Characterising the foraging ecology and mercury exposure of the Nationally Critical Whenua Hou diving petrel

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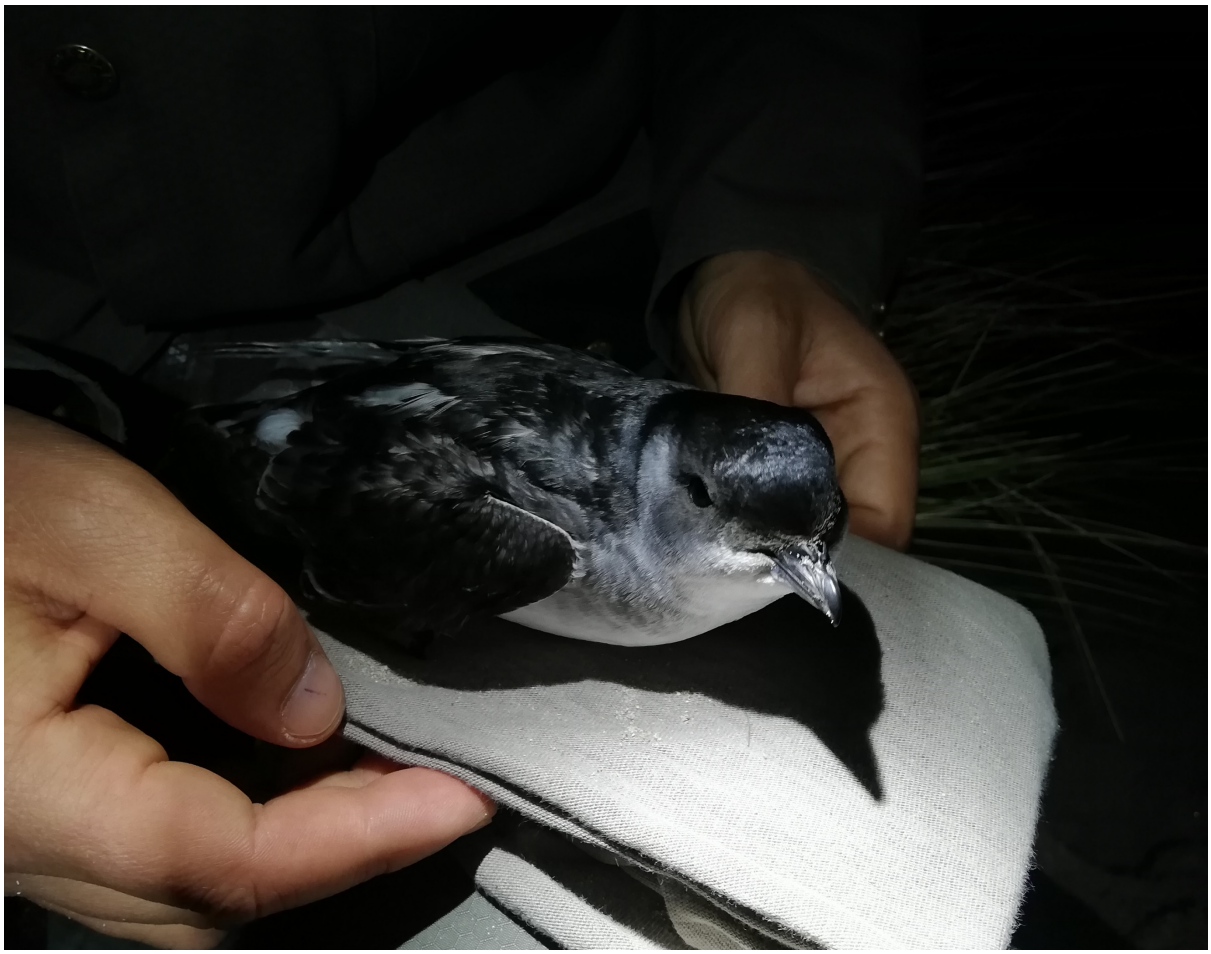

Whenua Hou diving petrel. Photo credit: Grace Tocker, 2019.

ABSTRACT

With seabird species in decline globally, significant research has gone into characterising their key prey species and foraging areas that need protection. Knowledge on the diet of a species has important implications for the development of conservation programmes. The sand dune system on Whenua Hou is home to the endemic Whenua Hou diving petrel (*Pelecanoides whenuahouensis*; hereafter WHDP) and a population of common diving petrels (*Pelecanoides urinatrix*; hereafter CDP). The WHDP is considered 'Nationally Critical' due to its small population size (~200 individuals) and restricted breeding range (0.018 km^2) on Whenua Hou. The foraging ecology of the WHDP is relatively unknown, as is its exposure to sources of marine pollution. This thesis aimed to characterise the foraging ecology of the WHDP, the potential interspecific competition with the sympatric CDPs, the prey present in the diets of each species, and their resulting exposure to mercury from the environment. In chapter 2, I used stable isotope analysis to infer the trophic dynamics of the WHDP. By sampling and analysing both blood and feathers, I was able to investigate potential differences in WHDP foraging ecology between the breeding and non-breeding seasons. I found a difference between the foraging ecology of male and female WHDPs, with results indicating females forage further out to sea than males and on prey of lower trophic value. I found that WHDPs forage an entire trophic level higher during the breeding season than the non-breeding season. As my sampling effort spanned three consecutive breeding seasons (2017-2019), I was able to detect interannual variation in the foraging ecology of WHDPs. The results revealed that WHDPs foraged at a higher trophic level during the breeding season of 2018 compared to that of 2017 or 2019. By characterising the isotopic niches of both the WHDPs and CDPs over the three years, I was able to demonstrate a degree of trophic segregation between the two species during the breeding season. In chapter 3, I designed and went through the initial development

stages for a novel multiplex-PCR assay to identify the prey species present in the diets of WHDPs and CDPs. The obstacles faced in the development of this protocol highlighted the suitability of DNA metabarcoding as an alternative method. In chapter 4, I analysed the mercury concentration in the same blood and feather samples used for stable isotope analysis. I demonstrated that male WHDPs had higher concentrations of mercury in their tissues than females, correlating with their foraging at a higher trophic level. The interannual variation in mercury concentration did not correlate with the trophic variation of WHDPs among years, indicating that the environmental fluctuations in mercury levels had a stronger effect on mercury exposure than diet. WHDP tissues consistently had higher concentrations of mercury than CDPs, correlating with their isotopic niche segregation and highlighting a potential threat to individual survival and reproductive success in WHDPs. Overall, my results describe patterns in the foraging ecology of the WHDP, as well as highlighting the potential threat from mercury exposure. This research can be used as a baseline for future investigations into the key prey species for the endangered WHDP and the impacts mercury exposure may be having on the population growth of this species.

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WHDP	Whenua Hou diving petrel
CDP	Common diving petrel
SGDP	South Georgian diving petrel
ENSO	El Niño/Southern Oscillation
NBS	Non-breeding season
LMM	Linear mixed-effect model
AIC	Akaike information criterion
KUD	Kernel utilisation density
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
A, T, C, G	Adenine, Thymine, Cytosine, Guanine

CHAPTER 1

General Introduction

1.1 Foraging ecology in declining seabird populations

Information on a species' diet is fundamental to understanding its ecological relationships and has important implications for the design and implementation of conservation programmes (Oli, Taylor and Rogers, 1993; Jones, Moss and Sanders, 2005; Margalida, Bertran and Heredia, 2009; Klare, Kamler and MacDonald, 2011). Knowledge of dietary preferences can be beneficial to the conservation of threatened species by helping to increase population distribution and improve breeding performance through supplementary feeding (Margalida, Bertran and Heredia, 2009). Diet studies can also shed light on the degree of conflict with domestic livestock (Oli, Taylor and Rogers, 1993). Alternatively, if the species is considered an invasive pest, dietary analysis allows the assessment of potential impacts on other components of the ecosystem (Jones, Moss and Sanders, 2005).

Many methods have been developed over the years to investigate foraging ecology. Identification and quantification of prey species becomes more complex and problematic with generalist predators as they feed on a range of different species (Symondson, 2002). Traditionally, diet has been analysed through direct observation of feeding events, stomach contents of deceased individuals and morphological analysis of prey remains in fecal samples (Corbett, 1989; Ciucci *et al.*, 1996; Burns *et al.*, 1998; Symondson, 2002). Direct observations can be relatively easy when a large predator kills one or two prey every few days (Symondson, 2002, Margalida, Bertran and Heredia, 2009). However, it becomes much more difficult with smaller predators feeding in cryptic environments on a much higher

number of prey, such as invertebrate predators or in cases concerning marine ecosystems (Pierce and Boyle, 1991; Symondson, 2002; Braley *et al.*, 2010; Klare, Kamler and MacDonald, 2011; Zeale *et al.*, 2011). Even when you are fortunate enough to witness a predator-prey interaction, it is difficult to know whether it was a common or rare event (Symondson, 2002).

To overcome these difficulties, a range of techniques have been developed. These include morphological analysis of prey remains in stomach samples, regurgitates, and feces (Oli, Taylor and Rogers, 1993; Symondson, 2002; Barrett *et al.*, 2007; Thalinger *et al.*, 2016). One example where fecal sample analysis was crucial for conservation management was for the endangered lesser long-nosed bat (*Leptonycteris yerbabuenae*) in Mexico (Stoner, O-salazar and Quesada, 2003). Previous studies had suggested that the wrong plant species were the main resources for these bats. Through analyzing the pollen present in scat samples, Stoner *et al.* (2003) were able to correctly identify the primary resources for the lesser long-nosed bats, which had important implications for their conservation. However, morphological analyses are biased towards prey with hard parts capable of withstanding digestion, whereas gelatinous species are often under-represented (Votier *et al.*, 2003; Braley *et al.*, 2010; Oehm *et al.*, 2017; Cavallo *et al.*, 2018). For example, stable isotope analysis, an alternative method that does not rely on hard parts remaining, revealed that jellyfish make up as much as 90% of the diet of certain fish species that use them as shelter (Cavallo *et al.*, 2018). This highlights the value of biochemical assays like stable isotope analysis in the field of feeding ecology. Additionally, with the advancement of technology, ecologists have been able to utilize modern molecular methods, such as diagnostic multiplex PCRs and DNA metabarcoding, to greatly increase the degree of taxonomic resolution discernable from the analysis of fecal samples (Vestheim and Jarman, 2008; Thalinger *et al.*, 2016; Waap *et al.*, 2017; Cavallo *et al.*, 2018).

Combined results from previous studies demonstrate that each method can be effective for analyzing the diet of certain species and completely unsuitable for the study of others. Direct observations can work for large terrestrial predators in open environments, such as lions or vultures, but are impossible for smaller species that forage either underground or underwater, such as seabirds (Symondson, 2002; Klare, Kamler and MacDonald, 2011; Zeale *et al.*, 2011).

Many seabird species are critical bio-indicators for the status of marine environments (Montevecchi 1993, Burger 1993, Burger and Gochfeld, 2002; Thalinger *et al.*, 2016; Fischer *et al.*, 2017). Diet is the crucial link connecting variation in ocean conditions with the myriad of measurements made on seabirds, such as reproductive success, survival, body condition and stress levels (Karnovsky, Hobson and Iverson, 2012). This requires insight into the type, amount, and quality of prey consumed (Karnovsky, Hobson and Iverson, 2012). This depth of understanding into seabird diet is key to the conservation of these marine top predators, with their important roles as bio-indicators, top-down population regulators, and nutrient cyclers between pelagic and coastal ecosystems (Karnovsky, Hobson and Iverson, 2012; Fischer *et al.*, 2017; Waap *et al.*, 2017; Cavallo *et al.*, 2018). Assessing seabird diet has the potential to reveal factors causing population declines and identify key prey species and foraging locations requiring protection (Karnovsky, Hobson and Iverson, 2012). This is important because of the widespread decline in seabird populations of 70% over the last 60 years (Paleczny *et al.*, 2015). Currently, 47% of all seabird species have declining population trends and 31% are considered globally threatened (BirdLife International 2018).

The foraging ecology of seabirds is difficult to determine using traditional methods. Direct observations of feeding events are almost impossible, and dietary samples are only obtainable during the breeding season when they return to land (Barrett *et al.*, 2007). To understand how seabird diet changes outside of the breeding season, it is necessary to use methods that

illuminate long-term trophic patterns from tissue samples obtained during the chick-rearing period (Barrett *et al.*, 2007; Cherel, Connan, *et al.*, 2014; Connan *et al.*, 2014). This can be achieved using stable isotope analyses on tissues with different turnover rates (Thompson *et al.*, 1998; Quillfeldt, McGill and Furness, 2005; Carravieri *et al.*, 2014). The stable isotope ratio of a given tissue reflects the diet at the time of synthesis (Bond and Jones, 2009; Bond, 2010; Polito *et al.*, 2011). Blood and feather samples are commonly used to study trophic shifts in seabirds as they provide insight into trophic level variation through different times of the year (Bearhop *et al.*, 2002; Quillfeldt, McGill and Furness, 2005; Bond, 2010).

1.2 A review of methods for dietary and trophic characterisation of seabirds

1.2.1 Traditional

Dietary analyses allow characterization of food web interactions and are used to inform conservation and management models (Cavallo *et al.*, 2018). Seabirds play a major role in structuring marine trophic webs through top-down ecosystem regulation by consuming around 70 million tonnes of the oceans biomass annually (Waap *et al.*, 2017). In seabirds, dietary studies originally consisted of direct observation of feeding or stomach content analysis of deceased individuals (Barrett *et al.*, 2007; Thalinger *et al.*, 2016; Oehm *et al.*, 2017). Morphological analysis of hard parts remaining in regurgitated pellets and feces also contributed to our understanding of the diet of many seabird species (Thalinger *et al.*, 2016).

Unlike many other Procellariiformes, diving petrels do not regurgitate food spontaneously upon capture, therefore it is necessary to use a stomach lavage technique to obtain stomach samples from live individuals (Reid *et al.*, 1997). To do this, a thin plastic tube is inserted into the stomach of a captured bird and salt water is slowly introduced using a syringe (Reid *et al.*, 1997; Bocher, Cherel and Hobson, 2000; Karnovsky, Hobson and Iverson, 2012). The

tube is removed when the buccal cavity is full of water and the bird is then inverted over a sieve while the abdomen is massaged to facilitate regurgitation (Bocher, Cherel and Hobson, 2000). This process is repeated up to three times to fully evacuate the stomach (Jahncke, Garcia-Godos and Goya, 1999). The samples can then be preserved and sorted to find identifiable prey fragments and estimate proportion by fresh mass in the diet (Reid *et al.*, 1997; Bocher, Cherel and Hobson, 2000; Cherel *et al.*, 2002). This method, though not destructive, is an invasive and stressful sampling experience for birds to go through (Oehm *et al.*, 2017). In the study by Reid *et al.* (1997), three of the 138 birds sampled died after sampling via stomach lavage. Therefore, sample types which can be obtained non-invasively are preferred, such as pellets, feces, or spontaneous regurgitations where possible (Thalinger *et al.*, 2016; Oehm *et al.*, 2017; Cavallo *et al.*, 2018).

Stomach samples are subject to bias from several sources. The sample will be over-represented by larger prey with hard parts capable of withstanding the early stages of digestion and under-represented by smaller gelatinous species which are digested rapidly into an amorphous gel (Votier *et al.*, 2003; Braley *et al.*, 2010; Karnovsky, Hobson and Iverson, 2012; Thalinger *et al.*, 2016; Oehm *et al.*, 2017; Cavallo *et al.*, 2018). In the previously mentioned study by Reid *et al.* (1997), they examined the deceased birds' stomachs and to see what remains after the three rounds of lavage. This revealed that 10-20% of the stomach contents remained after lavage, which could potentially bias the perceived diet (Reid *et al.*, 1997). Furthermore, although small species of fish do contain hard parts (otoliths) known to resist digestion, studies show that small otoliths do get fully digested if enough time is spent in the seabird's digestive tract resulting in the under-representation of small fish species in the diet (Karnovsky, Hobson and Iverson, 2012).

Morphological analysis of gut contents is beneficial to dietary analyses as it provides means of direct observation of a recent feeding event, however there are many factors

limiting what can be ascertained through stomach content analysis and, in seabirds, this method is temporally limited to the chick-rearing season when they are accessible on land (Barrett *et al.*, 2007; Polito *et al.*, 2011; Karnovsky, Hobson and Iverson, 2012). Other methods must be employed to uncover a how the diet of a seabird may change over the moulting period when they are at sea.

1.2.2 Stable isotope analysis

Stable isotope ratios have been used to estimate trophic positions of a variety of species (Estrada *et al.*, 2003). The theory behind stable isotope analysis is that animals "are what they eat", with the isotope ratio in a given tissue reflecting the diet at the time of synthesis (Thompson *et al.*, 1998; Bond and Jones, 2009; Bond, 2010; Polito *et al.*, 2011; Carravieri *et al.*, 2014). Correctly interpreting stable isotope ratios requires knowledge of the turnover rates of sampled tissues (Estep and Vigg, 1985; Quillfeldt, McGill and Furness, 2005).

Commonly used tissues in seabird trophic analysis are feathers and blood as they can be sampled non-destructively and give access to dietary information spanning different time scales (Bearhop *et al.*, 2002; Quillfeldt, McGill and Furness, 2005; Bond, 2010; Carravieri *et al.*, 2014). Feathers are lost and replaced in predictable moulting patterns and they are metabolically inert after synthesis, preserving their chemical composition almost indefinitely (Thompson *et al.*, 1998; Bond, 2010; Carravieri *et al.*, 2014; Cherel, Connan, *et al.*, 2014). This provides a means of non-invasive sampling of a tissue that reliably reflects the individuals diet from a set time in their recent life history (Thompson *et al.*, 1998; Carravieri *et al.*, 2014). For moulted feathers, stable isotope ratios provide insight into seabird diet away from the breeding colony, a time period not covered by traditional diet sampling methods (Barrett *et al.*, 2007; Bond and Jones, 2009). Blood has a much shorter turnover rate of

approximately 15 days in seabirds (Bearhop *et al.*, 2002). This allows the stable isotope signature of diet during the breeding season to be assessed.

