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Trophic Relationships among Pelagic Predators of the deep seas of the Madeira Islands

By

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"*O valor das coisas não está no tempo que elas duram, mas na intensidade com que acontecem. Por isso existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis*"

"*The value of things is not the time they last, but the intensity with which they occur. That is why there are unforgettable moments and unique people!*"

Fernando Pessoa

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Trophic Relationships among Pelagic Predators of the deep seas of the Madeira Islands

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Preface

The recent development of molecular technologies is improving our knowledge of predator-prey community interactions in many fields, such as agricultural pest control, plant-pollination webs and species extinctions. Seabirds are widely studied animals and are believed to be important models of the pelagic ecosystems they feed within. These animals occupy top-positions in marine food webs and play potentially an important role in top-down ecosystem regulation. Seabirds are also extraordinary animals that form colonies of thousands of individuals, often on remote islands. Under these conditions, seabirds compete for space but also for resources in order to coexist and for successful reproduction. Dietary analysis of seabirds thus provides unique ways to gain insights into marine ecosystem functioning and predator interactions, while providing valuable information for the conservation and protection of these animals.

This thesis contains six chapters. The first chapter is a general introduction, where I review the importance of dietary studies to community ecology and focus on current molecular technologies and methodological approaches for dietary analysis of predators. In this chapter I also include a brief section describing the study sites and the ecology of the study species. I outline our current knowledge regarding predator-prey interactions of procellariiformes and some important aspects of their prey.

Chapter 2 is the result of a collaborative study of the diet of Cory's shearwater, published in Molecular Ecology. In this chapter, a combined approach using morphological and molecular analyses was used to discriminate prey in Cory's shearwater. I did all the molecular parts of this study, including writing of relevant sections, all laboratory procedures and molecular identification of the prey.

Chapter 3 focuses on the problems associated with the identification of unknown species in metabarcoding studies, particularly of short barcodes retrieved in high-throughput sequencing studies. The chapter provides a set of new methodological approaches including different methods for identification, of wide applicability, that maximize confidence in taxonomical assignment of vertebrate and invertebrate prey. The chapter was submitted for publication.

Chapter 4 examined the predator-prey relationships of three sympatric small petrels using the approaches developed in chapter 3 to identify prey from non-invasive faecal remains. The chapter focuses on trophic partitioning between the study species and further provides an extensive list of the prey taxa identified around the Selvagens islands.

Chapter 5 relates the diet of a predator of mesopelagic prey with the moon cycle and investigates whether prey species composition and diversity are affected by nocturnal light, as assumed previously.

Chapter 6 is a general discussion, where I discuss the principal conclusions of each chapter and refer to important methodological constraints and future directions regarding molecular dietary analysis of marine predators. I finish with some general thoughts on the importance and the need to develop this field in ecology.

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To my husband, it is difficult to find words that can express how grateful I am for everything you have done for me. Without you nothing of this would have ever been possible. Thank you so much for always wanting everything that is best for me, even when it is sometimes not the best for us. I thank you so much and I am so happy that I have you in my life.

Table of Contents

Summary

This thesis provides a detailed study of the diet of various procellariiformes using new molecular approaches. Dietary studies remove fundamental blocks to our understanding of the structure of food webs, and provide insights into the demographic regulation of populations and the structuring of communities. The study species were the Band-rumped Storm-petrel (*Hydrobates castro*), Bulwer's petrel (*Bulweria bulwerii*), Cory's shearwater (*Calonectris borealis*) and White-faced Storm-petrel (*Pelagodroma marina*). The breeding colonies of the Madeiran-archipelago are Important Bird Areas (IBA) in the North-Atlantic, but little is known about the predator-prey relationships of its seabird populations. This probably relates to difficulties associated with obtaining robust prey estimates and the need to develop new methodologies to improving the resolution of species identification. Here, new molecular approaches were developed to recover prey from faeces and stomach contents using DNA-barcoding and high-throughput sequencing (HTS). The results obtained show clear improvements to the identification of the diets of procellariiformes, considerably outperforming morphological analysis, and retrieving prey identities from non-invasive faecal remains. Such approaches further showed that sympatric small seabirds of the sub-tropical NE-Atlantic significantly segregated their resources, while showing similar prey types with the species distributed in the Pacific, indicating that these petrels maintain foraging specialization across their distribution range. Foraging efficiency in seabirds has been widely hypothesized to change according to the moon cycle. Predators either optimise foraging during moonlit nights or reduce foraging effort because less accessible prey migrate downward the water column to avoid visual predators. I tested whether prey composition and diversity differ between moon-phases. However, I found no evidence for a significant influence of the moon on the diet of Bulwer's petrel, contradicting previous ecological assumptions. The results highlight the potential of DNA methodologies to the understanding of marine food webs and predator-prey relationships and will certainly make important contributions to marine community ecology.

CHAPTER 1:

General Introduction

General Introduction

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Inter- and intra-specific interactions within a community drive evolutionary processes of speciation and diversity. To understand current diversity it is important to ask ourselves the following questions: How do species interact in a community and what are the underlying mechanisms of species coexistence? How are communities regulated and how do they respond to environmental cues and changes?
Perhaps the most intuitive approach to answering these questions is to understand the trophic relationships between organisms.

Since the earliest dietary studies this field has grown from simple theoretical models of predator-prey interactions (Lotka-Volterra equations) to current dynamic food webs models (Williams & Martinez 2000). Methods used to describe the diet of animals have also evolved dramatically. Techniques have ranged from early morphological studies of prey remains to isotopic analysis of tissue and blood (Hobson et al. 1994), and from taxon specific molecular approaches (Symondson et al. 1993) to current metabarcoding techniques using high-throughput sequencing technologies (Taberlet et al. 2012).

Of all top-predators studied so far, seabirds are one of the most studied animals in the field of trophic ecology. This relates to some extent to seabirds life traits, but most importantly with their potential role in regulating ecosystem top-down processes and interactions with fisheries. Seabirds often form large colonies of thousands of individuals that assemble to reproduce (Coulson 2002). This enables researchers to collect huge amount of dietary data in a comparatively short time. Seabirds are also estimated to consume ca 70 million tonnes of marine biomass (Brooke 2004), approaching the total catch of marine fisheries (Karpouzi et al. 2007). High mortalities in seabirds, for example albatrosses, are thought to result from interactions during fishering operations, with birds commonly trapped in nets or on long lines (Inchausti & Weimerskirch 2001).

Dietary studies in seabirds have been classically conducted through morphological inspection of prey remains (Duffy & Jackson 1986). However, morphological analysis of stomach contents has known biases resulting from the prey being more or less susceptible to digestion. Stable isotope analysis of prey remains, in

predator blood, has provided an alternative approach to study the diet of seabirds. This technique has contributed a lot to our knowledge of the trophic positions of predators, providing information on the foraging habitats of seabirds (e.g. coastal vs. oceanic). However, stable isotope analysis has low resolution to discriminate between prey species in the diet of seabirds.

Molecular dietary analysis provides a more effective alternative to morphological analysis of prey. This analysis uses the information contained in DNA to identify prey. It is based on the principle that short fragments of DNA are sufficiently variable to distinguish species (Hebert et al. 2003a; b). As such, DNA-based analysis is currently the technique with the highest taxonomical resolution for dietary assessments of predators. Despite its potential, and the emerging high-throughput sequencing technologies that allow us obtaining millions of sequences from a single sample, this approach has rarely been applied to the diet of seabirds (Bowser et al. 2013; Jarman et al. 2013).

In this thesis, I studied the diet of various petrel species using two different molecular approaches. In one I performed standard DNA barcoding on individual prey items collected from the stomach contents of Bulwer's petrels. In the other less invasive approach I used current high-throughput sequencing technologies to discriminate taxa from the faeces of three sympatric species breeding in the northeastern Atlantic: Bulwer's petrels (*Bulweria bulwerii*), Band-rumped storm petrel (*Hydrobates castro*), Cory's shearwater (Calonectris borealis) and White-faced storm petrel (*Pelagodroma marina*). In this general introduction, I mainly focus on the two most important components of the thesis: (1) molecular dietary analysis, by presenting a brief review on the methods used, including recent high-throughput technologies and how they improve dietary assessment in seabirds and (2) the study species and sites, where I review their biology and ecology. I further outline some important aspects of their foraging ecology, including a review of trophic partitioning and mesopelagic prey. At the end of this introduction, I outline the principle objectives of this thesis and the hypotheses.

1.1 Molecular dietary analysis

Molecular dietary analysis is improving dramatically our knowledge of predator-prey interactions in both terrestrial and marine ecosystems.

Dietary studies in seabirds have been historically conducted through morphological analysis of prey remains in predator's stomach contents. This approach relies, generally, on visual inspection of hard-part remains that are less affected by digestion and that possess distinctive characters to discriminate species identities (Duffy & Jackson 1986). For example, the otoliths and vertebra of fish, the beaks of cephalopods or the exoskeletons of crustaceans resist digestion and are easily recovered from the stomach contents of seabirds. These structures sometimes possess unique shapes that vary between species and that can be used for taxonomical assignments (Clarke et al. 1986; Sadighzadeh et al. 2012). Stomach content analysis in earlier studies involved scarifying animals to collect the contents. This has, however, raised ethical concern so that currently stomach contents are collected through stomach flushing procedures (Wilson 1984) or are collected from spontaneous regurgitations. Although morphological stomach content analysis stands to these days as the most widely-used methods to describe the prey types of seabirds in detail, this technique is quiet invasive for the birds. Moreover, there are important inherent biases that can affect ecological hypotheses and conclusions. It has been shown that hard-structures are recovered differentially in stomach content. For example, cephalopod beaks are known to significantly accumulate in the stomach contents of chicks, while substantially different fish estimates are obtained depending whether vertebra or otoliths are analyzed (Xavier et al. 2011; Alonso et al. 2013). Often, diagnostic characters cannot be identified to species, and a high level of expertise and comprehensive reference collections are needed for robust taxonomic assignments.

Stable isotope analysis (SIA) has been applied as an alternative methodology to assess predators diet (Hobson et al. 1994). SIA involves measuring the traces of $\delta^{15}N$ and $\delta^{13}C$ in the blood of predators to determine their trophic positions. This technique relies on the assumption that the amounts of $\delta^{15}N$ in animal's tissue correlate with a stepwise accumulation of this compound in food webs. Seabirds that consume predacious fish have, therefore, higher signatures of $\delta^{15}N$ than, for example, planktivorous seabirds. The accumulation of stable $\delta^{13}C$ is less than that of $\delta^{15}N$, but provides information on the marine habitats seabirds were foraging on, e.g. inshore versus pelagic offshore systems (Sydeman et al. 1997). While stable isotope analysis allows us to infer the trophic positions of predators and foraging grounds without the known biases found with stomach content analysis, this technique has a very low

taxonomic resolution for identification of the prey species of seabirds (Jacob et al. 2005).

Molecular techniques applied to dietary analysis of predators bypass these problems. This field has grown dramatically the last two decades ago, from initial species-specific approaches in which predators are screened for certain prey types (Symondson & Liddell 1993) to current high-throughput sequencing techniques (HTS) that allow identifying multiple unknown organisms from a single sample (Taberlet et al. 2012).

Currently, one of the most widely used molecular approachs to determining the diet of predators is based on the principle of DNA barcoding (Hebert et al. 2003a;b). This technique involves DNA isolation, amplification of a universal barcode through Polymerase Chain Reactions (PCR) and finally, taxonomical assignments of unknown sequences. The principle aim of DNA barcoding is to provide a universal approach for species identifications without the need for taxonomic expertise. A major advantage of the DNA barcoding initiative is the implementation of a comprehensive public database of sequences - the Barcode of Life Database (BOLD) so that unknown specimens can be compared with references collected from almost everywhere in the world.
As seabirds often form colonies on remote islands with very little access it is not always feasible to build our own reference collections. Molecular dietary analysis, through DNA barcoding, represents therefore unique opportunities to accurately identify prey in these top-predators.

Molecular dietary analysis of seabirds can be applied in two different ways, through DNA barcoding: (1) sensu stricto or (2) sensu lato (reviewed in Valentini et al. 2009). Ecologist can, for example, isolate individual pieces of soft tissue or vertebra from the stomach contents of seabirds and perform standard DNA barcoding. In this case a DNA sequence is retrieved for each of the isolated items. With recent advances of HTS technologies, ecologists can alternatively take a mixture of the stomach content and identify all the prey therein contained through metabarcoding. In the latter, millions of DNA sequences are obtained from a single sample (Taberlet et al. 2012).

1.2 Metabarcoding for dietary analysis

The concept of DNA metabarcoding is founded on studies intending to describe ecological communities and assess biodiversity (Fonseca et al. 2010; Yu et al. 2012). The principle difference with the classical DNA barcoding approaches is that it allows multiple species to be identified from a single sample through the development of high-throughput sequencing (HTS) technologies. The samples used in metabarcoding, typically contain mixtures of DNA from target organisms but also DNA from external sources (human DNA, bacteria, etc). The DNA in environmental samples is usually degraded and is commonly referred as eDNA.

Current HTS systems generate millions of sequences in a single run (e.g. Roche/454 GS FLX, Illumina/Solexa Genome Analyzer, Life Technologies/Ion Torrent™). This means, in principle, that even the most degraded DNA molecules can be positively identified in a sample. This represents a huge step towards comprehensive dietary analysis of predators, where detailed information on predator-prey relationships has been until now limited by the existing methodologies. It also opens ways to assess the diet from non-invasively obtained predator remains such as faeces. Although for a few birds it has been possible to recover recognizable prey remains within faeces, these samples are generally considered non-informative for dietary assessments of seabirds (reviewed in Barrett et al. 2007). The inclusion of faeces for dietary assessment of seabirds is of enormous advantage, as stomach-flushing procedures are still invasive, laborious and stressful to the birds. Moreover, contrary to the standard barcoding approach, where a Sanger sequence is obtained for each item collected (e.g. in stomach contents), metabarcoding can be applied at a whole community level as hundreds of faecal samples can be obtained non-invasively and multiplexed within a single HTS run. Despite its potential for dietary studies, there are important drawbacks to the use of metabarcoding approaches on faecal remains, especially in the diet of seabirds. The principal technical constraint relates to the type of barcode used. The DNA molecules of prey in faeces are extremely degraded. Small DNA fragments are easier recovered via PCR from faeces than longer fragments (Deagle et al. 2010). As such, metabarcoding uses substantial shorter barcodes than the standard COI fragment of Folmer et al. (1994). A major problem in such metabarcoding studies is therefore whether it is

possible to find primers that will amplify fragments short enough to survive digestion in a predator yet taxonomically informative enough for species identification.

1.3 Molecular markers

Choosing the appropriate barcode is crucial for successful recovery of DNA in faeces and taxonomic assignments of unknown specimens. The most common markers used in HTS studies of vertebrate and invertebrate predators are short fragments of the Cytochrome Oxidase subunit I gene (COI)(Hajibabaei et al. 2006; Zeale et al. 2011) and the mitochondrial 16S rRNA (Palumbi 1996, Deagle et al. 2010. It is important to note that each barcode has its limitations and that barcodes should be chosen provide the best coverage for the study system.

The following criteria are usually applied for metabarcoding of eDNA:

- Length: shorter barcodes are recovered more successfully from degraded biological material than longer fragments (Zaidi et al. 1999).

- Phylogenetic signal: While the fragment has to be short, it also has to have sufficient variable sites to allow discrimination between species.

- Primer universality: Although variable barcodes are important for successful taxonomical discrimination, barcodes also need to have conserved flanking regions to design primers that will amplify a wide range of different taxa.

- Taxonomic coverage: To taxonomically assign unknown sequences, comprehensive references libraries have to be available for that barcode.

High-throughput sequencing has been extensively applied in dietary analysis of insectivorous vertebrates and invertebrates (Clare et al. 2014; Brown et al. 2014; Pinol et al. 2014). In fact, short barcodes targeting the COI have improved prey detection from faeces and guts of insectivores showing reasonably robust prey assignments in countries where extensive barcoding has taken place (e.g. Clare et al. 2014, King et al.

2015). However, the same might not hold for seabirds consuming highly diverse groups of prey such as fish, cephalopods and crustaceans.

Sutherland (2000) was the first author to perform dietary analysis on faecal remains from birds, recovering short ribosomal (12 rRNA) DNA fragments. Deagle et al. (2007; 2010) studied for the first time the diet of seabirds from faeces. The authors optimised different sets of primers amplifying short mitochondrial 16S rRNA barcodes of a wide range of prey. These barcodes were further successfully applied in high-throughput sequencing of other seabirds (Bowser et al. 2013). Deagle et al. (2007) referred to the fact that the COI of Folmer et al. (1994) was too variable to find conserved primer-binding sites for amplifying unbiasedly short barcodes among different orders of fish and cephalopods. This was also demonstrated in Deagle et al. (2014) comparing in silico the performances of short COI and ribosomal barcodes for metabarcoding.

Of the ribosomal genes, the 16S rRNA is a preferred candidate for molecular dietary analysis of seabird diets. It possesses hypervariable regions with sufficient phylogenetic signal to discriminate taxa, but also conserved regions flanking the former (Stiegler et al. 1981), where conserved primers can be designed. Because of these characteristics, the 16S rRNA has become an important barcode for phylogenetic reconstructions of vertebrate and invertebrate groups, becoming one of the most represented markers in the Genbank database. For some marine taxa, such as cephalopods, the sequence coverage is even higher than for COI (assessed February 2015).

1.5 Algorithms for prey descrimination in metabarcoding

Two major methods for species identification have been applied in dietary metabarcoding studies: sequence similarity thresholds (OTU-picking) or more complex evolutionary models. OTU-picking methods assign sequences to clusters based on user defined similarity thresholds. A representative sequence of each cluster (MOTU) can be than compared to a reference database (GenBank or BOLD), whereas MOTUs that do not match any references are putative new species. This methodology has been extensively applied in dietary metabarcoding studies of vertebrates and invertebrates

and also birds (Bowser et al. 2013; Jarman et al. 2013; Clare et al. 2014). Though OTU-picking methods perform relatively well on large HTS datasets, its accuracy for species delimitation is highly sensitive to PCR artifacts (e.g. chimeras) and sequencing errors, which occur frequently in current HTS platforms (Quince et al. 2009). Another important limitation of OTU-picking methods relates with the fact that it remains unclear whether a MOTU corresponds to a species, particularly when species are inferred from metabarcodes for which inter-specific thresholds have not been comprehensively tested.

Phylogenetic placement methods, contrary to OTU-picking, inform on the evolutionary relationships of a large collection of queries on a known phylogeny and are, therefore, not limited to a taxonomic identity. Such methods use a backbone alignment of full-length sequences, a backbone tree inferred from the full-length alignment (e.g. 16Sar/16Sbr, Palumbi 1994), and a collection of short query sequences that are assigned to branches of the backbone tree. Within these methods, the Evolutionary placement algorithm (EPA) (Berger et al. 2011) has shown high accuracy and best performance compared to other methods. EPA assigns queries on a reference tree based on Maximum likelihood. It starts optimising the branch lengths and other Maximum likelihood parameters (part of the ML model) on the reference tree. Each query is then inserted idependently on the reference tree and the insertion likelihood computed after optimizing the lengths of adjacent edges. Such query assignments bear parallelization in mind and circumvent computational problems associated with inferring a comprehensive phylogenetic tree simultaneously for reference sequences and queries in metabarcoding studies. Moreover, the short length of queries that presents a major problem for re-constructing stable phylogenies is improved in phylogentic placement methods as these short sequences are compared with full-length reference sequences.

1.5 Study sites

The study sites were the Selvagem Grande (30°09’N, 15°52’W) and the Deserta Grande (32°30’N 16°30' W) islands (Figure 1.1), located in the subtropical northeastern Atlantic. Both islands are part of the autonomous region of Madeira (Portugal). Due to their location, species colonization processes and bird populations, both islands are

protected nature reserves, integrated in the European network NATURA 2000 as well as Important Bird Areas (IBA).

Deserta Grande is located southeast from Madeira at a distance of approximately 40 km. It is the largest and the highest of the Desertas islands, which consist of another two islands: Ilhéu Chão and Bugio. The Desertas islands are estimated to have originated through volcanic activity ca 3.6 MY ago (Geldmacher et al. 2000).

Selvagem Grande is the most important and the largest island of the Selvagens archipelago, consisting of another two smaller islands (Selvagem pequena and Ilhéu de Fora) and numerous associated reefs. The Selvagem archipelago is closer to the Canary islands than to Madeira, located approximately 300 km southward off Madeira and 160 km northward off the Canary islands. Selvagem Grande was estimated to originate through volcanic activity ca 27 MY ago (Geldmacher et al. 2001).

The study sites share floral and faunal affinities with other volcanic oceanic islands located in the northeastern Atlantic: Azores, Canary and Cape Verde - comprising together the biogeographically region of Macaronesia.

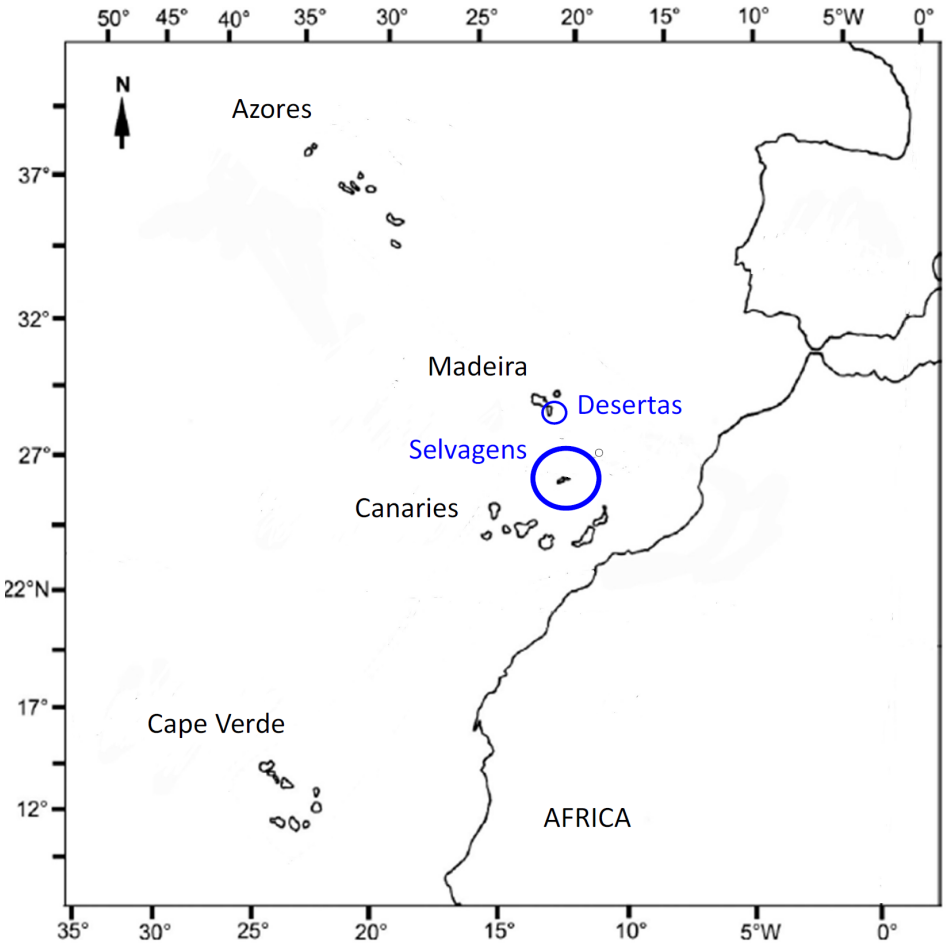

Figure 1.1. Study Sites: Desertas and Selvagens islands ate indicated in blue

1.6 Study species

The study species are three small seabirds of the order procellariiformes: Bulwer's petrels (*Bulweria bulwerii*, Jardine & Selby 1828), Band-rumperd Storm petrels (*Hydrobates castro*, Harcourt 1851; del Hoyo and Collar 2014), Corys´shearwater (*Calonectris borealis*, Cory 1881) and White-faced Storm petrel (*Pelagodroma marina*, Latham 1790) (Figure 1.2).

Figure 1.2. Study Species

1.6.1 Band-rumped Storm petrel

Band-rumped Storm petrels are members of the family Hydrobatidae. The species *Hydrobates castro* was formerly include in the genus Oceanodroma (Harcourt, 1851). In fact, the taxonomical classification of this genus has been widely studied, as genetically distinct breeding populations have been found throughout the world (Smith et al. 2007) The colonies of the Azores, for example, show evidence of sympatric speciation, where two distinct seasonal populations of Band-rumped Storm petrels are in fact two different species*: Hydrobates castro* and the endemic *Hydrobates monteroi* (Monteiro & Furness 1998; Bolton et al. 2008*). Hydrobates castro* is distributed throughout the subtropical regions of the eastern Atlantic and Pacific Oceans. The species is classified as "Least Concern" by the IUCN red list of threatened species

because of its high population numbers despite current trends of population decrease (Birdlife International 2013)

The breeding sites in the Atlantic are located in northwestern Portugal (Berlengas), the Macaronesia groups of islands and St Ascension in the subtropical southern Atlantic. At the Desertas islands two sympatric seasonal populations have been distinguished. The populations are termed "hot " and "cool" accordingly to its temporal breeding chronologies. The hot population starts laying eggs in June. The eggs begin to hatch at July and the chicks begin to fledge by September. The cold population starts its breeding cycle four months later in October. Nunes (2000) has shown that these populations show distinct phenologies (are generally larger), while a recent molecular study has found significant genetic differentiation between the populations (Smith et al. 2007).

The breeding chronology of *Hydrobates castro* at Selvagem is less studied. Previous investigations reported an extended breeding period (Mougin et al. 1990), rather than two separate populations. However the study of Smith et al. (2007) also distinguished genetically two seasonal populations on Selvagem Grande.

1.6.2 Bulwer's petrel

Bulwer's petrel is the smallest member of the family Procellariidae. This species is pan-tropical outside the breeding season, occurring throughout the world's oceans (Onley & Scofield 2007). The species is classified as of "Least Concern" under the IUCN red list of threatened species and its population trend is currently stable (BirdLife International 2012). Breeding colonies of Bulwer's petrel are found throughout the five Macaronesian archipelagos in the northeastern Atlantic and in many other islands of the Pacific Ocean. The breeding colonies of the Desertas and Selvagem Grande islands are the largest in the Atlantic. The estimated population size is of 10,000 pairs on these islands and corresponds to roughly 90% of the total populations breeding in the Atlantic Ocean (Nunes 2000).

The Breeding chronology of Bulwer's petrels is highly synchronous among populations of the Deserta Grande and Selvagem Grande islands. Egg laying starts in June and chicks start to hatch in middle of July. In September, chicks start to fledge (Nunes & Vincent 1998).

1.6.3 Cory's shearwater

Cory's shearwater *Calonectris borealis* belongs to the family Procellariidae. The breeding colonies of this shearwater are distributed throughout the Azores, Madeiran archipelago and Canary Island and Berlengas islands off the Portuguese coast. The population trend of Cory's shearwater is decreasing (BirdLife International 2014), but the species is listed as of Least Concern by the IUCN red list of threatened species because of its large population size (over 250,000 breeding pairs). The population of Selvagem Grande is the largest population of Cory's shearwater in the Atlantic (Granadeiro et al. 2006). This population showed a dramatic decline of ca 90% in 1970s caused by human interactions (culling by fisherman). Since then the colony has been protected by strict national policies and is recovering at an annual rate of ca 4-5%. By contrast the colonies of the Canary Islands and Azores are declining (BirdLife International 2014).

The population of Cory's shearwater breeding at Selvagem Grande presents remarkable attendance behavior. This population is unique in attending the colony during the day and present remarkable attendance cycles that might be related to possible social interactions caused by their high population densities (Granadeiro et al. 2009).

This species further presents dual foraging strategies during reproduction, where parents significantly shift between foraging grounds, feeding either on distant productive waters near the African coast or on oceanic waters surrounding the island to provision chicks.

1.6.4 White-faced Storm petrels

White-faced Storm petrel is the only member of the monotypic genus *Pelagodroma* belonging to the family Hydrobatidae. This species occurs throughout the temperate, subtropical and tropical Atlantic, Pacific and Indian Ocean. During the breeding season, White-faced Storm petrel forms numerous colonies in the southern hemisphere, occurring at remote oceanic islands such as Tristan de Cunha (St Helena)

and at coastal sites of Australia and New Zealand (BirdLife International 2014). The breeding populations of the northern Atlantic are considerably smaller and are essentially restricted to the Selvagens, Canary and Cape Verde islands (BirdLife International 2012).

White faced storm petrel shows a high geographic differentiation between breeding colonies, with a total of six subspecies described so far. P. m. hypoleuca (Webb, Berthelot & Moquin-Tandon 1842) is the only subspecies in the northern hemisphere and is endemic to the Selvagens and Canary islands. The global population of P. m. hypoleuca is almost entirely restricted to the Selvagens islands, with population estimates of 36,000 breeding pairs at Selvagem Grande (Campos & Granadeiro, 1999) and very few breeding pairs (50) in the Canary Islands (Rodríguez et al. 2003). P.m. hypoleuca is heavily predated by Yellow-legged Gulls at Selvagem Grande (Matias & Catry 2010), while its localized distribution implies conservation concern as this sub-species is probably vulnerable to extinction.

The breeding cycle of White-faced Storm petrel in the North Atlantic starts in the month of March, with the egg-laying phase lasting until the beginning of June. By mid-August all chicks have fledged (Campos & Granadeiro 1999).

1.7 Foraging methods

Bulwer's petrel, Band-rumped Storm petrel and White-faced Storm petrel are surface foragers, feeding on prey available at the ocean's surface. Cory's shearwater is a shallow diver, with maximum diving depths of ca 5 meters below the surface (Villard et al. 2011)

There are few records of the diving depths of the study species in the literature, with Bulwer's petrels diving the deepest, ca 2.4 m (Mougin & Mougin 2000). The diving depths of Band-rumped Storm petrels averaged 0.85 meters (Bried 2005). No record of the diving depths of White-faced Storm petrel is available. Clearly foraging depth can potentially affect the spectra of prey available to them.

1.8 Trophic partitioning in seabirds

Hutchinson (1957) conceptualized the ecological niche in an n-dimensional space of environmental variables, further distinguishing two spaces: the fundamental niche and the realized niche. The realized niche differs from the fundamental niche in that it represents the environmental space in which species coexist and interact with each other. Following Hutchinson's conceptualization of niche, Schroener (1974) proposed that species coexistence could be explained through trophic partitioning and defined three main dimensions in which species segregate resources: vertical and horizontal habitats, and prey type.

Evidence for trophic segregation among seabirds has been obtained across various communities of seabirds in polar, temperate and tropical regions of the Oceans. Seabirds are shown to segregate their resources depending on age, sex and among species in a community (Forero et al. 2005; Spear et al. 2007). Trophic segregation has shown to result from differences in the foraging grounds, prey types and sizes (Navarro et al. 2013, Spear et al. 2007, Ashmole 1968). The latter differ between seabirds depending on the time and foraging methods of seabirds (Miller et al. 2010).

Some of the most comprehensive studies to date describing partitioning by prey type among seabirds were conducted in the Pacific (Ashmole & Ashmole 1967; Harrison et al. 1983; Spear et al. 2007), as well as polar (Croxall et al. 1997; Ainley et al. 1992) and temperate regions (Pearson 1968; Ainley & Boekelheide 1990). The study of Spear et al. (2007) is the most comprehensive, addressing predator –prey relationships in thirty different species of procellariiformes in the Pacific. This study showed significant trophic partitioning of prey types. Studies on the predator-prey relationships of the sub-tropical Northern Atlantic are substantially fewer, and the most comprehensive studies addressing trophic partitioning have been assessed through stable isotope analysis (Young et al. 2010; Roscales et al. 2011)

The tropical open oceans are low productivity systems, characterized by unpredictable food resources that are patchily distributed (Balance et al. 1997; Weimerskirch 2007). Yet, the diet of seabirds in these systems is shown to be significantly more diverse than that of seabirds from temperate and polar regions (Spear et al. 2007; Croxall et al. 1997), which are generally more productive systems. This pattern also contrast with the foraging strategies of seabirds, as tropical species are proficient flyers and restricted to foraging on the sea surface (Balance et al. 1999). By

contrast, procellariiformes of high-latitudinal and polar region dive substantially more and have presumably more opportunities to take different prey types.

Although it is difficult to evaluate competitive pressures in natural systems, particularly due to the difficulties in obtaining comprehensive data on the prey availability, the existence of significant trophic partitioning and overlaps among seabird communities suggest that seabirds potentially compete for resources in many systems. Studying the diet composition of seabirds in a community is a fundamental step towards gaining insight into its foraging ecology and dynamics.

1.9 Mesopelagic organisms in the diet of seabirds

The term mesopelagic refers to organisms that are distributed at depths between 200 -1000 meters from the surface of the Oceans. Some mesopelagic groups such as myctophid fish are ubiquitous throughout the Worlds oceans, constituting ca 65% of the fish biomass in the pelagic deep seas (Hulley 1998). This fish are also main prey of many procellariiformes (Harrison et al. 1983; Spear et al. 2007).

To date, it is not entirely clear how pelagic birds that feed mainly on the sea surface can have such high contributions of mesopelagic organisms in their diets (Croxall & Prince 1994). One possible explanation is that birds are scavenging on dead floating remains at the sea surface (Croxall & Prince 1994). However, many of the prey identified have too dense tissues to float on the sea-surface so that this explanation has been rejected for some petrels (Croxall and Prince 1994).

In fact, oceanographic evidence points out that many mesopelagic organisms perform diel vertical migration (DVM). The most common pattern is a nocturnal DVM where organisms ascend to the surface at night and descent during the day. Patterns of DVM are believed to result from predator avoidance, where organisms can take advantage of enriched surface waters to feed, while less visible to predators (Zaret & Suffern 1976). Seabirds that forage on mesopelagic organisms are, therefore, thought to do so at night (Harrison & Seki 1987; Imber et al. 1992)

An important factor known to affect the distribution of mesopelagic organisms at the sea surface is the light intensity of the moon. During moonlit nights mesopelagic organisms are shown to remain in mesopelagic layers to avoid being eaten by surface predators (Clarke 1973; Kampa 1974; Benoit-Bird et al. 2009a,b).

Imber (1975) proposed that the foraging efficiency of seabirds varies with the moon cycle due to differences in prey availability. The foraging efficiency hypothesis has been proposed to explain some important aspects of the ecology of procellariiformes. For example, most petrels that attend colonies at night (ca 90% do so) generally arrive at later hours during moonlit nights, as a result of spending more time finding few available prey.

Despite the moon cycle potentially influencing the foraging success of seabirds and the enormous amount of dietary data collected in seabirds, few studies have actually investigated foraging across the moon cycle. The influence of the moon on the foraging of seabirds has been mainly inferred from activity patterns of seabirds at sea using GLS data loggers (Awkerman et al. 2005; Phalan et al. 2007; Yamamoto et al. 2008; Cruz et al. 2013; Dias et al. 2012). The dietary composition has not been investigated in relation to the moon phase.

1.10 Principal objectives

This thesis provides a detailed description of the range of prey species consumed by a community of petrels breeding in the northeastern Atlantic using novel molecular methodologies. These data are important to augment our knowledge of the trophic interactions of procellariiformes, as well as the influence of environmental clues, such as the moon cycle, on predator-prey relationships. The methodologies developed in thesis present new molecular approaches to the study of seabird diets and can be broadly applied to almost any marine predator.

The specific aims of this thesis were:

1. Augment taxonomic resolution of prey identified in stomach contents where previous morphological analysis failed to make positive identifications, and combine molecular and morphological identifications to assess the diet of Cory's shearwater (Chapter 2)
2. Develop a methodological approach, including new primers, two-step PCR amplification procedures, and application of recent phylogenetic-aware

algorithms, to improve detection and identification of prey from non-invasive faecal remains of three small petrels (Chapter 3, Chapter 4).

3. Provide detailed information on the predator-prey interactions of coexisting petrels and assess trophic segregation in small petrels breeding in the North Atlantic (Chapter 4)
4. Obtain a list of the small pelagic ichthyofauna in the waters surrounding the Madeiran archipelago, using data obtained through dietary analyses of petrels (Chapter 2, 4, 5)
5. Determine the effect of the moon cycle on the range of prey consumed by Bulwer's petrels (Chapter 5)

1.11 Hypothesis

The following main hypothesis were tested in this thesis:

A. *Phylogenetic approaches improve robustness in taxonomical assignments of short 16S rRNA queries as opposed to similarity-based approaches and can be applied to assess the diet of marine predators*

A crucial step of metabarcoding studies is the identification of unknown short barcode sequences. Recent studies point to a significant difference in diversity estimates depending on the approaches used for taxonomical assignments: 1. Similarity-based approaches with reference sequences, 2. Phylogenetic approaches that estimate the likelihood of insertion of queries on a reference tree.

Similarity-based approaches rely mostly on the similarity percentages of query sequences with reference sequences and use generally a specified percentage threshold (e.g. 2%) to distinguish species. This approach assumes that short barcodes differing by more than 2% are different taxa. Phylogenetic approaches differ substantially from the former, by calculating the probability of each query belonging to a specific branch in the reference tree. In this thesis, a short barcode of the 16S rRNA (Palumbi 1996) was recovered from the faeces of petrels. A percentage threshold separating species is not comprehensively available for this barcode. As such, a principal assumption in this thesis was that the similarity percentage of 16S rRNA barcodes differed substantially

depending on the taxonomic groups and that a threshold would be unlikely to accurately discriminate between species. Queries obtained upon studying the diet of Bulwer's petrels were identified based on the Evolutionary Placement Algorithms (EPA) (Berger et al. 2011) and Maximum likelihood reference trees. The similarity percentages of queries with the nearest neighbour in BLAST were obtained for comparison. This approach is addressed in Chapter 3.

B. *Sympatric petrels segregate their resource-use either as a dynamic response to limited resources or as a result of trophic specializations differing inter-specifically. In either case, trophic segregation will lead to a potential reduction of competition.*

Few studies have investigated trophic partitioning in coexisting seabird communities in tropical and sub-tropical regions, but the ones that did so often documented trophic segregation by habitat, prey type or sizes. These patterns are often attributed to potential mechanisms reducing competitive interactions. From studies conducted at different latitudes in nearshore and offshore systems, it is possible to understand that species show different segregation patterns. For example, at higher latitudes significant prey overlap has been observed among petrels. Significant overlap among birds has been also described in upwelling environments. Such patterns have been commonly attributed to the existence of dominant and superabundant taxa in these systems. Sub-tropical and tropical systems are, however, not characterized by superabundant or dominant species, and have higher diversity of rare species. Such systems provide therefore more opportunities for petrels to forage on different prey and therefore segregate resource-use. Trophic specialization among different petrel species therefore potentially leads to resource partitioning in these tropical systems.

Trophic segregation among three sympatric petrels was examined by recovering prey identities from faeces using current high-throughput sequencing technologies. To obtain robust identifications, queries were assigned to the lowest taxonomical rank using the approaches described in chapter 3. The above hypothesis was addressed in Chapter 4.

C. *The moon phase has a significant effect on the composition of taxa available to Bulwer´s petrels at night, as this petrel is shown to consume a wide range of mesopelagic species that are known to respond to moon light intensity.*

Bulwer's petrels, as do other procellariiformes, have a high proportion of mesopelagic species in their stomach contents. These types of prey are normally found below 200 meters from the ocean's surface. These organisms are thought to be available to surface foragers during the night, when mesopelagic organisms are known to perform vertical migration to the sea surface. However, during moonlit nights, mesopelagic species are thought to avoid being at the surface as a response to visual predators. Based on such evidence, it was assumed in this thesis that Bulwer's petrel potentially forages on other prey types during moonlit nights compared with darker nights when mesopelagic species are thought to be abundant. Another possibility, if Bulwer's petrel maintains mesopelagic prey types throughout the moon cycle, is that the range of species consumed will significantly differ in moonlit versus new moon nights, as the diversity of mesopelagic prey should significantly differ between moon phases. To test this hypothesis, prey were identified from the stomach contents of Bulwer's petrels across different moon phases. To estimate the numerical frequency of prey a combined approach was applied using morphological analysis of hard parts and Sanger sequencing of DNA extracted from tissue remains.

1.12 References

Ainley DG & Boekelheide RJ (1990) Seabirds of the Farallon Islands: Ecology, dynamics and structure of an upwelling system community. Stanford University Press, Palo Alto, CA.

Ainley DG, Ribic CA & Fraser WR (1992) Does prey preference affect habitat choice in Antarctic seabirds. Marine Ecology Progress Series 90:207-221.

Alonso H, Granadeiro JP, Ramos JA, Catry P (2013) Use the backbone of your samples: fish vertebrae reduces biases associated with otoliths in seabird diet studies. Journal Of Ornithology 154: 883-886

Ashmole NP & Ashmole MJ (1967) Comparative feeding ecology of sea birds of a tropical oceanic island. Peabody Museum of Natural History, Yale University

Ashmole NP (1968) Body Size, Prey Size, and Ecological Segregation in Five Sympatric Tropical Terns (Aves: Laridae). Systematic Biology 17: 292-304

Awkerman J, Fukuda A, Higuchi H & Anderson D (2005) Foraging activity and submesoscale habitat use of waved albatrosses Phoebastria irrorata during chick-brooding period. Marine Ecology Progress Series 291: 289–300

Ballance LT & Pitman RL (1999) Foraging ecology of tropical seabirds. In: Adams, NJ & Slotow RH (eds) Proc. 22 International Ornithology Congress, Durban: 2057-2071. Johannesburg.

Ballance LT, Pitman RL & Reilly SB (1997) Seabird community structure along a productivity gradient: Importance of competition and energetic constraint. Ecology 78:1502-1518.

Barrett RT, Camphuysen K, Anker-Nilssen T, Chardine JW, Furness RW, Garthe S, Huppop O, Leopold MF, Montevecchi WA & Veit RR (2007) Diet studies of seabirds: a review and recommendations. ICES Journal of Marine Science 64: 1675–1691.

Benoit-Bird KJ, Au WWL & Wisdoma DW (2009a) Nocturnal light and lunar cycle effects on diel migration of micronekton. Limnology and Oceanography 54: 1789-1800

Benoit-Bird KJ, Dahood AD & Würsig B (2009b) Using active acoustics to compare lunar effects on predator–prey behavior in two marine mammal species. Marine Ecology Progress Series 395: 119-135

Berger SA, Krompass D & Stamatakis A (2011) Performance, Accuracy, and Web Server for Evolutionary Placement of Short Sequence Reads under Maximum Likelihood. Systematic Biology 60: 291-302

BirdLife International (2012) *Bulweria bulwerii*. The IUCN Red List of Threatened Species. Version 2014.3 (www.iucnredlist.org, downloaded on 03 March 2015)

BirdLife International (2014) *Calonectris borealis*. The IUCN Red List of Threatened Species. Version 2014.3. (www.iucnredlist.org, downloaded on 03 March 2015)

BirdLife International (2013) *Hydrobates castro*. The IUCN Red List of Threatened Species. Version 2014.3. (www.iucnredlist.org, downloaded on 03 March 2015)

BirdLife International (2012) *Pelagodroma marina*. The IUCN Red List of Threatened Species. Version 2014.3. (www.iucnredlist.org, downloaded on 03 March 2015)

Bolton M, Smith AL, Gomez-Diaz E, Friesen VL, Medeiros R, Bried J, Roscales JL & Furness RW (2008) Monteiro's Storm-petrel Oceanodroma monteiroi: a new species from the Azores. Ibis 150: 717-727.

Bowser AK, Diamond AW & Addison JA (2013) From Puffins to Plankton: A DNA-Based Analysis of a Seabird Food Chain in the Northern Gulf of Maine. PloSONE (8): e83152.

Bried J (2005) Diving Ability of the Madeiran Storm Petrel Waterbirds 28:162-166.

Brooke ML (2004) The food consumption of the world's seabirds. Proceedings of the Royal Society B Supplement 271: S246–S248.

Brown DS, Burger R, Cole N, Vencatasamy D, Clare EL, Montazam A & Symondson WO (2014) Dietary competition between the alien Asian Musk Shrew (Suncus murinus) and a re-introduced population of Telfair's Skink (Leiolopisma telfairii). Molecular Ecology 23: 3695-3705.

Campos A & Granadeiro JP (1999) Breeding Biology of White-faced Storm-Petrel *Pelagodroma marina* in Selvagem Grande Island, North-east Atlantic. Waterbirds 22: 199-206

Clare EL, Symondson WOC & Fenton MB (2014) An inordinate fondness for beetles? Variation in seasonal dietary preferences of night-roosting big brown bats (Eptesicus fuscus). Molecular Ecology 23: 3633-3647

Clarke MR (1986) A handbook for the identification of cephalopod beaks. Clarendon Press, Oxford, England

Clarke TA (1973) Some aspects of the ecology of laternfishes (Myctophidae in the Pacific Ocean near Hawaii. Fishery Bulletin 71: 401-434

Coulson JC (2002) Colonial breeding in seabirds. In: Schreiber EA, Burger J (eds) Biology of marine birds. CRC Press, London, p 87–113

Croxall JP & Prince P (1994) Dead or alive, night or day: how do albatrosses catch squid? Antarctic Science 6: 155–162

Croxall JP, Prince PA & Reid K (1997) Dietary segregation of krill-eating South Georgia seabirds. Journal of Zoology 242:531–556

Cruz SM, Hooten M, Huyvaert KP, Proaño CB, Anderson DJ, Afanasyev V & Wikelski M (2013) At–Sea Behavior Varies with Lunar Phase in a Nocturnal Pelagic Seabird, the Swallow-Tailed Gull. PLoS ONE 8:e56889

Deagle BE, Chiaradia A, McInnes J& Jarman SN (2010) Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? Conservation Genetics 11: 2039-2048

Deagle BE, Gales NJ, Evans K, Jarman SN, Robinson S, Trebilco R, Hindell MA (2007) Studying seabird diet through genetic analysis of faeces: a case study on macaroni penguins (Eudyptes chrysolophus). PLoS ONE, 2, e831

Deagle BE, Jarman SN, Coissac E, Pompanon F & Taberlet P (2014) DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biology Letters 10: 20140562

Dias MP, Granadeiro JP & Catry P (2012) Working the day or the night shift? Foraging schedules of Cory's shearwaters vary according to marine habitat. Marine Ecology Progress Series 467: 245–252

Duffy DC & Jackson S (1986) Diet studies of seabirds: a reviewof methods. Colonial Waterbirds 9:1-17

Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Fonseca VG, Carvalho GR, Sung W, Johnson HF, Power DM, Neill SP, Packer M, Blaxter ML, Lambshead PJD, Thomas WK & Creer S (2010) Second-generation environmental sequencing unmasks marine metazoan biodiversity Nature Communication 1: 98

Forero MG, González-Solís J, Hobson KA, Donázar JA, Bertellotti M, Blanco G & Bortolotti GR (2005) Stable isotopes reveal trophic segregation by sex and age in the southern giant petrel in two different food webs. Marine Ecology Progress Series 296: 107–13.

Geldmacher J, Hoernle K, van der Bogaard P, Zankl G & Garbe-Schoenberg D (2001) Earlier history of the ≥70-Ma-old Canary hotspot based on the temporal and geochemical evolution of the Selvagen Archipelago and neighboring seamounts in the eastern North Atlantic. Journal of Volcanology and Geothermal Research 111: 55-87

Geldmacher J, van der Bogaard P, Hoernle K & Schmincke H-U (2000) The A-r age dating of the Madeira Archipelago and hotspot track (eastern North Atlantic). Geochemistry Geophysics Geosystems 1: paper number 1999GC000018.

Granadeiro JP, Alonso H, Almada V, Menezes D, Phillips RA & Catry P (2009) Mysterious attendance cycles in Cory's shearwater, *Calonectris diomedea*: an exploration of patterns and hypotheses. Animal Behaviour 78: 1455-1462.

Granadeiro JP, Dias MP, Rebelo R, Santos CD, Catry P (2006) Numbers and population trends of Cory's Shearwater *Calonectris diomedea* at Selvagem Grande, northeast Atlantic. Waterbirds, 29, 56–60

Hajibabaei M, Smith MA, Janzen DH, Rodriguez JJ, Whitfield JB & Hebert P (2006) DNA minimalist barcode can identify a specimen whose DNA is degraded. Molecular Ecology Notes 6: 959–964.

Harrison CS & Seki MP (1987) Trophic relationships among tropical seabirds at the Hawaiian Islands. In: Croxall, J.P. (ed.). Seabirds. Feeding ecology and role in marine ecosystems. Cambridge; Cambridge University Press: 301-326

Harrison CS, Hida TS & Seki MP (1983) Hawaiian seabird feeding ecology. Wildlife Monographs 85: 3–71.

Hebert PD, Cywinska A, Ball SL & de Waard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B 270: 313–321.

Hebert PD, Ratnasingham S & de Waard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society B 270: S96-9

Hernández E, Martín A., Nogales M, Quilis V, Delgado G. & Trujillo O (1990) Distribution and status of Bulwer's Petrel (*Bulweria bulwerii*) Jardine & Selby, 1828) in the Canary Islands. Boletim do Museu Municipal do Funchal 42: 5–16.

