facilitate adaptation to rapid climate change, and continued monitoring of Storm Petrel food resources, diet, foraging ecology and migration fuelling decisions will be used as an ongoing case-study of the ecological impacts of climate change.

Table 5.I. A selection of Moller et al.'s (2004b) list of areas of research where further investigation of the effects of climate change on birds may be particularly rewarding, together with a brief summary of how the present thesis addresses the highlighted problems.

Problem	**Studies required**	**Contribution of the present thesis**
Geographical distribution of studies	Studies from other regions than northern temperate zones	Study area of Portugal-N Africa spans the temperate-sub-tropical boundary
Taxonomic distribution of studies	Studies of orders other than passerines	Storm Petrels are passerine-sized Procellariiformes
Spatial scale of weather conditions	The relative role of local and global weather systems	Direct comparison of SST and NAO effects on Storm Petrel fuelling decisions
Scientific approach	More experiments are needed	Use of novel methodologies to understand mechanisms underlying associations
Interspecific interactions	Changing impact of predators and parasites	Strategic responses to changes in prey availability
Effects of climate change on phenotypic plasticity	Degree of phenotypic plasticity under different environmental conditions	Upper and lower limits to fuel load
Trait-specific responses to climate change	Which traits respond to climate change and why?	Migratory fuelling and prey choice are behavioural traits mediating responses to climate change
Complex annual cycles	Relative role of environmental conditions during breeding, migration and wintering for adaptation	Focus on environmental conditions along the migration route
Heterogeneity in responses to climate change	Age and sex differences in response to climate change	Tested for sex-differences in migration timing, migratory fuelling and diet

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