Analysis of stable isotope ratios (generally carbon and nitrogen) is done through homogenization of the tissue sample measurement through continuous-flow isotope ratio mass spectrometry (Estep and Vigg, 1985; Estrada *et al.*, 2003; Quillfeldt, McGill and Furness, 2005; Carravieri *et al.*, 2014). Ratios of heavy to light isotopes are expressed using the equation:

$$\delta \mathrm{X} = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000$$

where X is the heavy isotope, R_{sample} represents the ratio of heavy to light isotopes in the sample, and $R_{standard}$ is the ratio of heavy to light isotopes in the reference standard (MacNeil, Skomal and Fisk, 2005).

The δ value for a stable isotope ratio increases predictably with increasing trophic level because of the selective retention of the heavy isotope and excretion of the light isotope (Thompson *et al.*, 1998; Estrada *et al.*, 2003; Quillfeldt, McGill and Furness, 2005; Barrett *et al.*, 2007; Bond and Jones, 2009; Rayner *et al.*, 2010). In marine food webs, there is an enrichment of approximately 3-5 ‰ in nitrogen and 0.8 ‰ in carbon per trophic level (Quillfeldt, McGill and Furness, 2005). The differences in nitrogen isotope ratios are commonly used to determine trophic level and diet composition (Quillfeldt, McGill and Furness, 2005; Bond and Jones, 2009; Navarro *et al.*, 2013). As carbon isotope ratios only increase a small amount per trophic level, they are primarily used to pinpoint foraging regions due to the existence of carbon isotope gradients in nature (Bond and Jones, 2009). Carbon ratios differ between terrestrial versus marine, inshore versus offshore, and pelagic versus benthic food webs (Quillfeldt, McGill and Furness, 2005). Inshore food sources are enriched in ^{13}C compared to offshore, therefore comparing carbon isotope ratios can inform

researchers about seabird foraging distance out to sea (Bocher, Cherel and Hobson, 2000; Bond and Jones, 2009; Navarro *et al.*, 2013; Cherel, Connan, *et al.*, 2014).

Stable isotopes provide advantage over traditional diet studies because information obtained represents assimilated, not just ingested prey (Bearhop *et al.*, 2004). Isotopic composition within consumer tissue represents long-term feeding behaviours, not just a 'snapshot' dietary sample (Estrada *et al.*, 2003; MacNeil, Skomal and Fisk, 2005). However, there are still considerable gaps in our knowledge of how elemental isotopes behave in biological systems (Bond and Jones, 2009). Complications include lipid content of tissue impacting carbon isotope ratios due to lipids having lower ^{13}C compared to carbohydrates (Bond and Jones, 2009). To account for this, lipids can be removed from lipid heavy tissues, but the impact this has on $\delta^{15}N$ ratios is unclear. Species are also unique in the way that isotopes are incorporated from their diet into their various tissues, resulting in the need for a species specific 'discrimination factor' to be applied to results from a stable isotope analysis when comparing the isotopic niche of multiple species (Bond and Jones, 2009). Additionally, due to the complex and broad diet of seabirds that could be comprised of infinite combinations of prey species, stable isotope analyses lack the resolution required to identify prey beyond broad trophic groups (Bond and Jones, 2009; McInnes, Jarman, *et al.*, 2017). Here we find the benefit of combining biochemical assays such as stable isotope analysis, with its temporal and spatial coverage, with complementary methods that allow assignment of prey species present in the diet, such as traditional morphological analysis or modern DNA-based identification (Connan *et al.*, 2014; McInnes, Jarman, *et al.*, 2017).

1.2 3 Modern molecular methods

Over the last 20 years, modern molecular methods have been developed to improve the accuracy and resolution attainable through dietary analysis techniques, revolutionizing our

understanding of food-web interactions (Pompanon *et al.*, 2012; Traugott *et al.*, 2013; Thalinger *et al.*, 2016; Oehm *et al.*, 2017; Cavallo *et al.*, 2018). Early DNA approaches involved amplifying DNA extracted from gut contents using general primers and separating the fragments through gel electrophoresis (King *et al.*, 2008; Pompanon *et al.*, 2012). The resulting amplification bands in the gel provide insight into the diversity present in the diet, although it is difficult to isolate cryptic bands and interpret specific species present in the diet using this method (Pompanon *et al.*, 2012).

Recent advancements have seen the development of two main strands within the field of DNA-based dietary analysis, both of which use PCR (polymerase chain reactions) to amplify DNA for subsequent analyses (Pompanon *et al.*, 2012; Traugott *et al.*, 2013). One branch uses specific sets of primers to identify the presence or absence of certain predetermined prey within a predators diet, as seen in the multiplex PCR approach (Pompanon *et al.*, 2012; Thalinger *et al.*, 2016; Oehm *et al.*, 2017). The other strand uses universal primers to amplify a conserved DNA segment from all taxa and sequencing these amplicons using high-throughput (next-generation) sequencing, thereby identifying the full complement of prey species present in the diet (Pompanon *et al.*, 2012; Kress *et al.*, 2015). The targeted DNA segment is a highly-conserved region, such as the mitochondrial COI gene, that offers enough variability to allow distinction between taxonomic groups (Thalinger *et al.*, 2016; Waap *et al.*, 2017; Cavallo *et al.*, 2018). The variability comes from accumulated mutations and SNPs (single nucleotide polymorphisms) within this conserved region that turns the segment into an identification 'barcode', essential for approaches such as DNA metabarcoding (Deagle, Kirkwood and Jarman, 2009; Soininen *et al.*, 2009; Patel *et al.*, 2010; McInnes, Jarman, *et al.*, 2017; Waap *et al.*, 2017; Cavallo *et al.*, 2018)

Diagnostic multiplex PCR analyses provide a valuable alternative to sequencing when trying to detect a defined set of prey items (Thalinger *et al.*, 2016; Oehm *et al.*, 2017). A

thorough knowledge of the potential prey species is required for the design of the primer sets for this staged PCR approach. Multiplexing of taxon-specific primers allows several prey taxa to be identified within one reaction based on differences in amplicon size (Thalinger *et al.*, 2016). Limitations of multiplex PCR include the inability to detect unexpected species present in the diet, potentially missing significant taxa (Thalinger *et al.*, 2016; Oehm *et al.*, 2017; Cavallo *et al.*, 2018). This method works best when investigating trophic interactions in environments with a limited and predictable number of prey species (Thalinger *et al.*, 2016), which is not usually the case for multi-taxa predators such as seabirds (Bond and Jones, 2009).

Applying next-generation sequencing to trophic analyses is incredibly efficient in situations with a high number of potential prey items (Symondson and Harwood, 2014) but can become time-consuming and expensive when dealing with high sample numbers (Thalinger *et al.*, 2016). Sequencing methods rely on extensive banks of DNA barcodes (i.e. GenBank, NCBI) to identify species from the sequenced fragments of DNA (Cowart *et al.*, 2015; Srivathsan *et al.*, 2016). This can be greatly limited when studying ecosystems that are poorly sequenced (Symondson and Harwood, 2014). However, as the constant development of sequencing technology has greatly reduced costs, ecologists have been quick to exploit this and turn it into a powerful new tool for dietary analyses (Pompanon *et al.*, 2012). As a result of these advancements, there has been a significant expansion of sequence databases and it is now possible to characterise the entire diet of a species (Pompanon *et al.*, 2012; Symondson and Harwood, 2014; Cavallo *et al.*, 2018). The benefits of DNA metabarcoding include providing a high level of taxonomic resolution, even when prey remains are not physically identifiable (Vestheim and Jarman, 2008; McInnes, Jarman, *et al.*, 2017; Waap *et al.*, 2017; Cavallo *et al.*, 2018). Although this method cannot be used to determine prey size or

abundance in the diet, it does give an indication of species occurrence (McInnes, Jarman, *et al.*, 2017).

Table 1.1: Summary of the benefits and limitations for the various methods of dietary analysis discussed above.

Method	**Cost ($)**	**Benefits**	**Limitations**
Traditional	Low	Affordable Visual evidence Potentially species specific Provides idea of relative abundance	Temporally limited 'Snapshot' Over-represented by hard parts Under-represented by gelatinous sp. Invasive (stomach lavage)
Stable Isotope	Moderate	Wide temporal spectrum Non-destructive/minimally invasive Informs about trophic levels (N) and foraging distribution (C) Assimilated, not just ingested Long-term feeding behaviours	Rarely informs species composition Requires in depth knowledge of element distribution and tissue turnover within body Complications with lipid content
Multiplex PCR	Moderate - High	More affordable at high sample # Allows species identification where no hard parts survive digestion Gives an indication of species occurrence	Temporally limited Only identifies presence/absence of set species, not all prey Cannot identify prey size or abundance in diet
DNA Metabarcoding	Potentially Expensive	Identifies full complement of prey species Allows species identification where no hard parts survive digestion Gives an indication of species occurrence	Expensive with high sample numbers (although cost has reduced with technological advancement) Temporally limited Cannot identify prey size or abundance in diet

Due to the considerable variability in the methods with which dietary information can be collected and analysed, it is important to consider the central aims of the study when choosing among them (Barrett *et al.*, 2007). If trophic fluctuations over varying time periods

is of interest, biochemical approaches such as stable isotope analysis are well suited. If elucidating the full complement of prey taxa present in the diet is the focus, modern molecular methods such as DNA-Metabarcoding are valuable approaches. For studies interested in broadly characterizing the feeding ecology of their species, combining complementary techniques with different temporal and taxonomic resolutions allows dietary investigations to occur across varying time scales (Connan *et al.*, 2014).

1.3 With ingestion comes threat of contamination and pollution

1.3.1 Marine pollution

Sources of marine pollution are both terrestrial, via rivers and the wind, and aquatic, through marine dredging, mining, dumping and shipping (Todd, Ong and Chou, 2010). Rivers have deposited clays and silts in the oceans for millennia, however poor management of agricultural land, mining activities, deforestation and inland construction can greatly increase these sediment loads (Fabricius, 2005). Excess sediment in the oceans reduces light availability for photosynthetic organisms at the base of food webs (Todd, Ong and Chou, 2010). It also decreases the visual acuity of prey and predators (Weiffen *et al.*, 2006) and smothers benthic organisms (Rogers, 1983). Anthropogenic activity results in a large amount of extraneous material entering the marine ecosystem, affecting all components of the marine community from microorganisms to top animal predators (Nogales *et al.*, 2011).

Entrance of this excessive pollution into the marine environment results in a myriad of ecological disturbances, including eutrophication, heavy metal toxicity and plastic-related complications. Eutrophication of marine environments can occur due to excess nitrogen and phosphorus from untreated human and animal waste, fertiliser runoff, or industrial discharge (Ayoub, 1999; Gerber *et al.*, 2005). This influx of nutrients can increase primary production,

leading to phytoplankton blooms, hypoxia, and reduced growth rates or mortality for fish, echinoderms, crustaceans and molluscs (Breitburg, 2002; Gray, Wu and Ying, 2002). An extreme example of this is the 17,000 km^2 (and rising) area of oxygen-depleted "dead zone" caused by eutrophication in the Gulf of Mexico (Diaz and Rosenberg, 2008; Elser and Bennett, 2011). Heavy metals, such as mercury (Hg), are introduced to the environment through anthropogenic activities, including mining, vehicle emissions, leaching from landfills and manufacturing (Shazili *et al.*, 2006). Mercury pollution can inhibit recruitment, fertilisation, and development in marine invertebrates (Todd, Ong and Chou, 2010) and is a neurotoxin to vertebrates (Ceccatelli, Daré and Moors, 2010). Globally, millions of tonnes of marine litter (i.e. plastics, debris, garbage, and lost/abandoned fishing gear) enter and accumulate in the world's oceans (UNEP 2005). Microplastics (< 5 mm) are ingested by marine organisms, leading to internal blockages, toxic poisoning, and starvation due to false 'stomach-filling' (Wright, Thompson and Galloway, 2013). Macroplastics (> 5 mm) originate from discarded end-user products (i.e. plastic bottles, bags, and packaging) and abandoned fishing gear (Azzarello and Van Vleet, 1987). These macroplastics lead to entanglement, suffocation, drowning, and starvation (Derraik, 2002).

Seabirds are upper-trophic level predators in marine ecosystems and exhibit a diverse range of food preferences and foraging strategies (Lieske *et al.*, 2019). As they occur in many of the same areas utilized by humans, they are exposed to a myriad of direct and indirect threats (Lieske *et al.*, 2019). As mercury bioaccumulates within marine organisms and bioamplifies up the food web, top predators such as seabirds are exposed to the highest mercury levels within a particular ecosystem (Bryan, 1979; Monteiro and Furness, 1995). Marine debris is incredibly widespread in marine ecosystems, and due to its buoyancy and durability, it is consumed by a range of seabird species (Derraik, 2002; Cole *et al.*, 2013; Eriksen *et al.*, 2014; Lavers, Hutton and Bond, 2018). Wilcox *et al.* (2015) found that 59% of

seabird species ingest plastics from the environment. Commercial fishing equipment also poses a serious threat to seabirds, for example pursuit-diving species are particularly vulnerable to entanglement in submerged gill nets (Piatt and Nettleship, 1987; Davoren, 2007) and wide-ranging species such as albatrosses and petrels are most vulnerable to longline gear (Anderson *et al.*, 2011).

1.3.2 Mercury

The concentration of mercury in the biosphere has increased substantially as a result of anthropogenic activity since the industrial revolution (Fitzgerald *et al.*, 1998; Driscoll *et al.*, 2013; Outridge *et al.*, 2018). Anthropogenic emissions of gaseous mercury into the atmosphere reach even the most remote regions on Earth (Cherel *et al.*, 2018), resulting in mercury being a global scale pollution threat. As oceans play a major role in mercury cycling (Mason and Fitzgerald, 1993), it is important to understand how marine species respond to fluctuating levels of this potentially toxic contaminant. As top-level predators, seabirds are prominent biomonitors of the spatial and temporal patterns of mercury contamination in marine ecosystems (Monteiro and Furness, 1995).

High levels of mercury can impact the health of seabirds at both an individual and population level. For individual seabirds, organic methylmercury (MeHg) has a harmful effect on embryo development, as there has been shown to be maternal transfer of mercury to the egg (Kenow *et al.*, 2011). Methylmercury is classified as a neurotoxin to vertebrates, as high levels of exposure results in damage to the neural cells (Ceccatelli, Daré and Moors, 2010). Additional impacts of methylmercury in vertebrates include impaired physiological function (e.g., altering blood and organ biochemistry; Hoffman, Spalding and Frederick, 2005), endocrine disruption (Heath and Frederick, 2005; Tan, Meiller and Mahaffey, 2009), and altered reproductive behaviour (Frederick and Jayasena, 2011). From a population

perspective, chronic exposure to mercury can compromise survival and long-term fecundity, therefore contributing to population decline (Goutte *et al.*, 2014). Long-term exposure to environmentally relevant methylmercury levels has been shown to increase male-male pairing behaviour to 55% and decrease egg production to 30% in white ibises (*Eudocimus albus*; Frederick and Jayasena, 2011). This study also showed fledgling production in ibises exposed to methylmercury to have decreased by 35%. A study on wandering albatrosses (*Diomedea exulans*) has shown that high mercury levels increases the probability of non-breeding females (MeHg disrupts reproductive hormones; Tartu *et al.*, 2013) and decreases the probability of eggs hatching (Goutte *et al.*, 2014). Effects such as these indicate that mercury has the potential to significantly impact the health of individuals and population growth for seabird species.

1.4 Whenua Hou diving petrels

A species that would benefit from having its feeding ecology investigated is the newly described Whenua Hou diving petrel (*Pelecanoides whenuahouensis*; hereafter WHDP; (Fischer *et al.*, 2018). This species is a small burrow-breeding seabird considered nationally critical by the New Zealand Department of Conservation due to its low remaining population size (approx. 200 individuals) and extremely restricted breeding range (a single colony occupying 0.018 km^2 on Whenua Hou; Fischer *et al.*, 2017, 2018, 2020; Robertson *et al.*, 2017). The WHDPs share their breeding ground with a population of common diving petrels (*Pelecanoides urinatrix*, hereafter CDP; Fischer *et al.*, 2017). While predation from invasive mammals was the likely cause for historic declines, the reasons for a lack in population recovery of WHDP on pest free Whenua Hou remain unknown (Fischer *et al.*, 2017). Dietary characterisation will have important implications for the conservation of this threatened

species (Margalida, Bertran and Heredia, 2009) and will allow an evaluation of potential interspecific competition over shared resources with the CDP population. This will be achieved through combining complementary analytical methods, with different temporal and taxonomic resolutions, to allow dietary investigation to over varying time scales (Connan *et al.*, 2014). These methods include stable isotope analysis of blood and feather samples and modern DNA analysis of fecal samples. This combination will allow the full spectrum of prey species present in the WHDP and CDP diets to be detected as well as any fluctuations in trophic position between the breeding season and the non-breeding season (Bond and Jones, 2009; Carravieri *et al.*, 2014; Thalinger *et al.*, 2016; Oehm *et al.*, 2017). It is also important to investigate the exposure to mercury pollution for the threatened WHDP population given the potential impact on individual health and population growth (Ceccatelli, Daré and Moors, 2010; Goutte *et al.*, 2014).

1.4.1 Thesis objectives

My overall thesis objectives are to characterise the feeding ecology of the WHDP and elucidate any interspecific competition that may be occurring with the sympatric CDPs. I also aim to investigate the level of mercury contamination to which the WHDPs are exposed in an effort to explain the lack of population recovery after the pest eradication on Whenua Hou. My thesis consists of two data chapters and a method development chapter. In chapter 2, I use stable isotope analysis of blood and feather samples from WHDPs and CDPs to characterise the feeding ecology of the WHDP and any variation within the population, between seasons, and among years (2017-2019). I also aim to evaluate the level of interspecific competition between WHDPs and CDPs via isotopic niche overlap. In chapter 3, I develop a staged multiplex-PCR assay to identify prey taxa present in the faecal samples of WHDPs and CDPs to further evaluate whether they are targeting the same prey. In chapter 4,

I analyse the levels of Hg in blood and feather samples from WHDPs and CDPs to better understand a potential threat to WHDP population recovery. This is important due to the high incidence of WHDP egg infertility (Fischer J.H., unpublished data, Fischer *et al.*, 2017) and the slow population growth observed. Combined, these chapters will contribute our understanding of the WHDP and help design the conservation management plan for this species.