Hobson KA, Piatt JF & Pitocchelli J (1994) Using stable isotopes to determine seabird trophic relationships. Journal of Animal Ecology 63:786-798

Hulley AP (1998) Myctophidae. In Encyclopedia of Fishes 2. Edited by Paxton J, Eschmeyer WN. San Diego: Academic:127-128

Hutchinson GE (1957) Concluding remarks. Cold Spring Harbor Symposia on Quantitative Biology 22: 415– 427

Imber MJ (1975) Behaviour of petrels in relation to the moon and the artificial lights. Notornis 22: 302 –306

Imber MJ, Cruz JB, Grove JS, Lavenberg RJ, Swift CC & Cruz F (1992) Feeding ecology of the Dark-rumped Petrel in the Galápagos Islands. Condor 94: 437-447

Inchausti P & Weimerskirch H (2001) Risks of decline and extinction of the endangered Amsterdam albatross and the projected impact of long-line fisheries Biological Conservation 100: 377–386

Jacob U, Mintenbeck K, Brey T, Knust R & Beyer K (2005) Stable isotope food web studies: a case for standardized sample treatment. Marine Ecology Progress Series 287:251-253

Jarman SN, McInnes JC, Faux C, Polanowski AM, Marthick J, Deagle BE, Southwell C & Emmerson L (2013) Adélie Penguin Population Diet Monitoring by Analysis of Food DNA in Scats. PLoS ONE 8(12): e82227

Kampa EM (1974) Photoenvironment and vertical migrations of mesopelagic marine animal communities. In Biological rhythms in the marine environment (ed. P. J. DeCoursey), pp. 257-272. Columbia: University of South Carolina Press

Karpouzi VS ,Watson R & Pauly D (2007) Modelling and mapping resource overlap between fisheries and seabirds on a global scale: a preliminary assessment . Marine Ecology Progress Series 343: 87-99

King RA, Symondson WOC, Thomas RA (2015) Molecular analysis of faecal samples from birds to identify potential crop pests and useful biocontrol agents in natural areas. Bulletin of Entomological Research (http://dx.doi.org/10.1017/S0007485314000935)

Matias R & Catry P (2010) The diet of Atlantic Yellow-legged Gulls (Larus michahellis atlantis) at an oceanic seabird colony: estimating predatory impact upon breeding petrels. European Journal of Wildlife Research, 56: 861-869.

Miller AK, Kappes MA, Trivelpiece SG & Trivelpiece WZ (2010) Foraging-Niche Separation of Breeding Gentoo and Chinstrap Penguins, South Shetland Islands, Antarctica. Condor 112:683-695

Monteiro LR & Furness RW (1998) Speciation through temporal segregation of Madeiran storm petrel (Oceanodroma castro) populations in the Azores? Philosophical Transactions of the Royal Society B 353: 945-953

Mougin J & Mougin M (2000) Maximum diving depths for feeding attained by Bulwer's petrels (*Bulweria bulwerii*) during the incubation period. Journal of Zoology 250: 75– 77.

Mougin J-L, Jouanin C & Roux F (1990) Chronologie de la reproduction chez le Petrel-tempeate de Castro Oceanodroma castro (Hartcourt). L'Oiseau R.F.O. 60: 135-150.

Navarro J, Votier SC, Aguzzi J, Chiesa JJ, Forero MG, & Phillips RA (2013) Ecological Segregation in Space, Time and Trophic Niche of Sympatric Planktivorous Petrels. PLoS ONE 8: e62897

Nunes M & Vincente L (1998) Breeding cycle and nestling growth of Bulwer's petrel on the Desertas Islands, Portugal. Colonial Waterbirds 22: 198-204

Nunes M (2000) Madeiran Storm-Petrel (Oceanodroma castro) in the Desertas Islands (Madeira Archipelago): a new case of two distinct populations breeding annually? Arquipélago. Life and Marine Sciences Supplement 2: 175-179.

Nunes M (2000) New data on the Bulwer's petrel (*Bulweria bulwerii*) breeding biology in the Desertas Islands (Madeira Archipelago). Arquipélago. Life and Marine Sciences Supplement 2: 167-173

Onley D & Scofield P (2007) Albatrosses, Petrels and Shearwaters of the World (pp. 157–158).

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Mable BK, Moritz C (eds) Molecular Systematics. Sinauer, Sunderland, MA, pp 205–247

Pearson TH (1968) The feeding biology of sea-bird species breeding on the Farne Islands, Northhumberland. Journal of Animal Ecology 37:521−552

Phalan B, Phillips RA, Silk JRD, Afanasyev V, Fukuda A, Fox J, Catry P, Higuchi H & Croxall JP (2007) Foraging behaviour of four albatross species by night and day. Marine Ecology Progress Series 340: 271-286

Pinol J, San Andres V, Clare EL, Mir G & Symondson WOC (2014) A pragmatic approach to the analysis of diets of generalist predators: the use of next-generation sequencing with no blocking probes. Molecular Ecology Resources 14: 18-26

Quince C, Lanzén A, Curtis TP, Davenport RJ, Hall N, Head IM, Read LF & Sloan WT (2009) Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods 6: 639-641

Rodríguez B, De León L, Martín A, Alonso J & Nogales M (2003) Status and distribution of breeding seabirds in the northern islets of Lanzarote (Canary Islands). Atlantic Seabirds 5: 41–56

Roscales JL, Gómez-Díaz E, Neves V & González-Solís J (2011) Trophic versus geographic structure in stable isotope signatures of pelagic seabirds breeding in the northeast Atlantic. Marine Ecology Progress Series 434:1–13.

Rubolini D, Maggini I, Ambrosini R, Imperio S, Paiva VH, Gaibani G, Saino N & Cecere, JG (2014) The Effect of Moonlight on Scopoli's Shearwater *Calonectris diomedea* Colony Attendance Patterns and Nocturnal Foraging: A Test of the Foraging Efficiency Hypothesis. Ethology, 121: 284-299

Sadighzadeh Z, Tuset VM, Valinassab T, Dadpour MR & Lombarte A (2012) Comparison of different otolith shape descriptors and morphometrics for the identification of closely related species of Lutjanus spp. from the Persian Gulf. Marine Biology Research 8:802–814

Schoener TW (1974) Resource partitioning in ecological communities. Science 185:27-39

Smith AL, Monteiro L, Hasegawa O & Friesen VL (2007) Global phylogeography of the band-rumped storm-petrel (Oceanodroma castro; Procellariiformes: Hydrobatidae) Molecular Phylogenetics and Evolution 43:755-73

Spear LB, Ainley DG & Walker WA (2007) Foraging dynamics of seabirds in the Eastern Tropical Pacific Ocean. Studies in Avian Biology 35:1−99

Stiegler P, Carbon P, Ebel JP & Ehresmann C (1981) A General Secondary Structure Model for Procaryotic and Eucaryotic RNAs of the Small Ribosomal Subunits. European Journal of Biochemistry 120: 487–495

Sutherland RM (2000) Molecular analysis of avian diet. PhD Thesis. University of Oxford.

Sydeman WJ, Hobson KA, Pyle P & McLaren EB (1997) Trophic relationships among seabirds in central California: combined stable isotope and conventional dietary approach. Condor 99: 327–336

Symondson WOC & Liddell JE (1993) The detection of predation by Abax parallelepipedus and Pterostichus madidus (Coleoptera, Carabidae) on mollusca using quantitative elisa. Bulletin of Entomological Research 83: 641-647

Taberlet P, Coissac E, Hajibabaei M & Rieseberg LH (2012) Environmental DNA. Molecular Ecology 21: 1789–1793.

Valentini A, Pompanon F & Taberlet P (2009) DNA barcoding for ecologists. Trends in Ecology and Evolution 24: 110–117.

Villard P, Bonenfant C, Bretagnolle V (2011) Effects of satellite transmitters fitted to the Breeding Cory´s shearwaters. The Journal of Wildlife Management 75:709-714

Weimerskirch H (2007) Are seabirds foraging for unpredictable resources? Deep Sea Research Part II: Topical Studies in Oceanography 54: 211-223

Williams RJ & Martinez ND (2000) Simple rules yield complex food webs. Nature 404: 180-183

Wilson RP (1984) An improved stomach pump for penguins and other seabirds. Journal of Field Ornithology, 55, 109–112

Xavier JC, Phillips RA & Cherel Y (2011) Cephalopods in marine predator diet assessments: why identifying upper and lower beaks is important. ICES Journal of Marine Science 68: 1857−1864

Yamamoto T, Takahashi A, Yoda K, Katsumata N, Watanabe S, (2008) The lunar cycle affects at–sea behaviour in a pelagic seabird, the streaked shearwater, *Calonectris leucomelas*. Animal Behaviour 76: 1647–1652

Young HS, McCauley DJ, Dirzo R, Dunbar RD & Shaffer SA (2010) Niche partitioning among and within sympatric tropical seabirds revealed by stable isotope analysis. Marine Ecology Progress Series 416: 285-294.

Yu DW, Ji Y, Emerson BC, Wang X, Ye C, Yang C & Ding Z (2012) Biodiversity soup: metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring Methods in Ecology Evolution 3: 613–623

Zaidi RH, Jaal Z, Hawkes NJ, Hemingway J & Symondson WOC (1999) Can the detection of prey DNA amongst the gut contents of invertebrate predators provide a new technique for quantifying predation in the field? Molecular Ecology 8: 2081-2087.

Zaret TM, Suffern JS (1976) Vertical migration in zooplankton as a predator avoidance mechanism. Limnology and Oceanography, 21: 804–813

Zeale MRK, Butlin RK, Barker GLA, Lees DC & Jones G (2011) Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. Molecular Ecology Resources 11: 236–244

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CHAPTER 2:

An holistic ecological analysis of the diet of Cory's shearwaters using prey morphological characters and DNA barcoding

An holistic ecological analysis of the diet of Cory's shearwaters using prey morphological characters and DNA barcoding

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Author contributions

JPG, PC, HA and SW designed the study; PC, JPG, JAR and WOCS provided guidance during the study; HA, JPG and PC collected the data in the field; HA and JX identified and quantified prey trough conventional methods; HA and JPG analysed the data; SW performed the molecular part of the study; HA, SW, PC, JPG, and WOCS wrote the manuscript.

2.1 Abstract

Knowledge of the dietary choices and trophic niches of organisms is the key to understanding their roles in ecosystems. In seabird diet studies, prey identification is a difficult challenge, often yielding results with technique-specific biases. Additionally, sampling efforts are often not extensive enough to reveal intra-populational variation. Immature animals, which may constitute up to 50% of a population, may occupy a significantly different trophic niche to more-experienced birds, but this remains largely unexplored. We investigated the diet of Cory´s shearwater (*Calonectris diomedea*) from Selvagem Grande, an island located off the northwest African coast, collecting a total of 698 regurgitate samples over three consecutive breeding seasons. The diet was assessed using two complementary approaches for prey identification: conventional morphological analysis (using teleost fish vertebrae, otoliths and cephalopod beaks) and DNA barcoding of the 16S rRNA mitochondrial gene, in cases where a positive identification could not be retrieved. Species assignments employed BLAST and distance based methods, as well as direct optimization of the tree length based on unaligned sequences in POY. This method resulted in robust tree estimates and species assignments, showing its potential for DNA barcoding of stomach contents using hypervariable markers such as the 16S. The molecular approach increased taxonomic resolution and revealed an additional 17 taxa. Diet differed significantly according to breeding status, sex, breeding phase (pre-laying and chick-rearing) and year. Such direct evidence of trophic segregation within the same population has rarely been shown in seabirds and highlights the importance of including such variables in ecosystem-based management approaches.

2.2 Introduction

Dietary studies are essential building blocks of the science of ecology. Only with the support of dietary studies can we properly assess the position of species in food webs, their role in energy flow within ecosystems, the importance of feeding resources for demographic regulation and the impact of predation on populations and communities. Despite this, the trophic niche of numerous ecologically important

species, such as top marine predators, remains poorly understood (e.g., Naito et al. 2013). Two primary problems in previous research have been difficulties with prey identification and failure to sample relevant population segments (Barrett et al. 2007; Bowen et al. 2013) that may potentially display niche differentiations (Polis 1984). Amongst seabirds, which are major pelagic consumers, much effort has gone into sampling the diet of breeding birds (often only at the chick stage) while virtually nothing is known about non-breeders (Barrett et al. 2007). This gap is particularly relevant when one considers that non-breeders (mostly immature individuals) may represent >50% of the fully grown individuals in a population.

Trophic niche differentiation between immature and adult reproducing individuals is to be expected in species where growth is protracted and niche is strongly influenced by body size (e.g., Lucifora et al. 2009). However, in other taxa, particularly in birds, size varies little amongst fledged individuals. Nevertheless, even for a relatively invariable body size, we may expect differences linked to, for example, (a) age-related improvements in foraging competence (Kitowski 2003; Daunt et al. 2007), (b) the competitive exclusion of subdominant (generally younger) individuals by more dominant conspecifics (Goss-Custard et al. 1982), or (c) differential spatial distribution arising from the need of reproducing individuals to regularly attend breeding sites. Despite these expectations, we currently know very little about whether there are ontogenetic changes of trophic niches in birds, or their possible causes and consequences.

Molecular techniques, such as DNA barcoding (Kochzius et al. 2010; Zhang & Hanner 2012), are revolutionising dietary studies and are now being extensively applied in dietary analyses of vertebrate and invertebrate carnivores and herbivores (reviewed in Symondson 2002; Pompanon et al. 2012). Prey species can be identified even from highly degraded tissue (as found in faeces and regurgitates), using PCR. Most of these studies have identified prey species from homogenised meta-samples (guts or faeces), with quantitative estimates of species consumed derived from sequences obtained for each identified prey using either a cloning and sequencing technique or Next Generation Sequencing. Nevertheless, differences among prey species in the mitochondrial copy numbers per cell, as well as in the binding efficiency of the primers (Symondson 2002; Pompanon et al. 2012), may lead to substantial biases. One way to overcome this problem is to use a combined approach, using morphological analyses for quantitative

estimates of prey (hard parts recovered from guts or faeces) plus augmentation of species identification using DNA barcoding of tissues (Barnett et al. 2010; Dunn et al. 2010). Applying this approach to pelagic top-predators has the potential to enhance understanding of trophic dynamics and, as such, marine conservation and ecosystem-based management.

Birds are amongst the best studied animal classes, yet few studies have used molecular techniques to improve our understanding of their trophic ecology (e.g., Deagle et al. 2007; Jedlicka et al. 2013). Recently, molecular approaches has been used to investigate the dietary habits of seabirds, but those few studies have analysed faeces only (Deagle et al. 2007; Bowser et al. 2013; Jarman et al. 2013; but see Jarman et al. 2002), which implies that quantification of identified prey remained relatively crude (Deagle et al. 2010). The first aim of the present paper is to develop the technique and illustrate the tremendous potential of using DNA barcoding combined with morphological tools to provide an unusually refined picture of the diet of birds (in this case, of a pelagic seabird).

Our study model is the Cory's shearwater (*Calonectris diomedea borealis*), an oceanic predator of the Northeast Atlantic, which breeds on several islands and islets from the Azores and Berlengas archipelagos in the north to the Canary archipelago in the south (Thibault et al. 1997). The feeding ecology of Cory's shearwaters has been studied at several colonies (Granadeiro et al. 1998; Paiva et al. 2010; Xavier et al. 2011; Neves et al. 2012), but little is known about their diet in the southern area of their breeding range (but see den Hartog & Clarke 1996; Paiva et al. 2010 for studies with limited sampling effort). More importantly, Cory's shearwaters are long-lived birds that only start reproducing at a mean age of 9 years and frequently skip breeding seasons, even after their first reproduction (Mougin et al. 1997). As such, a large proportion of Cory's shearwater populations is comprised of non-breeders, but their trophic ecology has never been investigated. We also have a poor understanding of male-female differences in the ecology of this dimorphic species (Navarro et al. 2009; Ramos et al. 2009) and most studies carried out found no evidence of spatial (Navarro et al. 2009) or foraging niche sexual segregation (Navarro et al. 2007; Ramos et al. 2009). Hence, the second broad objective of this paper is to characterise the diet of Cory's shearwaters in the southern part of its breeding range and assess within-population sources of variation,

with a particular interest in differentiation between breeders and non-breeders, indicative of ontogenetic shifts in the trophic niche of this seabird.

2.3 Methods

2.3.1 Study area and species

Fieldwork was conducted in the Selvagem Grande island (30° 09' N, 15° 52' W), where ca. 30,000 Cory's shearwater breeding pairs nest (Granadeiro et al. 2006). This sub-tropical oceanic island is located ca. 350 km from up-welling enriched shelf areas off the African coast. Cory's shearwaters are long-distance migrants returning in early March from their wintering sites in the south Atlantic (Thibault et al. 1997). During the extended pre-laying period, birds re-occupy their nest cavities, protecting them from prospecting birds and eventually finding a mate. Egg-laying takes place at the end of May with the chicks hatching at the end of July. The chick-rearing period lasts approximately 97 days, until early November, when chicks fledge (Thibault et al. 1997).

2.3.2 Diet sampling and analysis

Sampling was conducted in the pre-laying period of 2010 (11 to 20 April) and during the chick-rearing periods of 2008, 2009 and 2010 (28 July to 2 October). Shearwaters returning from the sea were captured by hand when entering the nest or preparing to feed their chick. Non-breeding birds were also sampled in the chick-rearing period of 2009. In the incubation period, when birds are more sensitive to disturbance and are more likely to have empty stomachs, only non-breeders were sampled (15 to 25 June of 2010). Non-breeding Cory's shearwaters tend to stay outside the nest cavities and to be very vocal and socially interactive, and are, therefore, easily selected for sampling.

Birds were sexed based on their distinctive vocalisations (Thibault et al. 1997) or using a discriminant function based on bill measurements, which has a 98.8% success rate (Granadeiro 1993).

Prey samples were collected from the birds using the water off-loading technique (Wilson 1984). By selecting different areas each day and marking the birds with wax markers, we guaranteed that birds and nests were only sampled once. Fresh prey items found in food samples were identified using specialised guides (Quéro et al. 2003) and stored in 70% ethanol. Digested fish were quantified and identified to the lowest possible taxon from vertebrae and other hard remains (otoliths, dentaries and scales), using our own reference collection and published guides (Tuset et al. 2008). Cephalopods were identified from their beaks and quantified based on the number of mantles, other fresh remains (tentacles, flesh) and fresh beaks (upper and lower beaks were counted).

A large number of teleost fish from the genus *Scomber* were identified as *Scomber colias* (41.1%, N = 538). None was identified as *Scomber scombrus* and it was only possible to identify the remaining individuals to genus level (*Scomber*). Given this result, we pooled *Scomber colias* and *Scomber* sp. in all further analyses. We calculated frequencies of occurrence (FO): the number of samples with a given prey type, expressed as a percentage of the total number of samples and numerical frequencies (NF): the number of individuals of a given taxon, as a percentage of the total number of prey items.

Given their small size (less than 3 mm), most unidentified crustaceans and insects found in the samples were unlikely to be their direct prey, and were probably part of the diet of fish captured by shearwaters (secondary predation). Considering their parasitic habits, crustaceans from the family Isopoda were also probably captured along with fish prey. None of these prey were included when calculating the numerical importance of prey. The exception were three larger crustaceans (more than 30 mm, Decapoda) that were considered to be part of the shearwater diet.

2.3.3 Genetic analysis

A total of 83 muscle samples (27 cephalopods and 56 teleost fish), either unidentified through conventional diet analysis (45 samples) or only identified to higher taxonomic levels (*Trachurus* sp. and Exocoetidae) (38 samples), were examined using DNA barcoding (16s rRNA). Although the cytochrome c oxidase subunit I (COI) has

gathered wide consensus as a genetic marker for species discrimination of unknown taxa (Hebert et al. 2003), the 16S barcode provided a higher sequence database coverage within the range of prey identified in Cory´s shearwater diet. For example, in teleost fish, all genera within the family Exocoetidae were covered for 16S, but only three for COI. A search on squid "Teuthida" in the GenBank database retrieved 359 matches against 305 (after excluding the family Loliginidae, which is by far the best represented in Genbank). Therefore, the 16S was more informative in the context of this study.

We collected pieces of tissue from prey associated with hard structures (e.g., vertebrae) that could not be identified morphologically and used these for DNA barcoding. To extract prey DNA, individual prey tissue was washed with ddH_2O to remove adherent ethanol. As in other barcoding studies, that identified prey remains in stomach contents (e.g., Barnett et al. 2010), we chose where possible the inner parts of the tissue, since tissue from complex meta-samples may be contaminated with DNA of other prey. The DNA was extracted using the DNeasy Blood and Tissue Extraction Kit (Qiagen) following the protocol for purification of total DNA from animal tissues. Individual prey DNA from regurgitates was amplified using the universal primers of Palumbi (1996): 16ar, 5'-CGCCTGTTTATCAAAAACAT-3' and 16br, 5'-CCGGTCTGAACTCAGATCACGT-3´, with an expected amplicon length of ca 550-620bp.

Polymerase Chain Reactions (PCR) were performed with the Multiplex PCR Kit (Qiagen) using the following PCR reagent mixtures: 10µl of Multiplex PCR Master Mix (1X), 0.25µM of each primer, 0.1mM of BSA, 3.6 µl ddH2O, 2.4µl (~50 – 100 ng/µl) of template DNA in a total volume of 20 µl. Thermal cycling conditions were as follow: 95ºC for 15min; 35 cycles of 94ºC for 30s, 52ºC for 90s, 72ºC for 90s, and a final extension at 72ºC for 10min. PCR products were cleaned using ExonucleaseI and Antarctic Alkaline phosphatase enzymes (New England, Biolabs) and sequenced using the EZ-seq services of Macrogen, Inc (Amsterdam, Netherlands).

A. Molecular identification of prey using BLAST

Sequences were compared with those in GenBank using the BLAST algorithm (Altschul et al. 1990). Each taxonomic assignment was based on the percentage of

similarity with the reference sequences in GenBank. Species were directly assigned when the query sequence produced an identical match to the reference sequence (100% of identity). For BLAST matches higher than 99.0%, species were assigned when the query sequence matched monotypic genera or when the distribution range of potential con-specifics was outside our study area, but only if no other species was retrieved with this value. Inter-specific divergences in teleost fish are > 2% (i.e., Kochzius et al. 2010, Zhang & Hanner 2012) and in cephalopods 1.3-12.7% (Dai et al. 2012). Therefore, the above criteria were expected to produce robust species identifications.

B. Molecular identification of prey using phylogenetic analysis

Specimens were assigned using phylogenetic inferences in cases where the percentage of similarity between the query sequence and the reference sequence was lower than 99.0%. Two methods were used: (a) distance based Neighbour-Joining (NJ) trees and (b) direct optimisation (DO) of the tree length.

NJ trees were constructed in Mega 6 (Tamura et al. 2013) using the Kimura-2-parameter model of evolution (Kimura 1980). The nodal support was obtained using a 1000 bootstrap replicates. NJ trees were estimated from eight different data sets of aligned sequences, each corresponding to the families that produced the nearest match with the query sequences. Sequences for which no positive identifications were obtained in BLAST were included and aligned with all available representative genera of that family using Clustal W (Thompson et al. 1994) as implemented in BioEdit (Hall 1999).

DO analysis were performed in POY v 5.0.0 (Varón et al. 2010). This program infers the tree directly from unaligned sequences and overcomes, therefore, potential uncertainties in sequence alignment (e.g., the hypervariable 16S mtDNA, where different numbers of indels between sequences can significantly impact tree estimates). To generate the POY tree we used the reference sequence that produced the nearest match in BLAST, including all other congener reference sequences of the same family. The tree estimated in POY did not include cephalopods since only two taxa could not be identified using BLAST (only represented in NJ trees). Sequences were trimmed to produce the exact same sequence terminals (374-396 base pairs), since sequences that

are absent in the terminals can account for erroneous indel event counts in POY (De Laet 2010).

Because POY uses empirical gap cost criteria to optimise the tree length, we first performed sensitivity estimates under five different affine gap costs regimes: (2,1,1), (2,1,2), (2,1,3), (2,1,5), (2,1,7) (substitution cost, gap extension, gap opening). Trees based on parsimony were constructed using 100 initial trees generated by random addition sequences using Subtree Pruning and Regrafting (SPR) and Tree Bisection and Reconnection (TBR) branch swapping. The tree producing the most congruent topology with what is known of the evolutionary relationships of fish was chosen as the "optimum" tree. Nodal support was calculated using a 1000 bootstrap replicates with alternate SPR and TBR swapping.

Assignments of families and genera were obtained using a strict criterion based on how query sequences clustered in the NJ and DO trees (Wilson et al. 2011). According to this criterion, a taxon (family or genera) is identified if the query sequence nests within a clade that comprises members of that taxon.

As some of our query sequences that produced 100% matches in BLAST showed high similarities with other congeners (>98%) (families Carangidae and Exocoetidae), those were also included for phylogenetic analysis to validate species assignments. Species were identified if the query sequences clustered monophyleticaly with the taxon that produced an identical match in BLAST and with no other congeners. Unidentified vertebrae based on morphological analysis but otherwise positively identified using DNA barcoding were later used to identify those species and quantify their occurrence in all samples.

2.3.4 Statistical analysis

We initially checked for overall differences in the diet between sexes and among years using permutational multivariate analysis of variance based on distance matrices, implemented using the package "vegan" (Oksanen et al. 2011) running in R software (R Development Core Team 2010). The method undertakes a partitioning of the sums of squares of a multivariate data set, using semi-metric and metric distance matrices to produce a "pseudo-F value". We tested for the effects of sex and year on the frequency

of occurrence of all prey items (with frequencies larger than 5% in one breeding phase). Whenever these tests provided significant results, we further explored the effects of these factors (and their interaction) on the occurrence of each prey using binomial GLMs (Generalized Linear Models), with a logit link function. The statistical significance of each factor was tested through log-likelihood ratio tests of increasingly simpler nested models, based on chi-squared distributions.

2.4 Results

A total of 698 regurgitates were collected from adult Cory's shearwaters. From these samples, a total of 2018 prey items were collected, 76.6% of which were successfully identified to the species or genus level.

Morphological inspection of the 2018 prey items retrieved 40 different prey types, but only 23 of those could be identified to species or genera. The use of DNA barcoding on morphologically unidentifiable specimens increased the prey list to 17 new taxa (12 species, 3 genera and 2 families).

2.4.1 Prey discrimination

DNA barcoding of the16S ribosomal RNA gene produced longer fragments in fish than in cephalopods varying approximately between 550-600 and 460-500 base pairs, respectively. DNA sequences were submitted to GenBank (Table S2.3).

BLAST comparisons in GenBank allowed for positive identification of 35% of the sequences to the species level, while phylogenetic inferences successfully discriminated another 46% to the genus level. From these sequences, 14 (10 species, 2 genera and 2 families) matched taxa that have never been identified in the diet of Cory´s shearwaters using morphological characters (Table 2.1). We also confirm the presence of the neon flying-squid (*Ommastrephes bartramii*) in the diet of these birds, where the beaks of small specimens were difficult to distinguish from those of the European flying-squid (*Todarodes sagittatus*).

Table 2.1 Cory´s shearwater (*Calonectris diomedea*) prey identified using DNA barcoding of 16S mtDNA.

	Family	Genus/Species	Specimens	Percentage of Similarity	Phylogenetic analysis
Teleostei	Carangidae	*Trachurus* sp.	5		**
	Carangidae	*Trachurus picturatus* (Bowdich 1825)	2	100	***
	Coryphaenidae	*Coryphaena equiselis* Linnaeus 1758*	1	100	
	Diretmidae	*Diretmus argenteus* Johnson	1	99.8[a]	
	Exocoetidae	*Cheilopogon melanurus* (Valenciennes 1847)*	1	100	***
	Exocoetidae	*Cheilopogon pinnatibarbatus* (Bennett 1831)*	2	100	***
	Exocoetidae	*Cheilopogon* sp.	1		**
	Exocoetidae	*Exocoetus* sp.	16		**
	Exocoetidae	Unidentified	2		
	Halosauridae	*Halosaurus* sp.*	1		**
	Molidae	*Ranzania laevis* (Pennant 1776)*	2	100	
	Myctophidae	*Diaphus* sp.	1		**
	Myctophidae	*Lampadena atlantica* Maul 1969*	1	100	
	Neoscopelidae	*Neoscopelus macrolepidotus* Johnson 1863*	1	100	
	Scombridae	*Katsuwonus pelamis* (Linnaeus 1758)*	2	100	
	Sparidae	*Boops boops* (Linnaeus 1758)*	2	99.1[b]	
	Sternoptychidae	*Argyropelecus* sp.*	1		**
	Synaphobranchidae	Unidentified	2		**
	Trichiuridae*	Unidentified	2		**
Cephalopods	Chiroteuthidae	*Chiroteuthis mega* (Joubin 1932)*	1	99.8[b]	
	Cranchiidae	*Taonius pavo (*Lesueur 1821)	1	100	
	Histioteuthidae	*Histioteuthis* sp.	1		**
	Ommastrephidae	*Ommastrephes bartramii* (Lesueur 1821)	8		**

Single (*) asterisk correspond to taxa not recorded previously in the diet of Cory´s shearwater (den Hartog & Clarke 1996, Granadeiro et al. 1998, Paiva et al. 2010, Xavier et al. 2011, Neves et al. 2012). Double (**) and triple (***) asterisks represent positive genus and species assignments based on the Neighbour joining (NJ) and DO trees. Similarity percentages with the GenBank reference sequences for species identifications using BLAST are shown (a) Monotypic species, (b) assignment based on the geographical distribution of the taxa

It is noteworthy that the values of similarity between species and genera varied substantially, depending on the families and prey groups analysed. While most teleost

families and cephalopods showed sequence homologies lower than 98% between conspecifics and congeners (within the reported divergences of vertebrate species), members of the families Exocoetidae and Carangidae presented very high homologies even between genera (ca. 99%). Therefore, identifications in both families were only obtained based on phylogenetic inferences. Regardless of the method employed for estimating trees (NJ or DO) the terminal topologies between the different trees were highly congruent (Figure 2.1, Figures S2.1, S2.2). Congeners clustered in highly supported monophyletic groups, with the exception of some members of the family Myctophidae and the genus *Cheilopogon*, that were paraphyletic and polyphyletic, respectively. Query sequences clustered, generally, with the reference sequences that produced the highest sequence homology in BLAST. Moreover, DO inferences resulted in a highly resolved tree at both internal and terminal nodes with an "optimal" tree obtained using the following settings: cost regime of substitutions = 2, indels = 1 and gap opening = 3. A total of seven major clades with high bootstrap support (85-100) were obtained, with each representing a different family of teleost fish.

Based on phylogenetic assignments using strict and liberal criteria we were also able to increase the taxonomic resolution of morphologically unidentified Exocoetidae and *Trachurus* specimens, identifying two species of *Cheilopogon* (*C. melanurus* and *C. pinnatibarbatus*) and the species *Trachurus picturatus*. Furthermore, morphologically unidentified members of the family Exocoetidae presented seven distinct Molecular Operational Taxonomical Units (MOTUs), revealing a high diversity among these prey items. Sequences of morphologically unidentified *Trachurus* specimens presented two distinct MOTUs, where most sequences clustered separately from *T. picturatus* and the reference sequences. The congruence in tree topologies as well as the taxonomic resolution obtained suggests that genetic variability within the 16S rRNA gene is sufficient to discriminate between species.

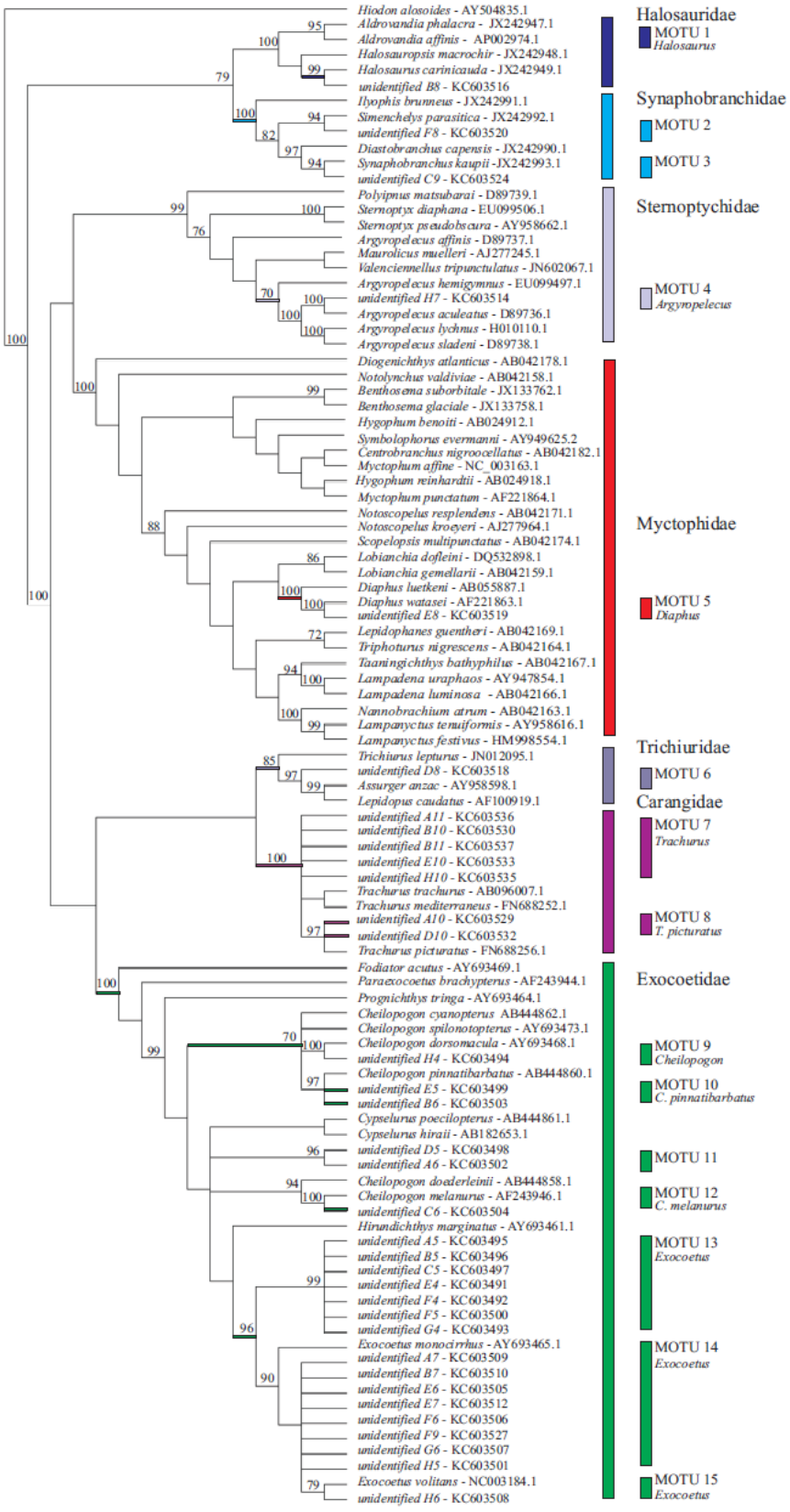
Hiodon alosoides - AY504835.1
Aldrovandia phalacra - JX242947.1
Aldrovandia affinis - AP002974.1
Halosauropsis macrochir - JX242948.1
Halosaurus carinicauda - JX242949.1
unidentified B8 - KC603516
Ilyophis brunneus - JX242991.1
Simenchelys parasitica - JX242992.1
unidentified F8 - KC603520
Diastobranchus capensis - JX242990.1
Synaphobranchus kaupii - JX242993.1
unidentified C9 - KC603524
Polyipnus matsubarai - D89739.1
Sternoptyx diaphana - EU099506.1
Sternoptyx pseudobscura - AY958662.1
Argyropelecus affinis - D89737.1
Maurolicus muelleri - AJ277245.1
Valenciennellus tripunctulatus - JN602067.1
Argyropelecus hemigymnus - EU099497.1
unidentified H7 - KC603514
Argyropelecus aculeatus - D89736.1
Argyropelecus lychnus - H010110.1
Argyropelecus sladeni - D89738.1
Diogenichthys atlanticus - AB042178.1
Notolynchus valdiviae - AB042158.1
Benthosema suborbitale - JX133762.1
Benthosema glaciale - JX133758.1
Hygophum benoiti - AB024912.1
Symbolophorus evermanni - AY949625.2
Centrobranchus nigroocellatus - AB042182.1
Myctophum affine - NC_003163.1
Hygophum reinhardtii - AB024918.1
Myctophum punctatum - AF221864.1
Notoscopelus resplendens - AB042171.1
Notoscopelus kroeyeri - AJ277964.1
Scopelopsis multipunctatus - AB042174.1
Lobianchia dofleini - DQ532898.1
Lobianchia gemellarii - AB042159.1
Diaphus luetkeni - AB055887.1
Diaphus watasei - AF221863.1
unidentified E8 - KC603519
Lepidophanes guentheri - AB042169.1
Triphoturus nigrescens - AB042164.1
Taaningichthys bathyphilus - AB042167.1
Lampadena urophaos - AY947854.1
Lampadena luminosa - AB042166.1
Nannobrachium atrum - AB042163.1
Lampanyctus tenuiformis - AY958616.1
Lampanyctus festivus - HM998554.1
Trichiurus lepturus - JN012095.1
unidentified D8 - KC603518
Assurger anzac - AY958598.1
Lepidopus caudatus - AF100919.1
unidentified A11 - KC603536
unidentified B10 - KC603530
unidentified B11 - KC603537
unidentified E10 - KC603533
unidentified H10 - KC603535
Trachurus trachurus - AB096007.1
Trachurus mediterraneus - FN688252.1
unidentified A10 - KC603529
unidentified D10 - KC603532
Trachurus picturatus - FN688256.1
Fodiator acutus - AY693469.1
Paraexocoetus brachypterus - AF243944.1
Prognichthys tringa - AY693464.1
Cheilopogon cyanopterus AB444862.1
Cheilopogon spilonotopterus - AY693473.1
Cheilopogon dorsomacula - AY693468.1
unidentified H4 - KC603494
Cheilopogon pinnatibarbatus - AB444860.1
unidentified E5 - KC603499
unidentified B6 - KC603503
Cypselurus poecilopterus - AB444861.1
Cypselurus hiraii - AB182653.1
unidentified D5 - KC603498
unidentified A6 - KC603502
Cheilopogon doederleinii - AB444858.1
Cheilopogon melanurus - AF243946.1
unidentified C6 - KC603504
Hirundichthys marginatus - AY693461.1
unidentified A5 - KC603495
unidentified B5 - KC603496
unidentified C5 - KC603497
unidentified E4 - KC603491
unidentified F4 - KC603492
unidentified F5 - KC603500
unidentified G4 - KC603493
Exocoetus monocirrhus - AY693465.1
unidentified A7 - KC603509
unidentified B7 - KC603510
unidentified E6 - KC603505
unidentified E7 - KC603512
unidentified F6 - KC603506
unidentified F9 - KC603527
unidentified G6 - KC603507
unidentified H5 - KC603501
Exocoetus volitans - NC003184.1
unidentified H6 - KC603508
Halosauridae
MOTU 1
Halosaurus
Synaphobranchidae
MOTU 2
MOTU 3
Sternoptychidae
MOTU 4
Argyropelecus
Myctophidae
MOTU 5
Diaphus
Trichiuridae
MOTU 6
Carangidae
MOTU 7
Trachurus
MOTU 8
T. picturatus
Exocoetidae
MOTU 9
Cheilopogon
MOTU 10
C. pinnatibarbatus
MOTU 11
MOTU 12
C. melanurus
MOTU 13
Exocoetus
MOTU 14
Exocoetus
MOTU 15
Exocoetus

Figure 2.1 Tree estimated in POY for identification of teleosts using direct optimisation (DO) method. Query sequences and Genbank accession numbers of morphologically unidentified specimens for which no reliable identification could be obtained in BLAST are shown (unidentified specimens code). Representative genera of the families that produced the nearest match are included in the tree. Query sequences clustering with Cheilopogon pinnatibarbatus, C. melanurus and Trachurus picturatus corroborate previous BLAST results (100% of similarity). The tree shows the number of different MOTUs (different prey) obtained in each family. Nodal support is presented for bootstrap values ≥ 70.

2.4.2 Diet composition

During the chick-rearing period, the diet of Cory's shearwaters was essentially composed of fish (FO range = 88.7 to 91.1%) and cephalopods (FO range = 27.2 to 46.8%). Chub mackerel (*Scomber colias*/sp.) was the most common prey (FO range = 35.6 to 51.2%) (Table 2.2). Pilot-fish (*Naucrates ductor*; FO range = 13.3 to 16.1%), sardine (*Sardina pilchardus*; FO range = 7.8 to 20.2%) and flying-fish (Exocoetidae; FO range = 7.4 to 14.4%) also occurred frequently. Among flying-fish, two genera were found, namely *Exocoetus* (FO range = 1.2 to 10%) and *Cheilopogon* (FO range = 1.1 to 3.2%). Subsequently, four species were identified: the tropical two-wing flying-fish (*Exocoetus volitans*), the bandwing flying-fish (*Cheilopogon exsiliens*), the Atlantic flying-fish (*C. melanurus*) and Bennett's flying-fish (*C. pinnatibarbatus*). The diet of Cory's shearwaters was diverse, being composed of at least 33 fish species from 20 different families (Table 2.2). Unidentified fish were found in 13.3 to 19.7% of the samples mostly because they were too digested or lacked identifiable hard remains.

The most common cephalopods were the neon flying-squid (*Ommastrephes bartramii*; FO range = 6.4 to 13.7%) and *Histioteuthis arcturi* (FO range = 1.1 to 5.3%). In addition, ten other cephalopod species from nine different families were present in the diet of Cory's shearwaters (see Table 2.2).

Crustaceans (FO range = 4.4 to 14.4%), mostly from the order Isopoda (FO range = 1.1 to 11.1%) and insects, from the family Halobatidae (FO range = 0 to 0.8%), were also present in the diet samples (Table 2.2). Fishery hooks were also found in three diet samples (FO = 0.5%).

Table 2.2 Frequency of occurrence (FO %) and numerical frequency (NF %) of prey, identified by a combined use of morphologic analysis and DNA barcoding, in the diet of Cory's shearwaters (*Calonectris diomedea*). Diet samples were collected in two different periods, pre-laying (only in 2010) and chick-rearing (in 2008, 2009 and 2010), at Selvagem Grande. Number of samples and prey is presented in brackets.

	Pre-laying		Chick-rearing					
	2010		2008		2009		2010	
	FO (30)	NF (318)	FO (180)	NF (416)	FO (248)	NF (631)	FO (188)	NF (553)
CEPHALOPODA	13.3	1.3	27.2	13.9	35.1	24.9	46.8	22.8
Chiroteuthidae								
Chiroteuthis mega (Joubin 1932)*	3.3	0.3						
Chiroteuthis sp.					1.2	0.5		
Cranchiidae								
Taonius pavo (Lesueur 1821)	3.3	0.3	0.6	0.2			1.1	0.5
Cranchia sp.								
Grimalditeuthidae								
Grimalditeuthis bonplandi (Verany 1839)			0.6	0.2				
Histioteuthidae								
Histioteuthis arcturi (Robson 1948)			1.1	0.5	1.2	0.5	5.3	2.0
Histioteuthis meleagroteuthis (Chun 1910)							0.5	0.2
Histioteuthis sp.			1.1	0.5	0.8	0.3		
Mastigoteuthidae								
Mastigoteuthis sp.					0.4	0.2		
Unidentified							2.1	0.7
Neoteuthidae								
Neoteuthis sp.							0.5	0.2
Ommastrephidae								
Ommastrephes bartramii (Lesueur 1821)*	3.3	0.3	7.7	3.8	13.7	9.2	6.4	2.7
Octopoteuthidae								
Taningia danae Joubin 1931			0.6	0.2	0.4	0.2		
Onychoteuthidae								
Ancistroteuthis lichtensteinii (Férussac 1835)					0.4	0.2		
Sepiidae								

Unidentified							0.5	0.2
Unidentified cephalopods	3.3	0.3	17.2	8.4	25.8	13.9	36.2	16.3
FISH	86.7	98.7	91.1	86.1	88.7	75.1	89.9	76.9
Belonidae								
Belone belone (Linnaeus 1761)			2.2	1.0	0.4	0.2	3.7	1.6
Caproidae								
Capros aper (Linnaeus 1758)					0.4	0.2	0.5	0.2
Carangidae								
Naucrates ductor (Linnaeus 1758)			15.6	15.9	16.1	14.9	13.3	10.7
Trachurus picturatus (Bowdich 1825)*			0.6	0.2	0.4	0.2		
Trachurus sp.	40.0	9.7	1.7	0.7	2.8	1.4	4.8	4.0
Clupeidae								
Sardina pilchardus (Walbaum 1792)			7.8	4.1	9.3	4.3	20.2	13.2
Sardinella sp.					0.4	0.2		
Congridae								
Conger conger (Linnaeus 1758)					0.8	0.3	0.5	0.2
Coryphaenidae								
Coryphaena equiselis Linnaeus 1758*					0.4	0.2		
Coryphaena sp.			0.6	0.2			1.1	0.5
Diretmidae								
Diretmus argenteus Johnson 1864*							2.7	0.9
Engraulidae								
Engraulis encrasicolus (Linnaeus 1758)			1.7	1.0	3.6	4.0	1.1	0.5
Exocoetidae								
Exocoetus volitans Linnaeus 1758			1.7	0.7				
Exocoetus sp.			8.3	4.1	1.2	0.5	4.8	1.6
Cheilopogon exsiliens (Linnaeus 1771)					1.2	0.5		
Cheilopogon melanurus (Valenciennes 1847)*					0.4	0.2		
Cheilopogon pinnatibarbatus (Bennett 1831)*					0.4	0.2		
Cheilopogon sp.			1.1	0.5	1.2	0.5	1.1	0.4
Unidentified	3.3	0.3	6.7	2.9	4.0	2.5	2.7	0.9
Halosauridae								
Halosaurus sp.*			0.6	0.2	0.4	0.2		
Unidentified			0.6	0.5			0.5	0.2

Macroramphosidae								
Macroramphosus scolopax (Linnaeus 1758)	36.7	83.3					1.1	0.4
Molidae								
Ranzania laevis (Pennant 1776)*							3.7	1.3
Myctophidae								
Diaphus sp.*					0.4	0.2		
Lampadena atlantica Maul 1969*							0.5	0.2
Unidentified			1.7	1.0			1.6	0.9
Neoscopelidae								
Neoscopelus macrolepidotus Johnson 1863*			0.6	0.2				
Scomberesocidae								
Scomberesox sp.	3.3	0.3	2.2	1.7	1.2	0.5	4.3	2.9
Scombridae								
Scomber colias Gmelin 1789	23.3	3.8	46.7	39.2	51.2	36.7	35.6	23.3
Katsuwonus pelamis (Linnaeus 1758)*			0.6	0.2	3.2	1.3	2.7	0.9
Sparidae								
Boops boops (Linnaeus 1758)*					0.4	0.2	1.1	0.5
Sternoptychidae								
Argyropelecus sp.*			0.6	0.5				
Synaphobranchidae*								
Unidentified			1.1	0.5	1.2	0.5	3.7	1.4
Trichiuridae*								
Unidentified			4.4	1.9	1.2	0.5	4.8	1.8
Unidentified fish	13.3	0.9	19.4	8.9	13.3	5.5	22.3	8.3
CRUSTACEA			4.4		6.5		14.4	
Decapoda	3.3	0.3					0.5	0.4
Isopoda			1.1		3.6		11.1	
Unidentified crustacean	13.3		3.3		2.8		2.7	
INSECTA					0.8			
Halobatidae					0.8			

Single (*) asterisk represent taxa first identified through DNA barcoding of the 16S mtDNA.

2.4.3 Diet of non-breeders

During the chick-rearing period of 2009, the diet of non-breeders and breeders differed significantly (pseudo-$F_{1,239}$ = 3.63, p = 0.02; Figure 2.3). Non-breeders preyed heavily on cephalopods (FO = 63.2%), compared to breeders of the same year (FO = 35.1%), and consumed less chub mackerel (Figure 2.3). During this period, neon flying-squid (FO = 36.8%), chub mackerel (FO = 31.6%), pilot-fish (FO = 21.1%) and horse/blue mackerel (FO = 10.5%) were the most frequent prey item of non-breeders (Figure 2.3). In the incubation period, non-breeders also consumed less fish (FO = 12.1%) while cephalopods were much more frequent in their diet (FO = 93.9%), particularly neon flying-squid (FO = 45.5%).

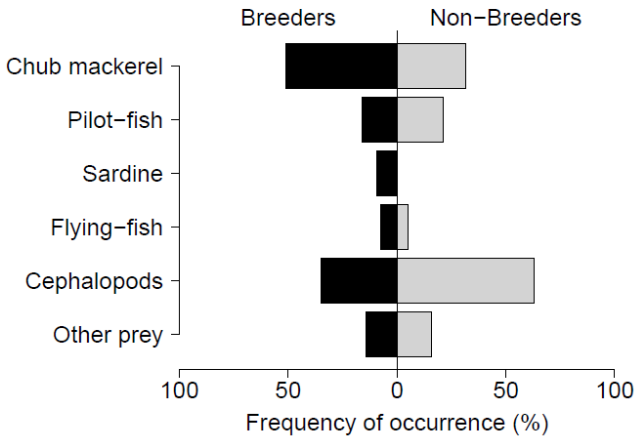

Figure 2.3 Diets of breeders (N = 248 diet samples) and non-breeders (N = 19) among Cory's shearwaters *Calonectris diomedea* during the chick-rearing period of 2009 (Frequency of occurrence, %).

2.4.4 Sex and inter-annual variations in diet

During the chick-rearing period, we found significant dietary differences between sexes (pseudo-$F_{1,458}$ = 10.04 p < 0.001; Table 2.3). Females delivered significantly more chub mackerel to their chicks than males. In contrast, males provided the chicks with more sardines (Table 2.3). We also found significant inter-annual variations in the diet of shearwaters (pseudo-$F_{2,458}$ = 8.13, p < 0.001), which were particularly noticeable in 2010, when the consumption of sardine and cephalopods was higher (Table 2.2), in comparison to previous years.

Table 2.3 Frequency of occurrence (FO %) of prey in the diet of male and female Cory's shearwaters (*Calonectris diomedea*), during the chick-rearing period of 2008, 2009 and 2010, at Selvagem Grande island. Sample size is presented in brackets. Differences among sexes were tested using a binomial GLM with a logit link function, controlling for the effect of year.

	Males (316)	**Females (191)**	**Sex**		**Year**	
	FO (%)	**FO (%)**	**F**	**p**	**F**	**p**
Fish	88.0 (278)	94.2 (180)	5.3	**< 0.05**	0.08	0.92
*Scomber colias/*sp.	38.0 (120)	58.6 (112)	21.7	**< 0.001**	5.9	**< 0.01**
Naucrates ductor	15.8 (50)	13.6 (26)	0.5	0.50	0.6	0.55
Sardina pilchardus	16.1 (51)	8.9 (17)	5.5	**< 0.05**	6.0	**< 0.01**
Trachurus sp.	4.7 (15)	2.1 (4)	2.3	0.13	0.4	0.69
Exocoetidae	10.1 (32)	5.8 (11)	2.9	0.09	0.9	0.41
Cephalopods	35.4 (112)	34.0 (65)	0.10	0.74	8.9	**< 0.001**

2.4.5 Seasonal variations in diet

The diet of shearwaters was substantially different among periods of the same year (pseudo-$F_{1,199} = 17.55$, $p < 0.001$). In the pre-laying period of 2010, trumpet fish (*Macroramphosus scolopax*; FO = 36.7%, NF = 83.3%) and horse/blue jack mackerel (*Trachurus* sp.; FO = 40.0%, NF = 9.7%) dominated the diet of Cory's shearwaters (Figure 2.2, Table 2.2). In this period, cephalopods were found to occur less frequently (FO = 13.3%, NF = 1.3%), than during the chick-rearing period of the same year (Figure 2.2, Table 2.2). Other fish, particularly sardine and pilot-fish were frequent during the chick-rearing period, but completely absent from the diet during the pre-laying stage (Figure 2.2, Table 2.2).

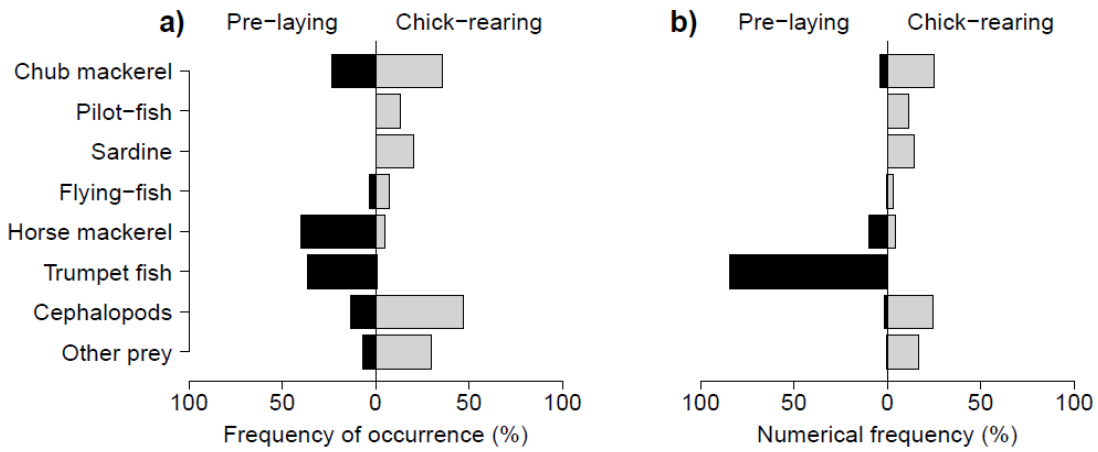

Figure 2.2 Diet of Cory's shearwaters *Calonectris diomedea* in the pre-laying (30 diet samples with 318 prey) and chick-rearing (188 diet samples with 553 prey) periods of 2010: a) Frequency of occurrence of each prey type (%), b) Numerical frequency of each prey type (%).

2.5 Discussion

This study of the diet of Cory's shearwaters from Selvagem Grande provides detailed information on the feeding ecology of this species. We used morphological and DNA barcoding methods in a complementary way to characterise and quantify the dietary composition of a pelagic seabird, showing the advantages of combining both techniques in diet studies of marine predators. The large number of samples collected during the provisioning period revealed dietary differences between birds of different breeding status and between sexes, something rarely shown to occur in seabirds.

2.5.1 The use of DNA barcoding in prey identification

DNA barcoding greatly improved our knowledge of Cory´s shearwater prey range, identifying species that would be overlooked in an analysis based solely on morphological traits. This was the case for small mesopelagic prey (such as myctophids), but also of some epipelagic and bathypelagic species that tend to be underestimated in morphological analyses due to a lack of representation in reference collections. Moreover, DNA barcoding proved to be effective in the identification of juvenile cephalopods, such as the neon flying-squid, a dominant prey in the diet of Cory's shearwater. Indeed, the identification of cephalopods from their beaks is

challenging, particularly for small individuals, as many diagnostic characters only develop later in life.

In generalist predators such as seabirds, prey species can only be identified if a comprehensive database of reference sequences across different prey groups exists (Hebert et al. 2003). Mitochondrial 16S rRNA sequences are the most widely used marker in marine systems and provided the most extensive database of sequences relevant to the potential prey of Cory´s shearwaters. We found that inter- and intra-specific variability of the 16S barcode was effective for accurate prey species discriminations in teleosts and squid, with GenBank reference sequences showing high percentage matches in BLAST and congeners clustering monophyletically in the NJ and DO trees. Depending on the studied species and on the potential prey, the 16S mtDNA gene seems to be a reliable marker for dietary analysis of marine predators.

The incompleteness of reference databases has been widely acknowledged as the main factor limiting accurate taxonomic assignments using DNA barcodes (Valdez-Moreno et al. 2012), but is also a limiting factor in morphological analyses. In the case of the families Trichiuridae and Synaphobranchidae only a few species and genera are represented in the GenBank database and, therefore, only family level assignments were obtainable. The expansion of the taxonomic and geographic scope of fish and cephalopod reference material in GenBank, particularly for oceanic species, is needed to disentangle the identification of closely related species.

Prey identified in the families Exocoetidae and Carangidae produced very similar BLAST matches to different genera and species in the GenBank database. In the case of Exocoetidae, query sequences produced matches with percentage of similarity > 98% even between different genera. Although most of our sequences could be reliably assigned to genus level based on the trees, many genera were polyphyletic suggesting that evolutionary relationships between these genera are unclear (especially *Cheilopogon*). These findings may be an artefact inherent to the use of single markers, which represent only a small snapshot of the evolutionary history of species. However, studies using mitochondrial and nuclear markers (cytb and RAG2) have also reported polyphyly of the genus *Cheilopogon* (Lewallen et al. 2011). Therefore, species assignments based on divergence thresholds should be interpreted with caution in these groups. Regardless of the success in species identification, DNA barcoding also allowed

identification of a high number of MOTUs, reflecting the diversity of flying-fishes around the Selvagens islands.

The complementary use of DNA and conventional methods allowed us to identify fish vertebrae of particular species (e.g., *Ranzania laevis*, *Katsuwonus pelamis*) and to use those in subsequent identification and quantification, bridging some of the gaps in our morphological reference collection. We should note that it is not always possible to collect viable tissue samples from digested prey remains (frequent in Procellariiform diet samples) in order to perform genetic analysis. Therefore, relying on a combined approach, we were able to maximise the identification and quantification of different types of prey.

2.5.2 Diet of non-breeders

The diet of non-breeding/immature seabirds has been seldom studied, mainly due to the difficulty of obtaining a sufficient number of samples at the breeding colony (Barrett et al. 2007; Granadeiro et al. 2009). Most researchers have relied on the isotopic analysis of tissues, and suggested that immatures feed at a lower trophic level (Forero et al. 2002; Votier et al. 2011), but the lack of taxonomic resolution of this approach prevented a better understanding of those differences.

At Selvagem Grande, a high number of non-breeding individuals, mostly composed by immature individuals, attend the colony during the breeding period (Granadeiro et al. 2009). We found that, during the chick-rearing period (August/September), the diet of non-breeders was substantially different from that of breeders, with a higher incidence of cephalopods (FO = 63.2% versus 35.1%) in the former group. Furthermore, in June of the same year, the incidence of cephalopods (mostly neon-flying squid) in the diet of non-breeders was even higher (FO = 93.9%). These results strongly point towards an ontogenetic shift in the trophic niche, the causes of which need to be evaluated by further studies. Non-breeders are less constrained by the need to attend the nesting colony and as such we would have expected them to feed more on distant (coastal) prey. However, the opposite pattern was revealed by our data, as squid in our system is more often captured in offshore waters (unpublished data). Does this differentiation reflect a difference in foraging abilities of breeders and non-

breeders? Does it reflect a different prey selection by adults when provisioning their offspring? Or could non-breeders be forced, by the competitively superior breeders, out of the rich feeding areas of the coastal upwelling (Ramos et al. 2013)? Our results urge more research in this area. Given the potential susceptibility of pelagic seabirds, such as the Cory's shearwater, to mortality linked to fishing vessels (Belda & Sanchez 2001) and to changes in the availability of their prey (Paiva et al. 2013), these results have clear implications. They suggest that different segments of seabird populations are likely to respond differently to ecosystem changes, or to the impacts of human activities, and those need to be taken into account, for example, in demographic modeling (Oro et al. 2010).

2.5.3 The influence of sex on diet

Direct evidence of sex-related dietary differences in pelagic seabirds is scarce (e.g., Xavier & Croxall 2005, Castillo-Guerrero et al. 2011) and most studies that investigated this issue were based on a small number of samples (e.g., Zavalaga et al. 2007; Xavier et al. 2011). Despite that, many studies (mostly based on stable isotopes or tracking) clearly showed the existence of a sex-related spatial or isotopic segregation in several seabird populations, often linked to sexual dimorphism (Phillips et al. 2011). We found clear dietary differences between sexes in Cory's shearwaters during the chick-rearing period, with males feeding more on sardines and less on chub mackerel than females. Despite the marked morphologic differences between sexes (Navarro et al. 2009; Ramos et al. 2009), sex-related differences in the diet or in foraging ranges of Cory's shearwaters were not found in previous studies (Navarro et al. 2007; Navarro et al. 2009; Xavier et al. 2011).

Male Cory's shearwaters are heavier, with larger bills and longer wings than females (Navarro et al. 2009; Ramos et al. 2009). It is possible that the higher wing load of males could provide them with greater mobility (Ramos et al. 2009) and enable them to increase their foraging range, in relation to females. Indeed, Cory's shearwaters from Selvagem Grande are known to prey on sardines mostly during long-distance foraging trips along the African coast (unpublished data). Weimerskirch et al. (2006) also described a greater foraging range of females in relation to males in red footed boobies

Sula sula, presumably due the larger size of females. However, sexual divergence in provisioning or foraging specialisation could also explain diet differences (Phillips et al. 2004) and this issue requires further investigation.

2.5.4 Inter-annual and seasonal variations in diet

There were inter-annual differences in the occurrence of some prey species in the diet of the shearwaters, namely sardines and cephalopods, which were more frequent in 2010. Cory's shearwaters are generalist predators (Thibault et al. 1997) and it is likely that these temporal variations may reflect a change in the abundance or availability of their main prey. However, inter-annual differences were smaller than variations linked to season and to foraging domain, found in this and in other studies (Paiva et al. 2010; Neves et al. 2012). Our results also contrast with previous studies at the Azores, where much more marked inter-annual variations in the consumption of fish and cephalopods were detected (Granadeiro et al. 1998; Paiva et al. 2010; Xavier et al. 2011; Neves et al. 2012). This suggests that the marine environment in the vicinity of the Selvagens Islands presented limited inter-annual changes in summer, which may be a general feature of these pelagic subtropical waters.

The diet of Cory's shearwaters was substantially different between the pre-laying and chick-rearing periods. During the pre-laying period, shearwaters fed mainly on trumpet fish and horse/blue jack mackerel. These prey species were of low importance during the chick-rearing period, when shearwaters increased the consumption of chub mackerel, sardine and pilot-fish. This variation in diet could be related to increased selectivity in prey choice, since parents are expected to select larger or higher-quality prey for their chicks (Wilson et al. 2004). Moreover, foraging areas explored by Cory's shearwaters are known to vary through the breeding season (e.g., Navarro et al. 2007), possibly contributing to these striking seasonal changes in diet.

2.6 Conclusions

Our study highlights the importance of combining different techniques to accurately describe the diet of a pelagic seabird. The use of DNA barcoding and

morphological analysis proved to be very efficient to study the diet of Cory´s shearwaters, by improving both the taxonomical resolution and the quantification of prey species. This approach is likely to be useful in future seabird dietary studies. We also show the occurrence of trophic segregation between birds of different breeding status and sex, highlighting the need to further investigate the dietary choices of different population segments. Understanding the sources of dietary variation within a seabird population will be important for instituting appropriate conservation or population management measures.

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Authors contributions

JPG, PC, HA and SW designed the study; PC, JPG, JAR and WOCS provided guidance during the study; HA, JPG and PC collected the data in the field; HA and JX identified and quantified prey trough conventional methods; HA and JPG analysed the data; SW performed the molecular part of the study; HA, SW, PC, JPG, and WOCS wrote the manuscript.

Data accessibility

Sequence data have been deposited to GenBank (accession numbers KC603479–KC603537). Input files (for POY, Mega 6 and R), R-codes and analyzed datasets have been deposited in Dryad.

2.8 References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403–410.

Barnett A, Redd KS, Frusher SD, Stevens JD, Semmens JM (2010) Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. *Journal of Experimental Marine Biology and Ecology*, **393**, 188–192.

Barrett RT, Camphuysen KCJ, Anker-Nilssen T, Chardine JW, Furness RW, Garthe S, Hüppop O, Leopold MF, Montevecchi WA, Veit RR (2007) Diet studies of seabirds: a review and recommendations. *ICES Journal of Marine Science*, **64**, 1675–1691.

Belda EJ, Sanchez A (2001) Seabird mortality on longline fisheries in the western Mediterranean: factors affecting bycatch and proposed mitigating measures. *Biological Conservation*, **98**, 357–363.

Bowen WD, Iverson SJ (2013) Methods of estimating marine mammal diets: A review of validation experiments and sources of bias and uncertainty. *Marine Mammal Science*, **29**, 719–754.

Bowser AK, Diamond AW, Addison JA (2013) From Puffins to Plankton: A DNA-Based Analysis of a Seabird Food Chain in the Northern Gulf of Maine. *PloS ONE*, **8**, e83152.

Castillo-Guerrero JA, Mellink E (2011) Occasional inter-sex differences in diet and foraging behavior of the Blue-footed Booby: maximizing chick rearing in a variable environment? *Journal of Ornithology*, **152**, 269–277.

Dai L, Zheng X, Kong L, Li Q (2012) DNA barcoding analysis of Coleoidea (Mollusca: Cephalopoda) from Chinese waters. *Molecular Ecology Resources*, **12**, 437–447.

Daunt F, Wanless S, Harris MP, Money L, Monaghan P (2007) Older and wiser: improvements in breeding success are linked to better foraging performance in European shags. *Functional Ecology*, **21**, 561–567.

Deagle BE, Chiaradia A, McInnes J, Jarman SN (2010) Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? *Conservation Genetics*, **11**, 2039–2048.

Deagle BE, Gales NJ, Evans K, Jarman SN, Robinson S, Trebilco R, Hindell MA (2007) Studying seabird diet through genetic analysis of faeces: a case study on macaroni penguins (*Eudyptes chrysolophus*). *PLoS ONE*, **2**, e831.

De Laet (2010) Letter to the editor. A problem in POY tree searches (and its work-around) when some sequences are observed to be absent in some terminals. *Cladistics*, **26**, 453–455.

den Hartog JC , Clarke MR (1996) A study of stomach contents of Cory's Shearwater, *Calonectris diomedea borealis* (Cory, 1881) (Aves: Procellariidae), from the Macaronesian Islands. *Zoologische Mededeelingen*, **70**, 117–133.

Dunn MR, Szabo A, McVeagh MS, Smith PJ (2010) The diet of deepwater sharks and the benefits of using DNA identification of prey. *Deep Sea Research Part I*, **57**, 923–930.

Forero MG, Hobson KA, Bortolotti GR, Donázar JA, Bertellotti M, Blanco G (2002) Food resource utilisation by the Magellanic penguin evaluated through stable-isotope analysis: segregation by sex and age and influence on offspring quality. *Marine Ecology Progress Series*, **234**, 289–299.

Goss-Custard JD, Durell SLVD, McGrorty S, Reading CJ (1982) Use of mussel *Mytilus edulis* beds by oystercatchers *Haematopus ostralegus* according to age and population size. *Journal of Animal Ecology*, **51**, 543–554.

Granadeiro JP (1993) Variation in measurements of Cory's Shearwater between populations and sexing by discriminant analysis. *Ringing and Migration*, **14**, 103–112.

Granadeiro JP, Alonso H, Almada V, Menezes D, Phillips RA, Catry P (2009) Mysterious attendance cycles in Cory's shearwater *Calonectris diomedea*: an exploration of patterns and hypotheses. *Animal Behaviour*, **78**, 1455–1462.

Granadeiro JP, Monteiro LR, Furness RW (1998) Diet and feeding ecology of Cory's shearwater *Calonectris diomedea* in the Azores, north-east Atlantic. *Marine Ecology Progress Series*, **166**, 267–276.

Granadeiro JP, Dias MP, Rebelo R, Santos CD, Catry P (2006) Numbers and population trends of Cory's Shearwater *Calonectris diomedea* at Selvagem Grande, northeast Atlantic. *Waterbirds*, **29**, 56–60.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series,* **41**, 95-98.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London - Biological Sciences,* **270**, 313–321.

Jarman SN, Gales NJ, Tierney M, Gill PC, Elliott NG (2002) A DNA-based method for identification of krill species and its application to analysing the diet of marine vertebrate predators. *Molecular Ecology*, **11**, 2679–2690.

Jarman SN, McInnes JC, Faux C, Polanowski AM, Marthick J, Deagle BE, Southwell C, Emmerson L (2013) Adélie Penguin Population Diet Monitoring by Analysis of Food DNA in Scats. *PloS ONE*, **8**, e82227.

Jedlicka JA, Sharma AM, Almeida RP (2013) Molecular tools reveal diets of insectivorous birds from predator faecal matter. *Conservation Genetics Resources*, **5**, 879–885.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, **16**, 111–120.

Kitowski I (2003) Age-related differences in foraging behavior of Montagu's harrier *Circus pygargus* males in south-east Poland. *Acta Ethologica*, **6**, 35–38.

Kochzius M, Seidel C, Antoniou A, Botla SK, Campo D, Cariani A, Vazquez EG, Hauschild J, Hervet C, Hjorleifsdóttir S, Hreggvidsson G, Kappel K, Landi M, Magoulas A, Marteinsson V, Nolte M, Planes S, Tinti F, Turan C, Venugopal MN, Weber H, Blohm D (2010) Identifying fishes through DNA barcodes and microarrays. *PLoS ONE,* **5**, e12620.

Lewallen EA, Pitman RL, Kjartanson SL, Lovejoy NR (2011) Molecular systematics of flyingfishes (Teleostei: Exocoetidae): evolution in the epipelagic zone. *Biological Journal of the Linnean Society,* **102**, 161–174.

Lucifora LO, García VB, Menni RC, Escalante AH, Hozbor NM (2009) Effects of body size, age and maturity stage on diet in a large shark: ecological and applied implications. *Ecological Research*, **24**, 109–118.

Moritz C, Cicero C (2004) DNA Barcoding: Promise and Pitfalls. *PLoS Biology*, **2**, e354.

Mougin J-L, Jouanin C, Roux F (1997) Intermittent breeding in Cory's Shearwater *Calonectris diomedea* of Selvagem Grande, North Atlantic. *Ibis*, **139**, 40–44.

Naito Y, Costa DP, Adachi T, Robinson PW, Fowler M, Takahashi A (2013) Unravelling the mysteries of a mesopelagic diet: a large apex predator specializes on small prey. *Functional Ecology*, **27**, 710–717.

Navarro J, González-Solís J, Viscor G (2007) Nutritional and feeding ecology in Cory's shearwater *Calonectris diomedea* during breeding. *Marine Ecology Progress Series*, **351**, 261–271.

Navarro J, Kaliontzopoulou A, Gonzalez-Solis J (2009) Sexual dimorphism in bill morphology and feeding ecology in Cory's shearwater (*Calonectris diomedea*). *Zoology*, **112**, 128–138.

Neves VC, Nolf D, Clarke M (2012) Spatio-temporal variation in the diet of Cory's shearwater *Calonectris diomedea* in the Azores archipelago, north-east Atlantic. *Deep Sea Research Part I*, **70**, 1–13.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H (2011) vegan: Community Ecology Package. R Package Version 2.0-2. http://CRAN.R-project.org/package=vegan

Oro D, Torres R, Rodríguez C, Drummond H (2010) Climatic influence on demographic parameters of a tropical seabird varies with age and sex. *Ecology*, **91**, 1205–1214.

Paiva VH, Xavier JC , Geraldes P, Ramírez I, Garthe S, Ramos JA (2010) Foraging ecology of Cory's shearwaters in different oceanic environments of the North Atlantic. *Marine Ecology Progress Series*, **410**, 257–268.

Paiva VH, Geraldes P, Marques V, Rodríguez R, Garthe S, Ramos JA (2013) Effects of environmental variability on different trophic levels of the North Atlantic food web. *Marine Ecology Progress Series*, **477**, 15–28.

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Mable BK, Moritz C (eds) Molecular Systematics. Sinauer, Sunderland, MA, pp 205–247.

Phillips RA, McGill RAR, Dawson DA, Bearhop S (2011) Sexual segregation in distribution, diet and trophic level of seabirds: insights from stable isotope analysis. *Marine Biology*, **158**, 2199–2208.

Phillips RA, Silk JRD, Phalan B, Catry P, Croxall JP (2004) Seasonal sexual segregation in two *Thalassarche* albatross species: competitive exclusion, reproductive role specialization or foraging niche divergence? *Proceedings of the Royal Society of London - Biological Sciences*, **271**, 1283–1291.

Polis GA (1984) Age structure component of niche width and intraspecific resource partitioning: can age groups function as ecological species? American Naturalist, 123, 541–564.

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SD, Taberlet P (2012) Who is eating what: diet assessment using next generation sequencing. Molecular Ecology, 21, 1931–1950.

Quéro J-C, Porché P, Vayne JJ (2003) Guide des Poissons de l'Atlantique Européen. Delachaux & Niestlé, Paris.

R Development Core Team (2010) R: A Language and Environment for Statistical Computing (2.10.1). R Foundation for Statistical Computing, Vienna

Ramos JA, Granadeiro JP, Phillips RA, Catry P (2009) Flight morphology and foraging behaviour of male and female Cory's Shearwaters. *Condor*, **111**, 424–432.

Ramos R, Granadeiro JP, Rodríguez B, Navarro J, Paiva VH, Bécares J, Reyes-González JM, Fagundes I, Ruiz A, Arcos P, González-Solís J, Catry P (2013) Meta-population feeding grounds of Cory's shearwater in the subtropical Atlantic Ocean: implications for the definition of Marine Protected Areas based on tracking studies. *Diversity and Distributions*, **19**, 1284–1298.

Symondson WOC (2002) Molecular identification of prey in predator diets. *Molecular Ecology*, **11**, 627–641.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA 6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, **30**, 2725–2729.

Thibault J-C, Bretagnolle V, Rabouam C (1997) *Calonectris diomedea* Cory's Shearwater. *Birds of the Western Palearctic Update*, **1**, 75–98.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.

Tuset VM, Lombarte A, Assis CA (2008) Otolith atlas for the western Mediterranean, north and central eastern Atlantic. *Scientia Marina*, **72**, 7–198.

Valdez-Moreno M, Quintal-Lizama C, Gómez-Lozano R, García-Rivas MdC (2012) Monitoring an alien invasion: DNA barcoding and the identification of lionfish and their prey on coral reefs of the Mexican caribbean. *PLoS ONE*, **7**, e36636.

Varón A, Vinh LS, Wheeler WC (2010) POY version 4: phylogenetic analysis using dynamic homologies. *Cladistics*, **26**, 72–85.

Votier SC, Grecian WJ, Patrick S, Newton J (2011) Inter-colony movements, at sea behaviour and foraging in an immature seabird: results from GPS-PTT tracking, radiotracking and stable isotope analysis. *Marine Biology*, **158**, 355–362.

Weimerskirch H, Le Corre M, Ropert-Coudert Y, Kato A, Marsac F (2006) Sex-specific foraging behaviour in a seabird with reversal sexual dimorphism: the red-footed booby. *Oecologia*, **146**, 681–691.

Wilson RP (1984) An improved stomach pump for penguins and other seabirds. *Journal of Field Ornithology*, **55**, 109–112.

Wilson LJ, Daunt F, Wanless S (2004) Self-feeding and chick-provisioning diet differ in the common guillemot *Uria aalge*. *Ardea*, **92**, 197–208.

Wilson JJ, Rougerie R, Schonfeld J, Janzen DH, Hallwachs W, Hajibabaei M, Kitching IJ, Haxaire J, Hebert PDN (2011) When species matches are unavailable are DNA barcodes correctly assigned to higher taxa? An assessment using sphingid moths. *BMC Ecology,* **11**, 1–14.

Xavier JC, Croxall JP (2005) Sexual differences in foraging behaviour and diets: a case study of wandering albatrosses. In: Ruckstuhl KE, Neuhaus P (eds) Sexual

segregation in vertebrates: ecology of the two sexes. Cambridge University Press, Cambridge, pp 74–91.

Xavier JC, Magalhães MC, Mendonça AS, Antunes M, Carvalho N, Machete M, Santos RS, Paiva V, Hamer KC (2011) Changes in diet of Cory's Shearwaters *Calonectris diomedea* breeding in the Azores. *Marine Ornithology*, **39**, 129–134.

Zavalaga CB, Benvenuti S, Dall'Antonia L, Emslie SD (2007) Diving behavior of blue-footed boobies *Sula nebouxii* in northern Peru in relation to sex, body size and prey type. *Marine Ecology Progress Series*, **336**, 291–303.

Zhang J, Hanner R (2012) Molecular approach to the identification of fish in the South China Sea. *PLoS ONE*, **7**, e30621.

2.9 Supplementary material

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

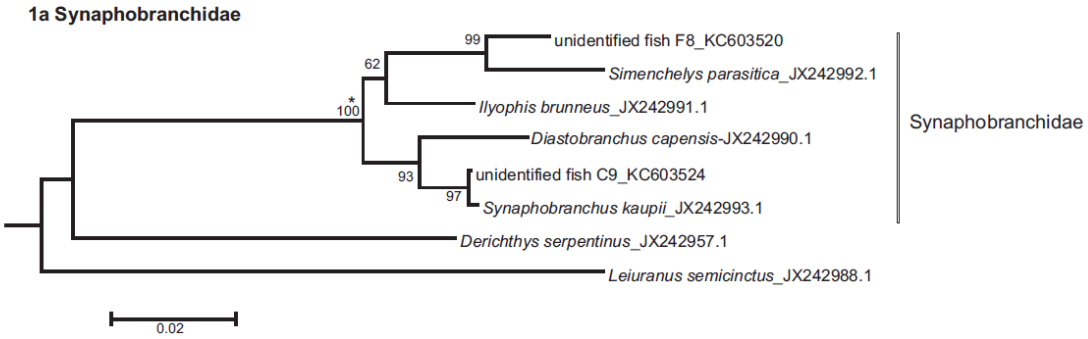

Figure S2.1. NJ tree based method for assignment of morphologically unidentified specimens in Cory's shearwater diet. Positive family level identifications were obtained for the families (a) Synaphobranchidae and (b) Trichiuridae. Specimens were positively assigned when monophyletic clusters with con-genera of the family were obtained. (*) . Represent positive assignments. Bootstrap values are above nodes and are presented for a cut-off value > 50.

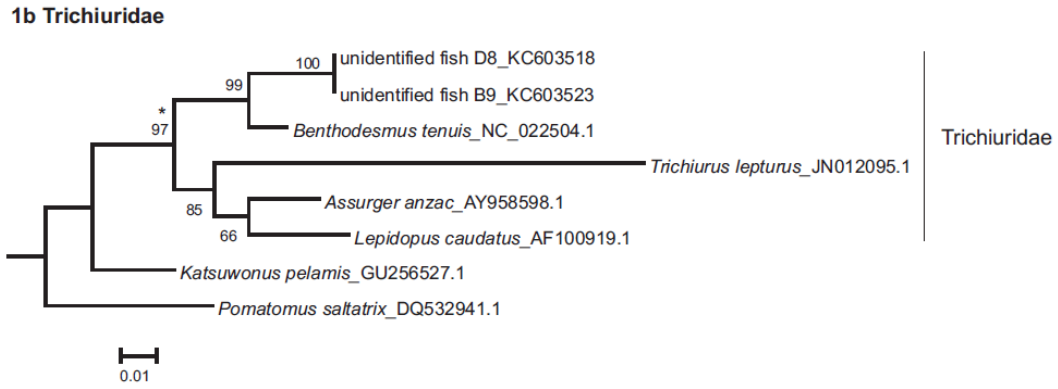

Figure S2.1 Continued

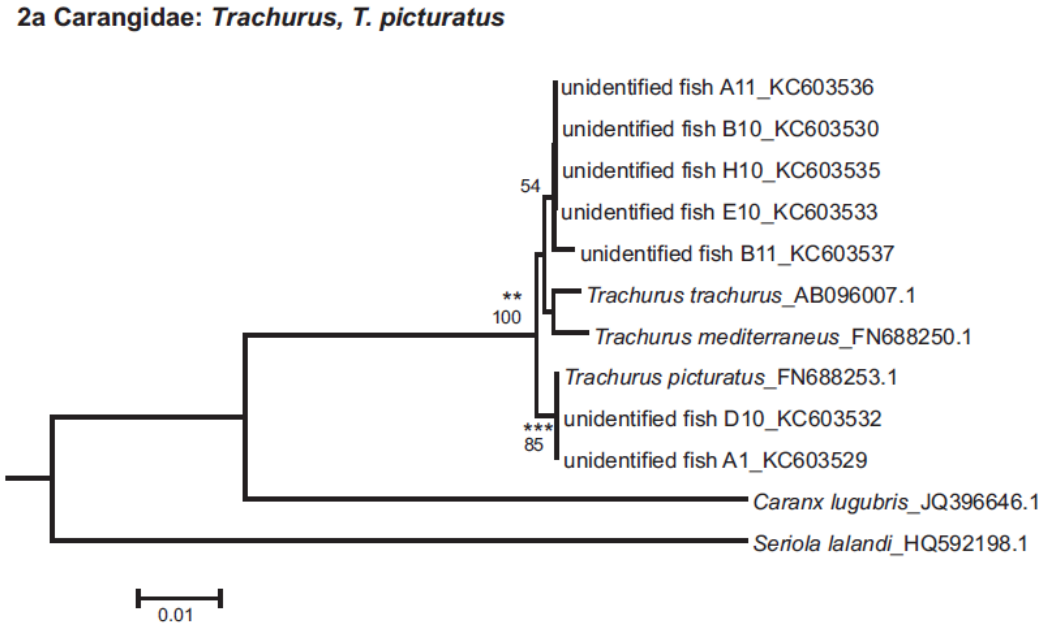

Figure S2. NJ tree based method for assignment of morphologically unidentified specimens in Cory's shearwater diet. Positive genus and species level identifications were obtained within the teleost families a) Carangidae b) Exocoetidae c) Halosauridae (d) Myctophidae (e) Sternoptychidae and cephalopods: f) Histioteuthidae g) Ommastrephidae. Specimens were positively assigned according to a strict criterion. (**) and (***) represent positive genus and species level assignments, respectively. Bootstrap values are above nodes and are presented for a cut-off value > 50

2b Exocoetidae: *Exocoetus sp, Cheilopogon sp, C. pinnatibarbatus, C. melanurus*

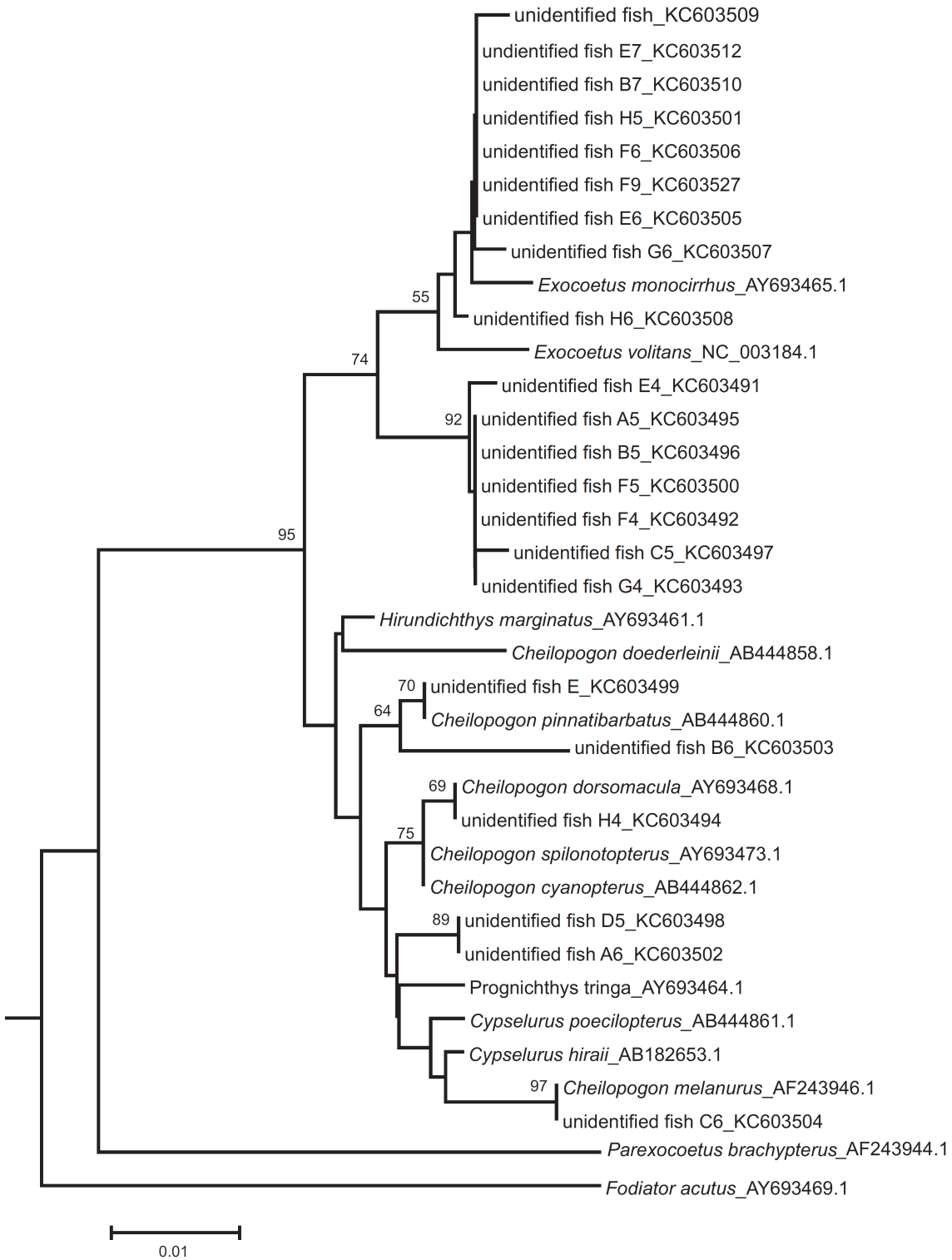

Figure S2.2 Continued

2c Halosauridae: *Halosaurus sp*

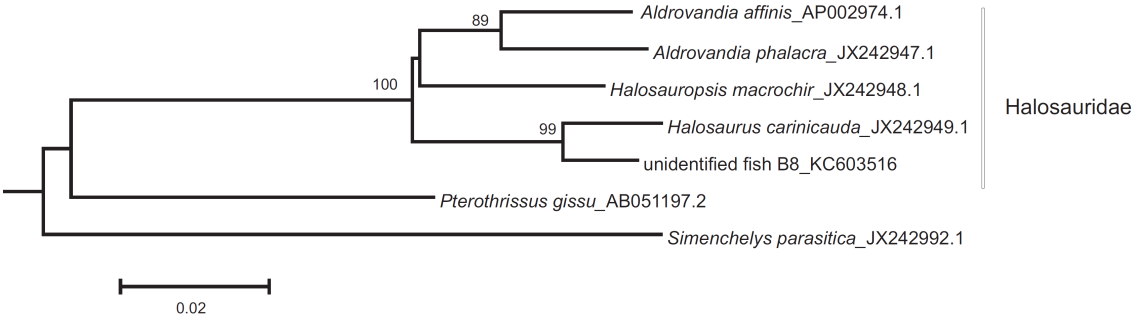

Figure S2.2 Continued

2d Myctophidae: *Diaphus sp*

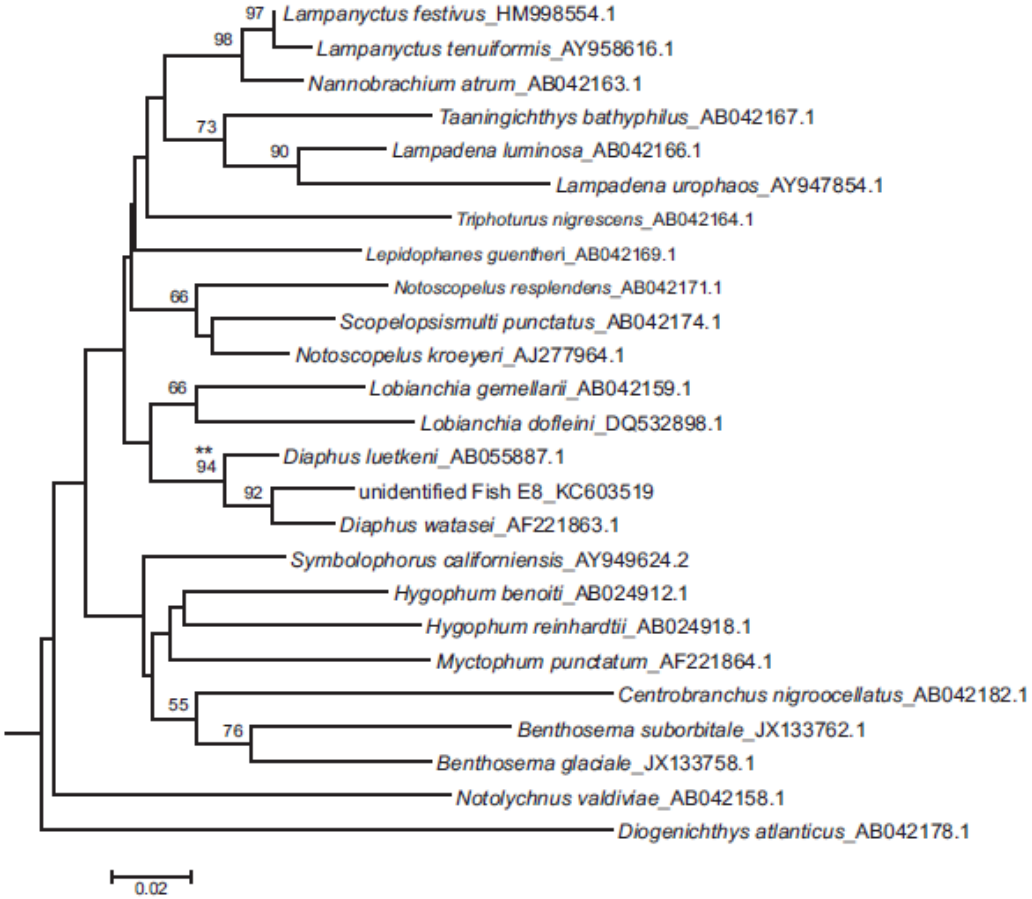

Figure S2.2 Continued

2e Sternoptychidae: *Argyropelecus sp*

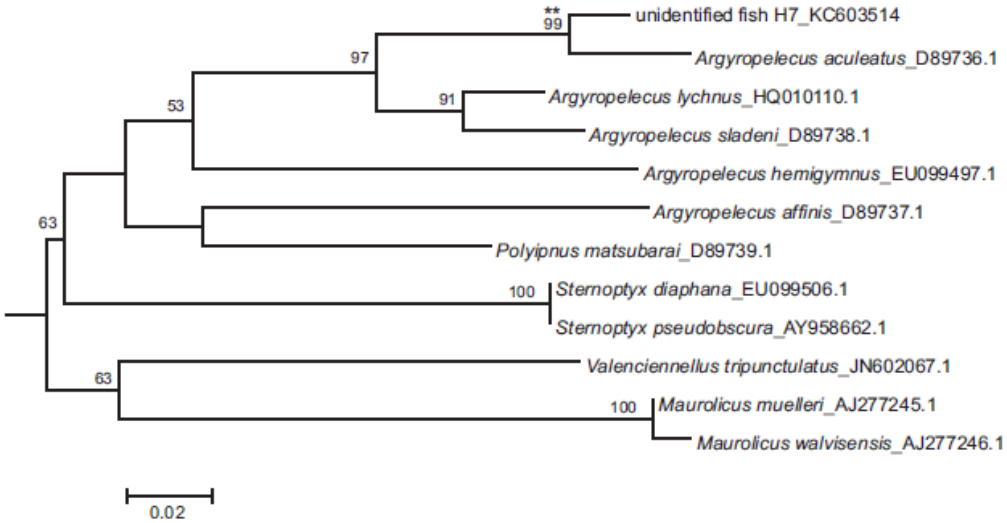

Figure S2.2 Continued

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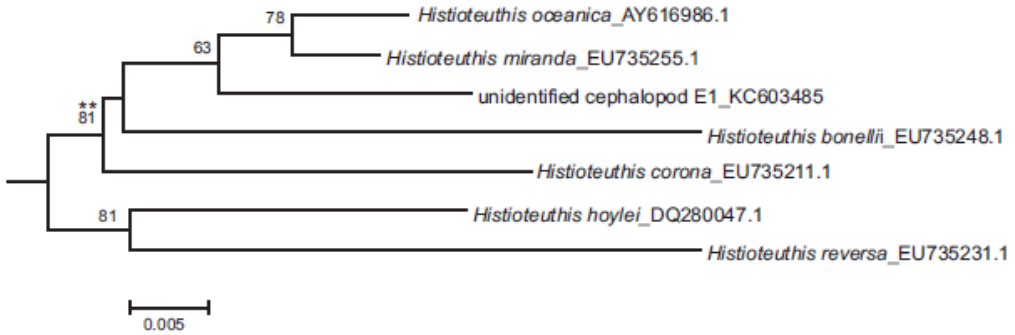

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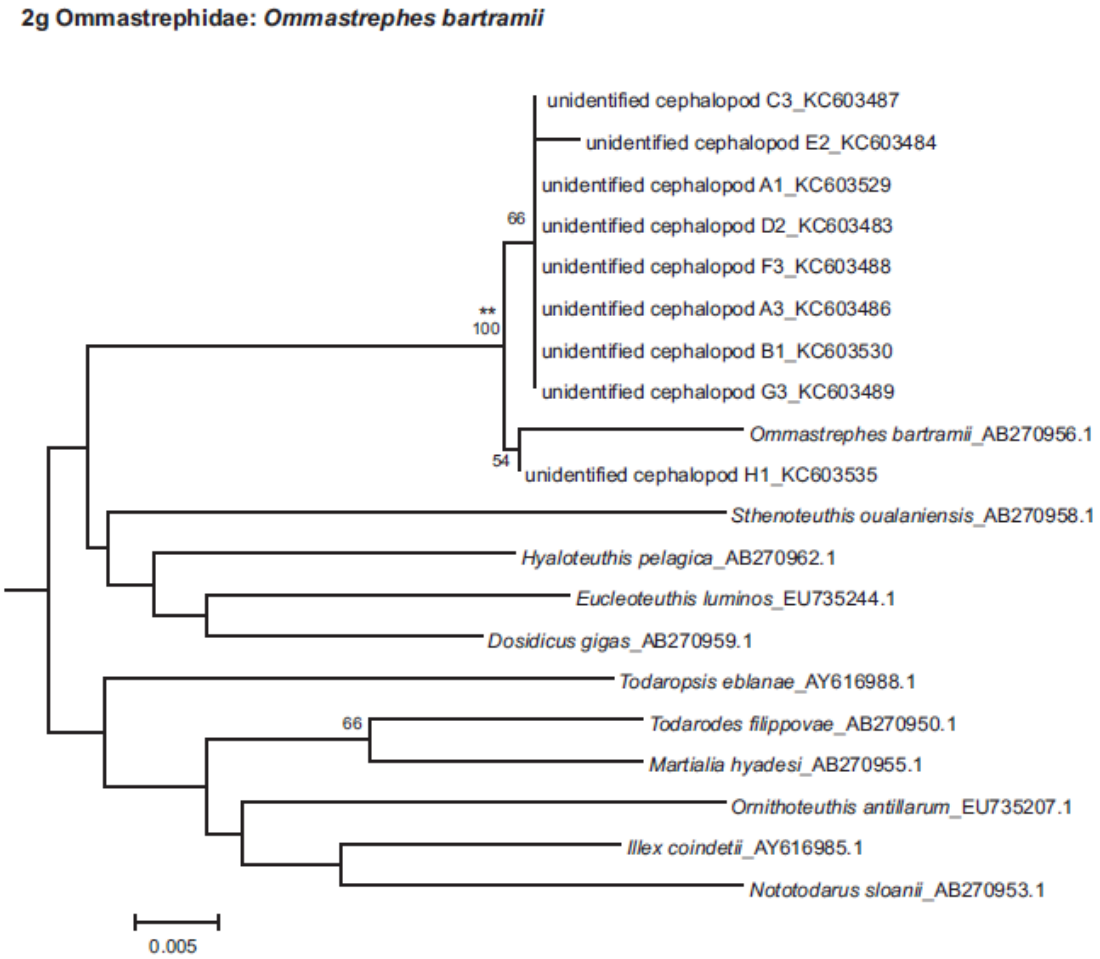

Figure S2.2 Continued

Table S2.3 Genbank accession numbers of prey identified using DNA barcoding**.**

Prey Group	Family	Genus	Species	Number of Individuals	Genbank accession number
Cephalopods	Histioteuthidae	*Histioteuthis*	n.i	1	KC603485
	Ommastrephidae	*Ommastrephes*	*Ommastrephes bartammi*	9	KC603479-KC603480,KC603482-KC603484, KC603486-KC603489
		Chiroteuthis	*Chiroteuthis mega*	1	KC603490
	Cranchidae	*Taonis*	*Taonis pavo*	1	KC603481
Fish	Carangidae	*Trachurus*	*Trachurus picturatus*	2	KC603529, KC603532
			n.i	5	KC603530, KC603533, KC603535-KC603537
	Coryphaenidae	*Coryphaena*	*Coryphaena equiselis*	1	KC603517
	Diretmidae	*Diretmus*	*Diretmus argenteus*	1	KC603521

Exocoetidae	*Cheilopogon*	n.i.	1	KC603494
		Cheilopogon melanurus	1	KC603504
		Cheilopogon pinnatibarbatus	1	KC603499
	Exocoetus	n.i.	16	KC603491-KC603493, KC603495-KC603497, KC603500-KC603501, KC603505-KC603510, KC603512, KC603527
	n.i	n.i	3	KC603498, KC603502-KC603503
Halosauridae	n.i.	n.i.	1	KC603516
Molidae	*Ranzania*	*Ranzania laevis*	2	KC603525-KC603526
Mytophidae	*Mytcophum*	*Myctophum atlantica*	1	KC603522
	Argyropelecus	n.i.	1	KC603514
	Diaphus	n.i	1	KC603519
Neoscopelidae	*Neoscopelus*	*Neoscopelus macrolepidotus*	1	KC603513
Scombridae	*Katsuwonus*	*Katsuwonus pelamis*	2	KC603511, KC603528
	Scomber	*Scomber australasicus*	1	KC603515
Sparidae	*Boops*	*Boops boops*	2	KC603531, KC603534
Synaphobranchidae	n.i	n.i	2	KC603520, KC603524
Trichiuridae	n.i	n.i	2	KC603518, KC603523
			59	

CHAPTER 3:

Phylogenetic placement of mitochondrial 16rRNA barcodes to identify vertebrate and invertebrate prey in a seabird, the Bulwer's Petrel

Phylogenetic placement of mitochondrial 16rRNA barcodes to identify vertebrate and invertebrate prey in a seabird, the Bulwer's Petrel

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Key-words: Evolutionary placement algorithm (EPA), high-throughput sequencing, trophic-interactions, Bulwer's petrel, faeces, stomach contents

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Running title: Evolutionary placement of prey in a seabird

Waap S, Catry P & Symondson WOC. Phylogenetic placement of mitochondrial 16rRNA barcodes to identify vertebrate and invertebrate prey in a seabird, the Bulwer's Petrel (submitted).