1.4.2 Study system

Whenua Hou is a 14 km^2 predator free island situated three kilometers north-west of Rakiura (Stewart Island, -46º S 167º E, Figure 1.1). There is one sandy beach on Whenua Hou, Sealers Bay, located on the north-eastern side of the island. This beach has an associated sand dune of 0.018 km^2 which is the only breeding ground for the sole remaining population of the nationally critical WHDP. There were once WHDP populations along the length of New Zealand, however the introduction of invasive mammalian pests resulted in the extirpation of all populations except the surviving ~200 individuals remaining on Whenua Hou (Taylor, 2000; Fischer *et al.*, 2018, 2020). There is also a breeding population of CDPs on Whenua Hou, with approximately 25 CDP burrows located in the same sand dunes (Fischer *et al.*, 2020). A subset of the WHDPs and CDPs breeding in the dune were sampled for this research. The breeding season for these diving petrels runs from September to January, and the blood and feather samples were taken during November of 2017, 2018 and 2019 coinciding with prospecting and incubation behaviours (Fischer *et al.*, 2020). Faecal samples were collected opportunistically throughout the breeding season. Johannes Fischer collected the 2017 samples and we collected the 2018 and 2019 samples together.

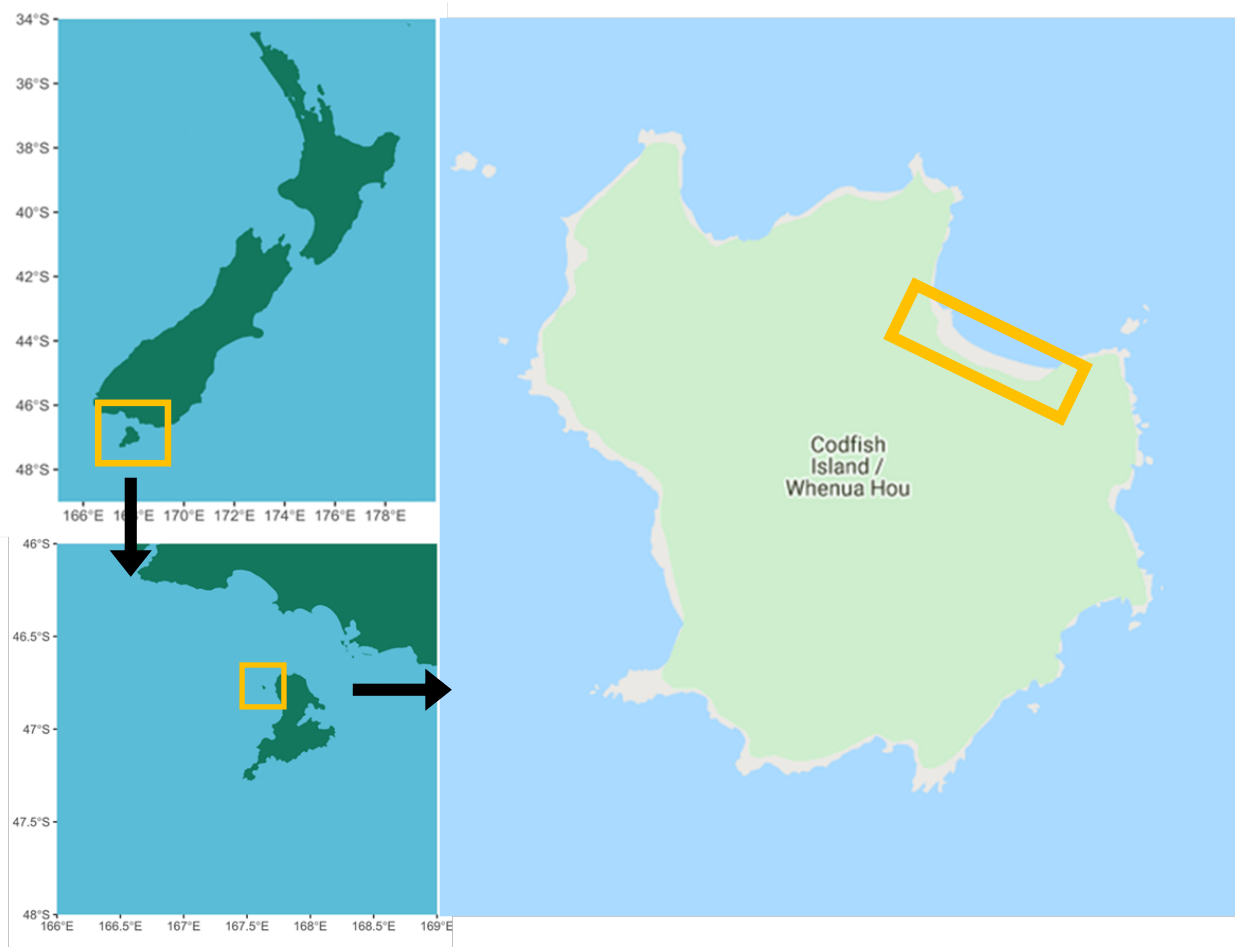

Figure 1.1: Map of New Zealand showing the location of Whenua Hou and the study site on Sealers Bay (Image of Whenua Hou obtained from Google Maps (Google, n.d.)).

1.4.3 Ethical statement

Fieldwork was designed to minimise invasiveness and handling time. All sampling procedures were in accordance with the Department of Conservation's standard operating procedure and guidelines for sampling avian blood and feathers. These sampling protocols have been shown to have no significant effect on individual health or survival (Taylor, 2010). All work was approved by the Animal Ethics Committee of Victoria University of Wellington (VUW AEC 23283, 27621) and carried out under permits of the Department of Conservation (entry permit M1718/01, M1819/01, M1920/02), the local Ngai Tahu iwi, and the Whenua Hou Komiti.

CHAPTER 2

Foraging ecology of the endangered Whenua Hou diving petrel: evaluating competition with abundant common diving petrels

2.1 Abstract

Understanding the foraging ecology of an endangered species is key to informing conservation management plans. Stable isotope analyses of blood and feather samples collected over three consecutive years (2017-2019) were used to infer the trophic dynamics of the Nationally Critical Whenua Hou diving petrel (*Pelecanoides whenuahouensis*; hereafter WHDP). Linear mixed-effect models were used to understand how various factors contribute to the variation seen in WHDP isotopic niche. Interspecific competition with the sympatric common diving petrels (*Pelecanoides urinatrix*; CDP) was assessed using kernel utilisation density estimates to calculate overlap in their isotopic niches. The isotopic niches of WHDPs were shown to be influenced by year and sex, with WHDPs foraging at a higher trophic level in the breeding season of 2018 compared to 2017 and 2019, and male WHDPs foraging at higher trophic levels than females. WHDPs were shown to forage an entire trophic level higher in the breeding season compared to the non-breeding season. Evaluating isotopic niche overlap between WHDPs and CDPs during the breeding season showed there to be a degree of niche segregation between the two species allowing them to coexist in the sand dunes on Whenua Hou. This research contributes to our understanding of the foraging ecology of the WHDP and highlights several areas of conservation concern for future investigation.

2.2 Introduction

The theory of ecological segregation hypothesises that coexisting species may partition their use of resources, in either temporal, spatial, or trophic dimensions, to eliminate or reduce competition (Hutchinson, 1959). This results in niche divergence and ultimately niche segregation. Niche segregation is crucial for community stability, particularly in densely populated communities of central place foragers sharing a breeding ground (Croxall and Prince, 1980). Seabird communities are a clear example of resource partitioning allowing ecologically similar species to breed in sympatry, often on predator free islands (Grémillet *et al.*, 2004; Cherel *et al.*, 2008). These species coexist either by foraging at different times of the day, foraging in different parts of the surrounding ocean or different depths in the water column, or by targeting different prey species (Phalan *et al.*, 2007; Masello *et al.*, 2010). When one of the species is rare and understudied, it is important to characterise its feeding ecology and to investigate the degree of trophic overlap with surrounding species that could indicate competition over shared resources (Ravache *et al.*, 2020).

Stable isotope analysis has greatly improved our understanding of the dietary needs and preferences of different seabirds (Peterson and Fry, 1987). This method is based on the assumption that the stable isotope ratios of a tissue, particularly those of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$), are reflective of the diet at the time of tissue synthesis (Bearhop *et al.*, 2002). As different tissues turnover at different rates, temporal variations in a species diet can be detected by sampling multiple tissue types (Bearhop *et al.*, 2002). Carbon isotope ratios reflect foraging location as carbon isoscapes exist throughout the environment (Kelly, 2000; Quillfeldt, McGill and Furness, 2005). In marine habitats, there is an enrichment of the heavier ^{13}C isotope in inshore food webs compared to those further offshore, and there is an enrichment of ^{13}C closer to the equator compared to lower latitudes (Peterson and Fry, 1987;

Cherel and Hobson, 2007). Nitrogen isotope ratios increase predictably with diet, as there is a rise in $\delta^{15}N$ of ~3 – 5 $^{0}/_{00}$ with each trophic level (Minagawa and Wada, 1984; Post, 2002). Several tissues can be sampled non-destructively from seabirds at their breeding grounds that can provide different temporal insights into their diet (Bearhop *et al.*, 2002). For example, whole blood samples are representative of the diet over the previous ~15 days (i.e. during the breeding season; Hobson and Clark, 1992), whereas feathers are inert once formed so their isotopic composition reflects diet at the time of feather growth (typically feathers are moulted and regrown during the non-breeding season; Nisbet *et al.*, 2002; Cherel *et al.*, 2006; Gladbach, McGill and Quillfeldt, 2007). Therefore, insights can be gained into any temporal and, if migratory, spatial variation in seabird diet between breeding and non-breeding seasons (Hobson and Clark, 1992).

As variation exists between individuals within a population, it is also important to know how various factors contribute to the variation seen in the foraging ecology of a species. The foraging ecology of an individual within a population can be influenced by factors including sex, age, body size, weight, breeding state, and body condition (Hobson, Alisauskas and Clark, 1993; Williams *et al.*, 2007; Harding *et al.*, 2008). It is important to identify the sources of variation within a population to characterise the inter-individual trophic dynamics. However, not all variation stems from within a population; many external drivers can also cause variation in feeding ecology. For example, oceans are under the influence of the El Niño/Southern Oscillation (ENSO; Yasunari, 1987), resulting in fluctuations of food availability, as well as being strongly influenced by climate change. Competitive pressure from sympatric species can also influence the trophic niche of a species. Seabird communities are often comprised of dozens of species, densely populated, foraging in the areas surrounding their breeding colony (Navarro *et al.*, 2013). If these species are not

segregating their foraging habits temporally, spatially or trophically, it can result in more dominant species outcompeting and extirpating others (Ravache *et al.*, 2020).

The sand dunes on Whenua Hou (Codfish Island, New Zealand) are the sole breeding site for the newly described, nationally critical Whenua Hou diving petrel (*Pelecanoides whenuahouensis*; hereafter WHDP; Fischer *et al.*, 2018). The WHDP was previously considered conspecific to the South Georgian diving petrel (*Pelecanoides georgicus*; hereafter SGDP; Fischer *et al.*, 2018). WHDPs typically forage within ~220 km of Whenua Hou during their breeding season (September to January) and migrate to the Southern Ocean between Australia and Antarctica (40-60°S and 110-150°E) during the non-breeding season (NBS; Fischer J.H. unpublished data). The specifics of the WHDP diet are unknown, however, it is assumed that they feed on a similar diet as SGDPs, primarily on euphausiids and copepods (Bocher, Cherel and Hobson, 2000). It is important to characterise the foraging ecology of the WHDP and the trophic variation that exists within the population. It is also key to understand how the foraging ecology of a small population (only ~200 individuals; Fischer *et al.*, 2020) responds to environmental change, both seasonally and interannually. The WHDPs share their breeding ground with a population of common diving petrels (*Pelecanoides urinatrix*, hereafter CDP; Fischer *et al.*, 2018). The CDP is a very abundant species with a circumpolar range in the southern hemisphere, breeding in several archipelagos of the Southern Ocean (Warham, 1990). In New Zealand alone, there are an estimated one million breeding pairs (Rayner *et al.*, 2017). Previous studies have shown dietary segregation between sympatric populations of CDP and SGDP (Croxall, Prince and Reid, 1997; Bocher, Cherel and Hobson, 2000), however, no studies have investigated the foraging ecology and potential interspecific competition of the diving petrels on Whenua Hou.

In this study I aimed to characterise the trophic dynamics of the WHDP, modelling the factors contributing to the intraspecific variation. I achieved this by utilising stable isotope analysis of blood and feather samples taken over three consecutive years (2017-2019) to quantify the trophic niche occupied by WHDPs. I constructed linear mixed-effect models using biologically relevant variables to explain the variation seen in isotopic ratios from different individuals. I then used the combination of blood and feather isotope ratios to investigate dietary shifts between breeding and non-breeding seasons as well as the results over three years to assess any inter-annual variation in WHDP feeding ecology. Using the same tissues collected from CDPs, I investigated the overlap of isotopic niches between WHDPs and CDPs as a proxy for interspecific competition.

2.3 Methods

A description of the study system can be found in section 1.4.2, along with the ethical approval for this research (section 1.4.3).

2.3.1 Sampling protocol

Whenua Hou diving petrels are known to come and go from their burrows just after dusk when they have the cover of darkness to evade predators (Fischer *et al.*, 2018). Therefore, sampling began in the last few minutes of daylight to have the greatest chance at catching birds when they are most active. Specifically designed burrow traps (Fischer J.H., unpublished data) were used to catch the birds as they were entering or leaving the burrows. The sampling process would take between 5-10 minutes for each individual caught, therefore no bird was handled for an excessive period of time that could be detrimental to their health (Taylor, 2010).

The blood samples were obtained via venipuncture of the metatarsal vein. First, the leg was sterilised with a 70% isopropyl alcohol wipe before piercing the skin with a 27-gauge medical grade needle. A micro haematocrit tube was used to draw up the droplet of blood that emerged. The blood was then transferred into a 2mL sample tube containing 0.9mL of 70% ethanol. Each sampling effort aimed to collect 0.1mL of blood for analysis. After the blood sample had been taken, a cotton pad was used to apply pressure to the leg until bleeding stopped.

Feather samples were taken by fully extending each wing and using stainless steel scissors to cut a 2cm sample from the sixth primary covert (PC6) feathers. The feathers were then placed into a labelled zip lock bag. Several body feathers were also sampled for genetic sex determination with Massey University, New Zealand.

Table 2.1: Number of blood and feather samples collected from Whenua Hou diving petrels and common diving petrels over the three-year sampling period on Whenua Hou.

Species	**Sample type**	**2017**	**2018**	**2019**	**Total**
Whenua Hou diving petrel	Blood	30 (m=15, f=15)	26 (m=13, f=13)	30 (m=16, f=14)	86
	Feathers	30 (m=15, f=15)	26 (m=13, f=13)	30 (m=16, f=14)	86
Common diving petrel	Blood	19 (m=7, f=12)	30 (m=10, f=20)	30 (m=14, f=15, ?=1)	79
	Feathers	21 (m=8, f=13)	30 (m=10, f=20)	30 (m=14, f=14, ?=1)	81

The blood samples were stored in 70% ethanol in a refrigerator at 4°C until ready to be processed. Storing blood samples in ethanol has been found to have no significant effect on stable isotope ratios (Hobson, Gloutney and Gibbs, 1997; Bugoni, McGill and Furness, 2008). The feather samples were kept in individual zip-lock bags and stored at room temperature.

2.3.2 Sample preparation

To prepare blood samples for shipping and analysis, they needed to be dried down to a solid to remove the hazardous ethanol solution. This process was carried out at the Ferrier Institute in Wellington with the help of Dr. Simon Hinkley. A Genevac EZ-2 Elite Personal Evaporator was used to evaporate the ethanol from each blood sample until a stable dry weight was reached. The dehydrated blood samples were then ground into a homogenous powder and stored in Eppendorf tubes at room temperature.

The preparation of the feather samples required a cleaning step followed by cutting the feathers into tiny pieces to homogenise each sample (Cherel *et al.*, 2018). Each feather was suspended in a solution of chloroform:methanol (2:1) and placed in an ultrasonic bath for 5 minutes to remove dust and dirt. This step was repeated, using fresh chloroform:methanol, to ensure the feathers are completely free from surface contaminants. The feathers were then passed through a solution of pure methanol and left to dry in an oven at 50 ºC overnight (Cherel *et al.*, 2018). Once dry, the feathers were cut into small fragments to homogenise the sample and stored in Eppendorf tubes at room temperature before analysis.

2.3.3 Sample analysis

The 2017 and 2018 blood and feather samples were analysed with Isotrace at Otago University, New Zealand. The 2019 samples were analysed by the Littoral Environnement et Sociétés laboratory (LIENSs, Université de La Rochelle, France). To test for potential differences in the results from two laboratories, approximately 10% of the 2017/2018 samples were reanalysed at LIENSs.

2.3.3.1 Isotrace protocol, Otago University

The blood and feathers samples were weighed out into ~0.8 mg aliquots in tin capsules for analysis. The relative abundance of carbon and nitrogen isotopes was determined using a continuous flow isotope ratio mass spectrometry (CF-IRMS) system comprised of Carlo Erba NA1500 (CE Instruments, Milan) elemental analyser interfaced with a Europa Scientific '20/20 Hydra' (Europa Scientific, UK) mass spectrometer.
Results are presented in the δ notation relative to the reference standard materials Vienna PeeDee Belemnite (VPDB) for carbon and atmospheric N_2 for nitrogen. Two laboratory standards (EDTA-OAS and IAEA MP153) were analysed for every 12 unknown samples in each sequence to check analytical precision and accuracy and allow instrument drift to be corrected if required. Based on the in-lab standards, the measurement error was found to be < 0.18 ‰ for $\delta^{15}N$ and < 0.27 ‰ for $\delta^{13}C$.