Author contributions

WOCS and PC significantly contributed to the experimental design and gave advice and critically reviewed the manuscript. I was the senior author and performed the laboratory work, data analysis and writing.

3.1 Abstract

A central problem in molecular analysis of predator diets is to identify prey species from short DNA barcodes, often with insufficient phylogenetic signal to accurately identify to species level. Most dietary studies have optimised similarity thresholds using short COI barcodes to taxonomically assign query sequences. Such barcodes are, however, very polymorphic and do not always include conserved primer binding sites that would amplify DNA from the wide range of prey eaten by predators with a highly diverse diet. Short non-protein coding barcodes of the relatively more conserved 16S rRNA gene have been proposed as alternative genetic markers, proving more potential primer sites, but have not been standardized for species discrimination among vertebrates and invertebrates.

Here, we phylogenetically placed short 16S rRNA reads (200-230 bp) on a reference tree of 16S rDNA Sanger sequences (494-502 bp) and overcame uncertainty in taxonomical prey assignments by obtaining Maximum Likelihood support in query placements using the Evolutionary Placement Algorithm (EPA). Short reads were obtained from high-throughput sequencing of faecal material from Bulwer's petrels, *Bulweria bulwerii*, while our reference tree included taxa identified from amongst the stomach contents of these birds. We show that this is a robust method for species discrimination in which EPA phylogenetically placed reads agreed with the highest neighbor in BLAST, while providing greater confidence in taxonomic rank assignments. We propose that molecular analyses of the diets of seabirds, and other marine predators, can benefit from this approach.

3.2 Introduction

Birds are important regulators of plant and animal communities and ecosystems (Sekercioglu 2006; Mooney et al. 2010). Therefore, analysis of species diversity in bird diets can elucidate their ecosystem function while revealing potentially influential dietary factors affecting their population dynamics and conservation. Dietary analysis of birds has been a traditional field in ecology, where prey remains are identified morphologically in stomach contents or faeces (Ralph et al. 1985; Duffy & Jackson

1986) or using other technologies such as fatty acid (Iverson et al. 2004) and isotopic signatures in predator blood and feathers (Hobson et al. 1994). Often, however, it is not possible to distinguish species specific diagnostic characters of hard part remains (Tollit et al. 2003), while isotopic and fatty acid signatures are generally low resolution techniques. In such cases, prey identification can be obtained through molecular taxonomy (Tautz et al. 2003; Vogler & Monaghan 2007) using DNA barcoding (Hebert et al. 2003) on prey remains.

Next-Generation Sequencing (NGS) technologies are revolutionizing research on predator-prey interactions through high-throughput sequencing of barcoding genes (Pompanon et al. 2012), though there have been few such studies conducted on birds (Deagle et al. 2007; Browser et al. 2013; Jarman et al. 2013). Here, large numbers of short barcodes ($>10^6$) can be obtained from a sample, allowing detection of prey even from highly degraded faecal material (*metabarcoding*, Taberlet et al. 2012), facilitating the use of non-invasive sampling strategies in molecular dietary studies (Symondson 2002).

Species identification is a critical step in all metabarcoding studies (Coissac et al. 2012). In most NGS dietary studies, queries are grouped into Molecular Operational Taxonomic Units (MOTUs) according to specific genetic distance threshold. Representative MOTUs are then taxonomically identified using identity percentages when compared with reference taxa, for example using BLAST (Altschul et al. 1990) (reviewed in Pompanon et al. 2012). MOTUs that do not produce positive species matches are generally classified as prey species that have not yet been sequenced. However, the extent to which a MOTU represents a species is debatable (Vogler and Monaghan 2007). Sequencing errors during NGS and PCR artifacts can further introduce biases in species counts using MOTUs (Quince et al. 2009).

Phylogenetically aware methodologies comparing anonymous reads to reference species have been proposed as a better proxy to infer the taxonomy of query sequences than sequence similarity-based approaches (Munch et al. 2008; Matsen et al. 2010; Berger et al. 2011). Short query sequences can be placed on a reference tree and assigned depending on its location on the tree. Positive placements occur when queries cluster within the terminal branches of the reference tree, whereas clustering at inner nodes suggests that the reference tree does not represent the entire diversity of queries (Berger et al. 2011) and assignments are conducted at broader taxonomic ranks. Current

phylogenetic algorithms for placement of short NGS reads on a reference tree include the pplacer (Matsen et al. 2010) and the Evolutionary Placement Algorithm (EPA) (Berger et al. 2011). Both compute the maximum likelihood of insertion of queries in a specific branch on the reference tree, providing more robust taxonomic identification.

Based on the premise that phylogeny-based approaches outperform those solely based on sequence similarity, we applied this approach to dietary analysis, specifically to assess the prey of seabirds. Birds and other top-predators possess specific constraints that make prey identification difficult when based solely on sequence similarity approaches. For instance, Bulwer's petrels, like many other birds, forage on highly diverse taxa, comprising numerous orders of fish and cephalopods. Such a diverse diet means that short standardized COI barcodes are too variable to provide common binding sites for the design of primers that will amplify the entire diversity of prey. Mitochondrial 16S rRNA barcodes provide, conversely, conserved sites flanking hypervariable DNA regions, thus allowing amplification of a broader range of taxa, while providing high taxonomical resolution (Deagle et al. 2014). Large numbers of 16S rRNA reference sequences are also available in public databases (e.g. GenBank) as this barcode has been commonly used for phylogenetic reconstructions of distantly related animals. However, 16S rRNA barcodes are not standard markers for species delimitation of vertebrates and invertebrates and similarity thresholds representing inter-specific variability have not been comprehensively tested (but see Kochzius et al. 2010; Zhang & Hanner 2012; Dai et al. 2012).

We performed high-throughput sequencing of short 16S rRNA barcodes from faecal material collected from Bulwer's petrels and constructed two different reference trees for fish and cephalopods to pylogenetically place query sequences. The principal aim of this study was to provide robust taxonomic assignments of Bulwer's petrel prey based on non-standard barcodes. We show that similarity percentage thresholds of 16S rRNA barcodes cannot be accurately obtained for taxonomic identification of vertebrate and invertebrate prey in Bulwer's petrels. However, EPA-based placements on both reference trees provided good support for prey assignments and in the taxonomic ranks identified.

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3.3 Material and Methods

3.3.1 Ethical statement

The fieldwork of this study was approved by the authorities involved and was undertaken under the research permits 107/2011 and 107/2012, provided by the Instituto da Conservação da Natureza e da Biodiversidade and by the Serviço do Parque Natural da Madeira, Portugal. Single stomach content flushing of birds has previously shown no significant effect on chick survival and growth (Clarke & Kerry 1994; Phillips 2006). Handling of birds for faecal collection showed no visible deleterious effects with all birds flying after release.

3.3.2 Sample collection

A total of 92 faeces were collected at Selvagem Grande (NE Atlantic, Portugal), comprising the chick-rearing phase of Bulwer's petrels during the years of 2011 (n=44) and 2012 (n=48). Adult birds were captured at night, put in artificial nests and released immediately after defecation. The faecal samples were retained for NGS sequencing.

Stomach contents were collected at the above colony in 2012 (n=44). Single-stomach contents were sampled in each chick using the water off-loading procedure described by Wilson et al. (1984). The samples were filtered through a sieve, washed with clean water and preserved in absolute ethanol. Prey remains were isolated for Sanger sequencing purposes.

3.3.3 Primer design

The primers were modified from Chord_16S_F/Chord_16S_R (Deagle et al. 2009) to comprehensively target putative prey in our study system. We modified both the 5´end and the 3´end of forward and reverse primers to enhance specificity with fish and cephalopods. For the two prey groups, therefore, we obtained different primer sets: modifiedChord_16S_F1/R1 and modifiedCeph_16S_F1/R1. Conserved primer binding

sites were identified through alignment of 16SrRNA sequences extracted from GenBank. We included representative sequences of the principal vertebrate and invertebrate orders occurring at our study site, focusing especially on taxa identified in the stomach contents of these birds. We also included degenerated base pairs in equal concentration mixtures to avoid mismatches with target species. To reduce predator amplification we integrated a mismatch with predator DNA at the 3´- end on both forward and reverse primers amplifying chordates. We additionally developed a blocking primer using a C3 spacer (Vestheim & Jarman 2008) to suppress amplification of Bulwer's petrel. We performed *in silico* PCRs on the whole mitochondrial database *mito* (available at http://www.grenoble.prabi.fr/trac/ecoPrimers/wiki/EcoPrimersDB) to evaluate the taxonomical coverage of each primer set using ecoPCR **(**Ficetola et al. 2010**).** *In silico* tests were performed under the option (e=2) allowing maximum two mismatches between the primers and the template sequences. As a single mismatch at the 3´end of primers can substantially lower the extension efficiency of PCR (Huang et al.1992), only exact matches between the template and the 3´end of primers were considered in our analysis. We also measured the taxonomical coverage of each primer pair (*Bc* coverage index) as the ratio of amplified Teleostei (fish) and Cephalopoda (Decapodiformes) species against the total number of species of the same taxonomical ranks in the *mito* database using the ecoTaxStat script (OBITools, Boyer et al. 2014)

3.3.4 DNA extraction, amplification and high-throughput sequencing

Faecal DNA was isolated using the QIAamp ® DNA Stool Mini Kit (Qiagen). Samples were centrifuged for 40 minutes at 10.000 rpm and the storage ethanol removed. DNA was extracted following the manufactures protocol, except that we added an additional proteinase K digestion step by re-suspending the faecal pellets in 1.2 ml of extraction buffer (0.5mM EDTA, SDS, Tris-HCL) and 20 µl of proteinase K (Qiagen). The samples were then incubated at 56ºC overnight.

Faecal DNA was amplified via a two-step PCR approach (Figure S3.1) similar to Berry et al. (2011). First-step PCRs used the primer sets modifiedChord_16S_F1/R1 and modifiedCeph_16S_F1/R1, each primer with a M13 tail at the 5´-end attached.

Amplicons generated in this step included the target fragment, the primers, and M13 tails at the terminals. In the second step, MID tagged adaptor sequences required by the ion torrent chemistry were added via PCR of amplicons generated in 1-step PCR (Figure S3.1). By so doing, multiple primers sets or loci can be used in NGS analysis at the same tag costs as single primers or loci.

PCR reactions were performed in single reactions in 10 µl of total volume using 1× Multiplex PCR Master Mix (Qiagen), 0.25uM of each primer and 2.5ul of DNA (1-step PCR). PCRs using the primer set modifiedChord_16S_F1/R1 included 10uM of blocking probes. For 2-step PCRs, we used the same reagent concentrations, except that we added as template 2µl of diluted amplicon (1:100) from 1-step PCR. PCR conditions in 1-step were as follows: 95ºC for 15 min; 35 cycles at 94 ºC for 30 sec, 60ºC for 1 min 30 sec and 72ºC for 45 sec; and a final extension of 2 min. PCR conditions in 2-step were 95ºC for 15 min; 15 cycles at 94 ºC for 30 sec, 60ºC for 1 min 30 sec and 72ºC for 1min 30 sec; and a final extension of 7 min.

The fluorescence of each band was quantified on a 2% agarose gel stained with EtBr and compared with a known concentration of ladder (Promega) using UVP VisionWorks ® LS Analysis Software. Amplicons were pooled into equimolar libraries (fish and cephalopods) and purified using the QIAquick Gel Extraction kit (Qiagen). High-throughput sequencing was conducted on an Ion Personal Genome Machine (IPM) using 400 bp chemistry at the Centre de Recerca en Agrigenòmica (CRAG), Barcelona.

3.3.5 Reference dataset, alignment and tree

To build a reference dataset for phylogenetic placement of query sequences, we first identified prey collected from the stomach contents of Bulwer's petrel chicks. Pieces of tissue were isolated and separately sequenced (Chapter 5). Two different approaches were used to amplify DNA from cephalopods and fish. For fish we performed standard barcoding using a COI cocktail (Ivanova et al. 2007) and retrieved species identities based on the criteria established in BOLD (Ratnasingham & Hebert 2007). We applied this approach also to cephalopods, but amplification success was very low and the BOLD database was further poorly represented for this group, with

queries matching references only at lower taxonomic ranks. Therefore, we obtained 16S rRNA sequences from cephalopod using the primer set 16Sar/16Sbr (Palumbi et al. 1996) and assigned Sanger sequences (see Figure 5.1 of Chapter 5). DNA extractions and PCRs reagent conditions followed those of Alonso et al. (2014). PCR conditions for fish were the same as in Ivanova et al. (2007) and in cephalopods the same as in Alonso et al. (2014). Sanger sequences were obtained through outsourcing at Macrogen, Inc (Amsterdam, Netherlands).

Once we obtained the taxonomical composition of taxa collected from stomach contents (see Chapter 5), we extracted representative mitochondrial 16S rRNA sequences from GenBank to build two different reference trees, for cephalopods and fish. For each tree, we further added other Genbank sequences to augment taxon sampling on the reference tree. We performed multiple sequence alignment in SATé-II. This program co-estimates alignments and trees simultaneously augmenting alignment accuracy of large datasets. Alignment and trees were inferred with MAFFT (Katoh & Standley 2013) MUSCLE (Edgar 2004) and FASTTREE (Price et al. 2010) under the GTR + Gamma model of evolution. Maximum likelihood reference trees were inferred in RAxML (Stamatakis et al. 2006) using the graphical GUI version 1.31 (Silvestro & Michalak 2012) under the GTR + Gamma model of evolution. Support values were obtained using thorough and rapid bootstrapping with 1000 replicates.

3.3.6 NGS data analysis

The program cutadapt-1.5 (Martin 2014) was used to remove adaptor sequences at the 3´- end of reads and remove sequence terminals with a lower quality than phred quality score=26. To obtain dietary composition of each sample, reads were de-multiplexed into forward and reverse MID tag combinations using barcode-splitter of the fastx-toolkit (Gordon 2010). M13 tails and primer sequences were removed for each sample using BioEdit (Hall et al. 1999). To group sequences into MOTUs we used the pipeline of UPARSE (Edgar 2013), where sequences were de-replicated, sorted according to their abundances, and singletons and chimera sequences removed. Sequences were grouped into MOTUs at 98% of similarity and mapped for each sample to obtain the frequency of occurrences (FO) of each prey sequence. A MOTU was

considered present in a sample providing > 5 sequences were obtained. We performed BLAST (Altschul 1990) comparisons to separate MOTUs into higher taxonomical groups, i.e. cephalopods and fish and removed sequences producing no match or contaminants that occur commonly in dietary analysis (bacterial, human and predator DNA) for downstream phylogenetic placement. MOTUs were aligned and assigned phylogenetically to the tips of the reference tree using the EPA web server (Berger et al. 2011). MOTUs showing uncertain placements on the reference trees, with maximum likelihood weights less than 0.8, were not positively assigned, but identified: 1. to species, if they clustered with reference taxa for ML likelihood weights > 0.99, providing reference congenera were represented on the tree or were monotypic, 2. to genera if queries were placed with ML likelihood > 0.90 but other congenera were not included in the tree for that family and 3. to families, if queries clustered monophyletically within clades containing the members of that family.

3.4. Results

3.4.1 Primers design

The primer sets and blocking primers in this study are presented in Table 3.1. *In silico* tests using ecoPCR showed that both primer sets amplify a broad range of fish and cephalopod species within the whole mitochondrial database (Bc coverage index =97.5, taxonomic rank=Teleostei; Bc coverage index=88.9%, taxonomic rank=Decapodiformes). Although primers were designed so as to augment specificity for cephalopods and fish, cross-amplification was recorded between both prey groups (i.e. fish primers also amplified some cephalopod sequences and cephalopod primers amplified fish sequences). The incorporation of the ion torrent adaptor sequences in the 2-step PCR recovered the same amplification success (measured by the presence of amplicon bands on agarose gel) as in 1-step PCR.

Table 3.1. Primers used in this study to amplify short 16SrDNA barcodes for NGS analysis. M13 forward and reverse tails were attached to the 5′end of primers and used in 1-step PCR. Adaptors plus MID tags were added to amplicons in 2-step PCR

Primers	Sequence (5′- 3)
modifiedChord_16S_F1	FR: 5′- CGAGAAGACCCTDTGRAG - 3′
modifiedChord_16S_R1	RV: 5′- GCTGTTATCCCTRGRGTAA - 3′
modifiedCeph_16S_F1	FR: 5′-AGGGACGAGAAGACCCTANTGAGC - 3′
modifiedCeph_16S_R1	RV: 5′- TCGCTGTTAYCCCTATG -3′
Blocking Probe_BB	5′- GTGGAACTTAAAAATCAGCGACCACCA[SpcC3]-3′
M13	FR: 5′- TGTAAAACGACGGCCAGT -3′
	RV: 5′- CAGGAAACAGCTATGAC - 3′
Adaptor	FR: 5′- CCATCTCATCCCTGCGTGTCTCCGACTCAG + 10 bp MID tags -3′
	RV: 5′- CCTCTCTATGGGCAGTCGGTGAT + 10 bp MID tags- 3′

3.4.2 Reference dataset

We constructed two different reference trees for phylogenetic placement of query sequences of cephalopods and fish. In total, 100 and 79 mitochondrial 16S rRNA reference Sanger sequences of fish and cephalopods were extracted from Genbank. The dataset comprised members of 16 and 23 different families from 9 and 2 different orders, respectively, in fish and cephalopods (Table 3.2). Reference alignments for phylogenetic reconstruction of fish included 502 bp and for cephalopods 494 bp sequences (including gaps). Taxon sampling of the reference dataset was conducted so

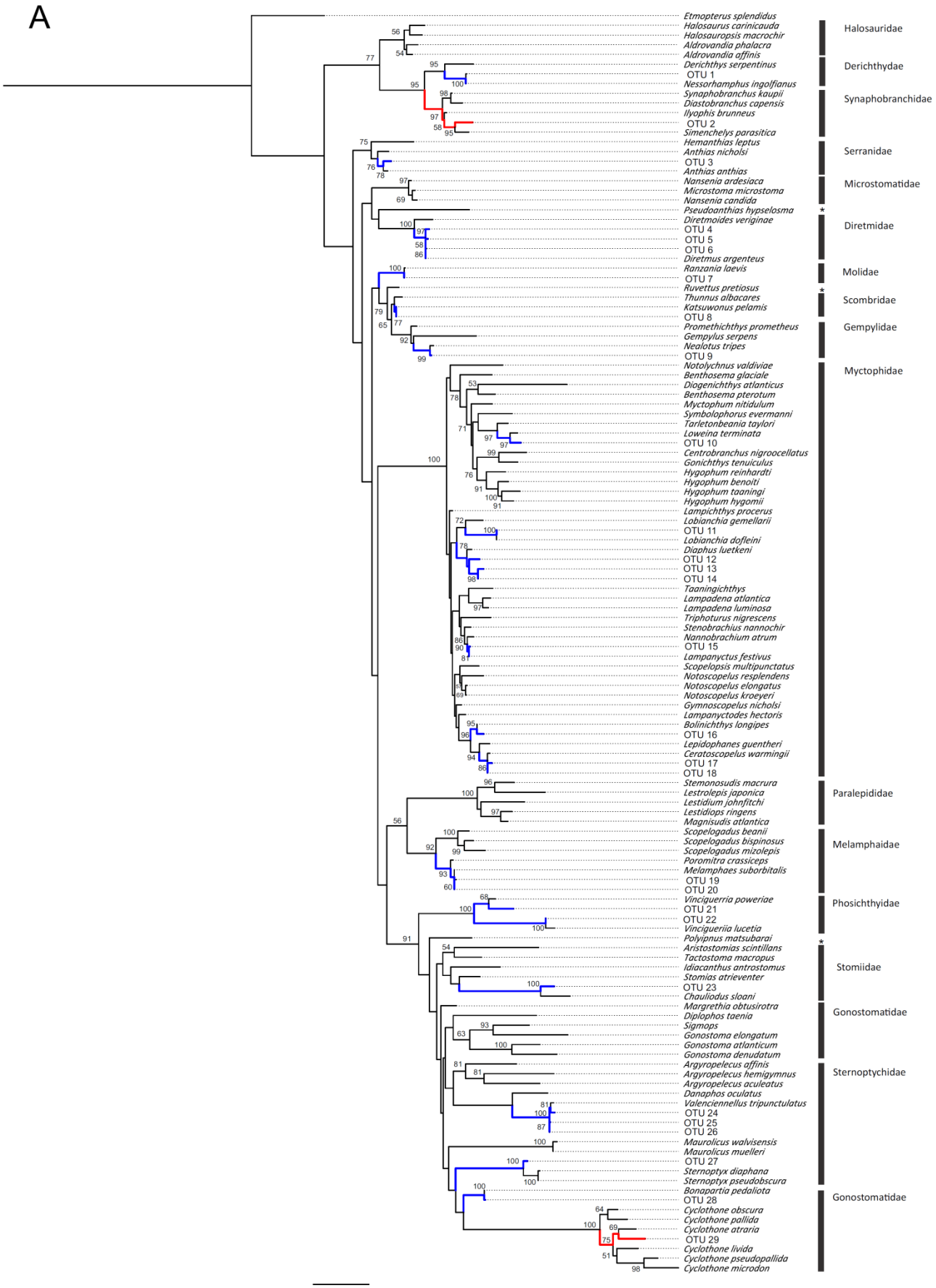
as to include all available reference taxa identified in stomach contents, but we further added other reference sequences to augment sampling of each prey family.

Both reference trees inferred from ML based analyses were resolved, producing monophyletic clades that corresponded each to a different taxonomic order (Figure 3.1). Although most families formed distinct monophyletic clusters, some taxa grouped polyphyletically with other family members (e.g. sternoptychids, gonostomatids and mastigoteuthids) indicating that short 16S rRNA barcodes lacked phylogenetic resolution at inner nodes for some family members. Conversely, clusters at terminal nodes were highly resolved showing high support with congenera rendered monophyletic on both reference trees. These results indicate that relationships retrieved in the reference trees are biologically meaningful and likely to produce robust species identifications of anonymous reads (Figure 3.1).

Table 3.2. Number of representative 16SrDNA Sanger sequences extracted from GenBank used to built two different reference trees, for fish and cephalopods. The number of sequences is presented for order and family taxonomical ranks. Genbank accession numbers are shown in supplementary (Tables S1a-b)

	Order	**Family**	**Nb of sequences**
Fish	Anguilliformes	Derichthydae	2
		Synaphobranchidae	4
	Aulopiformes	Paralepididae	5
	Argentiniformes	Microstomatidae	3
	Beryciformes	Diretmidae	2
	Perciformes	Gempylidae	4
		Molidae	1
		Scombridae	2
		Serranidae	4
	Myctophiformes	Myctophidae	34
	Notocanthiformes	Halosauridae	4
	Stomiiformes	Gonostomatidae	13
		Phosichthyidae	2

		Sternoptychidae	10
		Stomiidae	5
	Stephanoberyciformes	Melamphaidae	5
		Total	**100**
Cephalopoda	Teuthida	Architeuthidae	1
		Batoteuthidae	1
		Brachioteuthidae	1
		Chiroteuthidae	5
		Cranchiidae	7
		Cycloteuthidae	2
		Enoploteuthidae	9
		Gonatidae	6
		Histioteuthidae	6
		Joubiniteuthidae	1
		Lepidoteuthidae	1
		Lycoteuthidae	2
		Magnapinnidae	1
		Mastigoteuthidae	7
		Neoteuthidae	1
		Octopoteuthidae	2
		Ommastrephidae	13
		Onychoteuthidae	6
		Pholidoteuthidae	1
		Psychroteuthidae	1
		Pyroteuthidae	3
		Thysanoteuthidae	1
	Spirulida	Spirulidae	1
		Total	**79**


A
Etmopterus splendidus
Halosaurus carinicauda
Halosauropsis macrochir
Aldrovandia phalacra
Aldrovandia affinis
Halosauridae
Derichthys serpentinus
OTU 1
Derichthydae
Nessorhamphus ingolfianus
Synaphobranchus kaupii
Diastobranchus capensis
Ilyophis brunneus
OTU 2
Simenchelys parasitica
Synaphobranchidae
Hemanthias leptus
Anthias nicholsi
OTU 3
Anthias anthias
Serranidae
Nansenia ardesiaca
Microstoma microstoma
Nansenia candida
Microstomatidae
Pseudoanthias hypselosma
Diretmoides veriginae
OTU 4
OTU 5
OTU 6
Diretmus argenteus
Diretmidae
Ranzania laevis
OTU 7
Molidae
Ruvettus pretiosus
Thunnus albacares
Katsuwonus pelamis
Scombridae
OTU 8
Promethichthys prometheus
Gempylus serpens
Nealotus tripes
Gempylidae
OTU 9
Notolychnus valdiviae
Benthosema glaciale
Diogenichthys atlanticus
Benthosema pterotum
Myctophum nitidulum
Symbolophorus evermanni
Tarletonbeania taylori
Loweina terminata
OTU 10
Centrobranchus nigroocellatus
Gonichthys tenuiculus
Hygophum reinhardti
Hygophum benoiti
Hygophum taaningi
Hygophum hygomii
Lampichthys procerus
Lobianchia gemellarii
OTU 11
Lobianchia dofleini
Diaphus luetkeni
OTU 12
OTU 13
OTU 14
Taaningichthys
Lampadena atlantica
Lampadena luminosa
Triphoturus nigrescens
Stenobrachius nannochir
Nannobrachium atrum
OTU 15
Lampanyctus festivus
Scopelopsis multipunctatus
Notoscopelus resplendens
Notoscopelus elongatus
Notoscopelus kroeyeri
Gymnoscopelus nicholsi
Lampanyctodes hectoris
Bolinichthys longipes
OTU 16
Lepidophanes guentheri
Ceratoscopelus warmingii
OTU 17
OTU 18
Myctophidae
Stemonosudis macrura
Lestrolepis japonica
Lestidium johnfitchi
Lestidiops ringens
Magnisudis atlantica
Paralepididae
Scopelogadus beanii
Scopelogadus bispinosus
Scopelogadus mizolepis
Poromitra crassiceps
Melamphaes suborbitalis
OTU 19
OTU 20
Melamphaidae
Vinciguerria poweriae
OTU 21
OTU 22
Vinciguerria lucetia
Phosichthyidae
Polyipnus matsubarai
Aristostomias scintillans
Tactostoma macropus
Idiacanthus antrostomus
Stomias atrieventer
OTU 23
Chauliodus sloani
Stomiidae
Margrethia obtusirotra
Diplophos taenia
Sigmops
Gonostoma elongatum
Gonostoma atlanticum
Gonostoma denudatum
Gonostomatidae
Argyropelecus affinis
Argyropelecus hemigymnus
Argyropelecus aculeatus
Danaphos oculatus
Valenciennellus tripunctulatus
OTU 24
OTU 25
OTU 26
Maurolicus walvisensis
Maurolicus muelleri
OTU 27
Sternoptyx diaphana
Sternoptyx pseudobscura
Bonapartia pedaliota
Sternoptychidae
OTU 28
Cyclothone obscura
Cyclothone pallida
Cyclothone atraria
OTU 29
Cyclothone livida
Cyclothone pseudopallida
Cyclothone microdon
Gonostomatidae
0.2

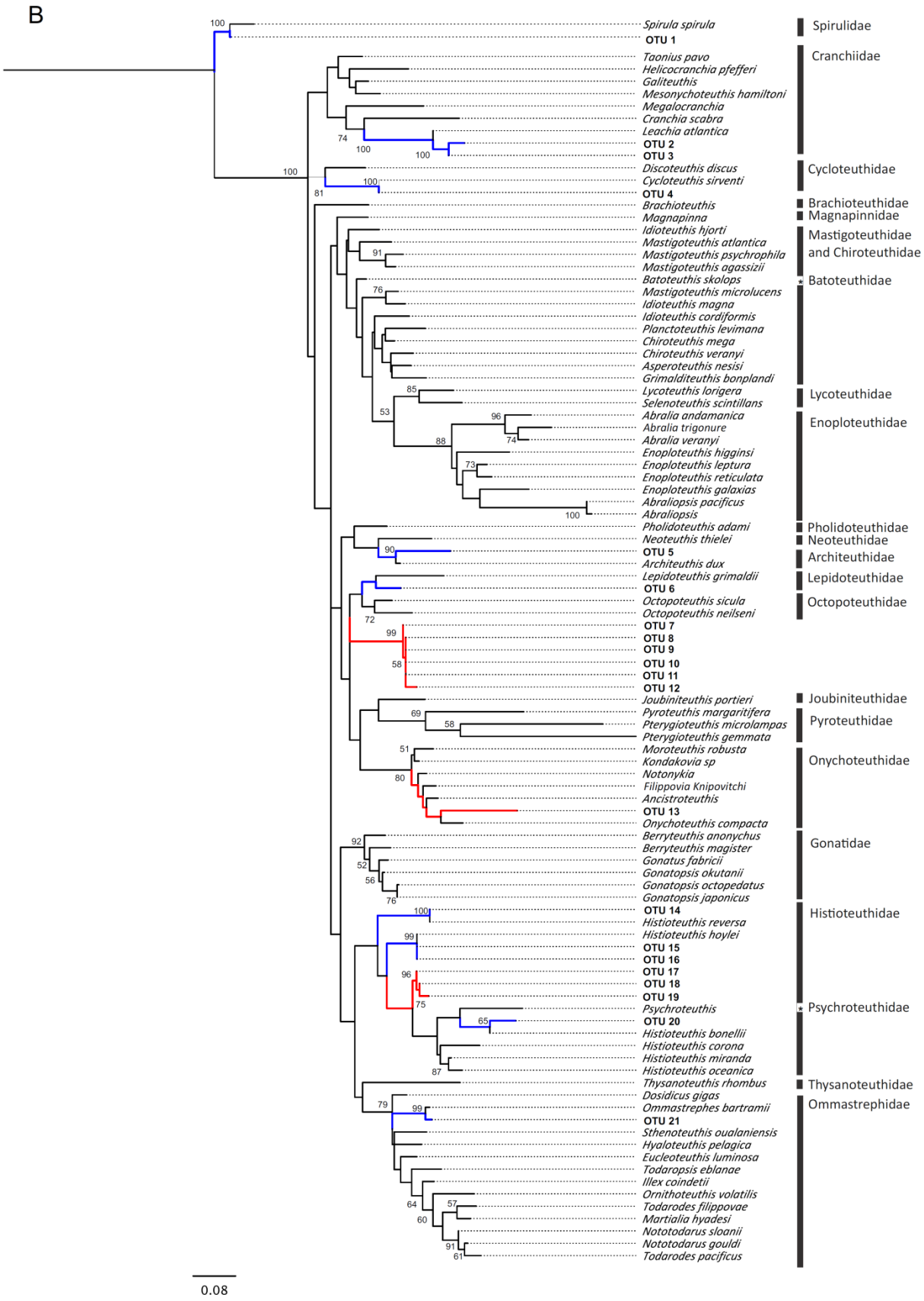


Figure 3.1. Phylogenetic placement of queries on reference trees. Maximum likelihood trees were inferred in RAxML to phylogenetically place fish (A) and cephalopod prey (B) using 1,000 bootstraps.

Bootstrap support is shown for values > 50. Confidence in the placement of queries on the trees was obtained by the Evolutionary Placement Algorithm (EPA), where blue lines indicate high support in query placement (likelihood weight ratio >0.90) and red lines indicate low support in query placement (likelihood weight ratio <0.90) - where other placements cannot be excluded. Clades corresponding to family ranks are shown. Reference taxa clustering outside a monophyletic clade (*).

3.4.3 EPA placement of query reads

The Ion torrent PGM run generated a total of 5.5 million reads, of which two million were included in the current study. Quality filtering reduced the dataset to 241,219 reads. Queries were pre-clustered at 98% similarity into 50 different MOTUs. Of these, 31 MOTUs formed distinct clusters in the reference trees corresponding to different taxonomic entities. Using Maximum likelihood weights of placement given by EPA and taking the completeness of the reference tree into account, we successfully assigned 16 putative species, 12 genera and 3 families to the nearest taxon (Table 3.3, Figure 3.1). With the exception of two monophyletic clusters of queries (OTU 7-12 and OTU 17-19) on the cephalopod reference tree, all other queries were placed on the terminal branches of the reference trees indicating that taxon sampling was mostly complete. Queries identified by the highest neighbor in BLAST were overall concordant with the taxonomic identifications using EPA, but showed discrepancy in the sequence similarity thresholds (Table 3.3). Cephalopod queries (OTU 7 – 12) that did not cluster at terminal edges or within supported monophyletic reference clades, showed similarity percentages of 85-87% with references in BLAST, but were not phylogenetically related with the same taxa on the tree, not even at broader taxonomic ranks (i.e. families).

Table 3.3. Taxa identified in Bulwer's faeces using EPA phylogenetic placement of query sequences on reference tree. Likelihood weights (>0.90) are shown for taxa identified with high confidence to the nearest taxonomic rank using EPA. The nearest neighbour in BLAST, as well as the identity percentages in BLAST are also shown. The frequency of occurrences (FO) of each prey are presented in % and corresponds to the number of samples containing a specific prey divided by the total number of samples containing prey. (*) indicates no positive identification.

	Family	Taxon	Identity % (BLAST)	ML (EPA)	FO (%)
Fish					
Anguilliformes	Derichthyidae	*Nessorhamphus ingolfianus*	99.56	1.00	1.79
	Synaphobranchidae	Synaphobranchidae	91.66		1.79
Beryciformes	Diretmidae	*Diretmus argenteus*	99.04-99.49	0.97-0.99	7.14
Myctophiformes	Myctophidae	*Bolinichthys*	98.08	0.96	1.79
		Ceratoscopelus	99.04	0.99	3.57
		Diaphus A	92.87-93.78	0.96 - 0.99	5.36
		Diaphus B	90.95		2.22
		Lampanyctus	100	0.95	1.79
		Lobianchia dofleini	99.53	1.00	1.79
		Loweina	91.7	0.99	1.79
Perciformes	Gempylidae	*Nealotus tripes*	98.06	1.00	3.57
	Serranidae	*Anthias*	94.17	0.99	1.79
	Scombridae	*Katsuwonus pelamis*	100	0.96	1.79
Stephanoberyciformes	Melamphaidae	Melamphaidae	98.62-100		3.56
Stomiiformes	Gonostomatidae	*Bonapartia pedaliota*	99.03	1.00	1.79
		Cyclothone	99.51		1.79
	Phosichthyidae	*Vinciguerria* A	94.2	0.99	1.79
		Vinciguerria B	96.39	1.00	1.79
	Sternoptychidae	*Sternoptyx*	96.04	0.99	5.36
		Valenciennellus tripunctulatus	96.88	1.00	3.57
	Stomiidae	*Chauliodus*	81.04	1.00	1.79

Tetraodontiformes	Molidae	*Ranzania laevis*	98.56	1.00	1.79
Cephalopods					
Oegopsina		Chiroteuthidae *	85.71-86.27		10.71
	Cranchiidae	*Leachia*	96.25	1.00	1.79
	Cycloteuthidae	*Cycloteuthis sirventi*	100	1.00	1.79
	Histioteuthidae	*Histioteuthis* *	96.5		3.57
		Histioteuthis hoylei	99.33-100	1.00	57.17
		Histioteuthis bonnelli	100	0.99	1.79
		Histioteuthis reversa	100	1.00	5.36
	Architeuthidae	*Architeuthis*	87.58	1.00	1.79
	Lepidoteuthidae	Lepidoteuthidae	91.62	0.90	5.36
	Ommastrephidae	*Ommastrephes bartramii*	100	1.00	25.00
	Onychoteuthidae	Onychoteuthidae	97.97		1.79
Spirulida	Spirulidae	*Spirula spirula*	100	1.00	3.57

3.4.4 Comparison with stomach contents

The diversity of prey identified (31 taxa) using high-throughput sequencing of Bulwer's petrel faeces was substantially greater than previously found through morphological studies using otoliths and cephalopod beaks at the same and other Macaronesian islands. Zonfrillo et al. (1986) identified a total of six cephalopod and fish retrieved from spontaneous regurgitations at Selvagem Grande, while Neves et al. (2012) detected 21 different fish and cephalopods. We also identified new families of fish (Derichthyidae and Synaphobranchidae) and cephalopods (Spirulidae), which were not known prey of Bulwer's petrels (Table 3.3). To understand whether highly digested faecal material is a good source of prey DNA we compared the FO of prey retrieved from faeces with that obtained from the stomach contents of chicks (Figure S3.2), but using Sanger sequencing of isolated tissue remains for the latter (Alonso et al. 2014). Although, a substantial higher number of taxa were identified, as expected, from the less digested material amongst the stomach contents, the FO of occurrences of the

principal families was very similar, dominated by sternoptychids, myctophids and histioteuthids (Figure S3.2). In the current study using NGS myctophids showed the highest species diversity, while sternoptychids and histioteuthids were mostly represented by *Sternoptyx* sp., *Histioteuthis hoylei* and *Histioteuthis reversa* (Table 3.3, Figure 3.2) Ommastrephids occurred in the faecal samples of 2011, but occurred only twice in 2012. This agrees with the abundances we found in stomach contents collected in 2012 using Sanger sequencing, where only a few ommastrephids were recorded. Although the FO of the main prey is highly concordant in both faeces and stomach contents, 39 % of the amplicons obtained from faeces retrieved only predator DNA and no prey, suggesting that a higher quantity of samples is needed to infer the diets of birds using faecal remains.

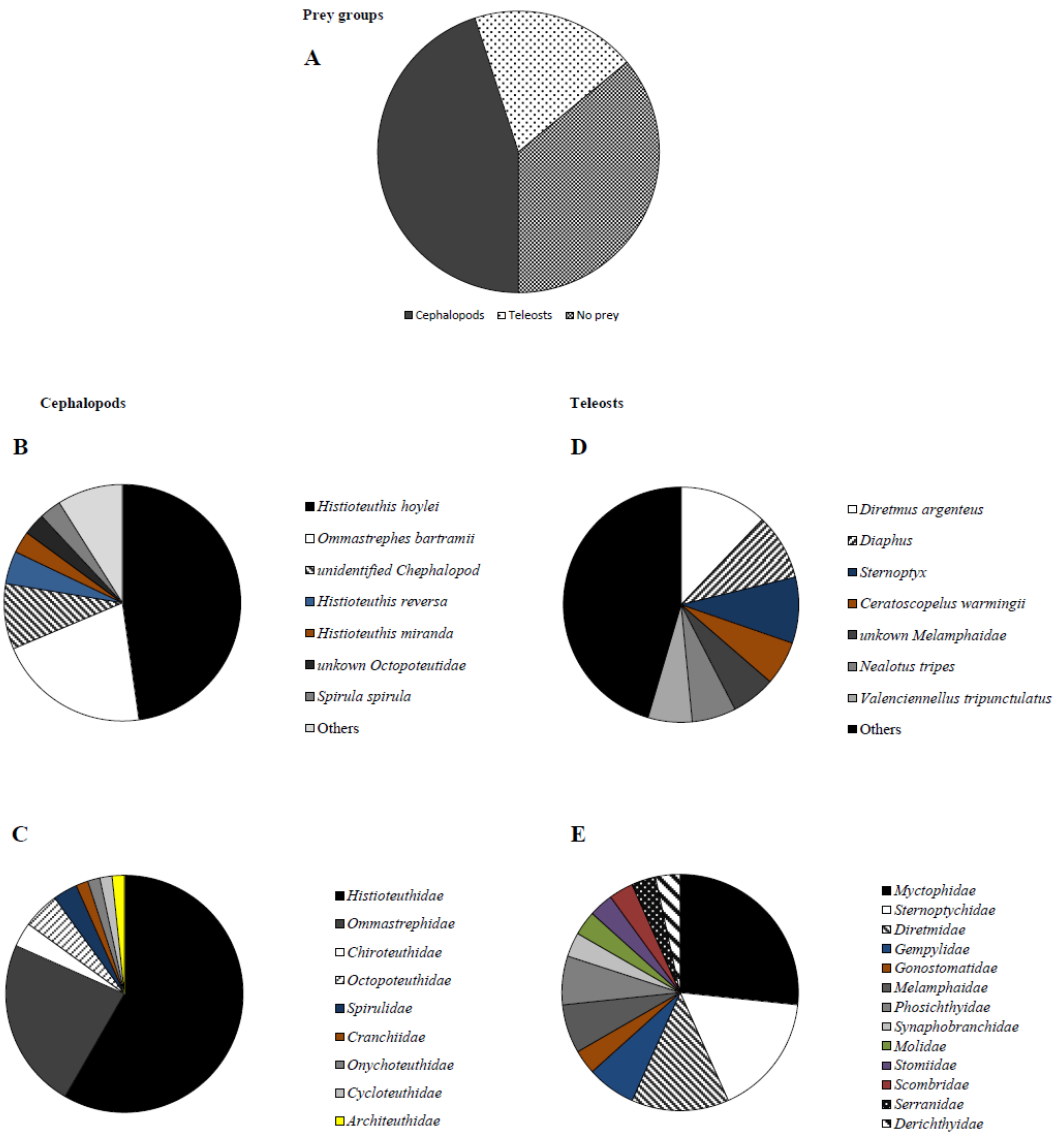


Figure 3.2. Frequency of occurence (%) of prey in faecal remains. A – Frequency of occurrences (FO) of the main prey groups, fish and cephalopods, in a total of 92 samples. FO of cephalopod (B) and fish (D) assigned to the nearest possible taxonomic rank using the Evolutionary Placement Algorithm. FO of families of cepahlopod (C) and fish (E). Taxa occurring in more than one faecal sample are shown, whereas "others" include single occurrences.

3.5 Discussion

This study provides an example of how phylogenetic-based approaches improve detection and confidence in prey taxonomical assignments in high-throughput sequencing studies using short non-standardized barcodes.

We used the 16S rRNA barcode to comprehensively amplify prey in Bulwer's petrels. Although other COI metabarcodes, such as ZBJ-ArtF1c/ZBJ-ArtR2c (Bohmann et al. 2011; Zeale et al. 2011) and LCO-1490/UniMinibarR1 (Brown et al. 2014) amplify a broad range of terrestrial invertebrate prey, the same may not apply to the diets of predators such as seabirds feeding on a taxonomically much more diverse range of taxa, that includes both vertebrates and invertebrates. For example, Browser et al. (2013) showed that 16S rRNA barcodes retrieved a substantially higher number of prey in seabirds than universal COI metabarcodes (Uni-MinibarF1/UniMinibarR1), while Deagle et al. 2014 points out that the variable nature of the COI molecule invariably precludes the design of general primers capable of amplifying broad taxonomical ranges and refers to ribosomal barcodes as alternative markers. More conserved mitochondrial ribosomal barcodes are potentially further good candidates for species discrimination of vertebrates and invertebrates biota as general primers have been successfully designed for phylogenetic inferences of distantly related taxa and such sequences are therefore well represented markers in public databases.

We found that most queries displayed positive matches in BLAST (Altschul et al. 1990), indicating that current vertebrate and invertebrate databases potentially represent most of the diversity of 16S rRNA queries in our study system. Nonetheless, query sequences identity percentages in BLAST varied substantially, between 81-100%. Query sequences often presented the same identity percentages with other taxa than they did with the nearest neighbor in BLAST, resulting in uncertainty in species assignments. We also found that short 16S rRNA barcodes showed substantial differences in the identity percentages when compared with full-length 16S rRNA

barcodes. Such findings support the contention that taxonomical identities cannot be accurately inferred solely from identity percentage thresholds with reference taxa. To provide robust prey assignments, we performed EPA phylogenetic placement on two different reference trees (fish and cephalopods). By so doing we successfully identified a total 31 different taxa from faeces and obtained taxonomic classifications for query sequences displaying high identity percentages in BLAST. The queries placed in the reference trees were highly consistent with the nearest neighbor in BLAST, indicating that EPA placement can be accurately applied in dietary analysis of birds. Based on ML values of placement and the position of query sequences on the reference tree, prey species can be delimited and identified to the nearest taxonomical rank depending on the completeness of reference trees.

In this study, we identified a substantially higher number of fish and cephalopods from faecal DNA than previous morphological studies on Bulwer's petrels using hard-part remains (Harrison 1983; Zonfrillo 1986; Neves et al. 2011). Moreover, detection of the principal prey in faeces (FO $\geq$ 4%) was similar to that detected in stomach contents using molecular tools (Figure S3.2), thus indicating that prey identifications can be accurately retrieved from faecal remains in petrels. Although the FO of the principal families of fish was very similar for both faeces and stomach contents, for cephalopods we obtained a considerable higher amount of ommastrephids, but fewer cranchiids than in stomach contents. Such differences might reflect real trophic differentiaton between the food assimilated by adults (faeces) and that fed to their chicks (stomach contents) (Figure S3.2), as reported for other pelagic seabirds, for example resulting from differences in the location where prey for self-feeding and for chick-provisioning are captured (Alonso et al. 2012), or result from methodological constraints. Differences in primer efficiency when amplifying short 16S rRNA barcodes is unlikely, as primers were highly conserved amongst both prey groups. In fact, ommastrephids were mostly present in the samples collected in 2011, while only two occurrences were reported in 2012 – the year we collected stomach contents at Selvagem Grande. Inter-annual differences in ommastrephids availability are also expected, as *O. bartramii* are known to be seasonal migrants (Clarke 1996)

3.6 Conclusion

The use of non-standardized barcodes such as the mitochondrial 16S rRNA in molecular dietary high-throughput sequencing studies often implies the use of your own DNA reference collections to provide for robust species identifications. However, it is not always feasible to access specimens, especially in oceanic habitats where sampling is expensive and time-consuming. Using a phylogenetic aware algorithm (EPA) we successfully assigned species entities from short 16SrRNA barcodes and augmented confidence in the ranks identified compared with the nearest neighbor in BLAST. We think that this approach can be extensively applied in future studies using high-throughput sequencing to investigate the diet of birds. Despite extensive records of prey identified in birds and the role of diet in ecosystem functioning, birds remain relatively understudied in the field of molecular trophic interactions.

3.7 Acknowledgements

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Conflict of Interest Statement

The authors declare no conflict of interest.

Data accessibility

All relevant files for phylogenetic placement of high-throughput sequencing reads have been deposited to Dryad, including sequence data, alignment and tree files. Sanger sequences obtained from stomach content remains were deposited to Genbank. Output from ecoPCR and ecoTaxStat were deposited to Dryad.

3.8 References

Alonso, H. Granadeiro, J.P, Paiva, V.H., Dias, A.S., Ramos, J.A. & Catry, P. (2012). Parent–offspring dietary segregation of Cory's shearwaters breeding in contrasting environments. Marine Biology, 159, 1197-1207

Alonso, H., Granadeiro, J.P, Waap S., Xavier, J., Symondson, W.O.C, Ramos, J.A. & Catry, P. (2014) An holistic ecological analysis of the diet of Cory's shearwaters using prey morphological characters and DNA barcoding. Molecular Ecology, 23, 3719-3733.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J.J. (1990) Basic local alignment search tool. Journal of Molecular Biology, 215, 403-410.

Berger, S.A, Krompass, D. & Stamatakis, A. (2011) Performance, Accuracy, and Web Server for Evolutionary Placement of Short Sequence Reads under Maximum Likelihood. Systematic Biology, 60, 291-302

Berry, D., Mahfoudh, K.B., Wagner, M. & Loy, A. (2011) Barcoded Primers Used in Multiplex Amplicon Pyrosequencing Bias Amplification. Applied and Environmental Microbiology, 21, 7846-7849.

Bowser, A.K, Diamond, A.W. & Addison, J.A. (2013) From Puffins to Plankton: A DNA-Based Analysis of a Seabird Food Chain in the Northern Gulf of Maine. PLoS ONE, 8(12), e83152.