2.3.3.2 LIENSs protocol

Blood and feather samples were weighed out (~0.5 mg) and packed into tin containers for stable isotope analysis. The relative abundance of carbon and nitrogen isotopes was determined with a Thermo Scientific Flash EA 1112 (Thermo Scientific, Italy) elemental analyser coupled to a Thermo Scientific Delta V Advantage (Thermo Scientific, Germany) continuous-flow mass spectrometer.
Results are presented in the δ notation relative to reference standard material VPDB and atmospheric N_2, for $\delta^{13}C$ and $\delta^{15}N$ respectively. Two laboratory standards (USGS-61 and USGS-62) were analysed for every 20 unknown samples in each sequence to check analytical precision and allow instrument drift to be corrected if required. Replicate measurements of internal laboratory standards indicated measurement errors < 0.15 ‰ for both $\delta^{13}C$ and $\delta^{15}N$ values.

Results are expressed in δ-notation using the equation:

$$\delta X = \left[\left(\frac{R_{Sample}}{R_{Standard}}\right) - 1\right] \times 1000$$

where X is the heavy isotope (^{15}N or ^{13}C), R_{Sample} is the ratio of heavy to light isotope in the sample, and $R_{Standard}$ is the ratio of heavy to light isotope in the reference standard (Peterson and Fry, 1987; MacNeil, Skomal and Fisk, 2005).

2.3.4 Data analysis

A paired t-test was run on the results from the samples repeated at both laboratories to ensure the protocols each carried out produced consistent results.

The $\delta^{13}C$ values from blood samples were retrospectively corrected for lipid content using the linear regression equations in Post *et al.* (2007), where $\Delta\delta^{13}C$ was calculated from C:N molar ratios. The different enrichment factors for blood and feather tissues were accounted for using the equations in Cherel *et al.* (2014), so that any trophic shifts between breeding and non-breeding seasons can be discerned from the analysis of the samples collected.

Linear mixed-effect models (LMMs) were constructed, using several biologically relevant variables, to analyse the factors contributing to the variation seen in the WHDP blood and feather stable isotope results. Stable isotope ratio values were z-transformed so beta values for each variable were comparable. The fixed effects in these models were sex (male or female), year (2017, 2018, or 2019), and breeding stage at time of sampling (prospecting or incubating). A random individual effect was also accounted for in each model as some individuals were sampled across multiple years. As blood isotopes represent recent foraging habits, the factor of breeding stage was applied. As feather stable isotope ratios represent foraging habits during the non-breeding season, they would be unaffected by the breeding stage of the individual at the time of sampling. For both the blood and feather stable isotope results, a 'null' model, where only random individual effects (ID) were considered, and a

'full' model combining all variables were generated. Models were run using the lme4 package in R (Bates, 2007; R Development Core Team, 2020). The Akaike Information Criterion corrected for small sample sizes (AIC_C) was used to rank the models and identify the relative importance of variables affecting the stable isotope ratios in blood and feather tissue from WHDPs (Burnham and Anderson, 2002). For each model, the AIC_C, the difference in AIC_C values relative to the best fit model (ΔAIC_C), and the weight of each model (ω) were calculated. Models with $\Delta AIC_C < 2.0$ were considered to have substantial support from the data (Burnham and Anderson, 2002).

From the model output, the resulting beta values for each variable were averaged across the models. The relative variable importance (RVI) for each factor was calculated by summing the ω values to discern which factors have the strongest effect on the variability of the stable isotope data. Factors were considered to have a strong effect on isotopic niche if the beta values plus or minus two standard errors did not intersect zero.

Interspecific competition was inferred through isotopic niche overlap between WHDPs and CDPs. This was calculated using the rKIN (kernel isotopic niches) package in R (Eckrich *et al.*, 2020), which uses kernel utilisation density (KUD) estimators to measure isotopic niche size (at 50% and 95% contours) and overlap between species. All analyses were performed using R 4.0.2 (R Development Core Team, 2020).

2.4 Results

Samples analysed at both institutions produced consistent stable isotope ratios allowing comparison of results across all three sampling years ($\delta^{13}C$: $t = -1.56$, $df = 19$, $p = 0.13$; $\delta^{15}N$: $t = 0.207$, $df = 19$, $p = 0.84$). Qqplots were used to confirm the normal distribution of the response variables (carbon and nitrogen stable isotope ratios).

2.4.1 Breeding season

The isotopic niche of the WHDP during the breeding season ranged from a $\delta^{13}C$ of -16.7 to -19.2 ‰ and a $\delta^{15}N$ of 9 to 14 ‰ (see Figure 2.1).

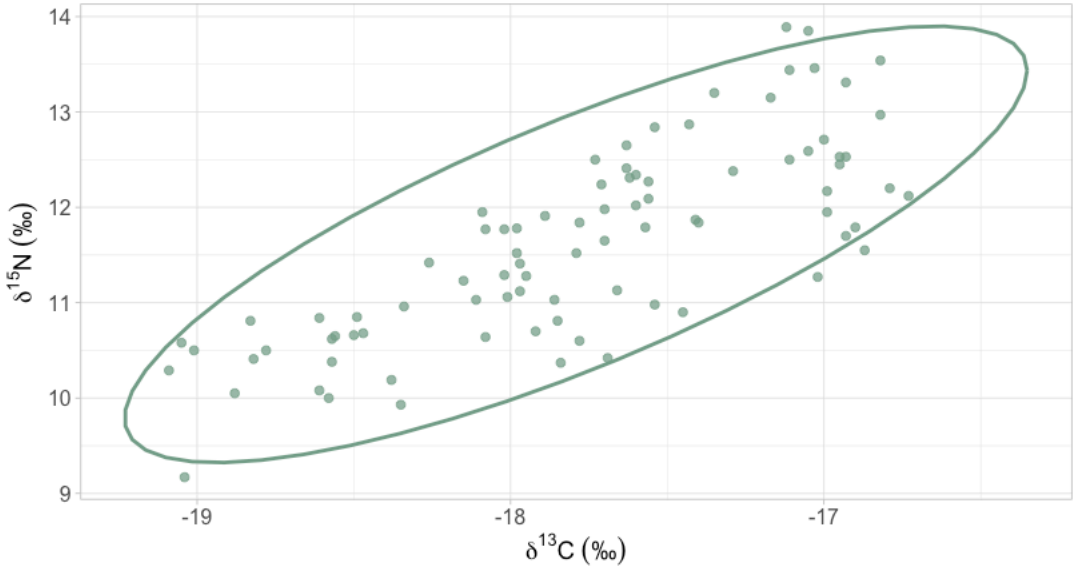

Figure 2.1: The isotopic niche of the WHDP during the breeding season, with the intraspecific variation visible in the spread of the results and an ellipse illustrating the 95% confidence interval.

The output from the LMMs showed that year and sex had a strong effect on the variation in $\delta^{13}C$ (Table 2.2). The reference factor for the effect of year was 2017, with the beta values showing the effect of samples being obtained in 2018 or 2019 relative to 2017. The results from the LMMs showed that ^{13}C was enriched during the breeding season for each consecutive year, however the increase was smaller from 2018 to 2019 (Figure 2.3). The reference state for the effect of sex was male, therefore the results showed female diet was depleted in ^{13}C compared to males. The effect of breeding stage, whether a bird was prospecting or incubating at the time blood was sampled, did not have a strong effect on blood $\delta^{13}C$.

Based on AIC, the top model combined the effects of year, sex, and ID. This model had an overall AIC weight of 0.57. The full model (year + sex + breeding stage + ID), however, also had substantial support from the data (ΔAIC = 1.27, AIC weight = 0.30)

Table 2.2: The model-averaged beta and RVI values for each fixed effect from the linear mixed-effect models attempting to describe the variation seen in the breeding season isotopic niche of WHDPs on Codfish Island, New Zealand. Results are expressed as β ± SE. (Indicates that β ± 2×SE does not intersect 0).*

	Fixed effect	$\beta_{Average}$	RVI
Breeding season $\delta^{13}C$	Year	*2018 0.78 ± 0.19 *2019 0.87 ± 0.20	1.00
	Sex	*-0.54 ± 0.21	0.87
	Breeding stage	-0.35 ± 0.32	0.36
Breeding season $\delta^{15}N$	Year	*2018 0.63 ± 0.20 2019 -0.05 ± 0.21	0.98
	Sex	*-0.49 ± 0.22	0.75
	Breeding stage	0.26 ± 0.33	0.29

Year and sex had strong effects on $\delta^{15}N$ during the breeding season (Table 2.2). The 2018 samples were enriched in ^{15}N compared to 2017, however the 2019 $\delta^{15}N$ dropped back down to the same level as 2017, so the $\delta^{15}N$ did not increase linearly with time (Figure 2.3A). WHDP females were depleted in ^{15}N compared to males. As with $\delta^{13}C$, breeding stage at time of sampling did not have a strong effect on $\delta^{15}N$.

The top model combined the effects of year, sex, and ID and had an AIC weight of 0.52. The full model also had a ΔAIC < 2.0 and was therefore supported by the data (Table S2.2).

2.4.2 Non-breeding season

The isotopic niche of the WHDP during the non-breeding season (NBS) ranged from a $\delta^{13}C$ of -20.7 to -23.7 ‰ and a $\delta^{15}N$ of 6.3 to 10 ‰ (Figure 2.2).

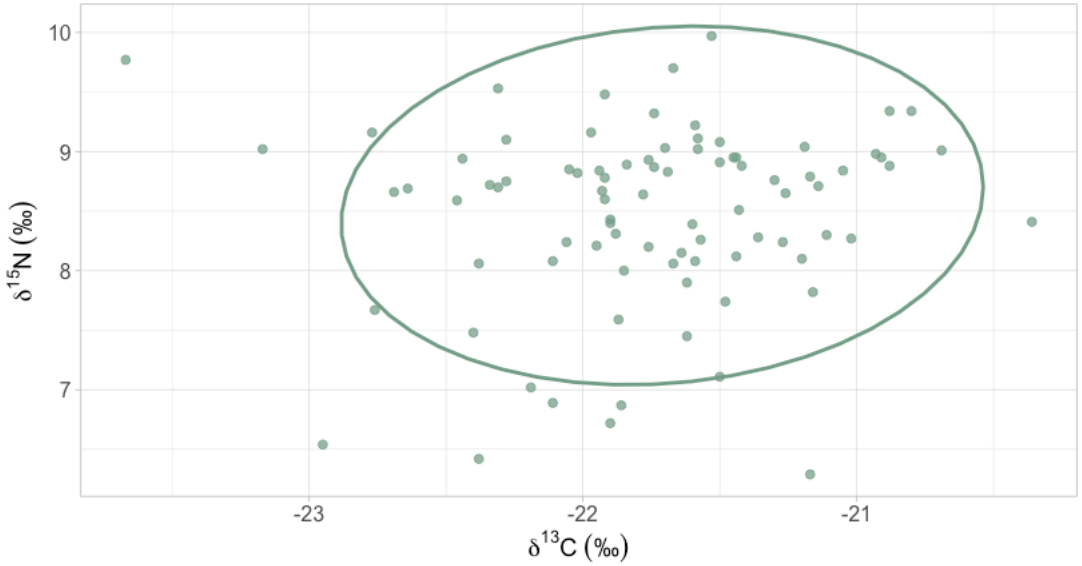

Figure 2.2: The isotopic niche of the WHDP during the non-breeding season, with the intraspecific variation visible in the spread of the results and an ellipse illustrating the 95% confidence interval.

The LMM results for modelling the factors influencing variation in NBS $\delta^{13}C$ showed that year had a strong effect (Table 2.3). The effect of year was strong between 2017 and 2019, with 2019 being enriched in ^{13}C, but was not strong for 2018 relative to 2017 (Table 2.3). There was no strong effect of sex on $\delta^{13}C$ during the NBS.

The top model combined the effects of year, sex and ID. This model had a weight of 0.46. The model for year and ID also had substantial support from the data (ΔAIC = 0.64, AIC weight = 0.33).

Table 2.3: The model-averaged beta and RVI values for each fixed effect from the linear mixed-effect models attempting to describe the variation seen in the non-breeding season isotopic niche for WHDPs on Codfish Island, New Zealand. Results are expressed as β ± SE. (Indicates that β ± 2×SE does not intersect 0).*

	Fixed effect	$\beta_{Average}$	RVI
Feather $\delta^{13}C$	Year	2018 -0.09 ± 0.22 *2019 0.50 ± 0.22	0.79
	Sex	0.37 ± 0.22	0.57
Feather $\delta^{15}N$	Year	2018 -0.08 ± 0.23 *2019 0.49 ± 0.23	0.72
	Sex	-0.43 ± 0.22	0.68

The LMM results showed that year had a strong effect on the variation in $\delta^{15}N$ during the NBS (Table 2.3). In these models, the effect of year was strong when comparing 2019 to 2017, with 2019 being enriched in ^{15}N, but was not strong between 2017 and 2018 (Figure 2.3B). There was no strong effect of sex on $\delta^{15}N$ in the NBS.

The top model combined the effects of year, sex and ID, with an AIC weight of 0.49. Two other models were also supported by the data, as they had a ΔAIC < 2.0 (Table S2.4)

2.4.3 Seasonal shift

The average isotopic niche occupied by WHDPs during the breeding season ranged from an approximate $\delta^{13}C$ of -17.5 to -18 ‰ and a $\delta^{15}N$ of 11 to 12 ‰ (Figure 2.3A). During the NBS, these values shifted to a $\delta^{13}C$ between -21.5 to -22 ‰ and a $\delta^{15}N$ between 8 and 9 ‰ (Figure 2.3B). This is a shift in $\delta^{13}C$ of ~ 4 ‰ and a shift in $\delta^{15}N$ of ~3 ‰.

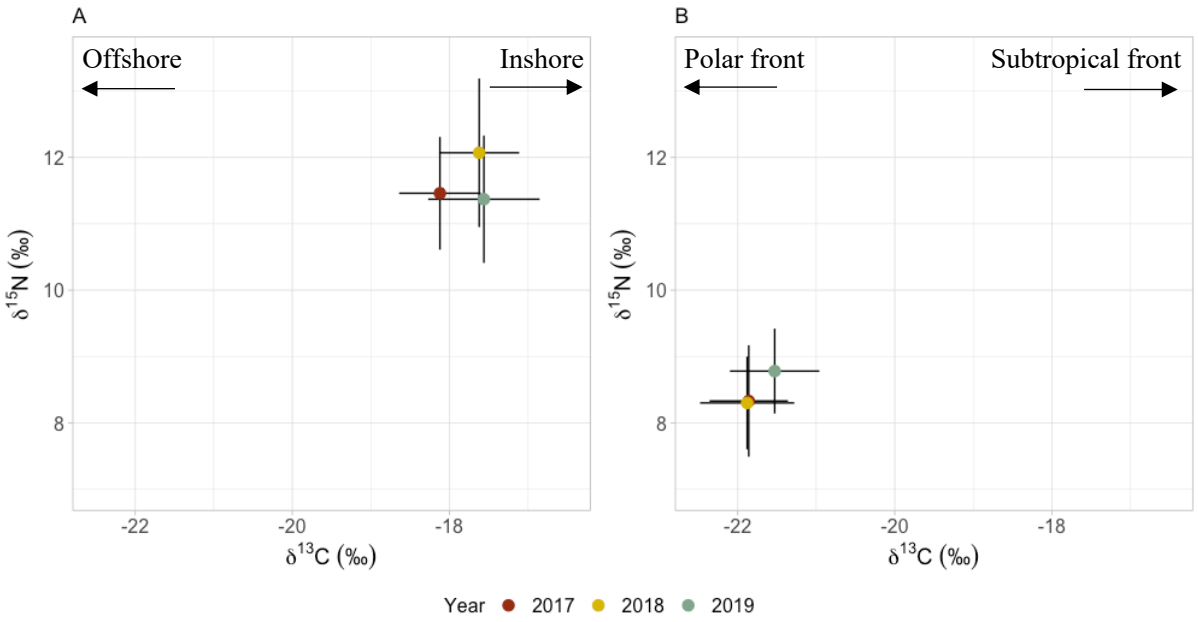

Figure 2.3: The mean ± SD of the carbon and nitrogen stable isotope ratios from WHDP blood samples over three consecutive years, showing the interannual variation in WHDP foraging behaviour during (A) the breeding season and (B) the non-breeding season.

2.4.4 Interspecific competition with CDPs

The shape and size of the isotopic niches of the WHDPs and CDPs varied between year and season (Figure 2.4). The core niche area (50% contour) of WHDPs during the breeding season was fairly consistent across all three sample years, rising slightly from a polygon area of 1.91 in 2017 to 2.42 in 2018 and 2.82 in 2019 (Table 2.4). The more conservative isotopic niche size estimate (95% contour) increased from 6.89 in 2017 to 8.42 in 2018 and 10.41 in 2019. In contrast, the CDP isotopic niche estimates appeared to be getting more compact in the breeding season, with a core niche area of 1.58 in 2017, 0.34 in 2018 and 0.60 in 2019. In the NBS, the WHDP core isotopic niche decreased from 1.66 in 2017 to 1.28 in 2018 and 1.06 in 2019. Similarly, the core CDP isotopic niche in the NBS decreased across the years, from 5.23 in 2017 to 4.35 in 2018 and 1.79 in 2019.