Brown, D.S., Burger, R., Cole, N., Vencatasamy, D., Clare, E.L., Montazam, A. & Symondson, W.O.C (2014) Dietary competition between the alien Asian Musk

Shrew (*Suncus murinus*) and a reintroduced population of Telfair's Skink (*Leiolopisma telfairii*). Molecular Ecology, 23, 3695–3705.

Clarke, J.R. & Kerry, K.R. (1994) The effects of monitoring procedures on Adélie penguins. CCAMLR Science, 1, 155-164

Clarke, M.R. (1996) The role of cephalopods in the World's Oceans: An Introduction. Philosophical Transactions of the Royal Society B, 351, 979-983

Coissac, E., Riaz, T. & Puillandre, N. (2012) Bioinformatic challenges for DNA metabarcoding of plants and animals. Molecular Ecology, 21, 1834–1847.

Dai, L., Zheng, X., Kong, L. & Li, Q. (2012) DNA barcoding analysis of Coleoidea (Mollusca: Cephalopoda) from Chinese waters. Molecular Ecology Resources, 12, 437–447

Deagle, B.E., Gales, N.J., Evans, K., Jarman, S.N., Robinson, S., Trebilco, R. & Hindell, M.A. (2007) Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (*Eudyptes chrysolophus*). PLoS ONE, 2(9), e831

Deagle, B.E, Kikwood, R. & Jarman, S.N. (2009) Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Molecular Ecology 18: 2022-2038

Deagle, B.E., Jarman, S.N., Coissac, W., Pompanon, F. & Taberlet, P. (2014) DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biology letters, 10, doi: 10.1098/rsbl.2014.0562

Duffy, D.C & Jackson S (1986) Diet studies of seabirds: A review of methods. Colonial Waterbirds, 9, 1-17

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res, 32(5), 1792-1797

Edgar, R.C. (2013) UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature Methods, 10, 996-998 dx.doi.org/10.1038/nmeth.2604

Ficetola, G.F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P. & Pompanon, F. (2010) An in silico approach for the evaluation of DNA barcodes. BMC Genomics, 1,1-434.

Gordon, A. (2010) FASTX-Toolkit. Available at http://hannonlab.cshl.edu/fastx_toolkit/ (accessed 20 May 2014).

Hall, T.A. (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series, 41, 95-98.

Harrison, C.S., Hida, T.S. & Seki, M.P. (1983) Hawaiian seabird feeding ecology. Wildlife Monographs, 85, 3-71

Hebert, P.D.N., Cywinska, A., Ball, S.L. & de Waard, J.R. (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B, 270, 313–322.

Hobson, K.A., Piatt, J.F. & Pitocchelli, J. (1994) Using stable isotopes to determine seabird trophic relationships. Journal of Animal Ecology, 63, 786–798

Huang, M., Arnheim, N. & Goodman, M.F. (1992) Extension of base mispairs by *Taq* DNA polymerase: implications for single nucleotide discrimination in PCR. Nucleic Acids Research, 20, 4567-4573

Ivanova, N.V., Zemlak, T.S., Hanner, R.H. & Hebert, P.D.N. (2007) Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes, 7, 544–548

Iverson, S.J., Field, C., Bowen, W.D. & Blanchard, W. (2004) Quantitative fatty acid signature analysis: a new method of estimating predator diets. Ecological Monographs, 74, 211–235

Jarman, S.N., McInnes, J.C., Faux, C., Polanowski, A.M., Marthick, J., Deagle, B.E., Southwell, C. & Emmerson, L. (2013) Adélie Penguin Population Diet Monitoring by Analysis of Food DNA in Scats. PLoS ONE, 8(12), e82227.

Katoh, K. & Standley, D.M. (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution, 30,772-780.

Kochzius, M., Seidel, C., Antoniou, A., Botla, S.K., Campo, D., Cariani, A., Vazquez, E.G., Hauschild, J., Hervet, C., Hjorleifsdóttir, S., Hreggvidsson, G., Kappel, K., Landi, M., Magoulas, A., Marteinsson, V., Nolte, M., Planes, S., Tinti, F., Turan, C., Venugopal, M.N., Weber, H. & Blohm, D. (2010) Identifying fishes through DNA barcodes and microarrays. PLoS ONE, 5**,** e12620

Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal, 17,10-12

Matsen, F.A., Kodner, R., & Armbrust, E.V. (2010) pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. BMC Bioinformatics, 11, 538.

Mooney, K.A., Gruner, D.S., Barber, N.A., Van Bael, S.A., Philpott, S.M. & Greenberg, R. (2010) Interactions among predators and the cascad- ing eVects of vertebrate insectivores on arthropod communities and plants. Proceedings of the National Academy of Sciences of the United States of America, 107, 7335–7340.

Munch, K., Boomsma, W., Huelsenbeck, J.P., Willerslev, E. & Nielsen, R. (2008) Statistical assignments of DNA sequences using Bayesian phylogenetics. Systematic Biology, 57, 750-757.

Neves, V.C., Nolf, D. & Clarke, M.R. (2011) Diet of Bulwer's petrel in the Azores, NE Atlantic. Waterbirds, 34, 357-362

Palumbi, S.R. (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Mable BK, Moritz C (eds) Molecular Systematics. Sinauer, Sunderland, MA, pp 205–247.

Phillips, R.A. (2006) Efficacy and effects of diet sampling of albatross chicks. Emu 106: 305–308

Pompanon, F., Deagle, B.E., Symondson, W.O.C., Brown, D.S., Jarman, S.N. & Taberlet, P. (2012) Who is eating what: diet assessment using next generation sequencing. Molecular Ecology, 21, 1931–1950.

Price, M.N., Dehal, P.S. & Arkin, A.P. (2010) FastTree 2: Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE, 5(3), e9490

Quince, C., Lanzén, A., Curtis, T.P., Davenport, R.J., Hall, N., Head, I.M., Read, L.F. & Sloan, W.T. (2009) Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods, 6, 639-641

Ralph, C.P., Nagata, S.E. & Ralph, C.J. (1985) Analysis of droppings to describe diets of small birds. Journal of Field Ornithology, 56, 165-174

Ratnasingham, S., & Hebert, P.D.N. (2007). BOLD: the barcode of life data system (www.barcodinglife.org). Molecular Ecology Notes, 7, 355–364.

Sekercioglu, C.H. (2006) Increasing awareness of avian ecological function. Trends in Ecology and Evolution, 21, 464–471.

Silvestro, D. & Michalak, I. (2012) raxmlGUI: A graphical front-end for RAxML. Organisms Diversity and Evolution, DOI: 10.1007/s13127-011-0056-0

Stamatakis, A. (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics, 22, 2688-2690

Symondson, W.O.C. (2002) Molecular identification of prey in predator diets. Molecular Ecology, 11, 627–641

Taberlet, P., Coissac, E., Hajibabaei, M. & Rieseberg, L.H. (2012) Environmental DNA. Molecular Ecology, 21, 1789–1793.

Tautz, D., Arctander, P., Minelli, A., Thomas, R.H. & Vogler, A.P. (2003) A plea for DNA taxonomy. Trends in Ecology and Evolution, 18, 70–74.

Tollit, D.J., Wong, M., Winship, A.J., Rosen, D.A.S. & Trites, A.W. (2003) Quantifying errors associated with using prey skeletal structures from faecal samples to determine the diet of Steller's sea lion (*Eumetopias jubatus)*. Marine Mammal Science, 19, 724–744.

Vestheim, H. & Jarman, S.N. (2008) Blocking primers to enhance PCR amplification of rare sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs. Frontiers in Zoology, 5, 12

Vogler, A.P. & Monaghan, M.T. (2007) Recent advances in DNA taxonomy. Journal of Zoological Systematics and Evolutionary Research, 45, 1-10.

Wilson, R.P. (1984) An improved stomach pump for penguins and other seabirds. Journal of Field Ornithology, 55, 109-112

Zhang, J. & Hanner, R. (2012) Molecular approach to the identification of fish in the South China Sea. PLoS ONE, 7, e30621.

Zonfrillo, B. (1986) Diet of Bulwer's petrel *Bulweria bulwerii* in the Madeiran archipelago. Ibis,128, 570-572

3.9 Supplementary material

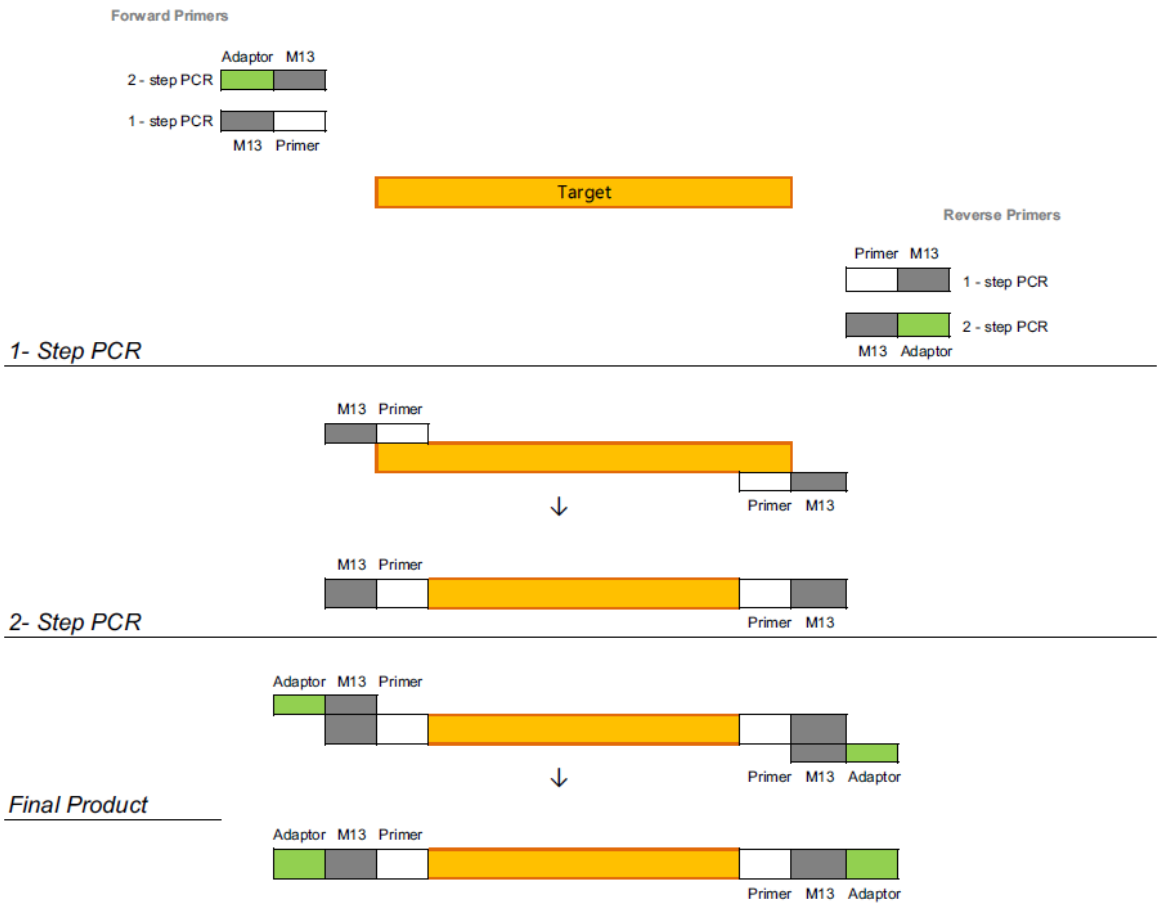


Figure S3.1. Schematic representation of the 2-step PCR approach.

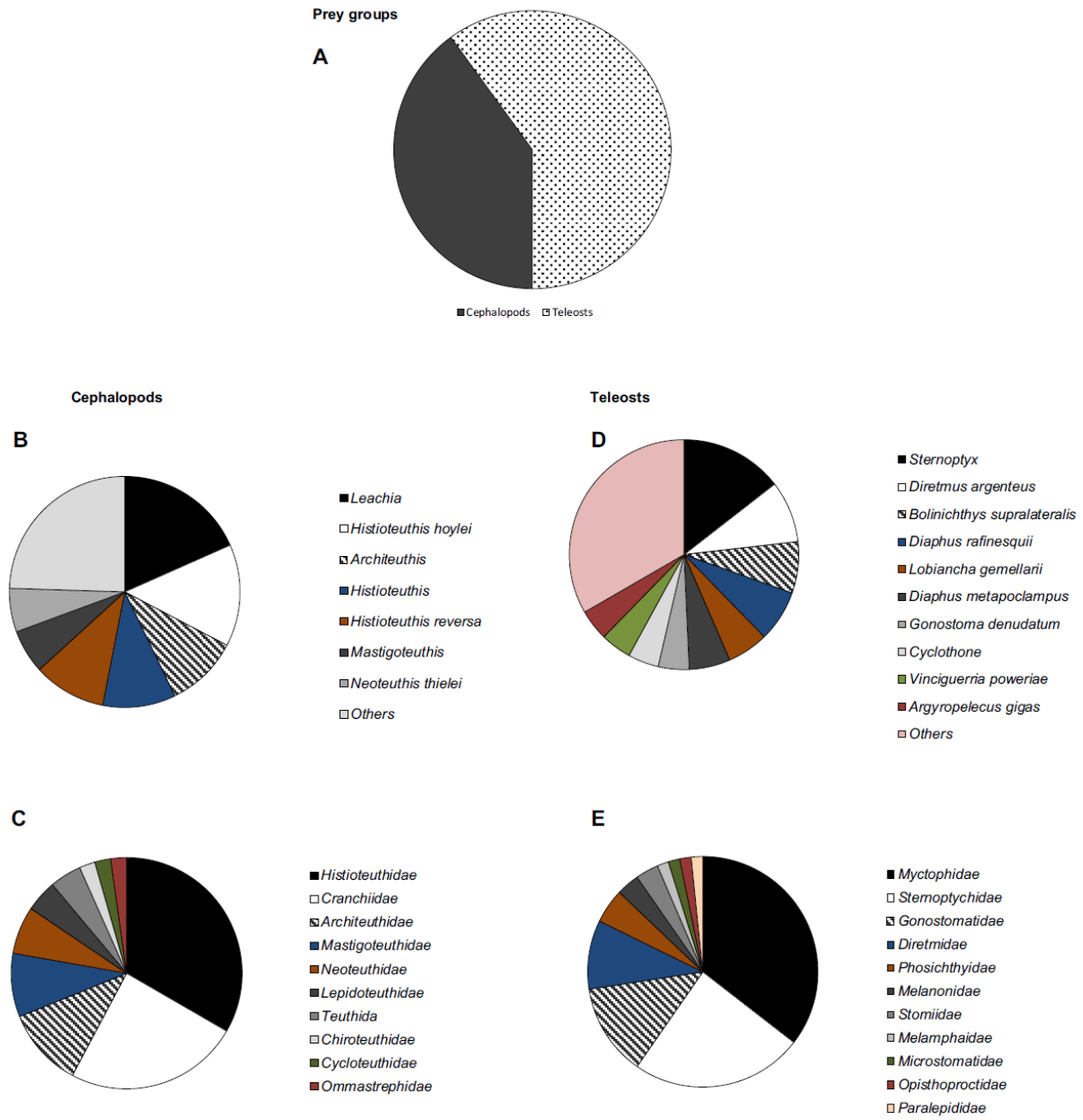


Figure S3.2. Frequency of occurence (%) of prey in stomach contents. A – Frequency of occurrences (FO) of the main prey groups, fish and cephalopods. FO of cephalopod (B) and fish (D), assigned to the nearest possible taxonomic rank using barcoding delimitation criteria in BOLD (for) and in Alonso et al. (2014) to assign 16S cephalopod Sanger sequences. FO of families of cepahlopod (C) and fish (E). Taxa occurring in more than 2% of stomach content are shown, whereas "others" include taxa occurring less than in 2% of the samples.

Table S3.1a-b. Genbank accession numbers of the reference taxa included in both reference trees, fish (a) and cephalopods (b)

S3.1 a)

Taxon	Accession number
Aldrovandia affinis	AP002974.1
Aldrovandia phalacra	JX242947.1
Anthias anthias	AY947617.1
Anthias nicholsi	HQ731420.1
Argyropelecus aculeatus	D89736.1
Argyropelecus affinis	D89737.1
Argyropelecus hemigymnus	EU099497.1
Aristostomias scintillans	KJ010559.1
Benthosema glaciale	AP012264.1
Benthosema pterotum	JX133755.1
Bolinichthys longipes	AB042165.1
Bonapartia pedaliota	AB026033.1
Centrobranchus nigroocellatus	AB042182.1
Ceratoscopelus warmingii	AB042168.1
Chauliodus sloani	AP002915.1
Cyclothone atraria	D84047.1
Cyclothone livida	D84052.1
Cyclothone microdon	D84053.1
Cyclothone obscura	D84055.1
Cyclothone pallida	D84056.1
Cyclothone pseudopallida	AB026041.1
Danaphos oculatus	HQ127667.1
Derichthys serpentinus	JX242957.1
Diaphus luetkeni	AP012231.1
Diastobranchus capensis	JX242990.1
Diogenichthys atlanticus	AB042178.1
Diplophos taenia	AB026031.1
Diretmoides veriginae	AP004426.1
Diretmus argenteus	KC603521.1
Gempylus serpens	DQ874735.1
Gonichthys tenuiculus	AB055888.1
Gonostoma atlanticum	D84049.1
Gonostoma denudatum	AB026039.1
Gonostoma elongatum	AB026036.1
Gymnoscopelus nicholsi	AP012250.1
Halosauropsis macrochir	JX242948.1
Halosaurus carinicauda	JX242949.1
Hemanthias leptus	FJ548772.1
Hygophum benoiti	AB024912.1
Hygophum hygomii	AB024915.1

S3.1 b)

Taxon	Accession numbers
Abralia andamanica	HQ845987.1
Abralia trigonure	X79584.1
Abralia veranyi	EU735259.1
Abraliopsis pacificus	AY616982.1
Abraliopsis sp	X79595.1
Ancistroteuthis lichtensteinii	EU735242.1
Architeuthis dux	KC701764.1
Asperoteuthis nesisi	EU421719.1
Batoteuthis skolops	EU735200.1
Berryteuthis anonychus	EU735238.1
Berryteuthis magister	AY681049.1
Brachioteuthis sp	EU735224.1
Chiroteuthis mega	KC860982.1
Chiroteuthis veranyi	EU735246.1
Cranchia scabra	DQ280046.1
Cycloteuthis sirventi	EU735204.1
Discoteuthis discus	EU735229.1
Dosidicus gigas	AB635421.1
Enoploteuthis galaxias	AJ223484.1
Enoploteuthis higginsi	AJ223485.1
Enoploteuthis leptura	EU735206.1
Enoploteuthis reticulata	X79572.1
Eucleoteuthis luminosa	AB635425.1
Galiteuthis sp	AY616987.1
Gonatopsis fabricii	EU735210.1
Gonatopsis japonicus	AY681021.1
Gonatopsis octopedatus	AY681024.1
Gonatus okutanii	EU735265.1
Grimalditeuthis bonplandi	AF110098.2
Helicocranchia pfefferi	AF110099.2
Histioteuthis bonellii	EU735248.1
Histioteuthis corona	EU735211.1
Histioteuthis hoylei	DQ280047.1
Histioteuthis miranda	EU735255.1
Histioteuthis oceanica	AY616986.1
Histioteuthis reversa	EU735256.1
Hyaloteuthis pelagica	AB270962.1
Idioteuthis cordiformis	KC860986.1
Idioteuthis hjorti	KC860990.1
Illex coindetii	AY616985.1

Hygophum reinhardti	AB024919.1
Hygophum taaningi	AB024916.1
Idiacanthus antrostomus	KJ010740.1
Ilyophis brunneus	JX242991.1
Katsuwonus pelamis	KM605252.1
Lampadena atlantica	KC603522.1
Lampadena luminosa	AB042166.1
Lampanyctodes hectoris	AB042170.1
Lampanyctus festivus	HM998554.1
Lampichthys procerus	AB042172.1
Lepidophanes guentheri	AB042169.1
Lestidiops ringens	KJ010558.1
Lestidium johnfitchi	AY952493.1
Lestrolepis japonica	KC441991.1
Lobianchia dofleini	DQ532898.1
Lobianchia gemellarii	AB042159.1
Loweina terminata	AB042184.1
Magnisudis atlantica	KJ128821.1
Margrethia obtusirostra	D84054.1
Maurolicus japonicus	JQ178227.1
Maurolicus muelleri	AJ277245.1
Melamphaes suborbitalis	KC442003.1
Microstoma microstoma	HQ127646.1
Myctophum nitidulum	AB042181.1
Nannobrachium atrum	AB042163.1
Nansenia ardesiaca	AP004106.1
Nansenia candida	HM998555.1
Nealotus tripes	AP012521.1
Nessorhamphus ingolfianus	JX242958.1
Notolychnus valdiviae	AB042158.1
Notoscopelus elongatus	KJ128846.1
Notoscopelus kroeyeri	AJ277964.1
Notoscopelus resplendens	AB042171.1
Polyipnus matsubarai	D89739.1
Poromitra crassiceps	KJ010747.1
Promethichthys prometheus	AP012504.1
Pseudanthias hypselosoma	JX094027.1
Ranzania laevis	KC603526.1
Ruvettus pretiosus	DQ532952.1
Scopelogadus beanii	AF221884.1
Scopelogadus bispinosus	AY947847.1
Scopelogadus mizolepis	AP002934.1
Scopelopsis multipunctatus	AB042174.1
Sigmops bathyphilus	AB026038.1
Simenchelys parasitica	JX242992.1
Joubiniteuthis portieri	EU735213.1
Kondakovia sp	EU735267.1
Leachia atlantica	EU735203.1
Lepidoteuthis grimaldii	DQ280048.1
Lycoteuthis lorigera	EU735257.1
Magnapinna sp	EU735227.1
Martialia hyadesi	AB270955.1
Mastigoteuthis agassizii	KC861000.1
Mastigoteuthis atlantica	KC861001.1
Mastigoteuthis magna	EU201156.1
Mastigoteuthis microlucens	EU201150.1
Mastigoteuthis psychrophila	KC861006.1
Megalocranchia sp	EU735228.1
Mesonychoteuthis hamiltoni	EU735261.1
Moroteuthis knipovitchi	DQ280050.1
Moroteuthis robusta	EU735241.1
Neoteuthis thielei	EU735215.1
Notonykia sp	EU735232.1
Nototodarus gouldi	AB270954.1
Nototodarus sloanii	AB270953.1
Octopoteuthis nielseni	AY616983.1
Octopoteuthis sicula	EU735217.1
Ommastrephes bartramii	AB270956.1
Onychoteuthis compacta	AJ223482.1
Ornithoteuthis volatilis	AB270961.1
Pholidoteuthis adami	EU735254.1
Planctoteuthis levimana	EU735247.1
Psychroteuthis sp.	EU735221.1
Pterygioteuthis gemmata	EU735208.1
Pterygioteuthis microlampas	EU735253.1
Pyroteuthis margaritifera	EU735209.1
Selenoteuthis scintillans	EU735230.1
Spirula spirula	AJ966785.1
Sthenoteuthis oualaniensis	AB270958.1
Taonius pavo	KC603481.1
Thysanoteuthis rhombus	EU735236.1
Todarodes filippovae	AB270950.1
Todarodes pacificus	AB270951.1
Todaropsis eblanae	AY616988.1

Stemonosudis macrura	AY952495.1
Stenobrachius nannochir	AB042162.1
Sternoptyx diaphana	EU099506.1
Sternoptyx pseudobscura	AY958662.1
Stomias atriventer	KJ010751.1
Symbolophorus evermanni	AY949625.2
Synaphobranchus kaupii	JX242993.1
Taaningichthys bathyphilus	AY949626.2
Tactostoma macropus	AY947849.2
Tarletonbeania taylori	AB042185.1
Thunnus albacares	KM588080.1
Triphoturus nigrescens	AB042164.1
Valenciennellus tripunctulatus	JN602067.1
Vinciguerria lucetia	HQ127632.1
Vinciguerria poweriae	HM143730.1

CHAPTER 4:

High-throughput sequencing technologies reveal trophic partitioning between sympatric small petrels in the sub-tropical eastern Atlantic

High-throughput sequencing technologies reveal trophic partitioning between sympatric small petrels in the sub-tropical eastern Atlantic

Key-words: Diet, high-throughput sequencing, mitochondrial 16S rRNA, Selvagens islands, small petrels, trophic segregation

Author contributions

This chapter includes contributions from: Catry P, Dias M, Granadeiro JP and Symondson WOC. PC, WOCS, JPG, MD contributed significantly to the experimental design and sample collection, WOCS, PC, JPG contributed with advice and critical review. I was the senior author and performed the laboratory work, sample collection, data analysis and writing.

4.1 Abstract

Colonial seabirds often assemble on small islands for reproduction. During this time seabirds are constrained in their foraging range and resource availability during their mating, incubation and chick rearing periods. The principle of competitive exclusion predicts that coexisting species with similar ecological functions differ in their resource-use. Despite these predictions, for many seabird communities we have still no information on their resource partitioning mechanisms. This is especially the case of small oceanic petrels, for which diet and foraging patterns are difficult to infer based on current methodologies. For example, tracking devices are too large to be deployed on small seabirds to assess their foraging grounds. Morphological stomach content analysis of prey remains is often not possible as prey are generally very small or at juvenile stages to allow robust identifications, especially at lower taxonomic ranks. Stable isotope analysis is a low-resolution technique, revealing the trophic position of predators in an ecosystem rather than providing detailed analysis of predator-prey interactions, which is essential for identifying fine-scale dietary differences between ecological similar species. Here we used a non-invasive approach to improve detection and taxonomic resolution in dietary analysis of small petrels, using molecular methodologies and high-throughput sequencing of the mitochondrial 16S rRNA from faecal remains of Bulwer's petrel (*Bulweria bulwerii*), Band-rumped petrels (*Hydrobates castro*), and White-faced Storm petrel (*Pelagodroma marina*) breeding in the northeastern Atlantic, at Selvagem Grande. The diet of these small petrels showed a high diversity of species, marked by high occurrences of rare prey types. We found a significant trophic segregation among the study species, each differing in the type of prey and contributions of major prey groups consumed (mesopelagic fish, reef-associated species, squids and crustaceans). The trophic patterns of these small species were overall consistent with the ones described in the Pacific, indicating that trophic specializations are maintained throughout the species distribution ranges and are potentially important mechanisms of trophic segregation among species.

Key-words: Band-rumped Storm petrel, Bulwer's petrel, Evolutionary Placement Algorithm (EPA), high-throughput sequencing, trophic segregation, White-faced Storm petrel,

4.2 Introduction

Competitive interactions for resources and reproduction are important drivers of speciation, coexistence and diversity (Darwin 1859; Brown & Wilson 1956; Dickman & Doebeli 1999). Competition is thought to be strongest in phylogenetically or ecologically related species, sharing the same environment and trophic position in time and space (ecological niche). Current competition theory predicts that species with the most favorable traits will eventually competitively exclude other species if resources are limited (Gause 1934; Hardin 1960). However, coexistence and diversity can persist if species segregate their resources (Hardin 1960; Pfenning & Pfenning 2009).

The extent to which resources are partitioned between coexisting species is therefore crucial for understanding and predicting community structuring and dynamics. Seabirds are useful models in which to study mechanisms of resource partitioning within communities. Almost all seabirds are colonial and central place foragers throughout the reproductive season (Coulson 2002), often at remote islands (King 1983). Although protected against major terrestrial predation pressures, colonial birds also have to share nesting space and resources with thousands of other breeding pairs for successful reproduction. Despite some debate as to whether bird high densities at colonies actually reduce prey availability (Croxall 1987), resource partitioning has been widely reported in colonial birds. For example, albatross display sexual niche segregation at colonies, either differing in their foraging habitats or prey types (Phillips et al. 2004; 2011). Coexisting seabirds have been further shown to differ significantly in their foraging activities along temporal and vertical niche axes, differing in their diel foraging patterns or diving depths (Forero et al. 2005; Miller et al. 2010; Navarro et al. 2013). Birds from large colonies also travel greater distances to forage than birds from smaller colonies (Lewis et al. 2001). Such a pattern follows Ashmole´s (1963)

predictions of local resource depletion resulting from density-driven interactions in resource-use (Lewis et al. 2001).

To date, niche partitioning in seabirds has been mainly assessed using tracking technologies (Phillips et al. 2004; 2007), stable isotope analysis (Young et al. 2010, Bocher et al. 2011) or both combined (Navarro et al. 2013). Tracking technologies provide detailed information on the geographic localization of foraging trips, while stable isotope analysis, using feathers or blood, provide information on the broad trophic positions of predators. Nonetheless, tracking devices for small birds are still under development but are currently too large to be deployed on these animals. As such, information on segregation by prey or habitat in small birds have to be inferred from isotopic data or visual inspection of prey remains in stomach contents. Both methodologies are, however, unable to provide the necessary fine-scale taxonomic resolution needed to accurately distinguish prey species. Morphological analysis of the diagnostic characters of prey remains is highly time-consuming technique, requiring a considerable expertise and a comprehensive reference collection for robust taxonomical assignments. Moreover, prey remains such as otolithes, vertebra and cephalopod beaks obtained from stomach contents usually show distinct digestion rates, which may provide a biased representation of the diet of seabirds (Xavier et al. 2011; Alonso et al. 2013). To some extent, stable isotope analysis overcomes such dietary biases, but is a low- resolution technique identifying prey at broad taxonomical ranks (Jacob et al. 2005). However, predators with similar functional traits, potentially consuming the same prey groups, but might differ substantially in their diets when analysed at the species level.

To overcome these limitations, a new branch of dietary analysis, metabarcoding of prey remains, is now emerging as a major field in the study of predator-prey interactions. The term metabarcoding refers to the detection and taxonomic identification of degraded biological material through high-throughput sequencing (HTS) of DNA barcodes (Taberlet et al. 2012). Given that HTS technologies can recover thousands of barcode sequences from a sample, even rare prey types (consumed in low abundances) can be recovered from highly degraded material, such as faeces. Although matching sequences to species is a controversial issue, numerous

bioinformatic tools have been developed to improve confidence in lower taxonomical assignments, including phylogenetically aware algorithms such as the Evolutionary Placement Algorithm (EPA) (Berger et al. 2011) and pplacer (Matsen et al. 2010). Metabarcoding can provide huge amounts of detailed dietary data and has the potential to revolutionise the field of marine community ecology.

Here we used current high-throughput sequencing technologies as well as Maximum Likelihood evolutionary algorithms (EPA) to investigate trophic partitioning between three small petrels: Bulwer's petrel (*Bulweria bulwerii*), Band-rumped Storm petrel (*Hydrobates castro*) and White-faced Storm petrel (*Pelagodroma marina*) breeding in the northeastern Atlantic. Dietary information on small procellariiformes is overall scarce, but particularly for the populations breeding in the North Atlantic. Therefore, a goal of this study was to describe the trophic interactions of small petrels in the North Atlantic and test whether the niche breath of these seabirds is consistent across its distribution range. A second goal was to provide a detailed analysis of the predator-prey interactions between small petrels through high-throughput sequencing of faecal remains. The most comprehensive studies on seabirds diet to date was that of Spear et al. (2007) in the Pacific where hundreds of specimens were killed to collect stomach contents. Although, the stomach contents of seabirds are now collected non-lethally through stomach flushing (Wilson 1984) this procedure is still too invasive and laborious for comprehensive inter-specific trophic assessments. HTS of faecal remains has been shown to greatly improve prey detection and resolution in seabirds compared with morphological identification (Bowser et al. 2013, Alonso et al. 2013).

Three small petrel species we studied coexist at their greatest numbers on Selvagem Grande and nearby islets, This island also hosts the largest population of Cory's shearwaters (*Calonectris borealis*) in the northeastern Atlantic (Granadeiro et al. 2006). The large colonies on these islands would be expected to exert strong competitive pressures if resources are limited. Moreover, trophic segregation by prey type and habitat has already been described for populations of Cory's shearwater at Selvagem Grande and other northeastern Atlantic populations (Alonso et al. 2014; Haug et al. 2015). However, no dietary information has been obtained for the three small petrels. Given that these species are the smallest seabirds, and sympatric throughout the

breeding cycle, we predict significant inter-specific partitioning of resources according to current competition theory.

4.3 Material and Methods

4.3.1 Ethical statement

This experiment was approved according to national regulations under the permits: 4/2011S, 1/2012S and 2/2012S (Instituto da Conservação da Natureza e da Biodiversidade (ICNB), Serviço do Parque Natural da Madeira – Portugal). Handling during the experiment was conducted and supervised by experts in the field and did not visibly harm any animal. All birds flew away after released.

4.3.2 Study sites and sample collection

This work was carried out at Selvagem Grande (30°09'N, 15°52'W), the largest of a small group of islands located ca. 300 km south of Madeira island, Portugal. The community of seabirds breeding in this island comprises five species of petrels: Cory's shearwater (*Calonectris borealis*), Band-rumped Storm petrel (*Hydrobates castro*), Bulwer's petrel (*Bulweria bulwerii*), Little shearwater (*Puffinus baroli*), and White-faced Storm petrel (*Pelagodroma marina*). The island represents one of the largest and most diverse breeding colonies of Procellariiformes in the northern Atlantic.

We obtained a total of 284 faecal remains of three different petrel species, Bulwer's petrel (n=99), White-faced Storm petrel (n=92) and Band-rumped Storm petrel (n=93). The three species under study here are sympatric through most of their breeding season, during the months of June to August/September. These seabirds are the smallest petrels breeding at Selvagem Grande.

Faeces of White-faced and Band-rumped Storm petrels were collected between June and July 2011 and 2012, whereas samples from Bulwer's petrel were collected during August 2011 and 2012. It is important to note that sample collection included the

chick-rearing period of White-faced Storm petrel and Bulwer's petrel, when birds are providing food to their chicks and therefore supposed to be more constrained in their foraging range. The breeding cycle of Band-rumped Storm petrel is less defined than both other species. Although few studies have been conducted on the breeding chronology of Band-rumped Storm petrel in the Madeiran archipelago, there is some morphological and genetic evidence for two distinct populations, breeding at four months intervals from April to September and from September to February (Nunes 2000; Smith et al. 2007). In this study, adults of Band-rumped Strom petrel were sampled in June 2011 and 2012, and therefore we assumed that birds were at the beginning of their breeding cycle.

Adults were caught when returning to their colonies at night, either directly from their nests or using mist-nets placed close to their nestling sites. As far as possible, faecal remains were collected opportunistically at the time birds were removed from the nets or processed. However, most samples were obtained from birds placed in clean containers of 25cm diameter for ca 1 h. Each container contained a clean metal grid, on which birds rested, and beneath it a filter paper. Faeces were removed from the filter paper using sterilized tools and preserved in absolute ethanol. Birds were immediately released.

4.3.3 Laboratory procedures and primers

Genomic DNA was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen). First, we performed a digestion step on the faecal samples with proteinase k, where we centrifuged the samples for 40 minutes at 10.000 rpm to remove the ethanol and suspended the faecal pellets in 1.2ml of lysis buffer containing 0.1M EDTA, 0.5M Tris-HCL, 2% SDS and 20 µl of proteinase K (Qiagen). Samples were incubated at 56ºC overnight. After this step we followed the manufactures guidelines except that we did not use Inhibitex tablets.

To amplify prey DNA, we used the primer sets: modifiedChord_16S_F1/R1, modifiedCeph_16S_F1/R1 and CrustF1/R1. Primer sets modifiedChord_16S_F1/R1 and modifiedCeph_16S_F1/R1, where modified from Deagle et al. (2009) and adapted

to our study system (Chapter 3). Primer set, Crust_16S_F1: 5´-GACGATARGACCCTATAA- 3´ and Crust_16S_R1: 5´- TCTGTTATCCCTARAG -3´was designed for this study to target crustaceans within the order Decapoda (shrimps, crabs), Euphausiacea (euphausiids) and marine Isopoda, which have been commonly found in the stomach contents of small petrels (D'Elbée & Hémery 1998). The taxonomic coverage (Bc coverage index) of primer set CrustF1/R1, was evaluated through in silico PCR tests using the software ecoPCR (Ficetola et al. 2010). To obtain the Bc coverage index for the order Decapoda we extracted whole mitochondrial genome sequences from Genbank database (118 different species). However, for marine isopods (Suborder: Valvifera) and euphausiids (Euphausiacea) no or few genome mitochondrial references are available, therefore we obtained all available partial 16SrRNA from Genbank in these groups, including a total of 20 and 25 species in each. We used the same parameters as described in Chapter 3 to run ecoPCR (Ficetola et al. 2010) and ecoTaxStat (OBITools, Boyer et al. 2014).

To minimize predator amplification during PCR, we used three blocking oligonucleotides, containing a C3 spacer at the 3´prime end (Vestheim & Jarman 2002). To suppress Bulwer's petrel DNA we used previous designed blocking oligonuclotides (BB) (Chapter 3), and designed a new blocking probes to block DNA of White-faced Storm petrel and Band-rumped Storm petrel (5´-GTGGAACTTAAAAATTAAAGGCCACT-SpC3-3´).

PCRs were conducted using a two-step approach, where prey DNA was amplified in step-1 and MID tagged adaptors incorporated in step-2 (Chapter 3). Reagent concentrations and thermal cycling conditions followed those of Chapter 3.To obtain equimolar sample concentrations, we quantified the fluorescence of each amplicon against a specific concentration of ladder (Promega) on a 2% gel (EtBr stained) using UVP VisionWorks ® LS Analysis. Three different libraries of equimolar amplicon mixtures were obtained for each primer set. Libraries were purified using QIAquick Gel Extraction kit (Qiagen). HTS sequencing was performed through outsourcing at the Centre de Recerca en Agrigenòmica (CRAG), Barcelona. Ion torrent high-throughput sequencing was conducted in a single run on a Personal Genome Machine (PGM) using a 318 chip and 400 base pair chemistry.

4.3.4 HTS data processing and prey assignments

HTS data processing and taxonomical assignments of 16SrRNA queries followed the pipeline of Chapter 3. Adaptor, M13 tails and primer sequences were removed at the sequence terminals (3´end) and filtered for phred quality scores ≥ 26 using cutadapt-1.5 (Martin 2014) and BioEdit (Hall 1999). Reads were de-multiplexed according to individual MID tag combinations (forward and reverse) of each sample using barcode-splitter of the fastx-toolkit (Gordon 2010). Queries were de-replicated, removed for singletons and chimeras, and pre-clustered at 98% of similarity using the UPARSE pipeline (Edgar 2013).

We identified the nearest neighbor of each MOTU using the BLAST algorithm (Altschul 1990) and separated MOTUs into three categories corresponding to the principal prey groups: fish, cephalopods and crustaceans. To adopt a conservative approach, only MOTUs showing more than 5 sequences were considered present in a sample. We also removed non-target contaminant sequences matching bacterial or predator and sequences not matching with any reference in the BLAST database.

MOTUs were taxonomically assigned based on Maximum likelihood of placement on reference trees using the Evolutionary Placement Algorithm (EPA) (Berger et al. 2011). For each MOTU category we constructed a tree containing reference 16S rRNA Sanger sequences of fish, cephalopods and crustaceans extracted from Genbank. We included reference taxa previously identified in the stomach contents of Bulwer's petrels (Chapter 3) and added references of the families producing the nearest neighbor in BLAST following the methodologies of Chapter 3 for sequence alignment and tree construction. MOTUs were assigned to species ranks for Maximum Likelihood weights > 0.99 (if congenera were represented on the tree), to genera ranks for ML weights > 0.90 and to family ranks if MOTUs grouped within monophyletic family members. For 16SrRNA sequences matching isopods, copepods and euphausiids, we did not use the EPA algorithm as reference sequences were to limited in Genbank for phylogenetic inferences to species ranks. Therefore, these taxa were only identified at broad taxonomical ranks (orders) using the nearest neigbour in BLAST and were only assigned if no other taxa were retrieved for similarity percentages >0.80.

4.3.5 Statistical analysis

To identify dietary groups, we performed multidimensional scaling (MDS) using presence and absence data of the prey taxa identified in each individual bird. MDS returns a set of observation into a dimensional space, where the distances among points are optimised to reflect the dissimilarities between samples (individual birds). We performed MDS in two-dimensional space (k=2) on a matrix of "Bray-Curtis" distances between the study birds.

To test whether the dietary composition varied significantly among the study species, we performed permutational analysis of variance (perMANOVA) using the adonis function (Oksanen et al. 2015) in R development core team (2015). We tested for the effect of the factor "species" by comparing observed statistics against 999 permutation distributions. To understand which prey taxa most contribute to the dissimilarities between the study species, we performed similarity percentage analysis, SIMPER (Oksanen et al. 2015). All multivariate analysis were performed in R using the Vegan package (Oksanen et al. 2015)

The frequency of occurrences of each prey type was calculated as the proportions of a particular prey type divided by the total number of prey types identified in each bird species.

4.4 Results

4.4.1 Taxonomical coverage of primers set CrusF1/R1

New primer set CrusF/R showed a high B_c coverage index for the range of crustaceans targeted: Decapoda (B_c= 99.15% of a total of 118 species), Euphausiacea (B_c = 96% of a total of 25 species) and the sub-order of marine isopods Valvifera (B_c= 95% of a total of 20 species); therefore likely demonstrating unbiased species amplification within these major crustacean groups.

4.4.2 HTS analysis and taxonomical assignments of queries

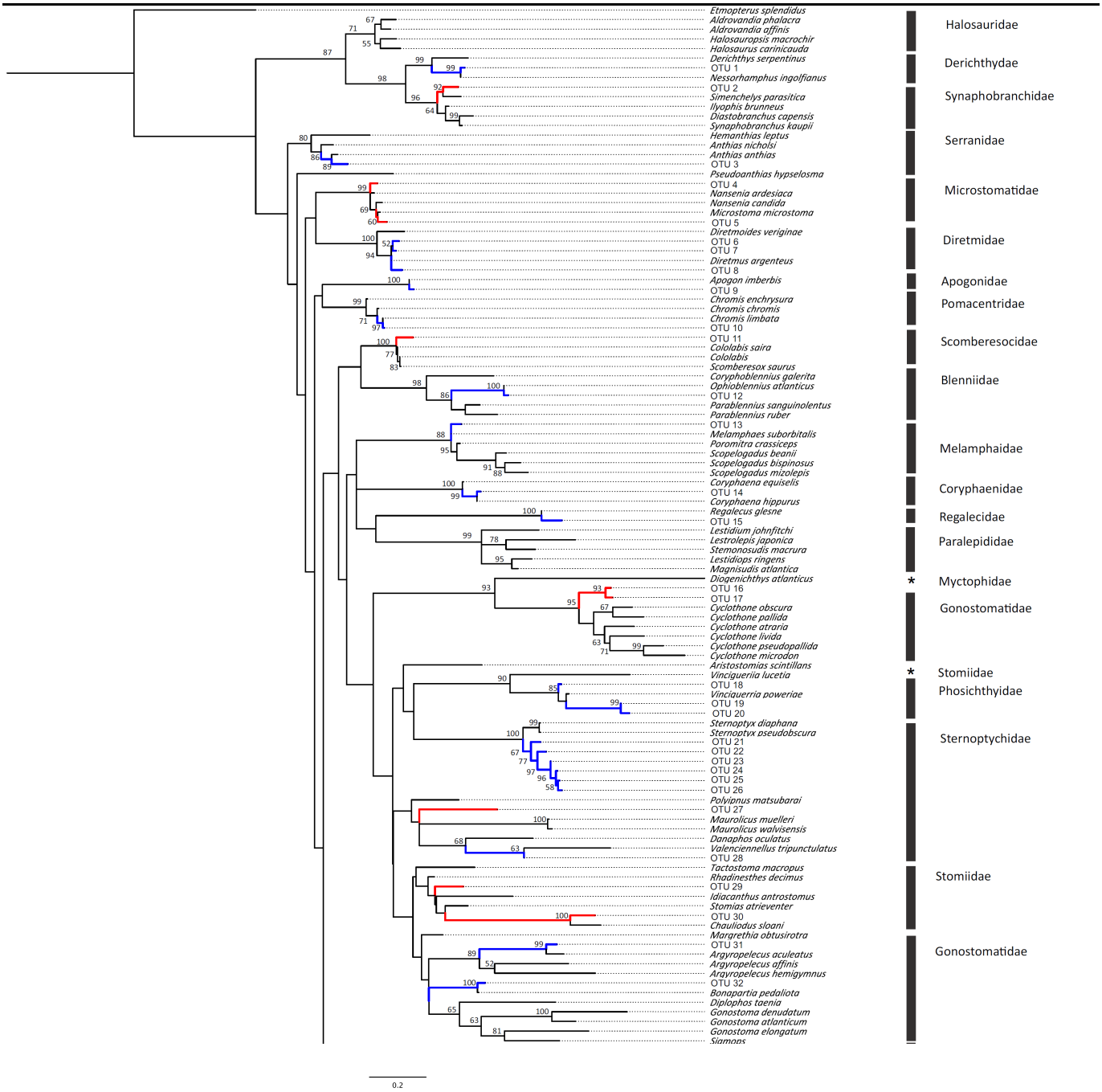
A total of 5.5 million reads were generated through ion torrent high-throughput sequencing. After pre-processing reads for quality and MID tag presence, our dataset included approximately 648,000 sequences, with sequence numbers varying between hundreds and thousands of sequences per faecal sample.

We obtained a total of 78 MOTUs producing unique matches in BLAST. Of these, 74 were successfully identified to the nearest taxonomical rank corresponding to 63 taxonomical identities, with 62% identified to species level, 21% to genera and 16% to families (Table 4.1, Figure 4.1a-c). EPA placement provided a higher robustness of prey taxonomic assignments than BLAST. For example, MOTUs displaying similarity percentages < 97% were often phylogenetically unrelated with the nearest neighbor identified in BLAST even at inner branches (families). Although MOTUs showing similarity percentages > 98% were overall successfully placed with the nearest neighbor of BLAST on the reference trees using EPA, few MOTUs showed uncertain placements. For example, queries clustering monophyletically with the fish family Scombridae, showed high similarity values with the nearest neighbour in BLAST, but also with other references in BLAST. These queries were not assigned to the nearest neighbour, or to any other reference producing high similarities in BLAST, based on EPA, showing that this algorithm provides robust taxonomic assignments.

Table 4.1. List of prey identified in the community of petrels at Selvagem Grande (NE Atlantic). The frequency of occurrences of prey is shown for each petrel species. Lower taxonomical ranks (species and genera) were assigned based on Maximum likelihood scores of placement on reference trees, given by the Evolutionary Placement Algorithm (EPA). Family ranks were identified based on monophyletic clusters containing members of that family for bootstrap support >0.80. Similarity percentages with the nearest neighbor of BLAST are shown. The frequency of occurrences of each taxa is shown and is expressed as the relative frequency of total items in each bird species. Circles indicate the depth range and habitat at which species are distributed, herein classified as mesopelagic (● > 200 m depth) and epipelagic (O at the surface) and reef-associated (△) (data obtained from FishBase (http://www.fishbase.org).

Order	Family	*Taxon*	EPA	BLAST	Bulwer´s petrel	Band-rumped Storm petrel	White faced Storm petrel
FISH							
Anguilliformes (eels**)**	Derichthyidae	*Nessorhamphus ingolfianus* ●	>0.99	99.26	2.2	-	0.7
	Synaphobranchidae	Unidentified ●		94.07	1.1	-	-
Beryciformes (squirrelfish and roughies)	Diretmidae	*Diretmus argenteus* ●	>0.99	98.39-99.20	3.3	2.6	0.7
Lampriformes	Regalecidae	*Regalecus glesne*	1	95.08	-	0.9	-
Myctophiformes (laternfish)	Myctophidae	*Bolinichthys* ●	0.93- >0.99	96.0-97.60	2.2	5.2	0.7
		Centrobranchus nigroocellatus ●	1	95.63-98.80	-	-	11.6
		Ceratoscopelus maderensis ●	1	98.8	-	-	2
		Ceratoscopelus warmingi ●			2.2	5.2	0.3
		Diaphus sp1 (luetkeni) ●	0.99	94.42	3.3	4.3	1.3
		Diaphus sp2 (molilis) ●	1	99.2	-	1.7	-
		Hygophum hygomii ●	1	98.8	-	0.9	1
		Hygophum reinhardtii ●	1	98.41-98.80	-	2.6	1.7
		Lampanyctus ●		95.6-98.80	1.1	11.2	0.7
		Lobianchia gemellarii ●	0.97	98.84	-	2.6	-
		Lobianchia dofleini ●	1	99.61	1.1	1.7	-
		Loweina ●	>0.99	91.9	1.1	0.9	-
		Myctophum nitidulum ●	1	97.2	-	-	0.7
		Notoscopelus resplendens ●	1	98.8	-	7.8	1.7
		others			-	3.6	-
Osmeriformes	Microstomatidae	Unidentified ●		97.99	-	0.9	0.3
Perciformes (perch)	Apogonidae	*Apogon imberbis* △	1	99.2	-	-	0.3
	Blenniidae	*Ophioblennius atlanticus* △	1	99.21	-	-	2.3
	Coryphaenidae	*Coryphaena hippurus* O	>0.99	99.3	-	-	0.3
	Gempylidae	*Nealotus tripes* ●	1	97.58	2.2	0.9	-
	Pomacentridae	*Chromis limbata* △	>0.99	99.22	-	-	1.7
	Serranidae	*Anthias anthias* △	>0.99	94.23	1.1	1.7	-
	Scombridae	Unidentified			1.1	-	1
	Sparidae	*Boops boops* △	1	98.8	1.1	-	3
Stephanoberyciformes	Melamphaidae	*Melamphaes suborbitalis* ●	0.82	99.2	1.1	-	-
Stomiiformes	Gonostomatidae	*Bonapartia pedaliota* ●	1	97.18	1.1	0.9	-

(dragonfish and hatchfish)							
		Cyclothone ●		98.37	1.1	6.9	0.3
	Phosichthyidae	*Vinciguerria* ●	0.96	96.3	2.2	2.6	1.3
		Vinciguerria poweriae ●				1.7	1.3
	Sternoptychidae	*Argyropelecus* ●	1	98.39	-	1.7	0.3
		Sternoptyx ●	0.93-0.95	94.65-96.98	3.3	17.3	3.6
		Valenciennellus tripunctulatus ●	>0.99	95	1.1	0.9	-
		others			2.2	-	-
	Stomiidae	*Chauliodus sloani* ●	1		1.1	-	-
Tetraodontiformes (pufferfish and sunfish)	Molidae	*Ranzania laevis* ○	1	97.99	1.1	-	-
	Tetraodontidae	Unidentified △		97.56	-	-	2.3
Unidentified fish					-	0.9	1.6
CEPHALOPODA							
Oegopsida	Architeuthidae	*Architeuthis*	>0.99	90.26	1.1	0.9	-
	Cranchiidae	*Helicocranchia pfefferi*	>0.99	92.19	-	0.9	-
		Leachia sp		96.53	1.1	-	-
	Cycloteuthidae	*Cycloteuthis sirventi*	1	98.93	2.2	-	0.3
	Histioteuthidae	*Histioteuthis sp1*		93.62-97.28	1.1	1.7	-
		Histioteuthis sp2			-	0.9	-
		Histioteuthis hoylei	1	99.47	14.4	2.6	0.3
		Histioteuthis reversa	1	99.47	4.4	-	-
	Mastigoteuthidae	*Mastigoteuthis magna*	>0.99	99.46	-	0.9	-
	Neoteuthidae	*Neoteuthis thielei*	1	98.97	-	0.9	-
	Octopoteuthidae	unidentified		92.86	2.2	-	-
	Ommastrephidae	*Ommastrephes bartramii*	1	99.48	18.9	-	1.3
	Onychoteuthidae	unidentified		97.38	1.1	-	-
Spirulida	Spirulidae	*Spirula spirula*		99.47	3.3	-	-
Unidentified Cephalopods					5.5	-	1.3
CRUSTACEA							
Decapoda: Brachyura	Grapsidae	*Pachygrapsus marmoratus*	>0.99	96.63-99.04	1.1	-	4
		Pachygrapsus maurus	>0.99	96.65-98.11	1.1	-	1.7
		Planes minutus	1		-	-	0.6
	Plagusiidae	*Plagusia depressa*	1	0.98	-	-	0.3
	Eriphiidae	*Eriphia verrucosa*	>0.99	99.03	-	-	0.7
		unidentified		78.67-84.88	-	-	0.3
	Portunidae	*Liocarcinus corrugatus*	1	98.51	-	-	0.3
		Portunus sp	1	91.63	-	-	0.7
		Portunus hastatus	1	99.01	-	-	7.9
		unidentified		78.74	-	-	0.3
Decapoda;	Aristeidae	*Aristeus antennatus*	>0.99	99.5	1.1	-	-
Euphausiacea				89.11-91.04	-	-	8.9
Copepoda				83.01	1.1	-	3.9
Isopoda				85.47	2.2	4.3	25


Etmopterus splendidus
Aldrovandia phalacra
Aldrovandia affinis
Halosauropsis macrochir
Halosaurus carinicauda
Derichthys serpentinus
OTU 1
Nessorhamphus ingolfianus
OTU 2
Simenchelys parasitica
Ilyophis brunneus
Diastobranchus capensis
Synaphobranchus kaupii
Hemanthias leptus
Anthias nicholsi
Anthias anthias
OTU 3
Pseudoanthias hypselosma
OTU 4
Nansenia ardesiaca
Nansenia candida
Microstoma microstoma
OTU 5
Diretmoides veriginae
OTU 6
OTU 7
Diretmus argenteus
OTU 8
Apogon imberbis
OTU 9
Chromis enchrysura
Chromis chromis
Chromis limbata
OTU 10
OTU 11
Cololabis saira
Cololabis
Scomberesox saurus
Coryphoblennius galerita
Ophioblennius atlanticus
OTU 12
Parablennius sanguinolentus
Parablennius ruber
OTU 13
Melamphaes suborbitalis
Poromitra crassiceps
Scopelogadus beanii
Scopelogadus bispinosus
Scopelogadus mizolepis
Coryphaena equiselis
OTU 14
Coryphaena hippurus
Regalecus glesne
OTU 15
Lestidium johnfitchi
Lestrolepis japonica
Stemonosudis macrura
Lestidiops ringens
Magnisudis atlantica
Diogenichthys atlanticus
OTU 16
OTU 17
Cyclothone obscura
Cyclothone pallida
Cyclothone atraria
Cyclothone livida
Cyclothone pseudopallida
Cyclothone microdon
Aristostomias scintillans
Vinciguerria lucetia
OTU 18
Vinciguerria poweriae
OTU 19
OTU 20
Sternoptyx diaphana
Sternoptyx pseudobscura
OTU 21
OTU 22
OTU 23
OTU 24
OTU 25
OTU 26
Polyipnus matsubarai
OTU 27
Maurolicus muelleri
Maurolicus walvisensis
Danaphos oculatus
Valenciennellus tripunctulatus
OTU 28
Tactostoma macropus
Rhodinesthes decimus
OTU 29
Idiacanthus antrostomus
Stomias atrieventer
OTU 30
Chauliodus sloani
Margrethia obtusirotra
OTU 31
Argyropelecus aculeatus
Argyropelecus affinis
Argyropelecus hemigymnus
OTU 32
Bonapartia pedaliota
Diplophos taenia
Gonostoma denudatum
Gonostoma atlanticum
Gonostoma elongatum
Halosauridae
Derichthydae
Synaphobranchidae
Serranidae
Microstomatidae
Diretmidae
Apogonidae
Pomacentridae
Scomberesocidae
Blenniidae
Melamphaidae
Coryphaenidae
Regalecidae
Paralepididae
*
Myctophidae
Gonostomatidae
*
Stomiidae
Phosichthyidae
Sternoptychidae
Stomiidae
Gonostomatidae
0.2

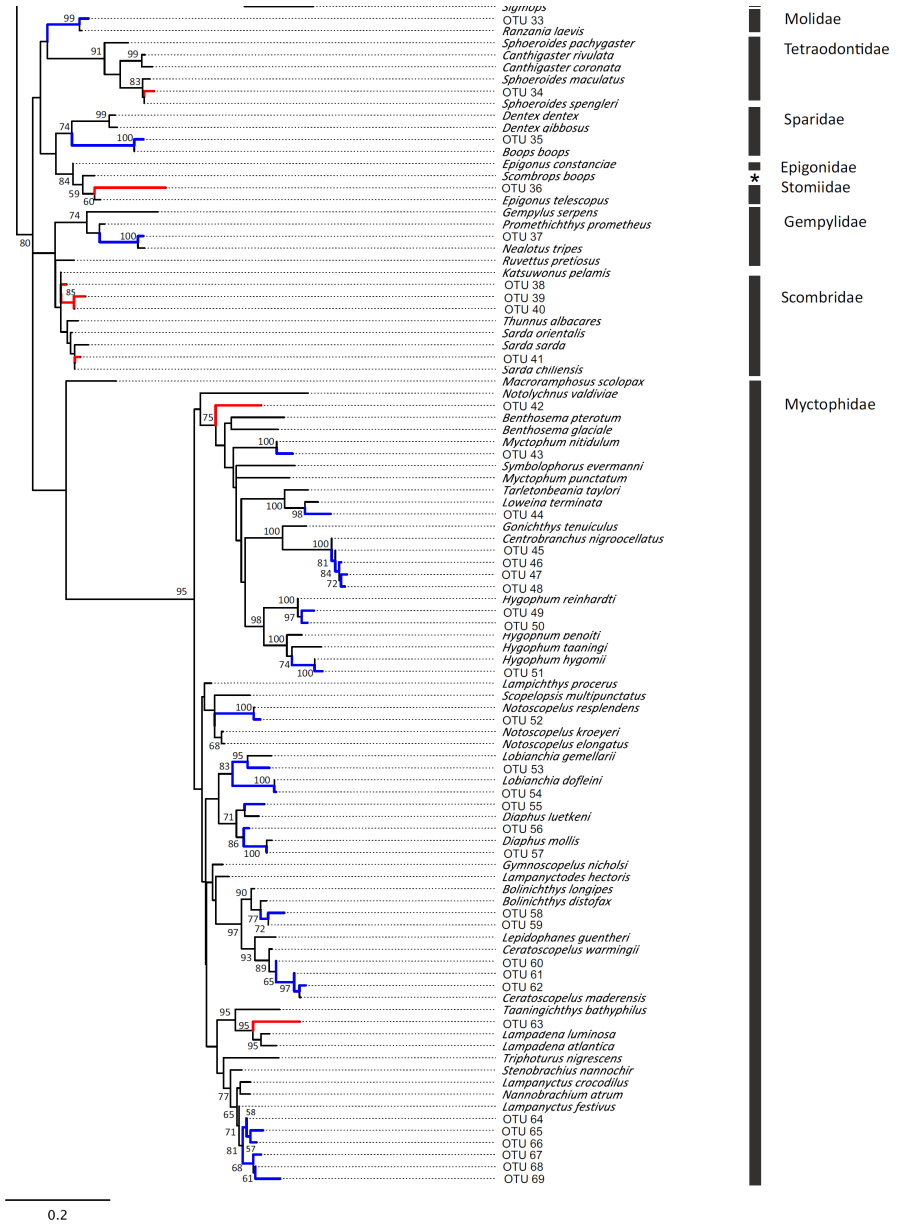


Figure 4.1a-c. Maximum Likelihood identification of queries through phylogenetic placement on three different reference trees: fish (a), cephalopods (b) decapod crustaceans (c). Bootstrap support, higher than 50%, is shown on the tree. Query placements obtained through the Evolutionary Placement Algorithm (EPA), are shown as blue lines – indicating high confidence in prey assignment (Likelihood weight ratio > 0.90) and red lines - indicating low confidence in prey assignments (Likelihood weight ratio < 0.90), where assignments with other references are equally probable. Clades corresponding to family ranks are shown. Reference taxa clustering outside a monophyletic clade (*). Genbank accession numbers of the reference taxa are included in supplementary material (Table S4.1 a-c)

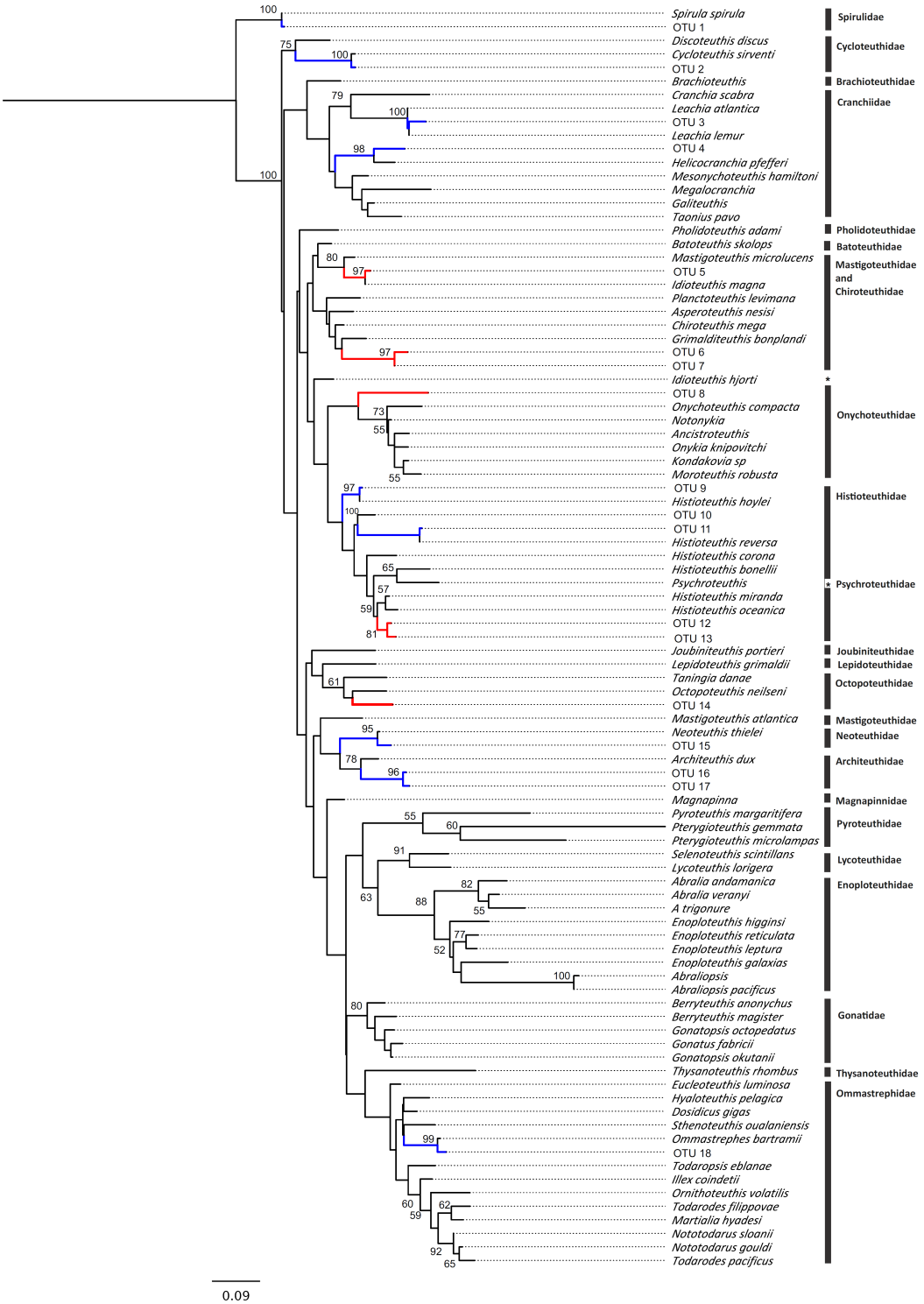


Figure 4.1b Continued

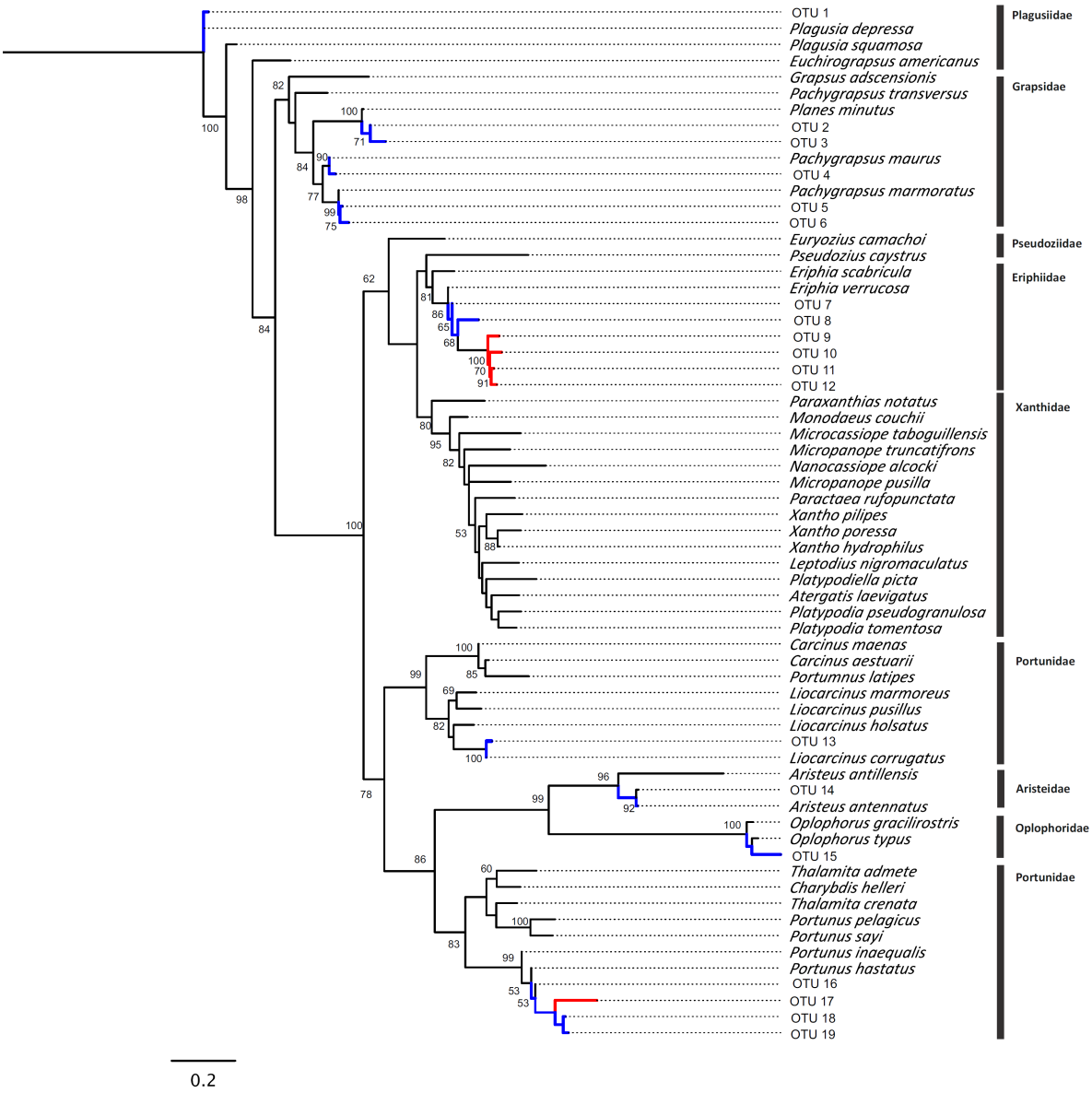


Figure 4.1c. Continued

4.4.3 Comparisons of diets

Of a total of 284 samples resulting in positive PCR amplications, HTS analysis revealed that only 55% of Bulwer's petrel, 79% of White-faced Storm petrel and 53% of Band-rumped Storm petrel samples actually contained prey. Dietary composition in all three birds showed high numbers of rare prey with over 37% of the taxa occurring only once in Bulwer's petrels, 34% in White-faced Storm petrel and 45% in Band-rumped Storm petrel. Many of these rare species were, however, included in the same

families (Table 4.1). Bulwer's petrels consumed substantially more cephalopods than both other birds (Figure 4.2) of which mesopelagic *Histioteuthis hoylei* and *Ommastrephes bartramii* were the most abundant species (Table 4.1). Conversely, White-faced Storm petrels showed a considerable higher consumption of crustaceans, especially crabs and isopods, than any other bird species, and notoriously less cephalopod (Figure 4.2, Table 4.1). This petrel was also unique in consuming species that are generally found in coastal or shallow habitats, such as various reef-associated fish and shore crabs, for example the crab *Eriphia verrucosa* and the Tidal Spray Crab (*Plagusia depressa*), which are also found in the stomach contents of gulls at Selvagens (Matias & Catry 2010)

Band-rumped Storm petrels consumed mainly mesopelagic fish, myctophids and sternoptychids, as so did Bulwer's petrels and White-faced Storm petrels, but differed to a wide extent in the range of species consumed (Table 4.1).

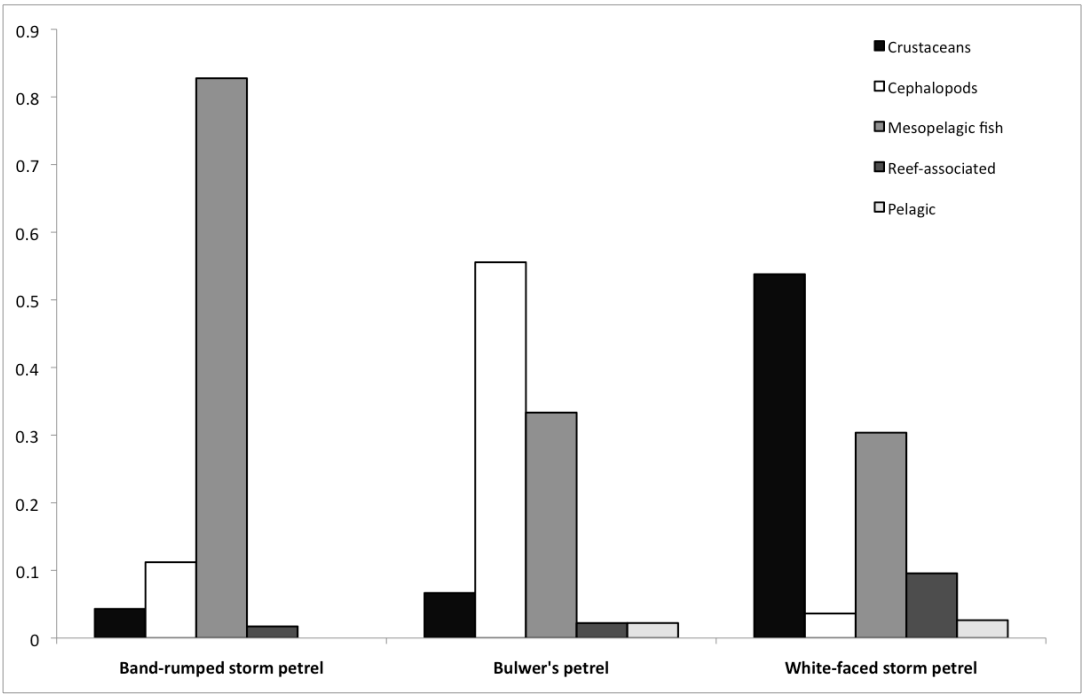


Figure 4.2: Frequency of the principal prey categories shown for each species. The proportions of prey shown were calculated as the number of individuals of a particular prey category divided by the total number of prey identified in each bird species.

4.4.4 Trophic segregation among petrels

To test for trophic segregation we plotted Bray-Curtis prey dissimilarities between individual birds in conceptual space using MDS (Figure 4.3). Overall, three main clusters corresponding to individuals of each bird species were distinguished, showing significantly higher inter-specific than intra-specific dissimilarities (perMANOVA, df=2, F=15.81, R^2=0.15, p<0.001). The most influential taxa contributing the most to the observed differences between clusters were the fish: *Sternoptyx* sp, *Centrobranchus nigroocellatus*, *Lampanyctus* sp and *Notoscopelus resplendens*; the cephalopods: *Histioteuthis hoylei* and *Ommastrephes bartramii*; and crabs and isopods, accounting for over 50% of dissimilarities between samples (SIMPER).

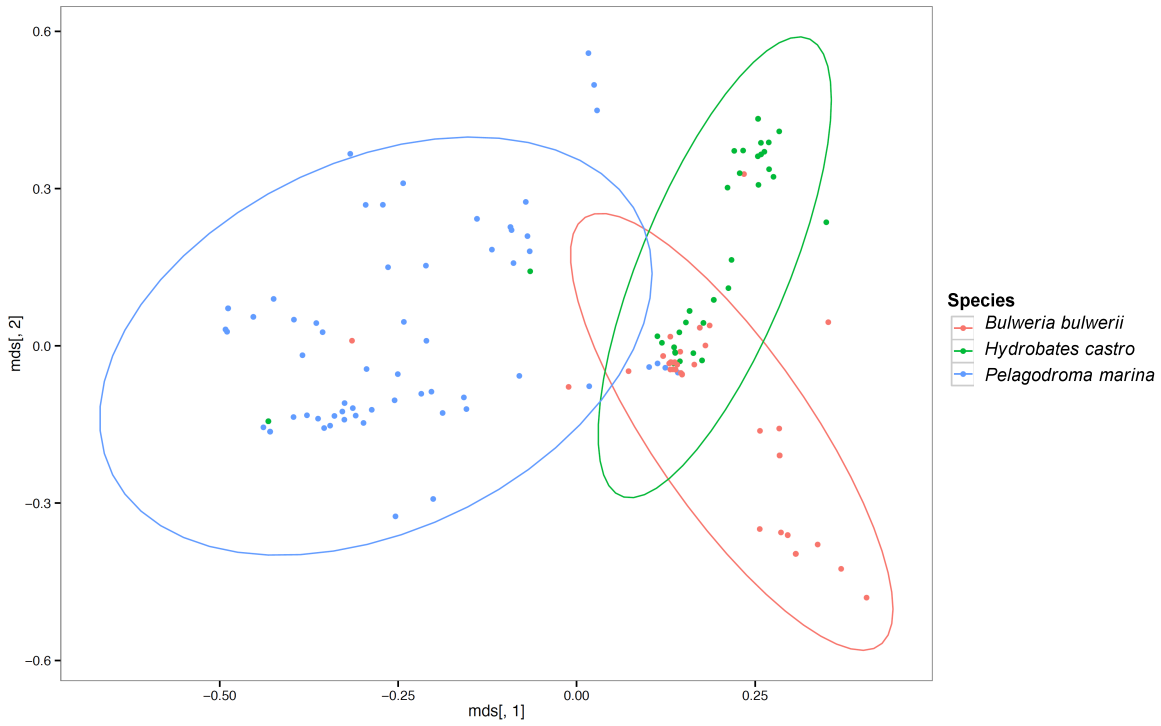


Figure 4.3: Multi-dimensional scaling based on presence / absence data (Bray-Curtis dissimilarities) of prey from faecas of Bulwer´s petrel (red points), Band-rumped strom petrel (green) and White-faced storm petrel (blue). 95% confidence ellipses show the dispersion among samples of each species.

4.5 Discussion

This study is to the best of our knowledge the first to address prey partitioning between sympatric small petrels (or any birds) using high-throughput sequencing (HTS) technologies on faeces. Prey species were discriminated based on short 16S rRNA barcodes and Maximum likelihood assignments of queries on reference trees. We obtained a similar composition of species from faeces as earlier studies conducted on stomach contents (Harrison 1986, Spear et al. 2007; Neves et al. 2011), indicating that species identification based on short 16S rRNA barcodes provides unbiased prey assignments. Moreover, contrary to former studies, HTS greatly improved prey identification of crustaceans to species ranks. Diversity within this group is under-represented in stomach content analysis, as exoskeletons and characteristic anatomical parts are often too degraded for robust lower taxonomical assignments.

The three species, for which we obtained data on trophic segregation by prey type, are the smallest birds within the community of birds breeding at Selvagem Grande. Given that these petrels coexist throughout their breeding cycles, current theory predicts that these species segregate in their dietary choices, in their foraging areas or a combination of both to reduce intra and inter-specific competition pressures.

Despite these assumptions many empirical studies have actually shown a significant isotopic overlap between similar functional birds. Bodey et al. (2014), for example, found significant inter-specific overlap between small petrels from the southern oceans. Forero et al. (2004) showed that phylogenetically-related birds breeding in Argentinean Patagonia presented a high degree of isotopic overlap. Both, however, explained trophic overlap differently, depending on the oceanographic context in which the species coexist. Bodey et al. 2014 refer to the unpredictability of oceanographic conditions in southern oceans as a probable cause for dietary overlap. At high latitudes, generally one or two superabundant prey types are dominant, such as for example krill, capelin and sandeels (Croxall & Prince 1980; Frederiksen et al. 2007; Buren et al. 2014). At offshore sites, these prey types are known to accumulate in patches. Birds that are constrained to forage near the colony for successful reproduction and chick rearing might have no other option than pursuing these prey, despite high competition pressures. The diet of the community of seabirds breeding at upwelling areas also typically shows superabundances of certain prey types, such as sardines and

anchovies (Bakun & Parrish 1991). In fact abundances of these prey types might be such that resources are not limited for these birds, therefore leading to high overlap in the diet (Forrero et al. 2004). In contrast, seabird communities at subtropical or tropical latitudes have shown substantially higher diversities of prey, not marked by few dominant prey species as in higher latitudes. This pattern is also evident in our study, with seabirds consuming a substantial number of species with rare occurrences (recorded only once in a sample). Coexisting birds can probably take advantage of higher species diversities and adapt to different prey types by changing foraging strategies.

Our findings suggest partitioning by prey type between these sympatric petrels, with White-faced Storm petrel segregating its diet the most. A similar pattern of niche segregation was previously described by Spear et al. (2007) who conducted a comprehensive study on the diet of thirty different species of procellariiformes in the tropical Pacific. As in our study, White-faced Storm petrels segregated the most from other petrels shown feeding on mesopelagic organisms mainly due to the high quantity of non-cephalopod invertebrates. The prey types we identified were very similar to the ones identified by Spear et al. (2007) in the diet of the same or similar birds.

The diet of White-faced Storm petrels spanned prey types across functional groups, foraging on fish, a few cephalopods and especially crabs and isopodes. Although this petrel consumed a high quantity of mesopelagic fish, as did both the Bulwer's petrels and Band-rumped Storm petrels, the White-faced Storm petrel was, nonetheless, unique in consuming epipelagic reef-associated species commonly found in the intertidal zones around Selvagem Grande or around more coastal areas such as the water masses surrounding the Canary islands off the African NW coast. It is noteworthy that the crabs identified in White-faced Storm petrels were probably plankton larvae or juveniles, as previously reported by Spear et al. (2007) who also identified crabs, but at megalopa larval stages, in the diet of White-faced Storm petrels. The presence of other littoral reef-associated species in the diet of White-faced Storm petrel is unexpected, as Storm petrels have generally not been observed foraging inshore. It has been, however, suggested that European Storm petrel (*Hydrobates pelagicus*) frequently does so, explaining why this species also presents littoral reef associated fish in their diets (D'Elbée & Hémery 1998; Poot et al. 2008).

Bulwer's petrels and Band-rumped Storm petrel relied on more similar prey groups, targeting almost exclusively mesopelagic fish and cephalopods, with very few crustaceans. The existence of mesopelagic species, commonly found in pelagic predator diets, has been hypothesized to result from foraging activity at night, when organisms from deep-scattering layers (DSL) migrate to the surface layers probably to feed. Data on at-sea activity of Bulwer's petrels in the Selvagem Grande supports these conclusions, showing a significant higher flight activity during darkness than daylight (Dias et al. in press). Therefore, differences in the diet of White-faced Storm petrel as compared with both other study species might result from a comparatively higher diurnal foraging activity of the former species. This would explain why White-faced Storm petrel consumed comparatively higher numbers of epipelagic species.

Despite similar functional prey groups, Bulwer's petrel and Band-rumped Storm petrel showed significant trophic partitioning at species ranks. Given a likely nocturnal foraging behavior in both of these birds, the differences suggest that these species probably reduce inter-specific competition by foraging on different feeding grounds. It is noteworthy that such conclusions have to be treated with caution, as Bulwer's petrels were sampled two months later, so that differences in the diet might result from seasonal shifts in prey availability. However, we have reasons to believe that season did not affect the prey types identified in both birds. In fact, the prey types most contributing to the trophic dissimilarities, were also important prey for small sympatric birds in the tropical Pacific. Moreover, as in our study, Bulwer's petrels consumed substantially higher frequencies of cephalopods than any other Storm petrel. White-faced Storm petrels relied to a high extent on crustaceans, while Storm petrels feeding on mesopelagic organisms preyed mainly on fish. Such results indicate that pelagic seabirds foraging on the deep open oceans show consistent trophic specializations across their distribution range. These trophic specializations are apparently the main factor promoting trophic segregation in these predators rather than a differential resource-use resulting from different oceanic habitats and breeding grounds.

4.6 Conclusion

High-throughput sequencing technologies provide important means to assess the diet of seabirds, allowing distinguishing prey at low taxonomical ranks so that trophic

segregation can be detected even between species that consume similar prey types. Moreover, by allowing detecting prey from faecal remains, it further opens ways to include large dietary datasets without physically harming animals. We identified a high diversity of prey and significant trophic segregation between coexisting species in the subtropical colonies of the northeastern Atlantic, while showing similar specializations with birds from in other sub-tropical regions. These results emphasize the importance of describing the prey types of predators to understand species interactions and niche withes in marine ecosystems.

4.7 References

Alonso H, Granadeiro JP, Ramos JA, Catry P (2013) Use the backbone of your samples: fish vertebrae reduces biases associated with otoliths in seabird diet studies. Journal Of Ornithology 154: 883-886.

Alonso H, Granadeiro JP, Waap S, Xavier J, Symondson WOC, Ramos JA. & Catry P (2014) An holistic ecological analysis of the diet of Cory's shearwaters using prey morphological characters and DNA barcoding. Molecular Ecology 23, 3719-3733.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJJ (1990) Basic local alignment search tool. Journal of Molecular Biology, 215, 403-410.

Ashmole NP (1963) The regulation of numbers of tropical oceanic birds. Ibis 103:458–473

Bakun A & Parrish RH (1991) Comparative studies of coastal pelagic fish reproductive habitats: The anchovy (Engraulis anchoita) of the southwestern Atlantic. ICES Journal of Marine Sciences 48:343-361.

Berger SA, Krompass D & Stamatakis A (2011) Performance, Accuracy, and Web Server for Evolutionary Placement of Short Sequence Reads under Maximum Likelihood. Systematic Biology 60: 291-302

Bocher P, Cherel Y & Hobson KA (2000) Complete trophic segregation between South Georgian and Common diving petrels during breeding at Iles Kerguelen. Marine Ecology Progress Series 208: 249-264.

Bodey TW, Ward EJ, Phillips RA, McGill RAR & Bearhop S (2014) Species versus guild level differentiation revealed across the annual cycle by isotopic niche examination. Journal of Animal Ecology 83: 470–478

Bowser AK, Diamond AW & Addison JA (2013) From Puffins to Plankton: A DNA-Based Analysis of a Seabird Food Chain in the Northern Gulf of Maine. PLoS ONE 8: e83152

Boyer F, Mercier C, Bonin A, Taberlet P & Coissac E (2014) OBITools: a Unix-inspired software package for DNA metabarcoding. Molecular Ecology Resources, submitted (http://metabarcoding.org/obitools/doc/welcome.html)

Brown WL & Wilson EO (1956) Character displacement. Systematic Zoology 5:49–64

Buren AD, Koen-Alonso M, Pepin P, Mowbray F, Nakashima B, Stenson G, Ollerhead N &Montevecchi, W. A. (2014). Bottom-Up Regulation of Capelin, a Keystone Forage Species. PLoS ONE (9) e87589

Coulson JC (2002) Colonial breeding in seabirds. In: Schreiber EA, Burger J (eds) Biology of marine birds. CRC Press, London, p 87–113

Croxall JP (1987) Seabirds: Feeding Ecology and Role in Marine Ecosystems (Cambridge Univ. Press, Cambridge, 1987).

Croxall JP & Prince PA (1980) Food, feeding ecology and eco- logical segregation of seabirds at South Georgia. Biological Journal of the Linnean Society 14:103-131

D'Elbee J & Hemery G (1998) Diet and foraging behaviour of the British Storm Petrel *Hydrobates pelagicus* in the Bay of Biscay during summer. Ardea 86: 1-10

Darwin C (1859) On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life. J. Murray. London

Deagle BE, Kikwood R & Jarman SN (2009) Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Molecular Ecology 18: 2022-2038

Dias MP, Alho M, Granadeiro JP & Catry P (in press) Wanderer of the deepest seas: migratory behaviour and distribution of the highly pelagic Bulwer's Petrel. Journal of Ornithology.

Dieckmann U & Doebeli M (1999) On the origin of species by sympatric speciation. Nature 400: 354-357

Edgar RC (2013) UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature Methods, 10, 996-998 dx.doi.org/10.1038/nmeth.2604

Ficetola GF, Coissac E, Zundel S, Riaz T, Shehzad W, Bessière J, Taberlet P & Pompanon F (2010) An in silico approach for the evaluation of DNA barcodes. BMC Genomics 1:1-434

Ficetola GF, Coissac E, Zundel S, Riaz T, Shehzad W, Bessière J, Taberlet P & Pompanon F (2010) An in silico approach for the evaluation of DNA barcodes. BMC Genomics 1: 1-434.

Forero MG González-Solís J, Hobson KA, Donázar JA, Bertellotti M, Blanco G, Bortolotti GR (2005) Stable isotopes reveal trophic segregation by sex and age in the southern giant petrel in two different food webs. Marine Ecology Progress Series 296: 107–113

Forero MG, Bortolotti GR, Hobson KA, Donazar JA Bertelloti M & Blanco G (2004) High trophic overlap within the seabird community of Argentinean Patagonia: a multiscale approach Journal of Animal Ecology 73: 789–801

Frederiksen M, Furness RW & Wanless S (2007) Regional variation in the role of bottom-up and top-down processes in controlling sandeel abundance in the North Sea. Marine Ecology Progress Series 337: 279-286.

Gause GF (1934) The struggle for existence. Williams & Wilkins. Baltimore.

Gordon A (2010) FASTX-Toolkit. Available at http://hannonlab.cshl.edu/fastx_toolkit/ (accessed 20 May 2014)

Gotelli NJ & Ellison AM (2013) EcoSimR 1.00 (http://www.uvm.edu/~ngotelli/EcoSim/EcoSim.html)

Granadeiro JP, Dias MP, Rebelo R, Santos CD, Catry P (2006) Numbers and population trends of Cory's Shearwater Calonectris diomedea at Selvagem Grande, northeast Atlantic. Waterbirds 29: 56–60

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41: 95-98.

Hardin G (1960) The competitive exclusion principle. Science 131:1292–1297.

Harrison CS, Hida TS & Seki MP (1983) Hawaiian seabird feeding ecology. Wildlife Monographs 85: 3-71.

Haug FD, Paiva VH, Werner AC, Ramos JA (2015) Foraging by experienced and inexperienced Cory's shearwater along a 3-year period of ameliorating foraging conditions. Marine Biology 162:649-660

Jacob U, Mintenbeck K, Brey T, Knust R & Beyer K (2005) Stable isotope food web studies: a case for standardized sample treatment. Marine Ecology Progress Series 287: 251– 253

King WB (1983) Seabird breeding habits. Oceanus 26:28 – 35

Lewis S, Sherratt TN, Hamer KC & Wanless S (2001) Evidence of intra-specific competition for food in a pelagic seabird. Nature 412: 816–819.

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal, 17:10-12

Matias R & Catry P (2010) The diet of Atlantic Yellow-legged Gulls (Larus michahellis atlantis) at an oceanic seabird colony: estimating predatory impact upon breeding petrels European Journal of Wildlife Research 56:861–869

Matsen FA, Kodner R & Armbrust EV (2010) pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. BMC Bioinformatics 11: 538.

Miller AK, Kappes MA, Trivelpiece SG & Trivelpiece WZ (2010) Foraging-Niche Separation of Breeding Gentoo and Chinstrap Penguins, South Shetland Islands, Antarctica. Condor 112:683-695

Navarro J, Votier SC, Aguzzi J, Chiesa JJ, Forero MG, & Phillips RA (2013) Ecological Segregation in Space, Time and Trophic Niche of Sympatric Planktivorous Petrels. PLoS ONE 8: e62897

Neves VC, Nolf D & Clarke MR (2011) Diet of Bulwer's petrel in the Azores, NE Atlantic. Waterbirds 34: 357-362

Nunes M (2000) Madeiran Storm-Petrel (*Oceanodroma castro*) in the Desertas Islands (Madeira Archipelago): a new case of two distinct populations breeding annually? Arquipélago. Life and Marine Sciences Supplement 2: 175-179

Oksanen J, Blanchet GF, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Henry M, Stevens H & Wagner H (2015) vegan: Community Ecology Package. R package version 2.2-1 (http://CRAN.R-project.org/package=vegan)

Pfenning KS & Pfenning DW (2009) Character displacement: ecological and reproductive responses to a common evolutionary problem. The Quarterly Review of Biology 84: 253–276.

Phillips RA, Croxall JP, Silk JRD & Briggs DR (2007) Foraging ecology of albatrosses and petrels from South Georgia: two decades of insights from tracking technologies. Aquatic conservation 17:S6-S21

Phillips RA, McGill RAR, Dawson DA, Bearhop S (2011) Sexual segregation in distribution, diet and trophic level of seabirds: insights from stable isotope analysis. Marine Biology 158: 2199-2208

Phillips RA, Silk JRD, Phalan B, Catry P & Croxall JP (2004) Seasonal sexual segregation in two Thalassarche albatrosses: competitive exclusion, reproductive role specialization or foraging niche divergence? Proceedings of the Royal Society of London, Series B 271:1283– 1291

Poot M (2008) Nocturnal and diurnal foraging of European Storm petrels *Hydrobates* sp along the Lisbon coast, Portugal. Airo 18:13-21

Smith AL, Monteiro L, Hasegawa O & Friesen VL (2007) Global phylogeography of the band-rumped storm-petrel (Oceanodroma castro; Procellariiformes: Hydrobatidae) Molecular Phylogenetics and Evolution 43:755-73

Spear LB, Ainley DG, Walker WA (2007) Foraging dynamics of seabirds in the Eastern Tropical Pacific Ocean. Studies in Avian Biology 35:1−99

Taberlet P, Coissac E, Hajibabaei M & Rieseberg LH (2012) Environmental DNA. Molecular Ecology 21:1789–1793

Vestheim H & Jarman SN (2008) Blocking primers to enhance PCR amplification of rare sequences in mixed samples – a case study on prey DNA in Antarctic krill stomachs. Frontiers in Zoology 5: 12

Wilson RP (1984) An improved stomach pump for penguins and other seabirds. Journal of Field Ornithology 55: 109-112

Xavier JC, Phillips RA & Cherel Y (2011) Cephalopods in marine predator diet assessments: why identifying upper and lower beaks is important. ICES Journal of Marine Science 68: 1857−1864

Young HS, McCauley DJ, Dirzo R, Dunbar RB & Shaffer SA (2010) Niche partitioning among and within sympatric tropical seabirds revealed by stable isotope analysis. Marine Ecology Progress Series 416:285−294

4.8 Supplementary material

Table S4.1 a-c. Genbank accession numbers of the reference taxa included in Chapter 4, (a) Fish, (b) Crustaceans and (c) Cephalopods.

S4.1 a	Fish - Taxon	Accession numbers
	Aldrovandia affinis	AP002974.1
	Aldrovandia phalacra	JX242947.1
	Anthias anthias	AY947617.1
	Anthias nicholsi	HQ731420.1
	Argyropelecus aculeatus	D89736.1
	Argyropelecus affinis	D89737.1
	Argyropelecus hemigymnus	EU099497.1
	Aristostomias scintillans	KJ010559.1
	Benthosema glaciale	AP012264.1
	Benthosema pterotum	JX133755.1
	Bolinichthys longipes	AB042165.1
	Bonapartia pedaliota	AB026033.1
	Canthigaster coronata	AP006743.1
	Canthigaster rivulata	AP006744.1
	Centrobranchus nigroocellatus	AB042182.1
	Ceratoscopelus warmingii	AB042168.1
	Chauliodus sloani	AP002915.1
	Chromis chromis	EF489731.1
	Chromis enchrysura	JQ707071.1
	Chromis limbata	EF489736.1
	Cololabis saira	EF458384.1
	Coryphaena equiselis	KC603517.1
	Coryphaena hippurus	KF719178.1
	Coryphoblennius galerita	EF521665.1
	Cyclothone atraria	D84047.1
	Cyclothone livida	D84052.1
	Cyclothone microdon	D84053.1
	Cyclothone obscura	D84055.1
	Cyclothone pallida	D84056.1
	Cyclothone pseudopallida	AB026041.1
	Danaphos oculatus	HQ127667.1
	Derichthys serpentinus	JX242957.1
	Diaphus luetkeni	AP012231.1
	Diastobranchus capensis	JX242990.1
	Diogenichthys atlanticus	AB042178.1
	Diplophos taenia	AB026031.1
	Diretmoides veriginae	AP004426.1
	Diretmus argenteus	KC603521.1
	Etmopterus splendidus	HM231283.1
	Gempylus serpens	DQ874735.1
	Gonichthys tenuiculus	AB055888.1
	Gonostoma atlanticum	D84049.1
	Gonostoma denudatum	AB026039.1
	Gonostoma elongatum	AB026036.1
	Gymnoscopelus nicholsi	AP012250.1

S4.1 b	Crustaceans - Taxon	Accession numbers
	Aristeus antennatus	KF768042.1
	Aristeus antillensis	AY601738.1
	Atergatis laevigatus	FJ548944.1
	Carcinus aestuarii	CAU74327
	Carcinus maenas	FM208763.1
	Charybdis lucifera	KF220511.1
	Eriphia scabricula	KC771005.1
	Eriphia verrucosa	EU863398.2
	Euchirograpsus americanus	AJ250648.1
	Euryozius camachoi	HM637970.1
	Grapsus adscensionis	FR871293.1
	Leptodius exaratus	KP256206.1
	Liocarcinus corrugatus	GQ268542.1
	Liocarcinus holsatus	GQ268540.1
	Liocarcinus marmoreus	GQ268547.1
	Liocarcinus pusillus	GQ268539.1
	Microcassiope taboguillensis	KF682967.1
	Micropanope pusilla	GU144440.1
	Micropanope truncatifrons	GU144434.1
	Monodaeus couchii	HM798510.1
	Nanocassiope alcocki	HM798516.1
	Oplophorus gracilirostris	KP075919.1
	Oplophorus typus	KP075923.1
	Pachygrapsus marmoratus	AY919094.1
	Pachygrapsus maurus	FR871308.1
	Pachygrapsus transversus	AM180259.1
	Paractaea rufopunctata	GU144442.1
	Paraxanthias notatus	HM798540.1
	Plagusia depressa	FN539000.1
	Plagusia squamosa	FN539001.1
	Planes major	KM510124.1
	Platypodia pseudogranulosa	HM798546.1
	Platypodia tomentosa	HM798547.1
	Platypodiella picta	AM076774.1
	Portumnus latipes	FM208764.1
	Portunus hastatus	FM208780.1
	Portunus inaequalis	FM208752.1
	Portunus pelagicus	DQ388052.1
	Portunus sayi	DQ388053.1
	Pseudozius caystrus	HM637984.1
	Thalamita admete	FJ152163.1
	Thalamita crenata	FM208754.1
	Xantho hydrophilus	HM798564.1
	Xantho poressa	JQ277185.1

Halosauropsis macrochir	JX242948.1
Halosaurus carinicauda	JX242949.1
Hemanthias leptus	FJ548772.1
Hygophum benoiti	AB024912.1
Hygophum hygomii	AB024915.1
Hygophum reinhardti	AB024919.1
Idiacanthus antrostomus	KJ010740.1
Ilyophis brunneus	JX242991.1
Katsuwonus pelamis	KM605252.1
Lampadena atlantica	KC603522.1
Lampadena luminosa	AB042166.1
Lampanyctodes hectoris	AB042170.1
Lampanyctus crocodilus	AP012258.1
Lampanyctus festivus	HM998554.1
Lampichthys procerus	AB042172.1
Lepidophanes guentheri	AB042169.1
Lestidiops ringens	KJ010558.1
Lestidiops ringens	KJ010622.1
Lestidium johnfitchi	Y952493.1
Lestrolepis japonica	KC441991.1
Lobianchia dofleini	DQ532898.1
Lobianchia gemellarii	AB042159.1
Loweina terminata	AB042184.1
Margrethia obtusirostra	D84054.1
Maurolicus japonicus	JQ178227.1
Maurolicus muelleri	AJ277245.1
Melamphaes suborbitalis	KC442003.1
Microstoma microstoma	HQ127646.1
Myctophum nitidulum	AB042181.1
Nannobrachium atrum	AB042163.1
Nansenia ardesiaca	AP004106.1
Nansenia candida	HM998555.1
Nealotus tripes	AP012521.1
Nessorhamphus ingolfianus	JX242958.1
Notolychnus valdiviae	AB042158.1
Notoscopelus elongatus	KJ128846.1
Notoscopelus kroeyeri	AJ277964.1
Notoscopelus resplendens	AB042171.1
Ophioblennius atlanticus	AY098846.1
Parablennius ruber	AY098834.1
Parablennius sanguinolentus	AY098837.1
Polyipnus matsubarai	D89739.1
Poromitra crassiceps	KJ010747.1
Promethichthys prometheus	AP012504.1
Pseudanthias hypselosoma	JX094027.1
Ranzania laevis	KC603526.1
Ruvettus pretiosus	DQ532952.1
Scomberesox saurus	AF243985.1
Scomberesox saurus	GQ412296.1
Scopelogadus beanii	AF221884.1
Scopelogadus bispinosus	AY947847.1
Scopelogadus mizolepis	AP002934.1
Scopelopsis multipunctatus	AB042174.1
Sigmops bathyphilus	AB026038.1
Simenchelys parasitica	JX242992.1

S4.1c	Cephalopods - Taxon	Accession numbers
	Abralia andamanica	HQ845987.1
	Abralia trigonure	X79584.1
	Abralia veranyi	EU735259.1
	Abraliopsis pacificus	AY616982.1
	Abraliopsis sp	X79595.1
	Ancistroteuthis lichtensteinii	EU735242.1
	Architeuthis dux	KC701764.1
	Asperoteuthis nesisi	EU421719.1
	Batoteuthis skolops	EU735200.1
	Berryteuthis anonychus	EU735238.1
	Berryteuthis magister	AY681049.1
	Brachioteuthis sp	EU735224.1
	Chiroteuthis mega	KC860982.1
	Cranchia scabra	DQ280046.1
	Cycloteuthis sirventi	EU735204.1
	Discoteuthis discus	EU735229.1
	Dosidicus gigas	AB635421.1
	Enoploteuthis galaxias	AJ223484.1
	Enoploteuthis higginsi	AJ223485.1
	Enoploteuthis leptura	EU735206.1
	Eucleoteuthis luminosa	AB635425.1
	Galiteuthis sp	AY616987.1
	Gonatopsis octopedatus	AY681024.1
	Gonatus okutanii	EU735265.1
	Grimalditeuthis bonplandi	AF110098.2
	Helicocranchia pfefferi	AF110099.2
	Histioteuthis bonellii	EU735248.1
	Histioteuthis corona	EU735211.1
	Histioteuthis hoylei	DQ280047.1
	Histioteuthis miranda	EU735255.1
	Histioteuthis oceanica	AY616986.1
	Histioteuthis reversa	EU735256.1
	Hyaloteuthis pelagica	AB270962.1
	Idioteuthis hjorti	KC860990.1
	Illex coindetii	AY616985.1
	Joubiniteuthis portieri	EU735213.1
	Kondakovia sp	EU735267.1
	Leachia atlantica	EU735203.1
	Lepidoteuthis grimaldii	DQ280048.1
	Lycoteuthis lorigera	EU735257.1
	Magnapinna sp	EU735227.1
	Martialia hyadesi	AB270955.1
	Mastigoteuthis atlantica	KC861001.1
	Mastigoteuthis magna	EU201156.1
	Mastigoteuthis microlucens	EU201150.1
	Megalocranchia sp	EU735228.1
	Mesonychoteuthis hamiltoni	EU735261.1
	Moroteuthis knipovitchi	DQ280050.1
	Moroteuthis robusta	EU735241.1
	Neoteuthis thielei	EU735215.1
	Notonykia sp	EU735232.1
	Nototodarus gouldi	AB270954.1
	Nototodarus sloanii	AB270953.1

Sphoeroides pachygaster	AB642280.1
Sphoeroides parvus	AP011914.1
Stenobrachius nannochir	AB042162.1
Sternoptyx diaphana	EU099506.1
Sternoptyx pseudobscura	AY958662.1
Stomias atriventer	KJ010751.1
Symbolophorus evermanni	AY949625.2
Synaphobranchus kaupii	JX242993.1
Taaningichthys bathyphilus	AY949626.2
Tactostoma macropus	AY947849.2
Tarletonbeania taylori	AB042185.1
Thunnus albacares	KM588080.1
Triphoturus nigrescens	AB042164.1
Valenciennellus tripunctulatus	JN602067.1
Vinciguerria lucetia	HQ127632.1
Vinciguerria poweriae	HM143730.1
Octopoteuthis nielseni	AY616983.1
Octopoteuthis sicula	EU735217.1
Ommastrephes bartramii	AB270956.1
Onychoteuthis compacta	AJ223482.1
Ornithoteuthis volatilis	AB270961.1
Pholidoteuthis adami	EU735254.1
Planctoteuthis levimana	EU735247.1
Psychroteuthis sp.	EU735221.1
Pterygioteuthis gemmata	EU735208.1
Pterygioteuthis microlampas	EU735253.1
Pyroteuthis margaritifera	EU735209.1
Selenoteuthis scintillans	EU735230.1
Spirula spirula	AJ966785.1
Sthenoteuthis oualaniensis	AB270958.1
Taonius pavo	KC603481.1
Thysanoteuthis rhombus	EU735236.1
Todarodes filippovae	AB270950.1
Todarodes pacificus	AB270951.1
Todaropsis eblanae	AY616988.1

CHAPTER 5:

Predator-prey interactions across the lunar cycle contradict foraging efficiency predictions in a pelagic bird, the Bulwer's petrels

Predator-prey interactions across the lunar cycle contradict foraging efficiency predictions in a pelagic bird, the Bulwer's petrels

Key-words: Bulwer's petrel, diel vertical migration, foraging efficiency hypothesis, mesopelagic prey, molecular analysis of diet.