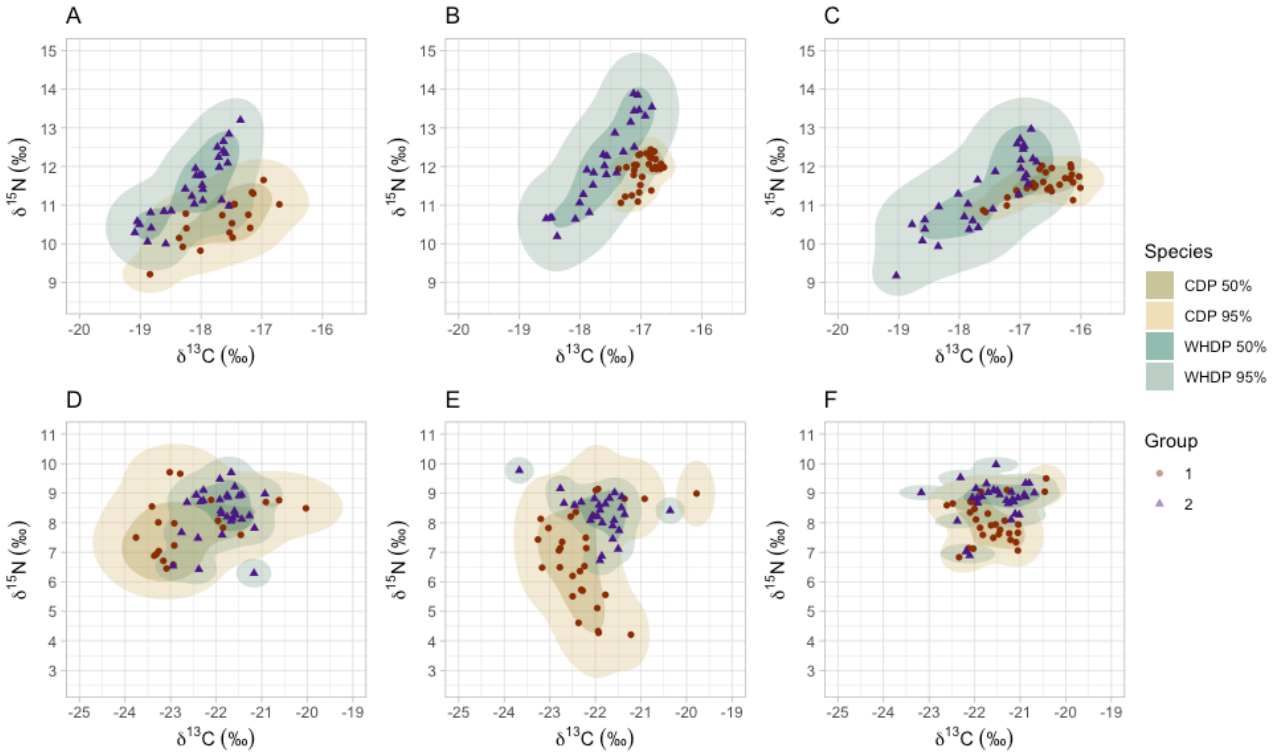

Figure 2.4: Kernel utilisation density plots for WHDP and CDP isotopic signatures for the breeding season (A) 2017, (B) 2018, (C), 2019, and non-breeding season (D) 2017, (E) 2018, (F) 2019. Both 50% and 95% contours are present to illustrate the overlap of data distribution.

Niche overlap was calculated from the perspective of the WHDP population and represents the proportion of the WHDP isotopic niche area that overlapped with the CDP isotopic niche. During the breeding season, overlap between the core isotopic niches was low, with a maximum of 8.6% of the WHDP core isotopic niche overlapping with the CDPs in 2019 (Table 2.4). When considering the 95% contours, niche overlap increased, with a maximum of 44.6% of the WHDP isotopic niche shared with CDPs in 2017. During the NBS, niche overlap was higher for both the core isotopic niche area (50% contour) and the wider 95% contour. The greatest isotopic niche overlap occurred in the 2017 NBS, with 47.6% of

the WHDP core area overlapping with that of the CDPs, and a 90.3% overlap when considering the 95% contours.

Table 2.4: The isotopic niche area of WHDPs and CDPs breeding on Whenua Hou and the proportion of the WHDP isotopic niche space that is overlapped by that of the sympatric CDPs at both 50% and 95% contours.

	Year	WHDP Area 50%	WHDP Area 95%	CDP Area 50%	CDP Area 95%	Overlap 50%	Overlap 95%
Breeding Season	2017	1.91	6.89	1.58	6.26	0.033	0.446
	2018	2.42	8.42	0.34	1.43	0.001	0.131
	2019	2.82	10.41	0.60	2.31	0.086	0.179
Non-breeding Season	2017	1.66	8.30	5.23	18.49	0.476	0.903
	2018	1.28	5.48	4.35	19.35	0.228	0.835
	2019	1.06	5.73	1.79	7.57	0.160	0.798

2.5 Discussion

2.5.1 Breeding season stable isotope ratios

During the breeding season, all seabirds are central place foragers, including WHDPs and CDPs (Masello *et al.*, 2010). Therefore, the variation in $\delta^{13}C$ between individuals is likely attributable to foraging at different distances from shore (Hobson, Piatt and Pitocchelli, 1994). Inshore marine food sources are usually enriched in ^{13}C compared to those further out to sea (Peterson and Fry, 1987; Kelly, 2000). My results show an increase in $\delta^{13}C$ each year, indicating that WHDPs may be utilising food sources closer to the breeding colony. My results also indicate that WHDP females may be foraging further from the breeding colony than males as they had a lower $\delta^{13}C$. Due to there being no strong effect of breeding stage on

$\delta^{13}C$, both prospecting and incubating WHDPs appear to forage a similar distance from the colony.

As $\delta^{15}N$ represents the relative trophic position of a species, the results indicate WHDPs foraged at a higher trophic level in 2018 compared to 2017 and 2019 (Minagawa and Wada, 1984; Kelly, 2000). As 2018 was an El Niño year (National Oceanic and Atmospheric Administration Climate Prediction Database repository; available from https://catalog.data.gov/dataset/climate-prediction-center-cpcoceanic-niño-index), this is an unusual trend because marine food webs generally experience reduced food supply during this climatic phase (Boersma, 1978). For example, other top marine predators such as Galápagos penguins (*Spheniscus mendiculus*) have experienced times of famine and dramatic population declines during strong El Niño years (Boersma *et al.*, 2013). This pattern may be explained by the WHDPs responding to the reduced food supply by feeding further up the food web and managing to avoid the intense competition for the limited food available. The enrichment of ^{15}N in male blood samples indicates that they foraged at higher trophic levels than females (Kelly, 2000). As there is no pronounced sexual dimorphism among WHDPs, there are no obvious physical differences to explain this observation (Fischer *et al.*, 2018).

2.5.2 Non-breeding season stable isotope ratios

During the NBS, WHDPs migrate to the Southern Ocean between Australia and Antarctica, foraging far from any land mass (Fischer J.H., unpublished data). Variation in $\delta^{13}C$ should therefore be interpreted as foraging at different latitudes rather than distances from shore (Quillfeldt, McGill and Furness, 2005). In 2017 and 2018, WHDPs appeared to be foraging in waters of the same latitude. In 2019, the WHDP feathers had a less negative $\delta^{13}C$ signature, potentially indicating a northern shift in NBS foraging area (Francois *et al.*, 1993; Trull and Armand, 2001). In contrast to the breeding season, there was no difference in the

isotopic niche of WHDP males and females during the NBS. This is supported by GLS tracking data showing no difference in the non-breeding ranges of male and female WHDPs (Fischer J.H., unpublished data).

The results for modelling the NBS $\delta^{15}N$ variation show that none of my variables had a particularly strong influence, and none of the models stood out from the rest as the four top models had similar Akaike weights to support them (Table S2.4; Burnham and Anderson, 2002). This suggests that other biological or environmental variables not included in this study were influencing the trophic position of WHDPs during the NBS.

2.5.3 Seasonal shift in isotopic niche

The effective $\delta^{13}C$ shift of ~ 4 ‰ between seasons is difficult to interpret directly, as WHDPs forage in completely different areas of the ocean during the breeding and non-breeding seasons. Given these ranges are on average > 3,500 km apart, this result is expected (Fischer J.H., unpublished data).

The seasonal shift in $\delta^{15}N$ of ~3 ‰ indicates that WHDPs feed on prey a whole trophic level higher during the breeding season compared to the NBS (Post, 2002). Feeding at higher trophic levels during the breeding season is expected as seabirds must balance the changing demands of self and offspring provisioning with the constraints imposed by central place foraging (Boersma, Rebstock and García-Borboroglu, 2015; Booth *et al.*, 2018). This could be explained by a shift from mainly preying on euphausiids, copepods and amphipods (i.e. lower trophic level prey; Henschke *et al.*, 2015) to including more fish larvae and cephalopods in their diet (higher trophic level prey; Hobson, Piatt and Pitocchelli, 1994; Pinkerton *et al.*, 2012). Similar patterns have been exhibited by other central-place foraging seabirds during their breeding season, such as northern rockhopper penguins (*Eudyptes moseleyi*; Booth *et al.*, 2018) and little penguins (*Eudyptula minor*, Zimmer *et al.*, 2011).

2.5.4 Interspecific competition with CDPs

During the breeding season, the core niche area (50% KUD contour) of the WHDPs only overlapped with that of the CDPs between 0.1-8.6%. This is surprising given these incredibly similar sympatric diving petrels share such a small breeding ground (Fischer *et al.*, 2018). However, it is likely that this offset in isotopic niche is a key factor allowing the WHDPs and CDPs to coexist on Whenua Hou (Navarro *et al.*, 2013). The position of the isotopic niches suggests that WHDPs foraged on prey items of slightly higher trophic value (e.g., including more fish and cephalopods in their diet) and foraged further from the breeding colony than CDPs (Figure 2.4; Kelly, 2000). The variation in niche sizes between species and over time suggests CDPs are becoming more specialised in their foraging behaviour as their niche size is decreasing, whereas WHDPs have a larger niche size that is gradually increasing (Table 2.4; Bolnick *et al.*, 2003). This indicates the WHDPs are more generalised in their foraging behaviour, suggesting they will be able to respond to changes in food availability or competitive pressure from other predators in their community (Terraube *et al.*, 2011).

There was a much greater overlap in the isotopic niches of WHDPs and CDPs during the NBS. The core niche of the WHDPs overlapped with the CDPs between 16-47.6%, and the 95% contour niche estimates overlapped between 79.8-90.3% (Table 2.4). The niche sizes of both species are decreasing over time, indicating increasingly specialised foraging behaviour (Bolnick *et al.*, 2003). As there was significant isotopic niche overlap between the WHDPs and CDPs during the NBS, there is the potential for strong competitive pressure from the more abundant CDP population on the rare WHDPs (Ravache *et al.*, 2020). However, since we do not know the non-breeding distribution of the CDP population on Whenua Hou, we cannot assume that their non-breeding grounds overlap for this competitive pressure to exist. Other CDP populations in New Zealand (Kauwahaia Island and Burgess Island; Rayner *et al.*, 2017) have been shown to migrate in the opposite direction to WHDPs during the NBS, to

the east and south towards the polar front. The population of CDPs in this study could migrate east, as the northern CDP populations have been shown to do, or they could migrate west with the sympatric WHDPs. Without information on the non-breeding distribution of the CDPs in this study, we cannot fully understand the competitive threat they may pose to the WHDPs during the NBS.

2.5.5 Consequences for species conservation

Stable isotope ratios during the breeding season suggest that females are foraging further from the breeding colony than males and potentially prey with lower trophic value (Table 2.2; Kelly, 2000). This could result in an energy deficit for females as they spend more energy on longer flights without the reward of more energy-rich prey (Dean *et al.*, 2013). A previous study investigated the potential for increased stress responses (corticosterone levels) in CDP individuals that had to travel further to meet energetic demands (Dunphy *et al.*, 2020), potentially reducing reproductive success (Kitaysky, Piatt and Wingfield, 2007).

The plasticity in observed diet, both between seasons and annually with marine oscillation patterns, suggests WHDPs may respond well to changing climatic conditions (Peers *et al.*, 2014). This indicates that climate change may not pose a direct threat to the WHDP population through changing ocean conditions and food availability (Grémillet *et al.*, 2012).

The shift in WHDP diet to a higher trophic level during the breeding season has potential positive implications for population growth. By adjusting their targeted prey to more energy-rich taxa, WHDPs are putting themselves in the best possible position to overcome the limitations set by central-place foraging and successfully provide for both themselves and their offspring (Williams and Rothery, 1990; Zimmer *et al.*, 2011; Booth *et al.*, 2018).

The competitive threat from the sympatric CDPs is low during the breeding season, as the core niche estimates for both species have a < 10% overlap (Table 2.4). This suggests niche

partitioning allows the coexistence of WHDPs and CDPs on Whenua Hou. However, there is considerable fishing activity within the breeding range of WHDPs (Fischer J.H., unpublished data). From a conservation perspective, overharvesting by fisheries can not only decrease prey availability for seabird communities, but also alter the ecological relationships among seabirds (Furness and Tasker, 2000; Votier *et al.*, 2004). Continuous or increasing pressure from fisheries could push sympatric seabirds from simply coexisting through niche partitioning to overtly competing for the remaining resources. Without distribution data for the Whenua Hou population of CDPs, their NBS migration patterns are unknown and the level of interspecific competition between CDPs and WHDPs is difficult to discern.

2.5.6 Study limitations

Stable isotope analysis is a commonly used method by seabird biologists to infer the foraging ecology of a species (Inger and Bearhop, 2008), however this method has several key limitations and biases that are not always accounted for (Barrett *et al.*, 2007). This study has accounted for these biases where possible, through retroactively correcting for the lipid content of blood using linear regression equations (Post *et al.*, 2007). Inter-tissue discrimination factors were also accounted for using equations from Cherel *et al*. (2014) to allow temporal comparison between the breeding and NBS. As with any mathematical correction, these introduced additional error to the data. The manner in which isotopes behave within a biological system is species specific and is only discernible through prolonged and controlled laboratory experiments (Hobson and Clark, 1992; Becker *et al.*, 2007). This is very difficult to maintain with seabirds, particularly endangered species, especially if researchers desire physiological responses comparable with populations in the wild (Bond and Jones, 2009).

This study maintained a high sample size over a three-year study period, however, annual trends and responses to ocean oscillations (e.g. ENSO) or climate change cannot be accurately depicted with only three consecutive years of data. With continued annual monitoring over several ENSO cycles, it would be possible to describe how WHDPs are responding to environmental fluctuation and the effects of climate change.

Finally, the stable isotope method has limitations in the accuracy of interpreting the results, particularly for $\delta^{13}C$ (Bond and Jones, 2009). I have chosen to interpret my $\delta^{13}C$ results simply as relative foraging location on a broad scale (Quillfeldt, McGill and Furness, 2005). However, ocean currents and changes in climate can cause the relocation of basal food webs, potentially resulting in a change of isotopic signature and the appearance of a spatial shift for a predator foraging in the same location. Additionally, as different combinations of prey species can result in the same isotopic signature in the consumer, it is therefore possible for two seabirds exploiting different food webs in the same location to have identical isotopic signatures (Bond and Jones, 2009).

2.5.6 Conclusions and future directions

In this study, I have characterised the variation in WHDP isotopic niche during the breeding and non-breeding periods over three consecutive years. My results demonstrated patterns of interannual variation and trophic shift between seasons. By sampling both the WHDPs and the sympatric population of CDPs, I assessed the potential for interspecific competition from the more abundant CDPs threatening the survival of the nationally critical WHDP population. My results highlighted several potential conservation concerns, for which future investigations are required to understand the potential responses of the WHDP population. Specifically, it would be beneficial to study the levels of stress hormones in breeding WHDPs to investigate whether there is a connection between corticosterone levels and rates of egg

infertility. Continuing the investigation into the trophic dynamics of the WHDPs for several more years is required to better understand the risks associated with a changing climate. It would be beneficial to use GLS tracking technology to follow the breeding and non-breeding distribution of the CDPs on Whenua Hou to further assess the threat of interspecific competition through niche overlap. Finally, as stable isotope analysis does not provide a fine scale resolution of the diet of a species, it would thus be beneficial to employ complementary methods to describe the key prey species in the WHDP diet. By understanding the specific prey combination targeted by WHDPs and CDPs, their true dietary overlap can be revealed.

CHAPTER 3

Developing a staged multiplex-PCR assay specific to seabird dietary analyses

3.1 Abstract

Understanding the key prey taxa in the diet of a threatened species is crucial to inform conservation management programmes. Seabirds are cryptic foragers and many methods have been developed to improve our understanding of their target prey species, from investigating remains in stomach contents to modern genetic analytical methods. The Whenua Hou diving petrel (*Pelecanoides whenuahouensis*, WHDP) is considered 'Nationally Critical' and understanding the key prey species in their diet will be essential for their conservation. In this chapter, I have designed a novel multiplex-PCR assay to detect various prey taxa present in the diets of WHDPs and the sympatric population of common diving petrels (*Pelecanoides urinatrix*, CDP). This assay started with a broad taxonomic resolution, aiming to identify the presence or absence of amphipods, decapods, euphausiids, maxillopods, cephalopods and fish in faecal samples collected from WHDPs and CDPs between 2017-2019. I had some success in designing primers to identify the presence of cephalopods and fish in dietary samples. However, with the myriad of obstacles in the design and development process, it seems that, with additional resources, a bulk sequencing approach such as DNA metabarcoding would be a more suitable method to detect the prey present in the diets of WHDPs and CDPs.

3.2 Introduction

Knowledge on the diet of a species is fundamental to understanding its ecological relationships and has important implications for the design and implementation of conservation programmes (Oli, Taylor and Rogers, 1993; Jones, Moss and Sanders, 2005; Margalida, Bertran and Heredia, 2009; Klare, Kamler and MacDonald, 2011). Insight into dietary preferences can be beneficial to the conservation of threatened species through hand-rearing captive or translocated individuals, and can improve breeding performance through supplementary feeding (Margalida, Bertran and Heredia, 2009).

Over time, methods investigating feeding ecology have developed from traditional observation to using modern molecular techniques to analyse dietary samples. Traditionally, diet has been analysed through direct observation of feeding events or using morphological analysis of the stomach contents from deceased individuals to identify prey species (Corbett, 1989; Ciucci *et al.*, 1996; Gonzales-Solis et al. 1997, Burns *et al.*, 1998; Symondson, 2002). This becomes much more difficult when investigating smaller predators feeding in cryptic environments, such as migratory marine predators (Pierce and Boyle, 1991; Symondson, 2002; Braley *et al.*, 2010; Klare, Kamler and MacDonald, 2011; Zeale *et al.*, 2011). Seabirds are generally only accessible for sampling when they are at their breeding colony, and their foraging ecology is largely unknown during their non-breeding season. Diet samples are obtained opportunistically, through spontaneous regurgitation or faecal samples, or invasively through stomach lavage techniques. However, morphological analyses of these samples are biased towards prey with hard parts capable of withstanding digestion, whereas gelatinous species are often under-represented (Votier *et al.*, 2003; Braley *et al.*, 2010; Oehm *et al.*, 2017; Cavallo *et al.*, 2018). This method is also temporally limited, as samples can only be obtained during the breeding period.