Author contributions

This chapter includes contribution from: Alonso H, Catry P, Dias M, Granadeiro JP and Symondson. PC, WOCS, JPG, MD contributed significantly to the experimental design, HA performed the morphological analysis of hard structures, PC, WOCS and JPG contributed with advice and critical review. I was the senior author and performed the laboratory work, sample collection, data analysis and writing

5.1 Abstract

The moon induces cyclical changes in oceanographic processes and nocturnal light levels that have long been known to affect animal reproduction and behavior. Several pelagic seabirds, for example, exhibit lunar rhythms in colony attendance and flight activity depending on the dates of new or full moons. Two hypotheses have been proposed to explain lunar rhythms observed in marine predators: 1. The *foraging efficiency* hypothesis states that foraging (by seabirds) is optimised during new moons and is based on current oceanographic evidence of moon-induced changes in the vertical migration patterns of organisms residing in deep oceanic layers, but ascending to the surface at night to feed. 2. Alternatively, predator avoidance can underlie lunar rhythms of activity, as birds are more visible to predators.

Evidence on the actual prey targeted by birds at different moon phases is virtually absent. Here, we evaluated foraging success in Bulwer's petrels across moon cycles, by specifically assessing potential shifts in diet. We combined DNA barcoding and morphological analysis to quantify and augment prey identification resolution using prey remains collected from stomach contents. Contrary to expectations by the *foraging efficiency hypothesis*, we found no evidence for shifts in prey species composition during different moon phases. Bulwer's petrels foraged almost exclusively on mesopelagic species and did not differ prey diversity among all moon phases. Our results contradict current oceanographic expectations of prey availability as mesopelagic species are thought to descend to deeper layers on moonlit nights, probably to avoid predation. Ours results reveal the importance of incorporating predator-prey interactions in moon phase-related foraging predictions.

5.2 Introduction

It is well known in the literature that moon affects animal behavior and reproduction on Earth (Skov et al. 2004). During full moons nocturnal animals might either augment activity at night, taking advantage of visual cues to mate and find food, or reduce activity to avoid predators (Brown et al. 2001; Kotler et al. 2010). The moon further exerts an important influence on environmental factors such as tides so that

many marine species have developed lunar periodic rhythms of 14.8 days and 29.5 days to optimise foraging, reproduction and dispersal (Queiroga et al. 2006; Zhang et al. 1999; Kaiser et al. 2011).

In the deep scattering layers (DSL) of the oceans, animals respond to solar light intensities, migrating upwards in the water column at night to feed at the surface, while descending to deeper layers during the day to avoid predators (Zaret & Suffern 1976). However, species of the DSL also react to changes in moonlight intensity, migrating closer to the surface during new moons than full moons (Clarke 1973; Kampa 1974; Benoit-Bird et al. 2009a;b).

It has been shown that pelagic predators also react to moon light intensity, differing significantly in their activity patterns at sea and at the colony (Horning & Trillmich 1999; Cruz et al. 2013). Many procellariiformes attend the colony at later hours during moonlit nights and show significantly higher flight activities than during new moon nights. Two hypotheses have been generally evoked to explain these patterns.

Imber (1975) suggested that the foraging efficiency of seabirds was lower during moonlit nights because of substantial less available prey that migrate down the water column to avoid visual predators. As a result seabirds spend more time searching for few available prey and return later to the colony at moonlit nights (Imber 1975; Klomp & Furness 1992) However, some authors have suggested the opposite, with predators increasing foraging efficiency during moonlit as a result of optimised prey detection during bright nights (Phalan et al. 2007; Cruz et al. 20013) (*The foraging efficiency hypothesis*). Although these observations generally agree with the foraging efficiency hypothesis, some authors have suggested that other factors may underlie the observed patterns, such as predator avoidance. Small petrels, for instance, are eaten by other predatory birds breeding at the same colonies and are expected to avoid the risk of encountering visual predators during moonlit nights, therefore delaying their return during moonlit nights (predation risk hypothesis)(Riou & Hamer 2008; Rubolini et al. 2014).

Despite predictable effects of the moon on the vertical migration patterns of species of the DSL, and its potential effects on ecosystem bottom-up and top-down processes, this topic has received very little attention in ecological studies. To date almost nothing is known about the range of species that react to moonlight in DSL or

how nocturnal active predators cope with putative absence of specific prey taxa on moonlit nights.

Dietary studies on top predators are a potentially effective way to study the impact of environmental factors on prey species availability, while providing insight into demographic regulation of predator populations, the structure of food webs and the organization of communities. As such, birds have been preferred models in the field of trophic ecology, primarily due to their important role in ecosystem regulation, with for example, ca 70 million tones of the ocean's biomass being consumed annually by seabirds (Brooke 2004), approaching the global catch by marine fisheries (Karpouzi et al. 2007).

A range of techniques have been developed to analyse prey remains in seabirds, including morphological analyses of stomach contents (Ralph et al. 1985; Duffy & Jackson 1986), isotopic signature analyses (Hobson et al. 1994), and more recently DNA-based techniques applied to faeces and stomach contents (Deagle et al. 2007; Bowser et al. 2013; Alonso et al. 2014, Chapter 4). Molecular techniques outperform both morphological and isotopic analysis, as DNA barcodes can be easily amplified from degraded prey tissue and assigned to taxonomical ranks based on reference sequences (Hebert et al. 2003; Vogler & Monaghan 2007). Morphological analysis, by contrast, has to rely on hard parts that are often too eroded and with insufficient inter-specific variation to assign to species. Isotope signatures can identify the trophic position of prey, are less subject to several known sources of bias in dietary studies, but have a low taxonomic resolution that cannot be solved by the application of dietary mixing models due to a high diversity of consumed prey (Jacob et al. 2005). To improve quantification estimates of the different prey consumed, recent studies have combined morphological identifications of hard parts with molecular techniques applied to undigested soft prey tissue, improving considerably both taxonomic resolution and prey ingestion estimates (Casper et al. 2007; Tollit et al. 2009; Alonso et al. 2014).

Here, we assessed the diet of a small seabird, the Bulwer's petrel, *Bulweria bulwerii*, at different moon phases during the breeding cycle, combining morphological and molecular analysis (DNA barcoding). DNA barcoding techniques have been rarely used to assess the diet of seabirds and have been only applied to few taxa, Macaroni and Little penguins (Deagle et al. 2007; 2010; Jarman et al. 2013), puffins (Bowser et al. 2013), and Cory's shearwater (Alonso et al. 2014).

Our aim was to investigate whether prey chosen by this pelagic bird varies with the moon phase. Previous studies on Bulwer's petrel showed that these birds are mainly predators of mesopelagic prey (Neves et al. 2011; Zonfrillo 1986; Spear et al. 2007), although some studies also report a considerable consumption of surface prey (Harrison et al. 1983). Given that mesopelagic prey are generally found in relatively deep oceanic layers, usually at depths above 200 meters, such prey are hypothesized to become only available to Bulwer's petrels at night, when species of the DSL ascend to the water surface to feed. Moonlight might therefore exert a negative effect on the range of prey species available to Bulwer's petrel, with birds shifting their diet to include other types of prey during full moons.

Specifically, we hypothesized the following: a. Foraging during new moons is optimal for Bulwer's petrels, as more species of the DSL are available at the sea surface, while birds can further visually benefit from bioluminescence from many mesopelagic species. b. Bulwer's petrels might respond to full moons and spend more time searching for prey or target different taxa that are not sensitive to moon light. As a consequence of the above, we predict a different signal of prey diversity, abundance and taxonomic composition according to moon phase. Furthermore, as molecular dietary analysis improves taxonomical resolution and prey detection and identification, we also expect to find taxa that were previously underestimated in morphological studies or not recorded in the diet of Bulwer's petrel, which may be key to identifying potential dietary differences between moon phases.

5.3 Material and Methods

5.3.1 Ethical statement

This study was approved under the permits 2/2012S, 5/2012D and 9/2013D, provided by the Instituto da Conservação da Natureza e da Biodiversidade and by the Serviço do Parque Natural da Madeira (Portugal). Stomach contents were sampled only once from each bird, with previous studies finding no significant affect on chick survival or growth (Clarke & Kerry 1994; Phillips 2006).

5.3.2 Field-work

A total of 141 stomach contents were collected from chicks of Bulwer's petrels at the subtropical Macaronesian islands of Deserta Grande (32°30'N 16°30' W) during the years of 2012 (n=28) and 2013(n=83) and at Selvagem Grande (30° 09' N, 15° 52' W) during 2012 (n=30). The two islands are situated approximately 270 km apart in similar oceanic environments. Chicks were sampled only once and a single stomach content flushing was performed using the water off-loading technique of Wilson et al. (1984). To remove salt we washed samples with clean water and filtered the contents through a sieve. Samples were preserved in absolute ethanol for molecular analysis of prey.

The prevailing atmospheric conditions during fieldwork were a clear sky or a skye with a very thin layer of clouds with minor influence on the nocturnal light intensity at the sea surface.

5.3.3 DNA isolation and amplification

DNA extractions were performed on prey tissue remains collected from the stomach contents of Bulwer's petrels using the DNeasy Blood & Tissue kit (Qiagen). Inner tissue layers were preferentially chosen for DNA extraction, as outer tissue remains might be cross-contaminated with DNA from other prey. Two different primer sets were used depending on the taxonomic groups targeted. For fish, we amplified the standard cytochrome *c* oxidase subunit I barcode (Folmer et al. 1994) using the M13 tail primer cocktail COI-2 and PCR conditions developed by Ivanova et al. (2007). For cephalopods we amplified a fragment of the 16S rRNA gene using the primer sets: 16ar and 16br (Palumbi 1996) and used optimised PCR conditions described in Alonso et al. (2014). The preferred use of the 16S rRNA gene for amplifying cephalopod DNA relates to a higher amplification success and a substantial higher representation of cephalopod taxa for the mitochondrial 16S than for COI (Alonso et al. 2014). PCRs were conducted with the Multiplex PCR kit (Qiagen) in total volumes of 12ul and final concentrations of: 1X Multiplex PCR Master Mix, 0.25 μM of each primer and 50-100ng/ul of DNA. PCR products were purified with the enzymes Exonuclease I and Antarctic Alkaline Phosphatase (New England, Biolabs). Amplicons were sent off for

Sanger sequencing at Macrogen, Inc (Amsterdam, Netherlands). Chromatograms were checked for quality with BioEdit (Hall 1999). Sequences were queried using the identification system engines in BOLD (Ratnasingham & Hebert 2007) and BLAST (Altschul et al. 1990) to obtain the nearest neighbor.

5.3.4 Identification and quantification of prey remains

For quantification and identification of prey, a combined morphological analysis of hard parts with molecular analysis of soft tissue remains was used (Alonso et al. 2014). Prey estimates based on morphological analysis are usually assigned at broader taxonomical ranks (e.g. families) compared with molecular analysis, often due to limited reference collections containing inter-specific diagnostic characters. As such, combining molecular and morphological prey counts might introduce uncertainty in prey estimates, for example, a tissue from a myctophid species may or may not be the same myctophidae identified from a vertebra, but might be counted as such when combing both methods. Hard structures also persist for longer than soft tissue in the stomach contents, so that some prey might be overrepresented using morphological prey counts. To take full advantages of both methodologies and minimize bias in prey estimates, we only considered hard-part remains that had tissue attached. In this way we ensured, as far as possible, that only the prey recently taken by Bulwer's petrels were considered in our analysis and that prey estimates obtained from both methods were comparable. For each hard structure we therefore obtained a DNA barcode. If no positive match could be obtained for the DNA barcode, either because there was no available reference or bad DNA quality, we identified the correspondent hard structure using morphological analysis (Alonso et al. 2013). It is important to note that for cephalopods, we obtained substantially higher numbers of tissue remains (tentacles, mantle) than fresh beaks (beaks with tissue attached). Most of the beaks obtained were very small, so that morphological identification could potentially result in incorrect species assignments (see Alonso et al. 2014). Given that that the number of fresh beaks was always inferior than the number of identified cephalopod species using DNA barcoding, prey estimates retrieved in this group result essentially from DNA barcoding.

All identifications and quantifications were obtained to the lowest possible taxonomic rank. Confidence in taxonomic identification of COI queries was based on

the identification algorithms of BOLD, while 16S barcodes were assigned phylogenetically based on Maximum Likelihood inferences with reference sequences obtained from Genbank. Multiple sequence alignment of queries and references were conducted in SaTé-II under MAFFT (Katoh & Standley 2013), MUSCLE (Edgar 2004) and FASTTREE (Price et al. 2010) using the GTR + γ nucleotide substitution model. Maximum likelihood tree inferences were performed in RAxML (Stamatakis 2006) using the CIPRES Science Gateway v.3.1 (Miller et al. 2010).

5.3.5 Statistical analysis

To assess whether the moon phase influence the prey consumed by Bulwer's petrels, we performed Multivariate analyses using a matrix of prey counts (number of times a specific taxon occurred in each sample). Samples were categorized into three levels: new moon (n=54), quarter moon (n=28) and full moon (n=54). Information on moon phase and the fraction of the moon illuminated were obtained from the United States Naval Observatory (http://aa.usno.navy.mil/data). Samples that were taken ± 2 days of the moon phase date were pooled within each moon phase category.

To visually check for multivariate patterns between observations, we performed metric multidimensional scaling (MDS) on Bray-Curtis distances (Kruskal & Wish 1978) between pairs of observations. To test whether the factor "moon phase" significantly affected prey choice in Bulwer's petrel we performed permutational multivariate analysis of variance (perMANOVA) using the adonis function (Oksanen et al. 2015). To estimate prey variability among sampling blocks (full-moon, new-moon and moon quarters) - beta-diversity, we calculated the homogeneity of multivariate dispersions in each moon phase using the function betadisper (Oksanen et al. 2015). Differences among moon phases were assessed using permutational tests of significance (permutest)(Oksanen et al. 2015).

We also obtained Shannon (H) and invSimpson (D) diversity indexes of the proportions of prey of a specific taxonomic rank divided by the total number of prey individuals in each moon phase and tested for significant differences using ANOVA (Analysis of variance)

All analyses were performed in R 3.1.2 (R Core Team 2014) using the vegan package (Oksanen et al. 2015).

5.4 Results

5.4.1 Prey identifications using morphological and genetic analysis

In total, 988 prey items (vertebra and tissue) were obtained from 141 stomach contents of Bulwer's petrels. The combined use of morphological analysis on hard part remains and molecular analysis of 800 16S rRNA and COI barcodes revealed that these prey items corresponded to 384 different individual prey.

Morphological analysis of vertebra only revealed 15 distinct taxonomic identities, of which 7 were identified to lower taxonomic ranks (genera and species), 2 to families and 6 remained unidentified. DNA barcoding substantially augmented prey detection and resolution, revealing 73 distinct taxonomic identities, 50 corresponding to fish and 23 to cephalopods. Of these, 57 were assigned to lower taxonomical ranks (genera and species) and 15 to family ranks, while only one remained unidentified. The nearest neighbors producing a positive match, using species level identification algorithms of BOLD, are shown in (Table 5.1). Phylogenetic assignments of 16S rRNA barcodes (cephalopods) were based on monophyletic insertion of queries with references on a Maximum likelihood tree (Figure 5.1).

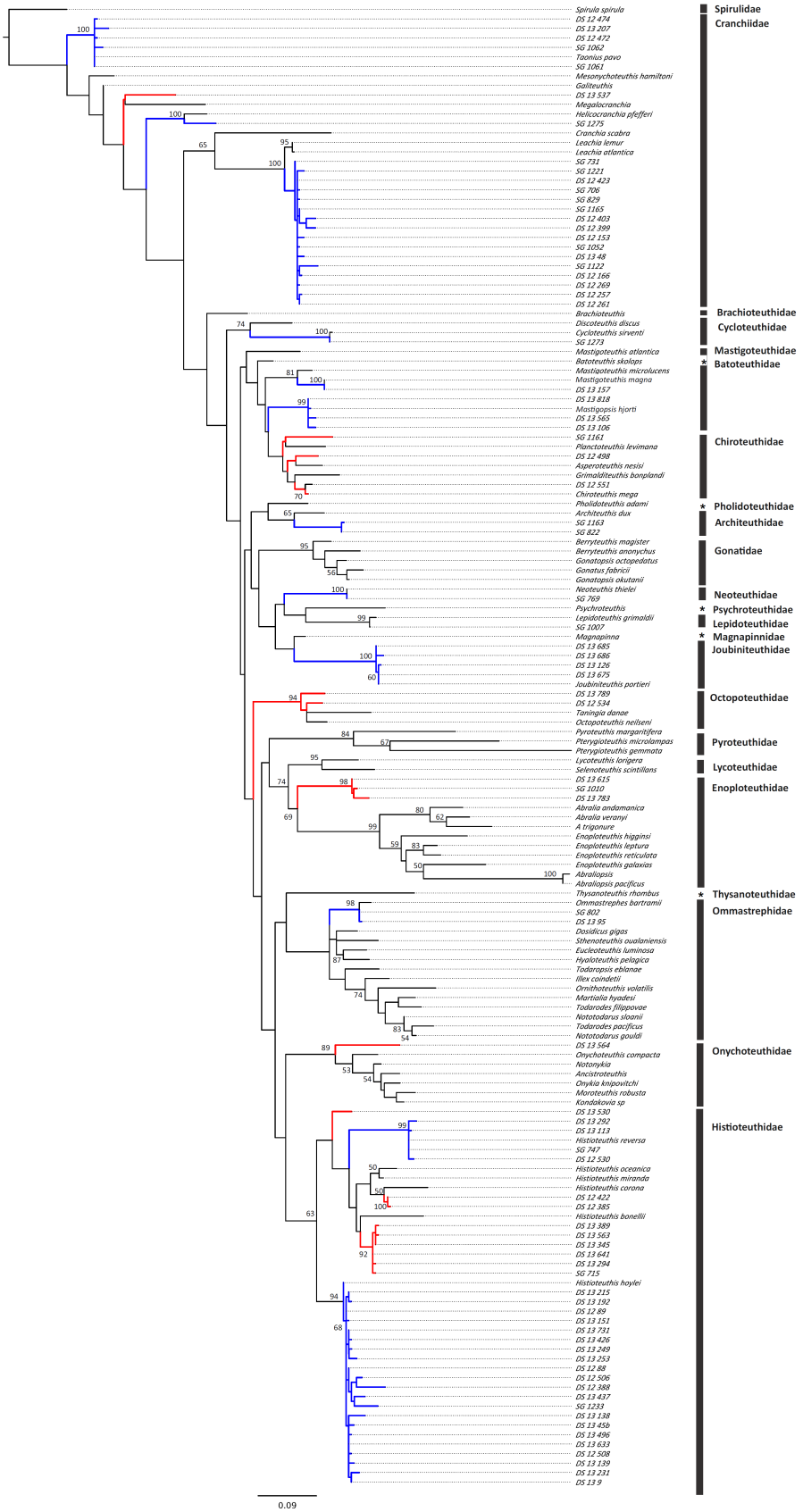


Figure 5.1. Maximum Likelihood RaxML tree to identify 16SrRNA query sequences of cephalopods. Positive identifications with high support (bootstrap support values > 80) are indicated in blue and

correspond to lower taxonomic rank assignments (genus and species level), except for the genus *Architeuthis* that was positively assigned despite a low bootstrap support = 65. Such assignment relates with its taxonomic uniqueness, as *Architeuthis dux* is the only member described for the family Architeuthidae. Red Lines indicate uncertain placement on the reference tree (low bootsrap support <80). In this case, queries were assigned only to family ranks if placed within a monophyletic reference cluster including members of that family. If queries clustered outside a family cluster, these were not identified. The voucher of each query sequence is indicated on the tree, showing the study site (DS, Desertas; SG, Selvagens) and the year of collection (2012, 2013)

Table 5.1. Numerical frequencies (%N) of prey in Bulwer's petrels stomach contents. Taxa were identified to the nearest taxonomical rank using phylogenetic assignments for 16S rRNA barcodes (Figure 1) and identification algorithms in BOLD for COI barcodes. The sequence similarity percentages of fish and cephalopods with references in BOLD and Genbank are show. The common names of representative taxa of each order are presented for fish. %N is expressed as the number of individuals in each taxonomic rank identified (Taxon) divided by the total number of individuals in all stomach contents (Total) and by the total number of individuals during each moon phase (New Moon, First Quarter, Full Moon, Last Quarter).

Order	Family	*Taxon*	BOLD/ Genbank	Total	Full Moon	New Moon	Quarter
FISH							
Anguilliformes (eels)	Synaphobranchidae	unidentified *		0.3	0	0	1.3
	Derichthyidae	*Derichthys serpentinus*	100	0.3	0.7	0	0
Aulopiformes (lizardfishes)	Alepisauridae	*Alepisaurus ferox*	99	0.3	0	0.6	0
	Paralepididae	*Magnisudis atlantica*	99.39	0.3	0	0.6	0
Beryciformes (squirrelfishes, roughies)	Diretmidae	*Diretmichthys parini*	100	0.3	0.7	0	0
		Diretmus argenteus	98.48-99.85	6.8	5.4	7.5	8
Clupeiformes (anchovies and herrings)	Opisthoproctidae	unidentified	89.72	0.3	0.7	0	0
Gadiformes (cods, grenadiers, hakes)	Macrouridae	*Malacocephalus*	99.84-100	0.3	0	0.6	0
	Melanonidae	*Melanonus zugmayeri*	99.84-99.85	0.3	0	0.6	0
Myctophiformes (laternfish)	Myctophidae	*Bolinichthys*	99.85	2.6	1.3	4.4	1.3
		Bolinichthys indicus	99.69	0.3	0	0	1.3
		Ceratoscopelus	99.69	2.1	2	2.9	1.7
		Diaphus brachycephalus	99.23	0.3	0	0.6	0
		Diaphus jenseni	100	0.3	0	0	1.3
		Diaphus sp1	97.24	0.3	0.7	0	0
		Diaphus sp2	99.69	0.5	1.3	0	0
		Diaphus metopoclampus	99.23	2.3	2.7	2.5	1.3
		Diaphus rafinesquii	99.62	2.3	0.7	3.8	2.7
		Hygophum reinhardtii	99.85	0.8	0.7	0.6	1.3
		Hygophum taaningi	100	0.5	1.3	0	0

		Hygophum hygomii	100	0.3	0	0.6	0
		Lampadena chavesi	99.38	0.5	0.7	0	1.3
		Lampanyctus	99.4	0.3	0	0.6	0
		Lepidophanes gaussi	100	1.1	0.7	0.6	2.7
		Lobianchia	99.54-99.85	2.6	1.3	3.8	2.7
		Notoscopelus resplendens	97.21	0.8	0.7	1.3	0
		unidentified	98.25	4.4	4.7	4.4	4
Notacanthiformes (spiny eels)	Halosauridae	*Aldrovandia affinis*	99.06	0.3	0.7	0	0
Osmeriformes (marine smelts)	Microstomatidae	unidentified	98.77	0.8	0.7	0.6	1.3
	Platytroctidae	*Searsia koefoedi*	99.66-99.82	0.3	0.7	0	0
Perciformes (perches)	Scombridae	*Naucrates ductor*	98.38	0.5	0	0.6	1.3
Stephanoberyciformes	Melamphaidae	*Melamphaes typhlops*	99.3	0.3	0	0.6	0
		Melamphaes	98.77	0.3	0.7	0	0
		Poromitra	98.46	0.3	0	0	1.3
		unidentified	90.03	0.6	1.4	0	0
Stomiiformes (dragonfishes, lightfishes, hatchetfishes)	Gonostomatidae	*Bonapartia*	99.69	0.3	0	0.6	0
		Cyclothone	99.69	0.8	0	1.9	0
		Gonostoma denudatum	99.55	1.3	1.3	1.9	0
		Margrethia obtusirostra		0.5	0	1.3	0
		unidentified	93.19	0.8	0	1.3	1.3
	Phosichthyidae	*Vinciguerria*	99.69-99.85	0.8	0	1.3	1.3
	Sternoptychidae	*Argyripnus atlanticus*	99.85	0.3	0	0.6	0
		Argyropelecus	99.53-99.69	5.2	4.7	4.4	8
		Sternoptyx	98.56-99.83	13.8	18.8	6.9	18.7
		Valenciennellus tripunctulatus	100	0.3	0.7	0	0
	Stomiidae	*Chauliodus*	98.57	0.8	0	0.6	2.7
		Stomias boa	100	1	0.7	1.9	0
		unidentified *		0.3	0	0.6	0
Syngnathiformes	Centriscidae	*Macroramphosus scolopax* *		0.5	1.3	0	0
Tetradontiformes (puffers and sunfish)	Molidae	*Ranzania laevis*	99.84	0.3	0.7	0	0
CEPHALOPODA							
Oegopsina	Architeuthidae	*Architeuthis*	93.83	4.9	8.1	3.8	1.3
	Chiroteuthidae	unidentified 1	94.29	0.5	0.7	0.6	0
		unidentified 2	99.6	0.3	0	0.6	0
		unidentified *3*	94.13-99.48	0.3	0.7	0	0
	Cranchiidae	*Helicocranchia pfefferi*	96.03	0.3	0	0.6	0
		Leachia	96.15-96.22	4.2	4.7	5	1.3
		Taonius pavo	98.60-99.2	0.8	0.7	1.3	0
		unidentified	94.28	0.3	0	0.6	0
	Cycloteuthidae	*Cycloteuthis sirventi*	0.962151394	0.8	0.7	0.6	1.3
	Histioteuthidae	unidentified 1	97.65	1.3	0	1.9	2.6
		unidentified 2	99.48-99.73	3.1	2.7	1.9	6.7
		undidentified 3	99.7	1.3	2	1.3	0
		Histioteuthis hoylei	99.18-99.59	12	14.8	10	10.7
		Histioteuthis reversa	99.59	1.6	1.3	2.5	0
	Joubiniteuthidae	*Joubiniteuthis sp*	99.55	0.8	1.3	0.6	0
	Lepidoteuthidae	*Lepidoteuthis grimaldii*	98.97	0.5	0	1.3	0
	Mastigoteuthidae	*Mastigoteuthis magna*	99.6	0.3	0	0.6	0

	Mastigoteuthis hjortii	97.69	2.1	1.3	3.1	1.3
Neoteuthidae	*Neoteuthis thielei*	99.79	0	0	0	0.3
Octopoteuthidae	unidentified	95.39	0.5	1.3	0	0
Ommastrephidae	*Ommastrephes bartramii*	99.8	1.6	0	1.3	5.3
Onychoteuthidae	unidentified	98.31	0.3	0	0.6	0
unknown Teuthida	*unknown Teuthida*	92.28	1.3	0.7	1.9	1.3

*Taxa identified exclusively using morphological analysis on vertebra

5.4.2 Prey composition

The main prey targeted by Bulwer's petrels were mesopelagic fish and cephalopods, dominated by myctophids, sternoptychids and histioteuthids (Figure 5.2a-b). Myctophids showed the highest diversity of prey, with 20 different prey types identified, whereas within sternoptychids and histioteuthids only four prey types were recorded (Table 5.1), of which *Sternoptyx* and *Histioteuthis hoylei* were the most frequently eaten species (Figure 5.2a). We found no obvious dietary shifts related to the moon cycle, with no clear pattern of greater numbers of species consumed during new moons. Except for two rare prey species (with a numerical frequency < 1%), *Ranzania laevis* and *Naucrates ductor* that are probably epipelagic, all other prey are known mesopelagic species residing in deeper oceanic layers. It should be noted that the neon-flying squid, *Ommastrephes bartramii*, despite residing in deep oceanic layers, is probably also found at the surface during the day (Murata 1988).

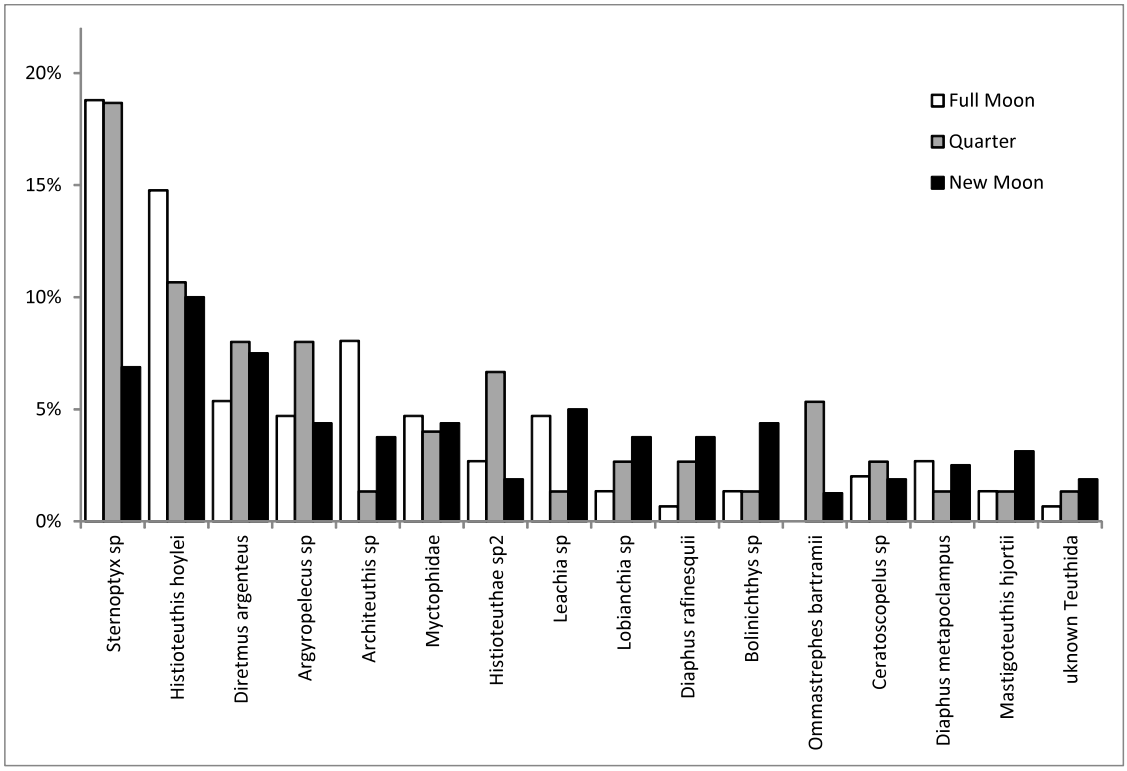


Figure 5.2 a-b. Numerical frequency (%N) of the taxa identified in Bulwer's petrels stomach contents at different moon phases; expressed as a function of total prey individuals in each moon phase (New moon=160, Quarter=75, Full-moon=149). a. Prey identified to the lowest taxonomical rank. b. Prey pooled into family ranks. Only the taxa contributing to equal or more than 5% of the total number of individuals found in all stomach contents are shown.

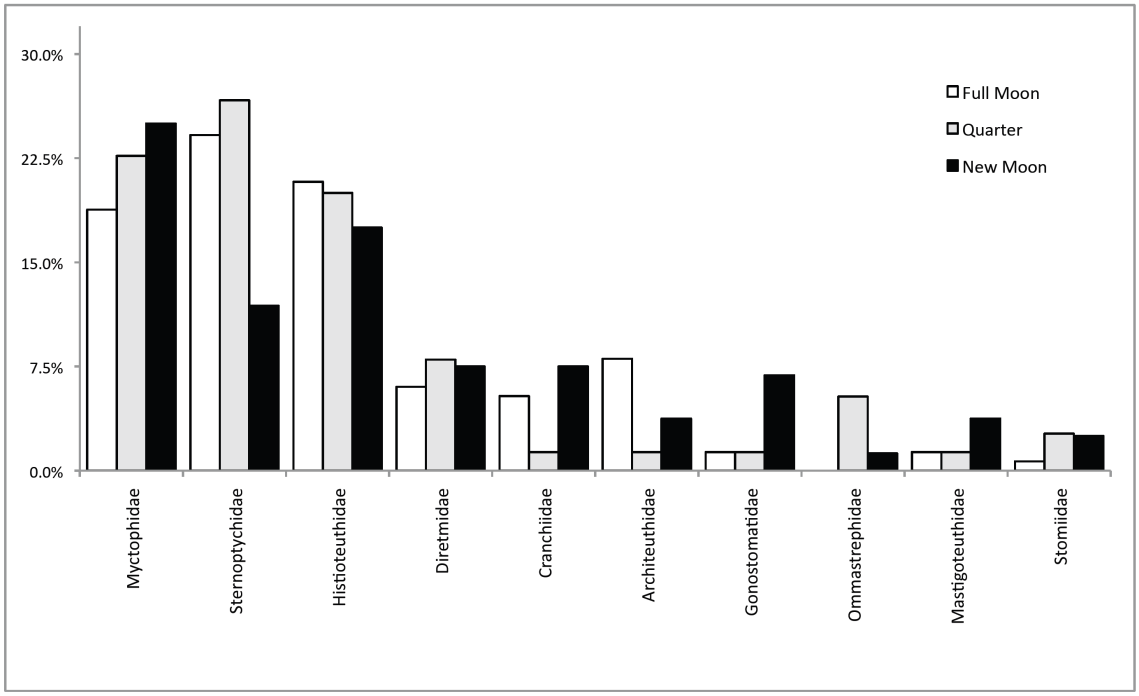


Figure 5.2 b. Continued

5.4.3 Influence of moon phase on prey consumed

MDS ordination showed no distinction between samples collected at different years, sites and moon-phases (Figure 5.3), suggesting that the moon cycle does not influence prey consumption in Bulwer's petrel. Given that samples were obtained in different years and at different sampling sites, we accounted for both confounding factors during perMANOVA, by adding both terms when testing for the effect of "moon phase" on prey distances. "Year" ($F_{1,136}$ = 1.384, R^2 = 0.007, p=0.40) and "Sampling sites" ($F_{1,136}$ =0.391, R^2=0.007, p= 0.391) did not significantly influence prey composition of Bulwer's petrel, while our results were consistent with MDS analysis showing no significant effect of moon phase ($F_{1,136}$=1.0939, R^2=0.01545, p=0.357) on prey dissimilarities.

Contrary to our initial hypothesis, beta-diversity between moon phases did not correlate significantly with the moon cycle (permutations=999; $F_{2,138}$=0.994, p=0.369) (Figure 5.4). Diversity indexes, of Shannon (ANOVA, $F_{2,138}$=0.548, p=0.58) and invSimpson (ANOVA, $F_{2,138}$=1.206, p=0.302) did also not vary significantly between samples collected at different moon phases.

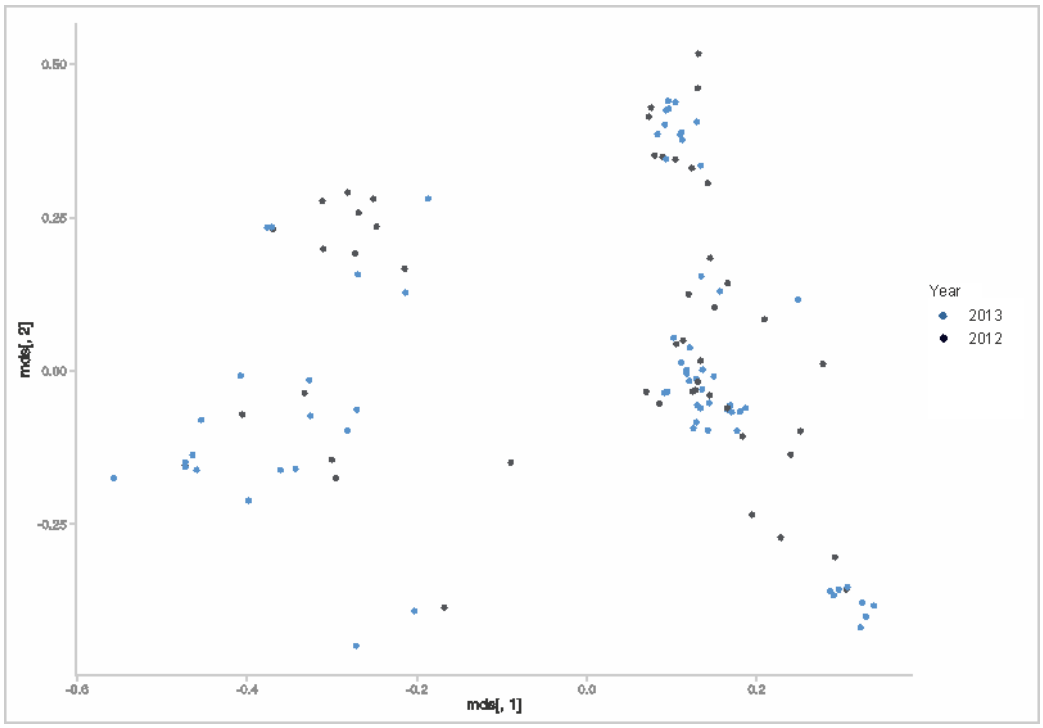


Figure 5.3.a-c- Metric multidimensional scaling (MDS) of "Bray-Curtis" dissimilarities showing prey variability among samples collected at a) different years (2011 and 2012) , b. at different locals (Selvagens and Desertas) and c) at different moon phases (Full Moon, New Moon Quarter

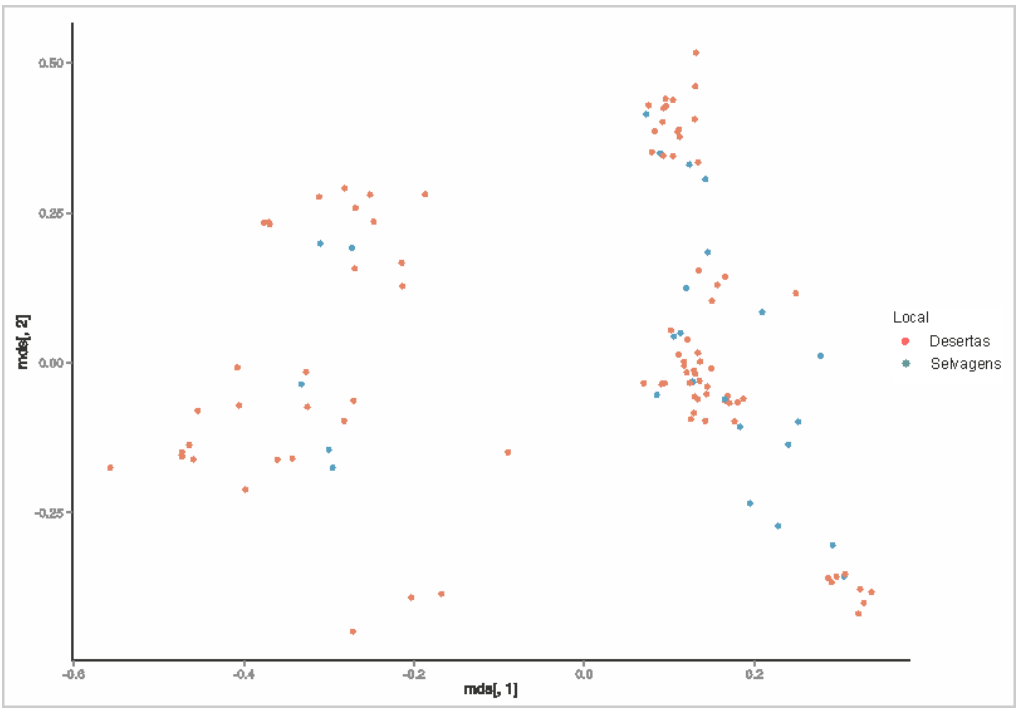


Figure 5.3.b- continued

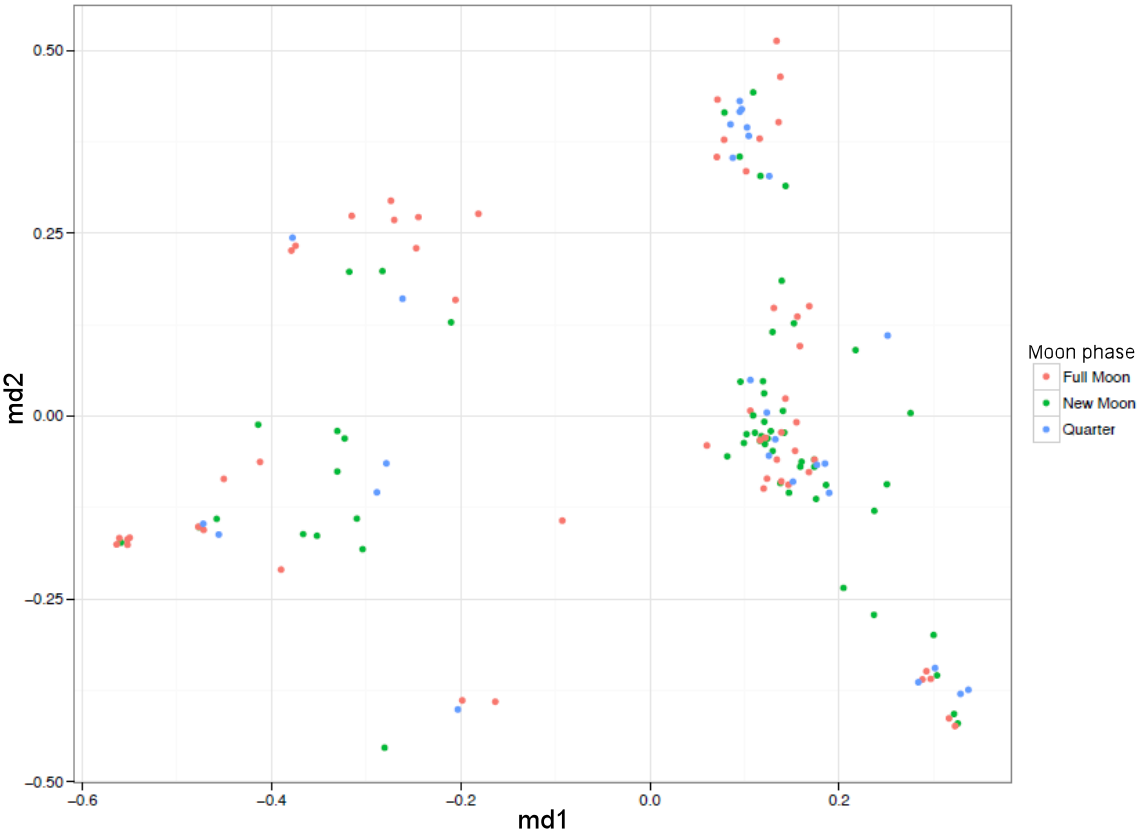


Figure 5.3.c- continued

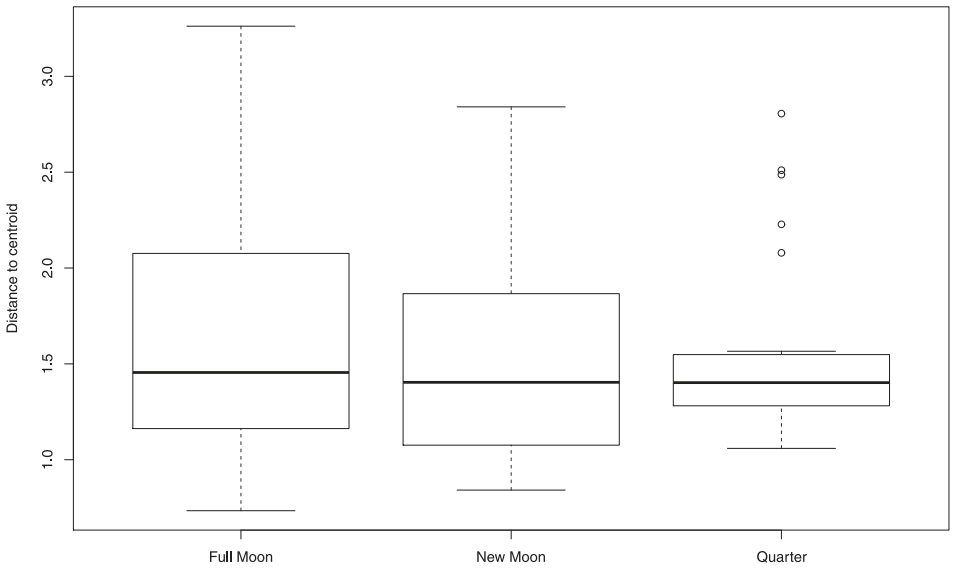


Figure 5.4. Boxplot of homogeneity of multivariate dispersions (betadiversity) at different moon phases. The graph shows the distances between group centroids (moon phases) in relation to multivariate space.