The biochemical method of stable isotope analysis can provide more temporal insight into foraging ecology. The stable isotope ratios of different tissues reflect the diet at the time of synthesis, and as tissues have different turnover rates, they can provide insight into diet with a wider temporal scope (Thompson *et al.*, 1998; Quillfeldt, McGill and Furness, 2005; Carravieri *et al.*, 2014, Bond and Jones, 2009; Bond, 2010; Polito *et al.*, 2011). From carbon and nitrogen stable isotope ratios, we can infer the trophic position and general foraging areas of a species (Quillfeldt, McGill and Furness, 2005), however we cannot detect specific prey species present in the diet (Bond and Jones, 2009; McInnes, Jarman, *et al.*, 2017).

Modern molecular methods have been developed over the last 20 years to improve accuracy and provide and unprecedented resolution of dietary analyses (Pompanon *et al.*, 2012; Traugott *et al.*, 2013; Thalinger *et al.*, 2016; Oehm *et al.*, 2017; Cavallo *et al.*, 2018). Recent advancements show the development of two main strands within DNA-based dietary analysis (Pompanon *et al.*, 2012; Traugott *et al.*, 2013). One approach, DNA metabarcoding, combines universal primers with high-throughput (next-generation) sequencing identify the full complement of prey species present in the diet (Kress *et al.*, 2015). The segment targeted by the universal primers is a highly conserved region of DNA that contains enough variability to distinguish between taxonomic groups, such as the mitochondrial cytochrome oxidase I (COI) gene (Thalinger *et al.*, 2016; Waap *et al.*, 2017; Cavallo *et al.*, 2018). The process of sequencing the DNA segments can be very expensive depending on the number of samples to be analysed and the amount of 'noise' created by bacterial or host sequences in the sample (Pompanon *et al.*, 2012). It is also limited by the taxonomic coverage of the reference sequence databases (i.e. GenBank, NCBI) which are required to positively identify species from the sequences obtained from the dietary sample (Cowart *et al.*, 2015; Srivathsan *et al.*, 2016).

The alternative approach combines specific primer sets in one polymerase chain reaction (PCR) to identify the presence or absence of predetermined prey taxa in a diet sample (multiplex-PCRs), providing a more-affordable alternative to next-generation sequencing (Pompanon *et al.*, 2012; Thalinger *et al.*, 2016; Oehm *et al.*, 2017). A thorough knowledge of the candidate prey species is required to design the primer sets for this PCR approach. Multiplexing of taxon-specific primers allows several prey taxa to be identified within one reaction based on differences in amplicon size (Sint *et al.*, 2014; Harper *et al.*, 2015; Thalinger *et al.*, 2016). This method has been utilised to characterise the diet of piscivorous kingfishers (*Alcedo atthis*) cormorants (*Phalacrocorax carbo*; Thalinger *et al.*, 2016), sardine larvae (*Sardina pilchardus*; Yebra *et al.*, 2019), and generalist arthropod predators such as the greater horseshoe bat (*Rhinolophus ferrumequinum*; Sint *et al.*, 2014). Limitations of multiplex PCR include the inability to detect unexpected species present in the diet, potentially ignoring significant taxa (Thalinger *et al.*, 2016; Oehm *et al.*, 2017; Cavallo *et al.*, 2018). This method works best when investigating trophic interactions in systems with a limited and predictable number of prey species (Thalinger *et al.*, 2016), which is not usually the case for multi-taxa predators such as seabirds (Bond and Jones, 2009). For systems where the number of candidate prey species exceeds the number of targets possible in a single multiplex-PCR, it is practical to design a staged experiment starting at a higher taxonomic level (e.g. order level) and gain taxonomic resolution with subsequent nested multiplex reactions (Thalinger *et al.*, 2016).

The sand dunes on Whenua Hou (Codfish Island, New Zealand) are the sole breeding site for the nationally critical Whenua Hou diving petrel (*Pelecanoides whenuahouensis*; hereafter WHDP; Fischer *et al.*, 2018). The WHDP was previously considered conspecific to the South Georgian diving petrel (*Pelecanoides georgicus*; hereafter SGDP; Fischer *et al.*, 2018). WHDPs forage within 221 km of Whenua Hou during their breeding season

(September to January) and migrate to the Southern Ocean between Australia and Antarctica (40-60 °S and 110-150 °E) during the non-breeding season (NBS; Fischer J.H., unpublished data). The specifics of the WHDP diet are unknown, however it is assumed that they feed on a similar diet as SGDPs, primarily euphausiids and copepods (Bocher, Cherel and Hobson, 2000). The WHDPs share their breeding grounds with a population of common diving petrels (*Pelecanoides urinatrix*, hereafter CDP; Fischer *et al.*, 2017). The CDP is a very abundant species with a circumpolar range in the southern hemisphere, breeding in several archipelagos of the Southern Ocean (Warham, 1990). Previous studies have shown dietary segregation between sympatric populations of CDPs and SGDPs in other archipelagos (Croxall, Prince and Reid, 1997; Bocher, Cherel and Hobson, 2000), however no studies have used modern molecular methods to characterise the prey species present in the diet of the diving petrels on Whenua Hou.

In this study I aimed to design a staged multiplex-PCR assay to detect the presence or absence of candidate prey species in the diets of WHDPs and CDPs on Whenua Hou. I collected faecal samples from WHDPs and CDPs over three consecutive breeding seasons (2017-2019) from which to extract DNA for dietary characterisation. I began designing the assay at a broad scale of taxonomic resolution to detect the presence of copepods, euphausiids, amphipods, decapods, cephalopods and fish in diving petrel dietary samples from a single PCR. The results from this first broad PCR assay will inform the design of subsequent stages at increasing taxonomic resolution. This method can be adapted to be suitable for the dietary investigation of other seabird species as fish, cephalopods and crustaceans are the key prey taxa for many seabird species (Thalinger *et al.*, 2016; Seabird Diet Database, 2018).

3.3 Methods

A description of the study system can be found in section 1.4.2, along with the ethical approval for this research (section 1.4.3).

3.3.1 Sampling protocol

Whenua Hou diving petrels come and go from their burrows just after dusk when they have the cover of darkness to evade predators (Fischer J.H., unpublished data). Therefore, sampling began in the last few minutes of daylight to have the greatest chance at catching birds when they are most active. Specifically designed burrow traps (Fischer J.H. unpublished data) were used to catch the birds as they were entering or leaving the burrows. The sampling process would take 5-10 minutes for each individual caught, therefore no bird was handled for an excessive period of time that could be detrimental to their health (Taylor, 2010). As diving petrels do not spontaneously regurgitate as a stress response to being captured, the only non-invasive dietary sampling method was opportunistic collection of faecal samples.

Plastic aprons were worn while handling the diving petrels to catch any faecal sample emissions. Disposable spoons were used to scoop up the faecal sample and transfer it into a 15 mL falcon tube containing ~5 mL of 70% ethanol. Spoons were only used for one sample and then discarded for sterility. Alcohol wipes were used to sterilise the plastic aprons between samples to avoid cross contamination of dietary DNA. Samples were kept refrigerated at 4 ˚C.

Table 3.1: Number of faecal samples collected from Whenua Hou diving petrels and common diving petrels over the three-year sampling period on Whenua Hou.

Species	2017	2018	2019	Total
Whenua Hou diving petrel	6 (m=4, f=2)	26 (m=13, f=13)	9 (m=4, f=5)	41
Common diving petrel	8 (m=5, f=3)	15 (m=7, f=8)	10 (m=4, f=6)	33

3.3.2 Method Development

3.3.2.1 Literature review of diving petrel diets

The first step in developing this assay was a review of studies that have detailed the diets of different diving petrel species (Table S3.1). This was limited to diving petrels as they are closely related to the CDPs and WHDPs central to this study. Diving petrels are known to feed largely on zooplankton, with some studies mentioning the presence of fish otoliths and cephalopod beaks in dietary samples (Imber & Nilsson 1980, Bocher et al. 2000, Bocher et al. 2003). The candidate prey species were sorted taxonomically to allow clear grouping of taxa for the design of this staged multiplex PCR (Table 3.2).

Table 3.2: List of potential prey species for primer design with indication of whether sequences are available for the genes COI, 12S, 16S and 18S.

Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	**COI**	**12S**	**16S**	**18S**
Chordata	Actinopterygii	Clupeiformes	Engraulidae	Engraulis	*E. ringens*	Y	N	Y	N
Chordata	Actinopterygii	Perciformes	Nototheniidae	Notothenia	*N. rossii*	Y	Y	Y	Y
Chordata	Actinopterygii	Beloniformes	Belonidae	Scomberesox	*S. saurus*	Y	N	Y	N
Chordata	Actinopterygii	Myctophiformes	Myctophidae	Krefftichthys	*K. anderssoni*	Y	N	Y	N
Chordata	Actinopterygii	Myctophiformes	Myctophidae	Myctophum	*M. nitidulum*	Y	Y	Y	N
Chordata	Actinopterygii	Atheriniformes	Atherinopsidae	Odontesthes	*O. regia*	Y	N	Y	N
Chordata	Actinopterygii	Scorpaeniformes	Normanichthyidae	Normanichthys	*N. crockeri*	N	N	Y	N
Chordata	Actinopterygii	Stomiiformes	Phosichthyidae	Vinciguerria	*V. lucetia*	Y	N	Y	N
Arthropoda	Malacostraca	Decapoda	Munididae	Pleuroncodes	*P. monodon*	Y	N	Y	Y
Arthropoda	Malacostraca	Decapoda	Porcellanidae			Y	Y	Y	Y
Arthropoda	Malacostraca	Decapoda	Hippidae	Emerita	*E. analoga*	Y	Y	Y	Y
Arthropoda	Malacostraca	Decapoda	Callianassidae	Callianassa		Y	Y	Y	Y
Arthropoda	Malacostraca	Decapoda	Hymenosomatidae	Halicarcinus	*H. planatus*	N	N	Y	N
Arthropoda	Malacostraca	Decapoda	Xanthidae			Y	Y	Y	Y
Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	Euphausia	*E. superba*	Y	N	Y	Y
Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	Euphausia	*E. mucronata*	Y	N	N	N
Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	Euphausia	*E. vallentini*	Y	N	Y	Y
Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	Nyctiphanes	*N. australis*	Y	N	Y	N
Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	Thysanoessa	*T. macrura*	Y	N	Y	Y

Arthropoda	Malacostraca	Euphausiacea	Euphausiidae	Thysanoessa		Y	N	Y	Y
Arthropoda	Malacostraca	Amphipoda	Cyllopodidae	Cyllopus		Y	N	N	Y
Arthropoda	Malacostraca	Amphipoda	Hyperiidae	Hyperiella	*H. antarctica*	Y	N	N	N
Arthropoda	Malacostraca	Amphipoda	Hyperiidae	Hyperoche	*H. medusarum*	Y	N	N	Y
Arthropoda	Malacostraca	Amphipoda	Hyperiidae	Primno	*P. macropa*	N	N	N	Y
Arthropoda	Malacostraca	Amphipoda	Hyperiidae	Themisto	*T. gaudichaudii*	Y	N	N	Y
Arthropoda	Malacostraca	Amphipoda	Hyperiidae	Themisto	*T. australis*	N	N	N	N
Arthropoda	Malacostraca	Amphipoda	Phronimidae	Phronima		Y	N	N	Y
Arthropoda	Maxillopoda	Pedunculata	Lepadidae	Lepas	*L. australis*	Y	N	Y	Y
Arthropoda	Maxillopoda	Calanoida	Calanidae	Calanoides	*C. acutus*	Y	N	N	Y
Arthropoda	Maxillopoda	Calanoida	Calanidae	Calanus	*C. simillimus*	Y	N	N	N
Arthropoda	Maxillopoda	Calanoida	Calanidae	Calanus	*C. propinquus*	Y	N	N	Y
Arthropoda	Maxillopoda	Calanoida	Clausocalanidae	Drepanopus	*D. pectinatus*	Y	N	N	Y
Arthropoda	Maxillopoda	Calanoida	Eucalanidae	Rhincalanus	*R. gigas*	Y	N	Y	Y
Arthropoda	Maxillopoda	Calanoida	Euchaetidae	Paraeuchaeta	*P. antarctica*	Y	N	N	Y
Arthropoda	Maxillopoda	Calanoida	Euchaetidae	Euchaeta		Y	Y	Y	Y
Mollusca	Cephalopoda	Oegopsida	Chiroteuthidae	Chiroteuthis		Y	Y	Y	Y
Mollusca	Cephalopoda	Oegopsida	Cranchiidae	Teuthowenia		Y	Y	Y	N
Mollusca	Cephalopoda	Oegopsida	Histioteuthidae	Histioteuthis	*H. atlantica*	Y	Y	Y	Y
Mollusca	Cephalopoda	Octopoda	Argonautidae	Argonauta		Y	Y	Y	Y

3.3.2.2 Round 1: low taxonomic resolution

The initial round of the multiplex assay was designed at a broad level of taxonomic resolution. The groups targeted were cephalopods, fish, and crustaceans. Due to the diversity of potential crustacean prey, this taxon was separated into euphausiids, amphipods/decapods, and maxillopods (copepods). Initially, the COI gene was used as a target for the primer sets developed as it is the most available marker for the target prey species (Table 3.2). The known COI sequences were imported from NCBI (National Center for Biotechnology Information) into Geneious Prime (Biomatters, New Zealand) for alignment and primer design.

3.3.2.2.1 Primer specifications

Primers were designed to be general to the targeted clade but specific enough that they would not pick up prey DNA from different taxa. The primer pairs needed to result in amplicons of different lengths (by ~20 bp, Thalinger *et al*., 2016) so that they would be discernible when separated by gel electrophoresis (Figure 3.1). As the target samples were faecal DNA, it would be expected to be degraded by the digestive process (Deagle, Eveson and Jarman, 2006). Therefore, primer length was restricted to amplicons of ~300 bp to increase the probability of amplifying an intact sequence of DNA. The minimum amplicon length was set at ~70 bp to avoid confusing amplification with primer-dimerisation (Das, Mohapatra and Hsu, 1999).

As the primer sets were going to be used in the same PCR, they needed to be optimised to the same reaction conditions (Thalinger *et al*., 2016). During the design process, this mainly concerns the annealing temperature for each primer. The annealing temperature of a primer depends on the GC content of the primer sequence and the length of the primer itself (Sachadyn, Sobiewska and Kur, 1998). The G (guanine) and C (cytosine) nucleotides have three hydrogen bonds to pair which means they separate at a higher

temperature than A (adenine) or T (thymine) nucleotides, which only have two available hydrogen bonding sites (Gould and Kollman, 1994). It is ideal for primers to have a GC content of around 50% and a length of between 18 – 26bp. This would produce a primer set with an annealing temperature of approximately 60°C (Dieffenbach, Lowe and Dveksler, 1993). All primer pairs were designed according to these specifications. Once designed, these primers were synthesised by Life Technologies (Auckland, New Zealand).

Table 3.3: Sequences of primers designed for the multiplex-PCR of diving petrel diets to detect the presence of amphipods/decapods, euphausiids, maxillopods, cephalopods and fish.

Primer	Sequence	Position on consensus sequence	Length	Annealing temperature (˚C)	GC content (%)	Amplicon size
Amph/Dec_F	GDGTAGATATAGCTTTCCCTCGTAT	236	25	58.1	41.7	158
Amph/Dec_R	GVACCTCTATGHCCTATATWAGAAG	393	25	56.2	39.1	158
Euph_F	WGCTGARTTAGGACAACCAGGWAS	81	24	61.3	47.8	78
Euph_R	AAAGCRTGRGCTGTAACWAYDAC	158	23	59.5	42.1	78
Maxill_F	AGTAATATTGCCCATGCTGGRG	371	22	59.4	47.6	287
Maxill_R	ATAGGGTCTCCTCCTCCACC	657	20	59.4	43.7	287
Ceph_F	GACTTCTCCCTCCATCYTTAAC	278	22	56.5	47.6	116
Ceph_R	GGDCCTGCATGAGATAGATTTC	393	22	57.7	47.6	116
Fish_F	GCHTCATCTGGNGTWGAAGC	311	20	59.0	55.6	194
Fish_R	TGTGAAATGGCAGGAGGTTT	504	20	57.6	45	194

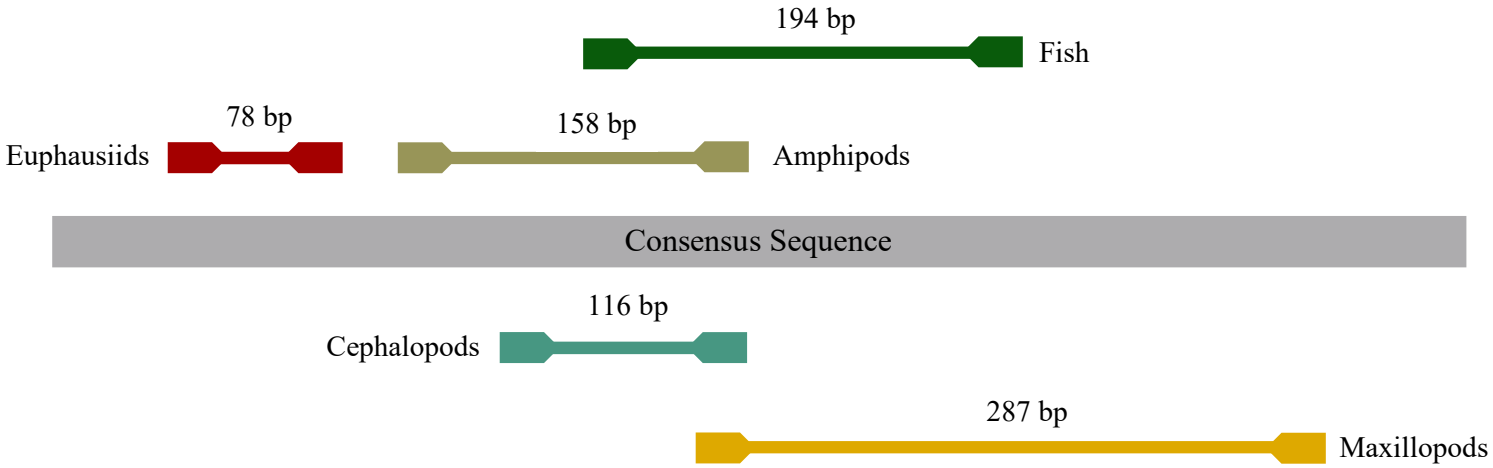

Figure 3.1: Position of novel primers along a consensus DNA sequence for the COI gene.

3.3.2.3 Competing uncertainties

When designing a novel PCR experiment, there are two competing uncertainties contributing to the success or failure of the experiment (Figure 3.2). First, whether the DNA extracted from samples has high enough quality to be successfully amplified through a PCR. If samples have been preserved, there is a strong possibility that the DNA will be degraded and that there will not be enough viable DNA to amplify (Frantzen *et al.*, 1998). That is why fresh samples are always preferable, however not always practical given the constraints of isolated field work. Different extraction methods also result in varying quality of DNA extracts (Abdel-Latif and Osman, 2017). It is important to trial different methods, such as extraction kits, high salt extraction, and phenol chloroform extraction, to ensure the highest quality extract possible. DNA quality can be quantified using NanoDrop Spectrophotometry to assess DNA concentration, purity, and level or unwanted organic contaminants. To confirm that the extracted DNA is suitable for PCR, a trial can be run using established universal primers. If amplification occurs, the DNA is suitable for PCR analyses and should work with correctly designed primers.

Once the quality of the DNA extraction has been confirmed, the novel primers can be trialled and optimised on this verified DNA (Figure 3.2). It is now important to address the second source of uncertainty, the viability of the designed primer pairs, as there are often discrepancies between the behaviour of primers *in silico* and their functioning in a lab-based PCR. It is essential to evaluate the results of a novel PCR, ensuring the amplification of the expected fragment size, the specificity of the primers (i.e., just one amplicon and only from the desired taxa), and to optimise the reaction protocol (i.e., temperature and reagent concentration) for the most successful outcome. If the PCR results in a fragment of the correct size, it is useful to have the amplicon sequenced to ensure the correct section of DNA was targeted.

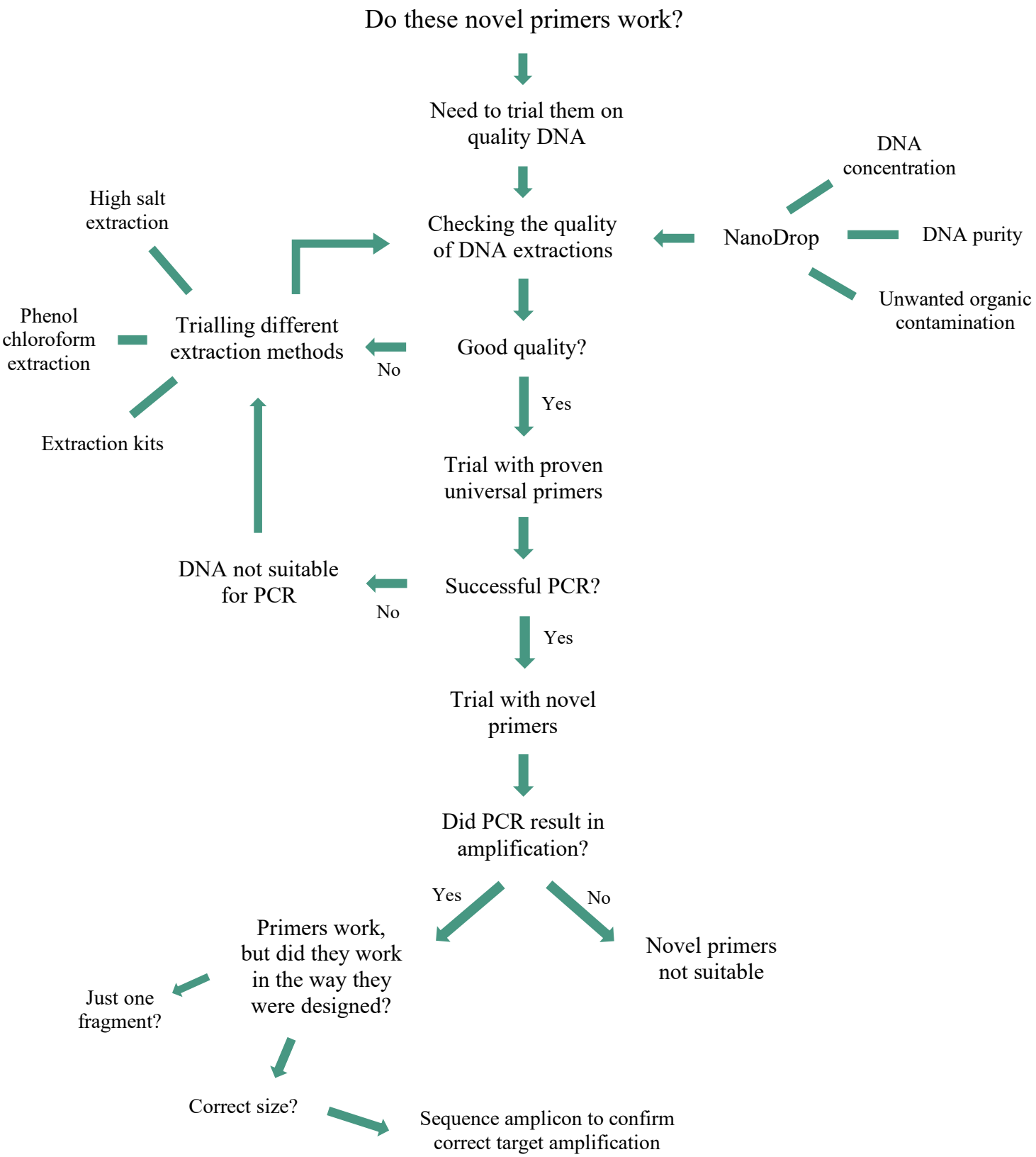

Figure 3.2: Process of dealing with the two sources of uncertainty associated with designing a novel PCR assay; quality of the DNA extract, and suitability of novel primers.

3.3.2.4 Extracting 'positive-control' DNA

With novel primers, it is important to trial and optimise them on their target DNA to ensure that they work the way they were designed (positive-control). DNA was extracted from frozen tissue samples of fish (tarakihi, *Nemadactylus macropterus*), cephalopod (squid, *Nototodarus sloanii*), and ethanol preserved specimens of euphausiid (krill, *Nyctiphanes sp.*), copepods (*Calanoides sp.*), and amphipods (*Hyperia sp.*) using the DNeasy Blood and Tissue DNA kits (QIAGEN, Hilden, Germany). Initially, the quality of the DNA extracts was assessed using a NanoDrop Microvolume Spectrophotometer (NanoPhotometer NP80, IMPLEN, Germany) to measure the concentration of DNA (ng/µL), DNA purity (desired 260/280 absorbance ratio > 1.8), and the presence of unwanted organic contaminants (desired 260/230 absorbance ratio > 2.0). The frozen fish and cephalopod tissue samples resulted in high quality DNA extracts according to the NanoDrop (Table 3.4), however the ethanol preserved crustacean specimens consistently resulted in poor quality DNA extractions.

Table 3.4: NanoDrop results for the positive control DNA extracts from samples under various preservation and extraction methods. Underlined extracts were used during primer trials.

Specimen	**Preservation method**	**Extraction method**	**Concentration (ng/µL)**	**260/280**	**260/230**
Fish	Frozen	DNeasy kit	332.1	1.90	2.27
Cephalopod	Frozen	DNeasy kit	158.95	2.10	2.05
Euphausiid	Ethanol	DNeasy kit	1.95	1.00	0.29
Copepod	Ethanol	DNeasy kit	3.25	1.18	0.27
Amphipod	Ethanol	DNeasy kit	2.10	0.98	0.98
Decapod	Frozen	DNeasy kit	16.35	1.97	1.44
Amphipod	Frozen	DNeasy kit	12.80	2.18	1.13
Decapod	Frozen	High salt	1286.90	2.15	2.19
Amphipod	Frozen	High salt	405.65	2.15	1.92

Fresh crustacean specimens were collected from rockpools at Tarakena Bay on Wellington's south coast to obtain better quality DNA extracts. Several glass shrimp (decapoda, *Palaemon affinis*) and amphipods (*Parawaldeckia sp.*) were collected, however no copepod or euphausiid samples were found. These fresh samples were frozen as soon as possible after collection to prevent DNA degradation. Initially, the DNeasy extraction kits were used to extract DNA from the new decapod and amphipod specimens, however this resulted in a low yield and significant levels of unwanted organic contamination (Table 3.4). Therefore, an alternative extraction method was trialled to improve the quality of DNA extracted. A modified high salt extraction protocol (Aljanabi & Martinez 1997) was used to extract DNA from the new decapod and amphipod samples, resulting in a greater yield and low level of contamination.

3.3.2.5 Trials with positive-control DNA

The four quality extractions (underlined in Table 3.4) were then trialled using 'universal' 16S primers (Chiar16S, initially designed for use on the broad Arthropoda phylum; Marquina, Andersson and Ronquist, 2019) to confirm they were suitable for PCR assays.

3.3.2.5.1 PCR protocol

The reaction mixture was optimised to contain 2 µL of DNA extract (diluted to ~10 ng/µL), 14.75 µL of PCR-grade water, 2.5 µL of reaction buffer (Bioline 10xNH_4), 2.0 mM of $MgCl_2$, 0.6 mg/µL of BSA, 0.4 µM of each primer, 0.2 mM of dNTPs, and 0.25 µL of BIOTAQ DNA polymerase. The amplification process was carried out on an Applied Biosystems Veriti Thermocycler (Thermo Fisher). Thermocycling conditions were 94 ˚C for 2 minutes, followed by 35 amplification cycles of 94 ˚C for 30 s, 48 ˚C for 30 s, and 72 ˚C for 1 minute, with a final elongation stage of 72 ˚C for 5 min. The PCR products were

then run on a 1.5% agarose gel at 80 V to separate the DNA amplicons. Amplicon size was determined using EasyLadder I (100-500 bp ladder, Bioline).

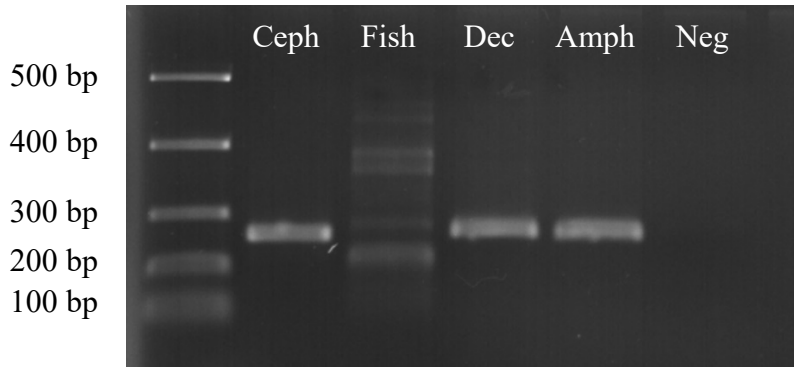

Figure 3.3: The resulting amplification from using the universal Chiar16S primers on DNA from cephalopod (Ceph), fish, decapod (Dec) and amphipod (Amph) extracts, and the negative control (Neg; no DNA).

Each of the DNA extracts resulted in amplification when using the universal Chiar16S primer set, indicating that the DNA is not too degraded to be amplified through PCR (Figure 3.3). An amplicon of approximately the same size (~270 bp) resulted from the PCR of cephalopod, decapod, and amphipod DNA. However, the fish DNA sample resulted in many weak spurious amplicons. This may indicate that the Chiar16S primers are not specific to one segment of the fish genome, but instead anneal to many different places. This would mean that the fish genome has multiple regions with very similar DNA sequences, which could prove difficult to design primers specific enough to result in one fragment size after amplification.

As the DNA extracts were shown to be suitable for PCR analyses, they could then be used to trial the novel primer sets and optimise the reaction conditions. Each primer set (Amph/Dec, Euph, Maxill, Ceph, and Fish) was tested against each DNA extract (cephalopod, fish, decapod, amphipod) to check that they amplify their target DNA and do

not result in false-positive identifications. Even though there were no usable DNA extracts from copepods or euphausiids, it was still important to confirm whether they amplified sequences from non-target DNA. The same reaction mixture and thermocycling conditions described in section 3.3.2.5.1 were used for each subsequent PCR.

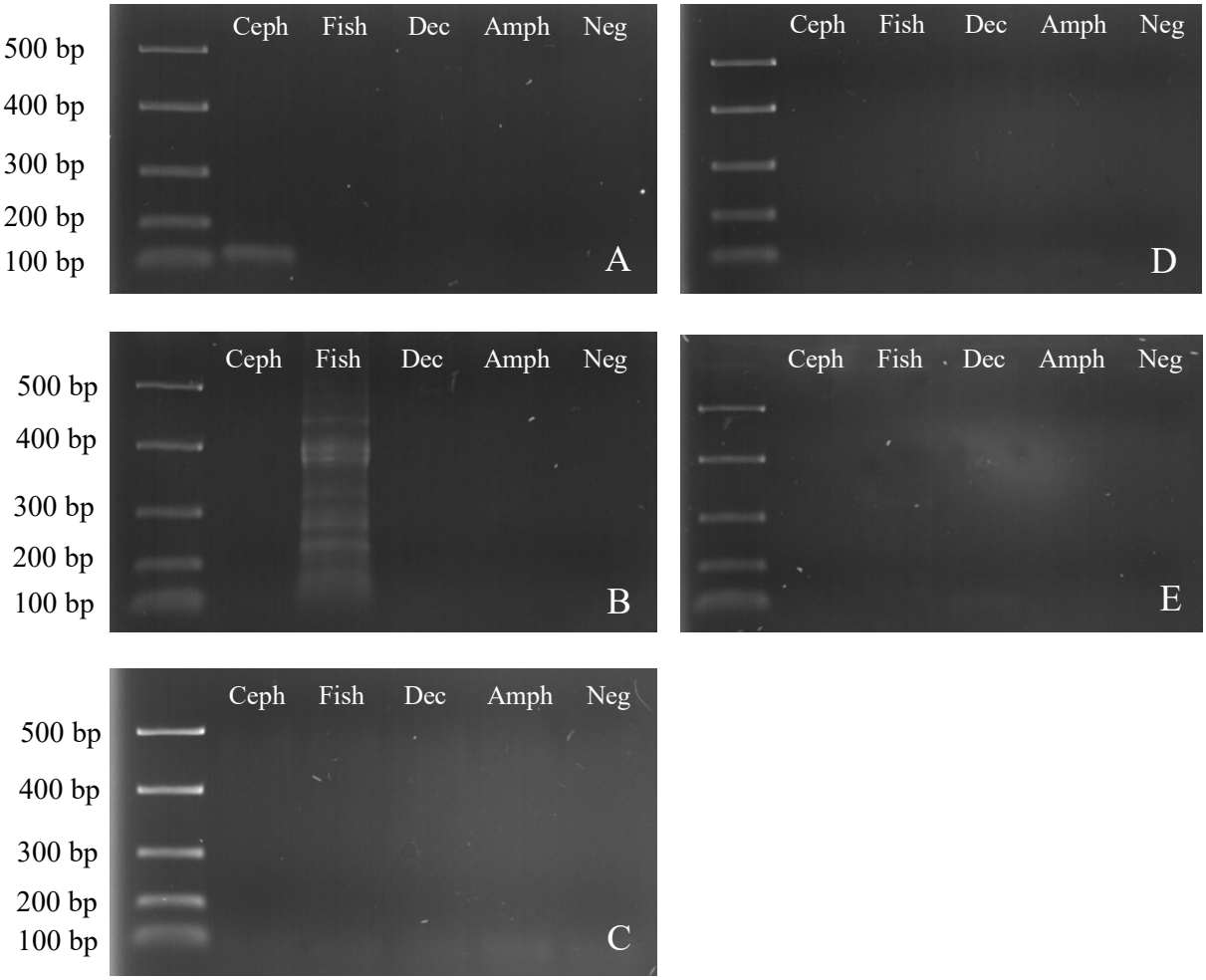

Figure 3.4: Specificity of novel primers designed to identify the presence or absence of DNA from their target taxa (cephalopods, fish, decapods, and amphipods). (A) Ceph primers, (B) Fish primers, (C) Amph/Dec primers, (D) Euphausiid primers and (E) Maxillopod primers trialled against DNA extracted from a cephalopod, fish, decapod and amphipod, and a negative control.

Figure 3.4A shows that the Ceph primers work the way they were designed, resulting in an amplification of ~116 bp for samples of cephalopod DNA, and no amplification for other prey taxa tested. Mixed results are seen for the Fish primers (Figure 3.4B) as amplification was specific to the fish DNA sample, however the primers did not amplify a single clear amplicon, but instead resulted in many spurious amplicons. This result was consistent with the outcome of using the universal Chiar16S primers on the fish DNA sample. The fish primers needed to be redesigned to obtain a single site of amplification. No amplification was observed using the Amph primers, therefore they too will need to be redesigned. The Euph and Maxill primers resulted in no amplification of the non-target DNA extractions which is a promising sign. However, this could have resulted from the PCR failing each time the Euph or Maxill primers were used as there were no positive control extracts available for these taxa. Access to fresh samples of euphausiids and maxillopods is needed to confirm function and specificity of these primer sets.

The fish primers were redesigned using the 16S gene as the sequences for this gene were available for all fish species in the pool of potential prey taxa (Table 3.2). The new Fish16S primers (Table S3.2) were designed with the same specifications as the first design round, therefore they should work when multiplexed with the other primer sets. The new Fish16S primers resulted in a single band specific to the fish DNA extract, however instead of the expected amplification of a 306 bp fragment, the assay resulted in an amplicon of ~220 bp (Figure 3.5A). This amplicon will be sent to Massey University for sequencing to determine what section of the fish genome these primers are preferentially targeting over their designed locus.