5.5 Discussion

This study is the first to investigate the influence of the moon on the diet of a marine predator of mesopelagic prey documenting, as such, that contrary to expectations nocturnal moonlight does not influence diet composition or diversity of Bulwer's petrels. This raises intriguing questions on how these birds capture prey, and on the variations of mesopelagic species in surface waters in relation to nocturnal ambient light.

Predatory foraging patterns correlated with the moon cycle have been already shown in marine mammals and birds (Yamamoto et al. 2008; Benoit-Bird et al. 2009; Rubolini et al. 2014). However, whether such patterns follow moon- induced changes in prey availability have been little explored.

Birds, for example, significantly increase flying activity and landings during moonlit nights, which is thought to correlate with either, a greater effort to find prey or, alternatively, to a reduction of foraging effort when darkness hampers detection probability and makes aerial foraging uneconomical (Awkerman et al. 2005; Phalan et al. 2007; Yamamoto et al. 2008; Regular et al. 2011; Dias et al. 2012). A similar

influence of the moon has been described for fur seals, with reduced diving activity but increasing diving depths during full moons, which is thought to correlate with lower availability of mesopelagic species near the surface (Horning & Trillmich 1999).

We obtained a detailed analysis of the prey composition of Bulwer's petrels using DNA-based methodologies combined with morphological prey estimates. The inclusion of molecular tools for dietary analysis of stomach contents substantially increased the range of identified prey species detected in previous studies in the subtropical NE Atlantic (Zonfrillo et al. 1986; Neves et al. 2011). Nonetheless, prey composition was very similar at broader taxonomic ranks, showing a major contribution of mesopelagic myctophiformes, stomiiformes and squids. Such prey ascend to near the surface at night during diel vertical migrations (Sutton & Hopkins 1996; Watanabe et al. 1999), suggesting that Bulwer's petrels are probably nocturnal foragers.

Evidence for a nocturnal foraging behavior was also obtained in the study of Dias et al. (in press) showing a substantial higher flight activity at sea of Bulwer's petrel during night than day. It has been debated as whether to the presence of mesopelagic organisms in seabird's stomach contents results from scavenging on dead remains. This is unlikely the case of Bulwer's petrels, as if so, Bulwer's petrel would probably forage during the day too, when floating dead remains are visually more detectable.

Contrary to all our predictions, Bulwer's petrel showed no significant prey shift in species diversity or composition across the lunar cycle, maintaining an almost exclusive consumption of mesopelagic prey during all moon phases. These results support the contention that moon plays no significant role in the foraging success of this bird. We therefore conclude that either, (1) the range of mesopelagic species eaten by Bulwer's respond differently to moonlight changes than assumed for species of the DSL or (2) Bulwer's petrel increases predatory effort during moonlit night to compensate for lower prey abundances.

Although the latter explanation agrees with the foraging efficiency hypothesis, we think that this is not the case in Bulwer's petrel. For instance, we would expect to find a higher frequency of epipelagic prey types during full moons than was found in this study. Only one epipelagic species, *Ranzania laevis*, was detected during the full moon, and this was only a single prey occurrence. The only other epipelagic species,

Naucrates ductor, was identified at all lunar phases other than full moons. Moreover, species such as the neon-flying squid, *Ommastrephes bartramii*, which are often found in surface waters (Murata 1988) and are a common prey of Cory's shearwater, which feeds on epipelagic prey (Alonso et al. 2014), these too were never consumed by Bulwer's petrel chicks during full moons.

A different response of prey to moon light than the one usually accepted may, therefore, be a more likely explanation. In fact, most of our current knowledge on the effect of the moon on species diel vertical migration patterns is based on few observations. While many mesopelagic species are thought to actively perform normal diel vertical migrations (nocturnally ascendent), some are shown to reverse their diel vertical migration patterns (nocturnally descent) (Ohman et al. 1983). Populations of the myctophid *Benthosema glaciale*, for example, perform diverse types of diel vertical migration: while some individuals ascend at night, others ascent during the day or simply remain at mesopelagic depths (Kaartvedt et al. 2009). The dietary patterns of Bulwer's petrel are unlikely the result of a particular prey type responding differently to moon light. The high diversity of prey across all moon phases suggests that these prey were all available to Bulwer's petrel regardless of the moon phase.

The response of organisms to moon light is, therefore, probably more complex than is generally assumed by the foraging hypothesis. Even if the moon plays and important role in shaping organismal distributions near the sea-surface, mesopelagic organisms might vary in their responses, or may be moved around by oceanographic currents (e.g. eddies) (Schneider et al. 2007) to such an extent that they are constantly locally available to pelagic predators throughout the moon cycle. We think that this is probably the case for Bulwer's petrels.

Our findings contradict previous hypothesis postulating higher prey species availability during new moons, with Bulwer's petrels showing no significant shift in prey species composition or diversity between lunar phases. Such results highlight the need to revise current predictions of foraging efficiency in marine predators.

5.6 Acknowledgements

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5.7 References

Alonso H, Granadeiro JP, Ramos JA & Catry P (2013). Use the backbone of your samples: fish vertebrae reduces biases associated with otoliths in seabird diet studies. Journal of Ornithology 154: 883- 886.

Alonso H, Granadeiro JP, Waap S, Xavier J, Symondson WOC, Ramos, JA & Catry, P (2014) An holistic ecological analysis of the diet of Cory's shearwaters using prey morphological characters and DNA barcoding. Molecular Ecology, 23: 3719-3733.

Altschul SF, Gish W, Miller W, Myers EW & Lipman DJJ (1990) Basic local alignment search tool. Journal of Molecular Biology, 215: 403-410.

Awkerman J, Fukuda A, Higuchi H, Anderson D (2005) Foraging activity and submesoscale habitat use of waved albatrosses Phoebastria irrorata during chick-brooding period. Marine Ecology Progress Series 291: 289–300.

Benoit-Bird KJ, Au WWL & Wisdoma DW (2009a) Nocturnal light and lunar cycle effects on diel migration of micronekton. Limnology and Oceanography 54: 1789-1800

Benoit-Bird KJ, Dahood AD & Würsig B (2009b) Using active acoustics to compare lunar effects on predator–prey behavior in two marine mammal species. Marine Ecology Progress Series 395: 119-135

Bowser AK, Diamond AW & Addison JA (2013) From Puffins to Plankton: A DNA-Based Analysis of a Seabird Food Chain in the Northern Gulf of Maine. PLoS ONE, 8(12): e83152.

Brooke ML (2004) The food consumption of the world's seabirds. Proceedings of the Royal Society B, 271: S246–S248

Brown JS, Kotler BP, Bouskila A (2001) The ecology of fear and the foraging game between owls and gerbils. Annales Zoologici Fennici, 38: 71–87

Casper RM, Jarman SN, Deagle BE, Gales NJ, Hindell MA (2007) Detecting prey from DNA in predator scats: A comparison with morphological analysis, using Arctocephalus seals fed a known diet. Journal of Experimental Marine Biology and Ecology, 347:144-154

Clarke JR & Kerry KR (1994) The effects of monitoring procedures on Adélie penguins. CCAMLR Science, 1: 155-164

Clarke TA (1973) Some aspects of the ecology of lanternfishes (Myctophidae) in the Pacific ocean near Hawaii. Fisheries Bulletin 71, 401–433.

Cruz SM, Hooten M, Huyvaert KP, Proaño CB, Anderson DJ, Afanasyev V, Wikelski M (2013) At–Sea Behavior Varies with Lunar Phase in a Nocturnal Pelagic Seabird, the Swallow-Tailed Gull. PLoS ONE 8:e56889

Deagle BE, Gales NJ, Evans K, Jarman SN, Robinson S, Trebilco R & Hindell MA (2007) Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (Eudyptes chrysolophus). PLoS ONE, 2(9), e831

Dias MP, Granadeiro JP & Catry P (2012) Do seabirds differ from other migrants in their travel arrangements? On route strategies of Cory’s shearwa- ter during its trans-equatorial journey. PLoS ONE 7: e49376.

Duffy DC & Jackson S (1986) Diet studies of seabirds: A review of methods. Colonial Waterbirds, 9: 1-17

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32: 1792-1797

Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299

Gauch HG (1982) Multivariate Analysis in Community Ecology. Press Syndicate of the University of Cambridge, Cambridge.

Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series 41: 95-98

Harrison CS, Hida TS & Seki MP (1983) Hawaiian seabird feeding ecology. Wildlife Monographs 47: 1–2.

Hebert PDN, Cywinska A, Ball SL & de Waard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society B, 270: 313–322.

Hobson KA, Piatt JF & Pitocchelli J (1994) Using stable isotopes to determine seabird trophic relationships. Journal of Animal Ecology, 63: 786–798

Horning M & Trillmich F (1999) Lunar cycles in diel prey migrations exert a stronger effect on the diving of juveniles than adult Galápagos fur seals. The Royal Society B, 266: 1127-1132

Imber MJ (1975) Behaviour of petrels in relation to the moon and the artificial lights. Notornis 22: 302 – 306.

Ivanova NV, Zemlak TS, Hanner RH & Hebert PDN (2007) Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes, 7: 544–548

Jacob U, Mintenbeck K, Brey T, Knust R & Beyer K (2005) Stable isotope food web studies: a case for standardized sample treatment. Marine Ecology Progress Series, 287:251-253

Jarman SN, McInnes JC, Faux C, Polanowski AM, Marthick J, Deagle BE, Southwell C & Emmerson L (2013) Adélie Penguin Population Diet Monitoring by Analysis of Food DNA in Scats. PLoS ONE 8(12): e82227

Kaartvedt S, Røstad A, Klevjer TA & Staby A (2009) Use of bottom-mounted echo sounders in exploring behavior of mesopelagic fishes. Marine Ecololgy Progress Series 395:109−118

Kaiser TS, Neumann D & Heckel DG (2011) Timing the tides: Genetic control of diurnal and lunar emergence times is correlated in the marine midge Clunio marinus. BMC Genetics, 12: 2-12

Kampa EM (1974) Photoenvironment and vertical migrations of mesopelagic marine animal communities. In Biological rhythms in the marine environment (ed. P. J. DeCoursey), pp. 257-272. Columbia: University of South Carolina Press.

Karpouzi VS, Watson R & Pauly D 2007. Modelling and mapping resource overlap between seabirds and fisheries on a global scale: a preliminary assessment. Marine Ecology Progress Series 343: 87-99

Katoh K & Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular Biology and Evolution, 30: 772-780.

Klomp NI & Furness RW (1992). Patterns of chick feeding in Cory's Shearwaters and the associations with ambient light. Colonial Waterbirds 15: 95–102.

Kotler BP, Brown J, Mukherjee S, Berger-Tal O & Bouskila A (2010) Moonlight avoidance in gerbils reveals a sophisticated interplay among time allocation, vigilance and state-dependent foraging. Proceedings of the Royal Society B, 277: 1469–1474.

Kruskal JB & Wish M (1978) Multidimensional Scaling. Sage Publications, Beverly Hills.

Lyons KG & Schwartz MW (2001) Rare species loss alters ecosystem function – invasion resistance. Ecology Letters, 4: 358-365

McCann K, Hastings A & Huxel GR (1998) Weak trophic interactions and the balance of nature. Nature, 395: 794-798.

Miller MA, Pfeiffer W & Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE). New Orleans, pp. 1–8.

Mougeot F & Bretagnolle V (2003) Predation risk and moonlight avoidance in nocturnal seabirds. Journal of Avian Biology, 31: 376-378.

Murata M (1988) On the flying behavior of neon flying squid Ommastrephes bartrami observed in the central and northwestern North Pacific. Nippon Suisan Gakkaishi, 54: 1167-1174

Neves VC, Nolf D & Clarke MR (2011) Diet of Bulwer's petrel in the Azores, NE Atlantic. Waterbirds, 34: 357-362

Ohman MD, Frost BW & Cohen EB (1983) Reverse diel vertical migration: an escape from invertebrate predators. Science 220:1404-1407.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH & Wagner H (2015) vegan: Community Ecology Package. R package version 2.2-1. http://CRAN.R-project.org/package=vegan

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Mable BK, Moritz C (eds) Molecular Systematics. Sinauer, Sunderland, MA, pp 205–247.

Phalan B, Phillips RA, Silk JRD, Afanasyev V, Fukuda A, Fox J, Catry P, Higuchi H & Croxall JP (2007) Foraging behaviour of four albatross species by night and day. Marine Ecology Progress Series, 340: 271-286

Phillips RA (2006) Efficacy and effects of diet sampling of albatross chicks. Emu 106: 305–308

Poos MS & Jackson DA (2012) Addressing the removal of rare species in multivariate bioassessments: The impact of methodological choices. Ecological Indicators, 18: 82–90

Price MN, Dehal PS & Arkin AP (2010) FastTree 2: Approximately Maximum-Likelihood Trees for Large Alignments. PLoS ONE, 5(3): e9490

Queiroga H, Almeida MJ, Alpuim T, Flores AAV, Francisco S, Gonzalez-Gordillo JI, Miranda AI, Silva I & Paula J (2006) Wind and tide control of megalopal supply to estuarine crab populations on the Portuguese west coast, 307: 21-36

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Ralph CP, Nagata SE & Ralph CJ (1985) Analysis of droppings to describe diets of small birds. Journal of Field Ornithology, 56: 165-174

Ratnasingham S & Hebert PDN (2007) BOLD: the barcode of life data system (www.barcodinglife.org). Molecular Ecology Notes 7: 355–364.

Regular PM, Hedd A & Montevecchi WA (2011) Fishing in the dark: a pursuit-diving seabird modifies foraging behavior in response to nocturnal light levels. PLoS ONE 6: e26763

Riou S & Hamer KC (2008) Predation risk and reproductive effort: impacts of moonlight on food provisioning and chick growth in Manx shearwaters. Animal Behaviour 76: 1743–1748

Rubolini D, Maggini I, Ambrosini R, Imperio S, Paiva VH, Gaibani G, Saino N & Cecere, JG (2014) The Effect of Moonlight on Scopoli's Shearwater Calonectris diomedea Colony Attendance Patterns and Nocturnal Foraging: A Test of the Foraging Efficiency Hypothesis. Ethology, 121: 284-299

Schneider DC (2007) Seabirds and fronts: a brief overview. Polar Research 8:17-21

Skov MW, Hartnoll RG, Ruwa RK, Shunula JP, Vannini M & Cannicci S (2004) Marching to a different drummer: crabs synchronize reproduction to a 14 th lunar tidal cycle. Ecology, 86:1164–1171

Spear LB, Ainley DG & Walker WA (2007) Foraging dynamics of seabirds in the Eastern Tropical Pacific Ocean. Studies in Avian Biology 35:1−99

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics, 22: 2688-2690

Tollit DJ, Schulze AD, Trites AW, Olesiuk PF, Crockford SJ, Gelatt TS, Ream RR, & Miller KM (2009) Development and application of DNA techniques for validating and improving pinniped diet estimates. Ecological Applications 19: 889–905.

Vogler AP & Monaghan MT (2007) Recent advances in DNA taxonomy. Journal of Zoological Systematics and Evolutionary Research, 45: 1-10.

Watanabe H, Moku M, Kawaguchi K, Ishimaru K & Ohno A (1999) Diel vertical migration of myctophid fishes (family Myctophidae) in the transitional waters of the western North Pacific. Fisheries Oceanography 8:115-127

Wilson RP (1984) An improved stomach pump for penguins and other seabirds. Journal of Field Ornithology, 55: 109-112

Yamamoto T, Takahashi A, Yoda K, Katsumata N & Watanabe S (2008) The lunar cycle affects at–sea behaviour in a pelagic seabird, the streaked shearwater, Calonectris leucomelas. Animal Behaviour 76: 1647–1652.

Zaret TM & Suffern JS (1976) Vertical migration in zooplankton as a predator avoidance mechanism. Limnology and Oceanography, 21: 804–813.

Zhang C, Abello P & Ernest N (1999) Endogenous tidal and semilunar moulting rhythms in early juvenile shore crabs Carcinus maenas: implications for adaptation to a high intertidal habitat. Marine Ecology Progress Series, 191:257-266

Zonfrillo B (1986) Diet of Bulwer's petrel *Bulweria bulwerii* in the Madeiran archipelago. Ibis,128: 570-572

CHAPTER 6:

General Discussion

General Discussion

In this thesis, I examined the trophic relationships of four petrels breeding in the northeastern Atlantic, as well as the influence of the moon cycle on predator-prey interactions of a strictly mesopelagic predator. I further, developed various laboratory procedures and tested different analytic approaches (OTU picking and phylogenetic placement methods) to optimise prey identification in these petrels. The study species were the Band-rumped Storm petrel (*Hydrobates castro*), Bulwer's petrel (*Bulweria bulwerii*), Corys' shearwater (*Calonectris borealis*) and White-faced Storm petrel (*Pelagodroma marina*). The avian fauna of the Madeira-Selvagens archipelago are most important colonies of the North Atlantic, but except for Cory's shearwater, and a single limited study describing the prey types of Bulwer's petrels (Zonfrillo 1986), nothing is known on the foraging strategies of these birds. By using different molecular methodologies, including standard DNA barcoding approaches on stomach contents and new high-throughput sequencing techniques on faecal remains, I therefore provide the most comprehensive and detailed study to date on the trophic ecology of the petrels breeding in the Macaronesian region.

6.1 Overview of main results

Chapter 2: An holistic ecological analysis of the diet of Cory's shearwaters using prey morphological characters and DNA barcoding

This chapter presents an innovative approach to study the diet of seabirds using morphological and molecular analysis to quantify prey in stomach content. DNA barcoding of items collected in stomach contents has been applied in the diet of marine mammals and predatory fish (Tollit et al. 2009; Barnett et al. 2010), but not in the diet of seabirds. Morphological analysis on stomach contents uses generally the hard-parts of prey to make positive taxonomic identifications. However, some hard-parts are better recovered from stomach contents than others, which can bias prey estimates. For

example, studies have shown that prey identified through otoliths are generally underrepresented if compared with vertebrae (Alonso et al 2013). Otoliths are however better represented in reference collections than vertebrae, so that using the latter for prey identification might reduce the spectrum of detected prey. Moreover, juvenile prey for which hard structures are still developing might be significantly underrepresented in dietary studies. Or, extremely digested prey that lack distinctive morphological characters might be identified only at broad taxonomic ranks. As such, dietary patterns between populations with similar diets might not be detected.

In this chapter, we found that many cephalopods could not be distinguished based on their beak morphologies. In fact Cory's shearwater consumed a substantial number of juvenile cephalopods and the beaks were not sufficiently developed to allow for robust identification. Many hard structures further remained unidentified due to incomplete reference collections. 16S rRNA barcoding (Palumbi 1996) showed a substantial improvement in almost all cases where morphology failed to make a positive identification. This barcode was used instead of the universal COI barcode (Folmer et al. 1994), due to greater amplification success for cephalopod DNA.

Given that the 16S rRNA gene is not standardized for species discrimination, we employed additionally phylogenetic analysis to discriminate prey taxonomically through direct optimization of the tree lengths in POY (DeLaet 2010).
Molecular analysis showed a substantial improvement for dietary analysis in seabirds revealing 17 new taxa, many of which have never been described before despite extensive dietary studies on Cory's shearwater (den Hartog & Clarke 1996, Granadeiro et al. 1998; Paiva et al. 2010; Xavier et al. 2011; Neves et al. 2012). Combining molecular information with hard structures further allowed us to augment our reference collections and use this information for subsequent prey identification and quantification. We identified a significant trophic segregation among the prey types of birds of different breeding status and sex. The feeding ecology of immature seabirds is very poorly known, and direct evidence of their diet is rare. These results therefore highlight the need for further investigation of the prey choices of immature seabirds and other population segments.

Chapter 3: Phylogenetic placement of mitochondrial 16rRNA barcodes to identify vertebrate and invertebrate prey in a seabird, the Bulwer's Petrel

Here I optimised new methodological and phylogenetic approaches to taxonomically assign prey, using as an example new Next Generation Sequencing (NGS) data obtained during analysis of the diet of Bulwer's petrels (*Bulweria bulwerii*). The main objective of this chapter was to develop suitable metabarcodes and optimise prey detection and identification from non-invasive faecal remains to study the diet of petrels.

Currentely, most dietary studies using high throughput technologies assign prey based on similarity percentage with reference sequences, either using own sequence collections or public sequence databases. Seabirds, for example, eat very diverse groups of prey, including numerous orders of vertebrates and invertebrates. To design primers that potentially bind to the DNA from such a diverse range of prey, we optimised prey detection from the mitochondrial 16SrRNA, which contrary to COI, presented conserved primer-binding sites for primer design. Short ribosomal genes, such as16SrRNA, have been already applied to metabarcoding studies describing the diet of seabirds and other marine predators and have been suggested by other authors as preferential metabarcodes for dietary analysis (Deagle et al. 2007; 2009; Deagle et al. 2014).

Inter-specific sequence similarity thresholds have been extensively studied for short COI barcodes (Hajibabaei et al. 2007; Zeale et al. 2011), but have not been comprehensively tested for vertebrate and invertebrates rRNA barcodes. In fact, divergence estimates using 16S rRNA show substantial differences among vertebrate and invertebrate species belonging to the same family and genera (Turan et al. 2009; Dai et al. 2012), so that a universal threshold separating all species might further not exist. To overcome such problems and obtain reliable prey identifications, I used a phylogenetically aware algorithm that places query sequences on a reference tree of full-length sequences (16Sar/16Sbr, Palumbi et al. 1994) based on the Maximum likelihood of each query sequences belonging to a specific branch on the tree. Phylogenetic placement algorithms have been applied to metabarcoding of microbial communities, but never to dietary analysis.

The Evolutionary placement algorithm (EPA) (Berger et al. 2011) was used for phylogenetic placement of queries in this chapter. EPA optimises the tree length of each query sequence on a reference tree of full-length sequences and assigns queries based on its likelihood of insertion on each branch of the tree. This algorithm has shown as good or better accuracy and performance than other phylogenetic placement methods (Berger et al. 2011; Matsen et al. 2010)

The results of this chapter suggest that prey can be accurately identified from short 16S rRNA barcodes and that phylogenetic algorithms to place queries on reference trees ensures greater confidence in taxonomical assignments, while providing further information on the evolutionary relationships of queries on known phylogenies. Query identifications based on the nearest neighbour in BLAST were often questionable as queries sometimes showed high similarities (>98%) with sequences other than the nearest neighbour. Phylogenetic-based analysis overcomes these problems as it relies on informative characters and Maximum likelihood support to infer species relationships. Depending on the completeness of the reference tree it was possible to assign queries to the lowest taxonomic rank regardless of its similarity percentage.

To the best of our knowledge, this chapter provides a first example of this approach to dietary analysis using high-throughput sequencing. Moreover, the chapter also includes modified 16S rRNA primers adapted to dietary analysis of petrels, and a methodological two-step PCR approach (Berger et al. 2011) to individually tag each amplicon for HTS, reducing significantly the costs per sample.

Chapter 4: High-throughput sequencing technologies reveal trophic partitioning between sympatric small petrels in the sub-tropical eastern Atlantic

In this chapter, I examined the diet of three sympatric small petrels: Band-rumped Storm petrel, Bulwer's petrel and White-faced Storm petrel breeding in the northeastern Atlantic using HTS of mitochondrial 16SrRNA retrieved from faecal remains. According to competition theory, ecological similar species segregate their resource utilization to reduce competition (Gause 1934; Hardin 1960). Significant dietary overlap between coexisting species indicates that species are competing for the same resources, whereas significant dietary segregation indicates either that species

have evolved different dietary specializations throughout time, or that species actively segregate resources, foraging, for example, in different habitats or at different times during the day or night. It is important to note that patterns of trophic partitioning have to be interpreted in the context of resource availability, as competitive interactions are expected if resources are limited.

By performing metabarcoding on faeces, 74 different taxa were detected, of which 83% were successfully identified to lower taxonomic ranks, including genera and species. Most of these birds showed a high diversity of prey species within the same families and spanned prey across different groups, eating fish, cephalopods and crustaceans, often during the same feeding bout. Compared to morphological analysis on the stomach contents of Bulwer´s petrel (performed in Chapter 3 and Chapter 5), metabarcoding of faeces showed substantial improvement in prey detection and resolution. This was mainly due to the fact that reference collections for the majority of hard structures collected (vertebra) were substantially more limited than 16SrRNA sequence databases (Genbank) and often lacked inter-specific diagnostic characters. Moreover, other hard structures such as otolithes were less present in Bulwer´s stomach contents, despite available morpholical databases (e.g. AFORO database, Lombarte et al. 2006)

To identify patterns of dietary segregation within and among different bird populations and species, large sample sizes are generally needed raising substantial ethical concern if these are collected either through lethal or invasive stomach content analyses. Currently, stable isotope analysis (SIA) is a widely used non-invasive method to assess trophic niche partitioning among seabirds. This technique has shown significant trophic segregation among various seabird species, breeding status and sex. Dietary composition can be further inferred through SIA by applying general mixing models to assess the contribution of specific prey taxa in each sample. However, for many seabirds such as Band-rumped Storm petrels, Bulwer´s petrels and White-faced Storm petrels, for which prey have been rarely described, SIA can be only conducted to identify broad taxonomic prey goups as reference prey against which stable isotope signatures can be compared are not available and need to be collected from extensive surveys covering the spectrum of potential prey available to seabirds.

The results of this study show that metabarcoding of faecal material is extremely promising in the field of trophic ecology. The species composition retrieved for each

petrel was high, varying (between 33 - 43 in each (Table 4.1)) and significant dietary segregation among the study species was further obtained, despite most fed on the same broad taxonomic groups.

The diets of these species was dominated by mesopelagic organisms, but differed significantly in the range and type of prey consumed. White-faced Storm petrels showed the most distinctive pattern, including, in contrast to both of the other species, a high percentage of crustaceans, especially of the order Brachyura (crabs) and Isopoda. The diet of Bulwer's petrel showed substantially higher contributions of cephalopods, while Band-rumped Storm petrels consumed almost exclusively mesopelagic fish. Similar diets have been described for the same species and genera in the oceanic Pacific (Spear et al. 2007), indicating that these species probably maintain trophic specialization throughout their distribution range.

Prey availability in oceanic habitats, such as the study site, is thought to be low compared with that found in nutrient-rich coastal environments that generate higher biological production (Ballance et al. 1997; Weimerskirch 2007). However, the diet of tropical and sub-tropical seabirds foraging in oceanic habitats often shows higher prey diversity than its higher latitudinal and polar counterparts, which generally exploit one or two superabundant prey types (Spear et al. 2007; Croxall et al. 1997). This distinctive dietary pattern is evident in this study, marked by the high percentages of rare prey and high diversity. The prey diversity in the open tropical oceans compared with polar oceanic systems might, therefore, provide a wider range of species on which seabirds might specialize. In fact, this probably explains why significant trophic partitioning has been generally observed in tropical communities (e.g Spear et al. 2007; Young et al. 2010; Roscales et al. 2011). Moreover, given the high similarities between the prey types consumed in the sub-tropical Pacific and in the sub-tropical North Atlantic (this chapter), it is probable that these predator-prey specializations are consistent across the distribution ranges of species.

However, to better understand the mechanisms underlying trophic partitioning in seabird communities it is still essential to widen our knowledge of the foraging grounds of the birds, allowing us to determine resource availability. For example, one important limitation of our study is the fact that most GPS tracking technologies are too large for deployment on these birds, so therefore we have no data on the foraging grounds of the three species during the breeding season. The species of crabs, as well as the reef-

associated fishes herein identified, are known to occur at the intertidal areas, around the Selvagem Grande or Canary Islands. These results are however unexpected as Strom petrels are rarely seen to forage near shore (but see D'Elbée & Hémery 1998; Poot 2008). The presence of isopods in the diet of birds is questionable, as isopods are parasites of many fish. However, White-faced storm petrels showed an extraordinary high contribution of isopodes (ca 20% in numerical frequencies) compared to any other petrel. Given that they eat a similar proportion of mesopelagic fish to the other petrels studied, of, probably, similar size, there are no reasons to think that the fish consumed by White-faced storm petrel had more parasites. Small crustaceans and other miscellaneous invertebrates often accumulate on floating seaweed. If white-faced storm petrels take organisms from this floating material this could explain our results, while it could also explain why other authors found substantial amounts of miscellaneous invertebrates amongst the stomach contents of White-faced storm petrels (Spear et al. 2007).

Chapter 5: Predator-prey interactions along the lunar cycle contradict foraging efficiency predictions in a pelagic predator, the Bulwer's petrels

Here I examined the predator-prey interactions of Bulwer's petrels in relation to the moon cycle through DNA barcoding of prey items collected from stomach contents. Petrels often show distinct foraging patterns, changing activities depending on the moon phase (Awkerman et al. 2005; Phalan et al. 2007; Yamamoto et al. 2008; Cruz et al. 2013; Dias et al. 2012). Such patterns strongly suggest that the moon plays an important role in the foraging of petrels.

Dietary analysis of stomach content as well as on faecal remains (Chapter 4) revealed that Bulwer's petrel consumed almost exclusively mesopelagic organisms. These organisms are thought to be available to Bulwer's petrels during the night, when species from the deep scattering layers of the ocean ascend to the sea-surface. This evidence suggests that Bulwer's petrels are probably nocturnal foragers as suggested in other studies, that showed substantially higher flight activity in Bulwer's petrel during the night (Dias et al. in press).

The hypothesis of this chapter was that diet of Bulwer's petrels differs significantly between new moon and full moon, as mesopelagic prey are thought to react to nocturnal light intensity by avoiding the sea surface during moonlit nights. We were, therefore, expecting significant different species composition or prey diversity between moon phases. However, no such evidence was found, with Bulwer's petrels consuming overall the same prey types and maintaining prey diversity across the moon cycle. These results contradict current the foraging hypothesis that predicts a significant effect of the moon on the foraging efficiencies of procellariiformes (Imber 1975; Phalan et al. 2007). The results also contradict general oceanographic assumptions on the diel vertical migration patterns of mesopelagic organisms (Clarke 1973; Kampa 1974). It is important to note that Bulwer's petrel foraged on a wide diversity of prey and it is therefore unlikely that the dietary patterns are the result of particular mesopelagic prey type reacting differently to the moonlight. Instead the enormous diversity of prey obtained throughout all moon phases suggests the contrary, that all these prey were also available to Bulwer's petrels during moonlit nights.

Although, we did not find any evidence for a significant influence of the moon on the prey types of Bulwer's petrels, it is important to consider the limitations of this chapter that can bias this conclusion. The most important one relates to the biomass of prey delivered to the chicks. Here we obtained the numerical frequencies of each prey consumed, but not the biomass. Bulwer's petrel could deliver higher biomasses of prey during new moon to their chicks, reflecting a higher foraging success. However, a recent study correlating body mass of Bulwer's petrel at Deserta Grande with the moon cycle found no significant correlation (Gatt 2014).

Scavenging on dead remains has been proposed to explain the existence of mesopelagic prey in the stomach contents of petrels (Croxall & Prince 1994). In this case we would not expect a significant influence of the moon in the diet of these animals. However, if Bulwer's petrel, were scavenging on dead remains then they would probably scavenge on other prey types too and would do so during the day, when floating remains are more visible.

6.2 Technical considerations

The use of molecular analyses to study the diet of procellariiformes showed a substantial improvement over conventional morphological analysis. A higher number of prey species, compared with morphological analysis of Bulwer´s stomach contents, were recovered and identified. This was clear whether analyses were conducted using standard DNA barcoding of individual items collected in stomach content (Chapter 2, 5) or using HTS of faecal remains (Chapter 4). For small petrels it has previously not been possible to obtain meaningful prey estimates from faeces, as in most cases these samples are composed of a soft matrix of degraded tissues without identifiable prey remains. The use of short 16SrRNA barcodes and phylogenetic placement methods showed that these samples contain relevant dietary information.

Although faecal remains are substantially more degraded than stomach contents, in future, with the development of HTS ecologists will probably make use of these environmental samples to comprehensively assess the trophic interaction of seabirds. Despite its enormous potential, HTS to assess the diet of seabirds has been rarely conducted (Bowser et al. 2013; Jarman et al. 2013). This is particularly strange if we take into consideration the amount of high-throughput sequencing studies on other vertebrates, such as bats (Bohman et al. 2011; Clare et al. 2013; 2014). In this thesis, HTS on faecal remains from small petrels successfully permitted the identification of high numbers of prey taxa and showed significant trophic segregation between all three petrels (Chapter 4). While HTS has been successfully applied to study the diet of other seabirds, such as penguins and puffins (Deagle et al 2007; Bowser et al. 2013; Jarman et al. 2013), this thesis is to the best of my knowledge the first study to demonstrate that metabarcoding can be successfully applied to understand community interactions of highly pelagic seabirds. Dietary analysis in these predators is challenging, as they often feed on inaccessible prey for which morphological references are often not available. Many of these birds further breed on very remote islands so that dietary information has been very limited to identify the contribution of particular prey through stable isotope mixing models. 16SrRNA metabarcoding of faecal remains bypassed such constraints, as most oceanic prey sequences were largely available in current databases (GenBank). However, there are some important constraints to this approach that deserve further investigations.

A principal constraint relates to the sample itself. Faeces contain highly degraded mixtures of DNA (eDNA). Faecal DNA is highly fragmented so that

fragments longer than 200 bp are often not recovered. However, such small fragments are also often too uninformative to allow accurately identification and separation of species (reviewed in Pompanon et al. 2012). Metabarcoding studies in bats or other insectivorous predators show that species can be identified accurately from small COI fragments, often providing sequence homology of above 98 % with reference sequences (Clare et al. 2013a;b). This threshold has been tested comprehensively for small COI barcodes, so that even if references are not available, different prey species can be still identified if sequences differ from each other by more than 2% - termed Molecular Operational Taxonomical Units (MOTU). For seabirds and other marine predators this approach is less appropriate. Seabird diets span substantially over different taxonomic groups, consuming numerous orders of vertebrates, cephalopods and crustaceans. It has not been possible to find a conserved region within the standard COI barcode where conserved primers can be designed to amplify all taxa, without significant bias, and separate species within and between prey groups. To accurately describe the diet of seabirds from faeces, other barcodes than the universal COI need to be found. This is difficult because the Consortium for the Barcode of Life (CBOL) focuses on COI to provide a universal database of reference sequences to identify unknown specimens.

In this thesis, I used as an alternative barcode the 16S RNA of Palumbi (1996). No comprehensive studies on the similarity thresholds distinguishing species are currently available for this marker, but some studies show that divergences among species and prey groups vary substantially (Dai et al. 2012). Instead of using similarity-based approaches to identify prey, a phylogenetic-aware algorithm was used to place queries on a reference tree.

16SrRNA references are extensive in the Genbank databases, as this marker has been widely used for phylogenetic inferences of vertebrates and invertebrates. Interestingly, for the cephalopods relevant to our study system, the sequence cover was actually higher than for the COI barcode.

This phylogenetic placement approach, using 16SrRNA barcodes, was very promising for the analysis of diet from seabird's faeces, as we retrieved very similar (but also additional) contributions of prey taxa compared with previous morphological studies on stomach contents (Spear et al. 2007) and even with DNA barcoding of prey items collected in stomach contents shown in Chapters 2 and 5. While 16SrRNA is seemingly a good barcode for HTS, it is important to refer some inherent difficulties

with this marker. A primary issue relates to the amount of predator DNA amplified. The primers for this study were modified from Deagle et al. (2009) to include polymorphisms at the 3' end with predator DNA. This procedure is known to significantly reduce amplification of non-targets. However, we still amplified a high amount of predator DNA. To reduce this amount, I further developed species-specific blocking probes to reduce amplification of seabirds. Even so, a high amount of predator sequences was obtained in the NGS data to the extent that over 40% of the Bulwer's samples were predator DNA. Fortunately, such is the coverage obtained using HTS, more than sufficient numbers of prey sequences were also obtained.

In future, however, more effective methodologies are needed to block predator DNA or alternatively new markers have to be found. Once the best barcode to study the diet of marine predators is agreed, it will be necessary to revise current sequence databases so as to include substantially more sequences from other barcodes than the COI.

6.3 General conclusion and Future directions

In this thesis, I investigated the trophic ecology of petrels. For some petrels breeding in the northeastern Atlantic, this was the most comprehensive study conducted so far. Some of the conclusions from this thesis do not conform to current predictions made on the foraging behavior of these birds nor do they fit with current oceanographic biological expectations. For example, it is assumed that mesopelagic species react to moon light, and it has been demonstrated that mesopelagic scattering layers concentrate at deeper depths during moonlit nights than during dark nights (Benoit-Bird et al. 2009a;b). However, it has also been often documented that seabirds forage actively on mesopelagic species during times when these species are thought not to be at the sea surface, for example during the day (Pitman & Balance 1990). Another important point is that many of the mesopelagic prey identified are rarely available in the first few meters from the surface, and not at the maximum diving depths of the study species.

By studying the diet of Bulwer's petrel, using new technologies and relating it to environmental factors such as the moon phase, it is clear that the foraging dynamics of seabirds are substantially more complex than assumed. In fact if Bulwer's petrel diet

models the environment it feeds on then many of our assumptions about species availability on the Oceans surface need further research.

Little is known about the diet and interactions of small petrels breeding in the sub-tropical or tropical North Atlantic. Dietary analysis in small seabirds is particularly difficult, as these species consume small prey where hard structures are generally difficult to recover and identify, especially when reference collections are not available, as in our study system. Moreover, current tracking devices have been too heavy to deploy on small birds, so that data on the foraging grounds utilized by small seabirds come essentially from direct observations or stable isotope analysis. In this thesis, we found consistent trophic patterns across seabird's distribution ranges. Future research is, however, needed to understand the interplay between trophic specialization, different breeding grounds and marine habitats in shaping predator-prey interactions. Molecular approaches will certainly make an important contribution to this field, especially if applied to the diet of seabird communities all over the world, including different latitudes (from polar to tropical) and environments (oceanic and coastal). Molecular approaches can be further used to complement current methodologies. For example, by combining tracking devices and activity loggers with molecular dietary analysis, it will be possible in the future to map the location of prey. Such information can much contribute to the design of marine protected areas for seabird and other predator communities.

Unless predator-prey interactions are thoroughly investigated, it is impossible to understand fully how communities evolve and ecosystem function, which is essential for predicting ecological responses to human interactions, invasive species and climatic change, as well as the dangers of species extinctions and ecosystem collapse.

6.4 References

Alonso H, Granadeiro JP, Ramos JA & Catry P (2013) Use the backbone of your samples: fish vertebrae reduces biases associated with otoliths in seabird diet studies. Journal Of Ornithology 154: 883-886

Ballance LT & Pitman RL (1999) Foraging ecology of tropical seabirds. In: Adams, NJ & Slotow RH (eds) Proc. 22 International Ornithology Congress, Durban: 2057-2071. Johannesburg

Bohmann K, Jomadjem A, Noer C, Rasmussen M, Zeale M, Clare EL, Willerslev E & Gilber MTP (2011) Molecular dietary analysis of two African free-tailed bats (Molossidae) using high throughput sequencing. PLoS ONE. 6: e21441

Awkerman J, Fukuda A, Higuchi H & Anderson D (2005) Foraging activity and submesoscale habitat use of waved albatrosses Phoebastria irrorata during chick-brooding period. Marine Ecology Progress Series 291: 289–300

Ballance LT, Pitman RL & Reilly SB (1997) Seabird community structure along a productivity gradient: importance of com- petition and energetic constraint. Ecology 78: 1502–1518

Barnett A, Redd KS, Frusher SD, Stevens JD & Semmens JM (2010) Non-lethal method to obtain stomach samples from a large marine predator and the use of DNA analysis to improve dietary information. Journal of Experimental Marine Biology and Ecology 393: 188–192

Benoit-Bird KJ, Au WWL & Wisdoma DW (2009a) Nocturnal light and lunar cycle effects on diel migration of micronekton. Limnology and Oceanography 54: 1789-1800

Benoit-Bird KJ, Dahood AD & Würsig B (2009b) Using active acoustics to compare lunar effects on predator–prey behavior in two marine mammal species. Marine Ecology Progress Series 395: 119-135

Berger SA, Krompass D & Stamatakis A (2011) Performance, Accuracy, and Web Server for Evolutionary Placement of Short Sequence Reads under Maximum Likelihood. Systematic Biology 60: 291-302

Bowser AK, Diamond AW & Addison JA (2013) From Puffins to Plankton: A DNA-Based Analysis of a Seabird Food Chain in the Northern Gulf of Maine. PLoS ONE, 8(12), e83152.

Clare EL, Symondson WOC, Broders H, Fabianek F, Fraser E, Mackenzie A, Boughen A, Hamilton R, Willis C, Martinex F, Menzies A, Norquay K, Brigham M, Poissant J, Rintoul J, Barclay R & Reimer J (2013a) The diet of Myotis lucifugus across Canada: assessing habitat quality and dietary variability. Molecular Ecology 23: 3618–3632

Clare EL, Symondson WOC & Fenton MB (2013b) An inordinate fondness for beetles? Variation in seasonal dietary preferences of night roosting big brown bats (Eptesicus fuscus). Molecular Ecology 23: 3633–3647

Clarke TA (1973) Some aspects of the ecology of lanternfishes (Myctophidae) in the Pacific ocean near Hawaii. Fisheries Bulletin 71: 401–433.

Croxall JP & Prince P (1994) Dead or alive, night or day: how do albatrosses catch squid? Antarctic Science 6: 155–162

Croxall JP, Prince PA & Reid K (1997) Dietary segregation of krill-eating South Georgia seabirds. Journal of Zoology 242:531–556

Cruz SM, Hooten M, Huyvaert KP, Proaño CB, Anderson DJ, Afanasyev V & Wikelski M (2013) At–Sea Behavior Varies with Lunar Phase in a Nocturnal Pelagic Seabird, the Swallow-Tailed Gull. PLoS ONE 8:e56889

Dai L, Zheng X, Kong L & Li Q (2012) DNA barcoding analysis of Coleoidea (Mollusca: Cephalopoda) from Chinese waters. Molecular Ecology Resources 12: 437–447.

D'Elbee J & Hemery G (1998) Diet and foraging behaviour of the British Storm Petrel Hydrobates pelagicus in the Bay of Biscay during summer. Ardea 86: 1-10

De Laet (2010) Letter to the editor. A problem in POY tree searches (and its work-around) when some sequences are observed to be absent in some terminals. Cladistics 26: 453–455

Deagle BE, Gales NJ, Evans K, Jarman SN, Robinson S, Trebilco R & Hindell MA (2007) Studying seabird diet through genetic analysis of faeces: a case study on macaroni penguins (Eudyptes chrysolophus). PLoS ONE, 2, e831

Deagle BE, Kikwood R & Jarman SN (2009) Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. Molecular Ecology 18: 2022-2038

Deagle BE, Jarman SN, Coissac E, Pompanon F & Taberlet P (2014) DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biology Letters 10: 20140562

den Hartog JC & Clarke MR (1996) A study of stomach contents of Cory's Shearwater, Calonectris diomedea borealis (Cory, 1881) (Aves: Procellariidae), from the Macaronesian Islands. Zoologische Mededeelingen 70: 117–133

Dias MP, Alho M, Granadeiro JP & Catry P (in press) Wanderer of the deepest seas: migratory behaviour and distribution of the highly pelagic Bulwer's Petrel. Journal of Ornithology.

Dias MP, Granadeiro JP & Catry P (2012) Working the day or the night shift? Foraging schedules of Cory's shearwaters vary according to marine habitat. Marine Ecology Progress Series 467: 245–252

Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Gales RP (1988) The Use of Otoliths as Indicators of Little Penguin Eudyptula minor Diet. Ibis 130: 418-426

Gatt MC (2014) The influence of moon phase on foraging success in the Bulwer's Petrel (*Bulweria bulwerii*). Msc thesis. The Manchester Metropolitan University, UK

Gause GF (1934) The struggle for existence.Williams & Wilkins. Baltimore

Granadeiro JP, Monteiro LR & Furness RW (1998) Diet and feeding ecology of Cory's shearwater Calonectris diomedea in the Azores, north-east Atlantic. Marine Ecology Progress Series 166: 267–276

Hajibabaei M, Singer GA, Clare EL & Hebert PD (2007) Design and applicability of DNA arrays and DNA barcodes in biodiversity monitoring. BMC Biology 5: 24

Hardin G (1960) The competitive exclusion principle. Science 131:1292–1297

Harvey JT & Antonelis GA (1994) Biases Associated with Nonlethal Methods of Determining the Diet of Northern Elephant Seals. Marine Mammal Science 10: 178-187

Imber MJ (1975) Behaviour of petrels in relation to the moon and the artificial lights. Notornis 22: 302 –306

Jarman SN, McInnes JC, Faux C, Polanowski AM, Marthick J, Deagle BE, Southwell C & Emmerson L (2013) Adélie Penguin Population Diet Monitoring by Analysis of Food DNA in Scats. PLoS ONE 8: e82227

Kampa EM (1974) Photoenvironment and vertical migrations of mesopelagic marine animal communities. In Biological rhythms in the marine environment (ed. P. J. DeCoursey), pp. 257-272. Columbia: University of South Carolina Press

Lombarte A, Chic Ò, Parisi-Baradad V, Olivella R, Piera J & García-Ladona E (2006) A web-based environment for shape analysis of fish otoliths. The AFORO database. Scientia Marina 70: 147–152

Matsen FA, Kodner R & Armbrust, EV (2010) pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. BMC Bioinformatics 11: 538

Neves VC, Nolf D & Clarke M (2012) Spatio-temporal variation in the diet of Cory's shearwater Calonectris diomedea in the Azores archipelago, north-east Atlantic. Deep Sea Research Part I 70: 1–13

Paiva VH, Xavier JC, Geraldes P, Ramírez I, Garthe S & Ramos JA (2010) Foraging ecology of Cory's shearwaters in different oceanic environments of the North Atlantic. Marine Ecology Progress Series 410: 257–268

Palumbi SR (1996) Nucleic acids II: the polymerase chain reaction. In: Hillis DM, Mable BK, Moritz C (eds) Molecular Systematics. Sinauer, Sunderland, MA, pp 205–247

Phalan B, Phillips RA, Silk JRD, Afanasyev V, Fukuda A, Fox J, Catry P, Higuchi H & Croxall JP (2007) Foraging behaviour of four albatross species by night and day. Marine Ecology Progress Series 340: 271-286

Pitman RL & Balance RT (1990) Daytime feeding by Leach´s storm petrel on a mid-water fish in the eastern tropical Pacific. The Condor 92: 524-527

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SD & Taberlet P (2012) Who is eating what: diet assessment using next generation sequencing. Molecular Ecology, 21, 1931–1950.

Poot M (2008) Nocturnal and diurnal foraging of European Storm petrels *Hydrobates* sp along the Lisbon coast, Portugal. Airo 18:13-21

Roscales JL, Gómez-Díaz E, Neves V & González-Solís J (2011) Trophic versus geographic structure in stable isotope signatures of pelagic seabirds breeding in the northeast Atlantic. Marine Ecology Progress Series 434:1–13

Spear LB, Ainley DG & Walker WA (2007) Foraging dynamics of seabirds in the Eastern Tropical Pacific Ocean. Studies in Avian Biology 35:1−99

Tollit DJ, Schulze AD, Trites AW, Olesiuk PF, Crockford SJ, Gelatt TS, Ream RR & Miller KM (2009) Development and application of DNA techniques for validating and improving pinniped diet estimates. Ecological Applications 19: 889–905

Turan C, Gunduz I, Gurlek M, Yaglioglu D & Erguden D (2009) Systematics of Scorpaeniformes Species in the Mediterranean Sea Inferred From Mitochondrial 16S rDNA Sequence and Morphological Data. Folia biologica (Kraków) 57: 219-226

Vogler AP & Monaghan MT (2007) Recent advances in DNA taxonomy Journal of Zoological Systematics and Evolutionary Research 45: 1–10.

Weimerskirch H (2007) Are seabirds foraging for unpredictable resources? Deep Sea Research Part II: Topical Studies in Oceanography 54: 211-223

Xavier JC, Magalhães MC, Mendonça AS, Antunes M, Carvalho N, Machete M, Santos RS, Paiva V & Hamer KC (2011) Changes in diet of Cory's Shearwaters Calonectris diomedea breeding in the Azores. Marine Ornithology 39: 129–134

Yamamoto T, Takahashi A, Yoda K, Katsumata N & Watanabe S (2008) The lunar cycle affects at–sea behaviour in a pelagic seabird, the streaked shearwater, Calonectris leucomelas. Animal Behaviour 76: 1647–1652

Young HS, McCauley DJ, Dirzo R, Dunbar RD & Shaffer SA (2010) Niche partitioning among and within sympatric tropical seabirds revealed by stable isotope analysis. Marine Ecology Progress Series 416: 285-294

Zeale MRK, Butlin RK, Barker GLA, Lees DC & Jones G (2011) Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. Molecular Ecology Resources11: 236–244

Zhang J, Kapli P, Pavlidis P & Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29: 2869–2876

Zonfrillo B (1986) Diet of Bulwer's petrel *Bulweria bulwerii* in the Madeiran archipelago. Ibis 128: 570-572