The amphipod/decapod primers were redesigned using the 18S gene as this was the second most sequenced region of the amphipod genome after COI (Table 3.2). The new Amph18S primers were designed following the same specifications in the first design attempt

(section 3.3.2.2.1) so they can be multiplexed with the other primer sets. Trials with this new primer set resulted in three distinct bands when used with the decapod DNA extract and no amplification for the fish, cephalopod or amphipod DNA extracts (Figure 3.5B). The middle of the three bands looks like it could be the expected amplicon of 187 bp (Table S3.2). Sequencing these amplicons will reveal whether this is the targeted segment as well as the origin of the two spurious bands. Various optimisation protocols (i.e. adjusting annealing temperature or magnesium concentration in the reaction mixture) may eliminate these spurious bands or result in amplification when used on the amphipod DNA extract.

Additional primers were also designed due to increasing evidence of jellyfish and salps (tunicates) in seabird diet from the genetic analysis of dietary samples (McInnes, Alderman, *et al.*, 2017; Cavallo *et al.*, 2018). These taxa were not previously thought to be important in seabird diet as traditional methods are biased towards prey with identifiable hard parts and often blind to the presence of gelatinous prey species (Karnovsky, Hobson and Iverson, 2012). The jellyfish primers (193 bp) were designed on the 16S gene and the salp primers (136 bp) were designed to target the 18S gene (Table S3.2). These primers were also designed to be used in the same multiplex-PCR assay as the first round of primers designed to increase the number of broad taxonomic groups identifiable in the same reaction. The same issue arose with the Jelly and Salp primers as with the Maxill and Euph primers – the difficulty in accessing fresh (live) samples to extract DNA and trial the primers on. These primers were tested against the DNA samples on hand to ensure they did not amplify DNA from non-target taxa (Figure 3.5). However, they were not able to be trialled on their target DNA to check whether they result in the expected amplicon size. The Jelly16S primers resulted in primer dimerisation in all reactions (Figure 3.5C; Das, Mohapatra and Hsu, 1999) seen in the consistent bands < 100 bp, including the negative control. These primers will need to be redesigned so that they amplify their target DNA and not each other. Interestingly, the

Salp18S primers resulted in an amplicon ~ 120 bp when trialled against the glass shrimp (Dec) DNA extract (Figure 3.5D). There was some weak primer dimerisation seen in the results from the fish, cephalopod, amphipod and negative reactions. It would be helpful to sequence the fragment amplified in the Dec PCR to understand what the Salp18S primers are targeting and whether they are suitable for identifying decapod DNA instead of salps as they were designed.

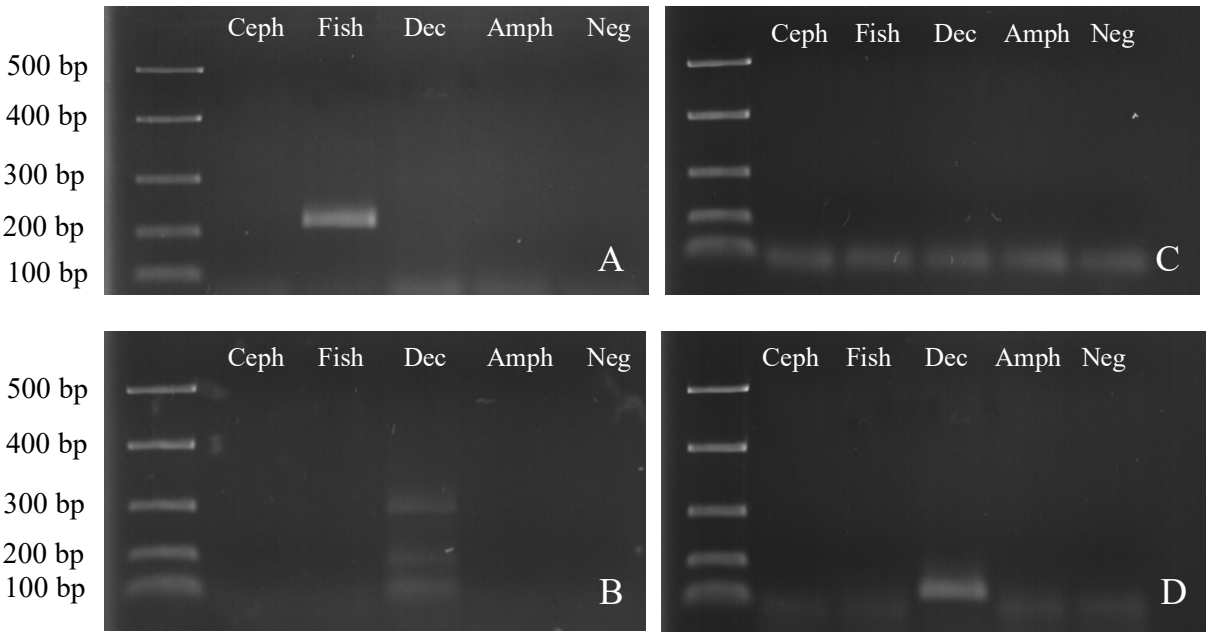

Figure 3.5: Specificity of novel primers designed to identify the presence or absence of DNA from their target taxa (cephalopods, fish, decapods, and amphipods). (A) Fish16S primers, (B) Amph18S primers, (C) Jelly16S primers, and (D) Salp18S primers trialled against DNA extracted from a cephalopod, fish, decapod and amphipod, and a negative control.

3.3.3 Future directions

The next stages in the design of this protocol are to continue to optimise the Euph and Maxill primer sets using high-quality salt extracted DNA once fresh samples are obtained. Even though they may need to be redesigned, it would be of interest to trial the Jelly16S and

Salp18S primers on target DNA extracts from fresh samples once they can be obtained. The annealing temperature and reagent concentrations will be optimised through repeated trials. This may eliminate the primer dimerisation observed in trials with the Jelly16S and Salp 18S primer sets. When consistent results of a single band are achieved, the PCR products will be sent to Massey University, with the successful cephalopod PCR product and the unexpected Fish16S product, for sequencing to confirm the target DNA sequence was amplified.

The next stage in the development of this method is to combine the primer pairs in a stepwise manner to check that they do not inhibit the performance of each other, as can occur if the primers from different pairs interact (Shuber, Grondin and Klinger, 1995). Ideally, all primer pairs will optimise to the same, or at least similar, reaction conditions (Shuber, Grondin and Klinger, 1995). When trialled on a mixture of the different DNA extracts, the primer cocktail should produce the same bands of amplification as when used individually (Figure 3.6).

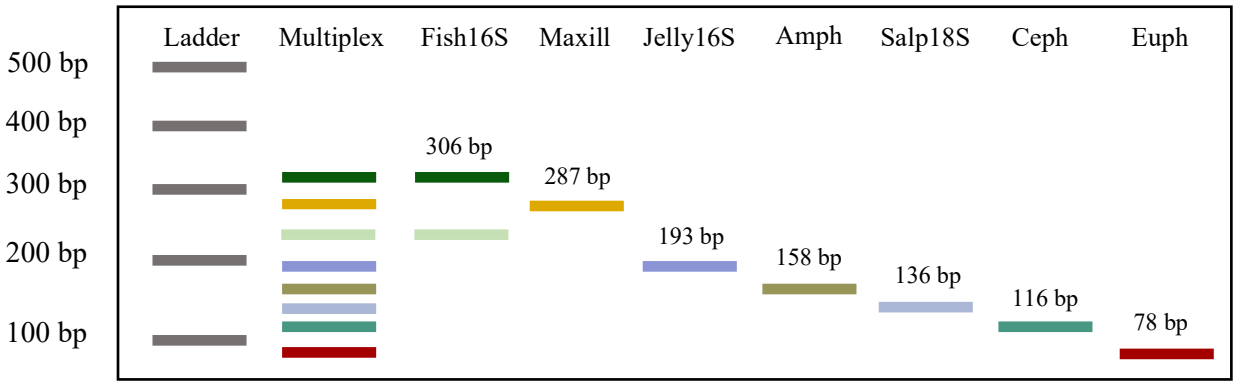

Figure 3.6: Theoretical 'in silico' PCR results for the initial, broad taxonomic resolution dietary analysis for the diving petrels on Whenua Hou. The light green band in the Fish16S column represents the unexpected amplicon of ~220 bp instead of the expected fragment of 306 bp.

Different methods will be trialled to extract the DNA from the faecal samples. As there were issues with extracting quality DNA from crustacean specimens preserved in ethanol (Table 3.4), there is a possibility that a quality DNA sample will not be able to be extracted from the faecal samples that are also stored in ethanol (Frantzen *et al.*, 1998). Initial trials using the high salt extraction protocol were not promising, however other methods will be investigated to try extract usable DNA from the faecal samples collected. Previous studies extracting DNA from seabird faecal samples had success using the QIAamp DNA Stool Mini Kits (QIAGEN; Deagle *et al.*, 2007; Deagle, Kirkwood and Jarman, 2009; Bowser, Diamond and Addison, 2013).

Dietary samples often contain high levels of the predator's own DNA, and the predominance of one source of DNA within a sample can bias molecular analysis (Polz and Cavanaugh, 1998; Green and Minz, 2005). Low concentrations of rare prey DNA within the diet can be masked by the predator's DNA during amplification, particularly because the prey DNA in stomach or faecal samples tends to be far more degraded than predator DNA (Deagle, Eveson and Jarman, 2006). The use of blocking primers was developed to counter issues like this, where predator specific blocking primers target predator DNA and inhibits amplification (Vestheim and Jarman, 2008). Based on the relative success of trialling this multiplex PCR on DNA extracted from WHDP and CDP faecal samples, the use of blocking primers may be a potential solution for any inhibition demonstrated by the overabundance of predator DNA (Vestheim and Jarman, 2008).

Depending on the results from running this initial stage of multiplex PCR on the faecal sample extracts, subsequent phases can be designed with increasing degrees of taxonomic resolution (Thalinger *et al.*, 2016). For example, if the Euph primers successfully amplify euphausiid DNA from the WHDP faecal samples, the next phase can be designed to distinguish between the genera Euphausia, Nyctiphanes and Thysanoessa (Table 3.2). This

nested multiplex PCRs can be used to determine whether certain taxa are present in the diets of not only WHDPs and CDPs but adapted to investigate the diets of any predator suspected of targeting the same broad taxonomic groups.

3.4 Evaluation of Method

3.4.1 Successes

I had success with the design of the Ceph primers as they are specific to cephalopod DNA and amplify a segment of DNA that is the same size as the segment they were designed to amplify. Sequencing the resulting fragment will confirm whether it was in fact the targeted fragment that was amplified. I also had some relative success with the Fish16S primers as they too were specific for fish DNA, however they resulted in an amplicon of an unexpected size. This PCR product will have to be sequenced to determine the region of the fish genome targeted by these novel Fish16S primers. The Fish16S primers may still be able to be included in the multiplex-PCR assay if no other primer pairs unexpectedly produce an amplicon of the same size.

3.4.2 Obstacles

One of the main obstacles I faced in designing this assay was the disparity between the expected results according to the *in silico* PCR in Geneious and the true PCR products obtained in the lab (Shuber, Grondin and Klinger, 1995). This resulted in the design and redesign process taking a long time, especially as the primers needed to optimise to the same reaction conditions so they could be combined in a single PCR assay (Shuber, Grondin and Klinger, 1995). Depending on the outcome of trialling the unconfirmed primers (e.g. Euph,

Maxill, Jelly16S and Salp18S) on positive control DNA, I may need to go through several redesign stages until the primers behave in the way they were designed.

Another significant obstacle to optimising my primers was my limited access to fresh samples within the prey taxa of diving petrels. Despite inquiring with organisations such as VUCEL (Victoria University Coastal Ecology Lab), NIWA (National Institute of Water and Atmospheric Research) and Te Papa Tongarewa for access to fresh or frozen samples of euphausiids, maxillopods (copepods specifically), jellyfish, and salps, I was unable to find samples on which to optimise my primers.

3.4.3 Reflection

This method has a time-consuming design process and optimisation phase, however once it is ready it has the potential to be an affordable analytical process when dealing with high volumes of samples (Shuber, Grondin and Klinger, 1995; Thalinger *et al.*, 2016). It takes a long time to design primer sets capable of being multiplexed, and even more time to optimise the primer sets when they behave differently in the lab than in the design software (Shuber, Grondin and Klinger, 1995). It is also difficult to optimise, particularly at high taxonomic resolution, when access to fresh samples of prey only found in the open ocean is required. However, once designed, it provides an adaptable dietary analysis method that is more affordable that high-throughput sequencing at high sample numbers (Thalinger *et al.*, 2016), particularly concerning dietary samples that have a high concentration of predator and bacterial DNA sequences. With additional time and resources to optimise a staged multiplex PCR protocol, this could be developed into a valuable tool for seabird biologists. Almost any seabird diet can be assessed with this initial broad-scale multiplex-PCR, and the subsequent stages can be adapted to be species specific following this method development plan. However, due to the myriad of obstacles faced in developing this method, a more promising

method may be to use the DNA metabarcoding approach (Kress *et al.*, 2015; Rennstam Rubbmark *et al.*, 2019). Although it would require more financial resources, the metabarcoding approach would allow identification of the majority of prey present in the diets of WHDPs and CDPs without the lengthy design and optimisation phase found to accompany the staged multiplex-PCR approach.

CHAPTER 4

Investigating the degree of mercury exposure for Whenua Hou diving petrels

4.1 Abstract

Mercury accumulates within organisms and bioamplifies up the food web, resulting in top predators such as seabirds being exposed to the highest levels of toxic mercury within an ecosystem. The Whenua Hou diving petrel (*Pelecanoides whenuahouensis*; hereafter WHDP) is considered 'Nationally Critical' due to its small population size (~200 individuals) and restricted breeding ground on Whenua Hou. As they have been shown to forage at a higher trophic level than the sympatric common diving petrels (*Pelecanoides urinatrix*; CDP), they are at risk of being exposed to high concentrations of mercury through their diet. Blood and feather samples from WHDPs and CDPs collected over three years (2017-2019) were analysed to understand the intra- and interspecific variation in mercury exposure for these species. The results indicate that male WHDPs are exposed to higher concentrations of mercury than females. They also suggest that interannual variation in mercury exposure may be more strongly determined by environmental fluctuations than by diet. The interspecific variation in mercury concentration correlates with the trophic data in chapter 2, as WHDPs foraging on prey of higher trophic value than CDPs also had higher concentrations of mercury in their tissues. This has the potential to compromise survival and reproductive success for the WHDPs and should be further evaluated for their conservation.

4.2 Introduction

The concentration of mercury (Hg) in the marine environment has increased substantially since the industrial revolution (Thompson, Furness and Walsh, 1992; Selin, 2009; Driscoll *et al.*, 2013; Outridge *et al.*, 2018). Hg becomes available in the biosphere through both natural and anthropogenic processes (Becker *et al.*, 2016; Cherel *et al.*, 2018). Natural sources of Hg include volcanic and geothermal activity (Ebinghaus *et al.*, 2002; Fitzgerald and Lamborg, 2007), but these emissions are dwarfed by those of anthropogenic origin (i.e. mining and fossil-fuel burning; Selin, 2009). When released into the atmosphere, Hg emissions reach even the most remote ecosystems (Fitzgerald *et al.*, 1998; Fort *et al.*, 2014; Cherel *et al.*, 2018), rendering it a global scale pollution threat (Driscoll *et al.*, 2013). Hg can be in elemental, inorganic, and organic forms (Burger and Gochfeld, 2002). Inorganic Hg is methylated by anaerobic bacteria into methylmercury (MeHg), the most toxic form (Jensen and Jernelov, 1969).

The properties of Hg result in bioaccumulation within organisms and bioamplification up the food web, exposing top predators, such as seabirds, to the highest Hg levels within an ecosystem (Bryan, 1979; Monteiro and Furness, 1995). Exposure to high levels of Hg can impact the health of seabirds at both an individual and population level. MeHg damages neural cells and has been shown to have a harmful effect on embryo development (Ceccatelli, Daré and Moors, 2010; Kenow *et al.*, 2011; Goutte *et al.*, 2014). Additional detrimental impacts include impaired physiological activity (e.g., altering blood and organ biochemistry; Hoffman, Spalding and Frederick, 2005), endocrine disruption (e.g., interfering with sex hormones; Heath and Frederick, 2005; Tan, Meiller and Mahaffey, 2009), and altered reproductive behaviour (Frederick and Jayasena, 2011). Chronic exposure to high levels of Hg can compromise survival and long-term fecundity, contributing to population decline

(Goutte *et al.*, 2014). Previous studies have shown MeHg to increase male-male pairing behaviour, decrease egg production, and reduce the probability of successfully fledging a chick (Frederick and Jayasena, 2011; Tartu *et al.*, 2013; Goutte *et al.*, 2014). These effects demonstrate the potential for Hg exposure to impact the health of individual seabirds and inhibit population growth.

As ingested Hg is assimilated in various ways throughout the body of a seabird, different tissues can highlight different aspects of Hg exposure. Hg dynamics in seabirds can be viewed as a multicompartment model, involving ingestion through diet, absorption in the intestine, transport in the blood stream, accumulation and storage in internal tissues (i.e. muscle, liver, kidney), with redistribution for excretion through feather growth, eggs and excreta (Lewis, Becker and Furness, 1993; Monteiro, Granadeiro and Furness, 1998). Seabirds have demonstrated the ability to demethylate Hg and store inorganic Hg in the liver (Thompson and Furness, 1989). Approximately 70-93% of the total Hg burden within seabirds is excreted into the plumage (Honda, Nasu and Tatsukawa, 1986; Braune and Gaskin, 1987; Burger, 1993). Dietary Hg accumulates in soft tissues between moults and is transferred into growing feathers during the non-breeding season (Monteiro, Granadeiro and Furness, 1998). Therefore, feathers exhibit a high concentration of Hg (Monteiro and Furness, 1995), correlate positively with levels in internal tissues (Lewis and Furness, 1991; Thompson, Hamer and Furness, 1991), and reflect the uptake and storage over the previous intermoult period (Furness, Muirhead and Woodburn, 1986; Honda, Nasu and Tatsukawa, 1986; Monteiro, Granadeiro and Furness, 1998). Alternatively, blood samples represent recent absorption of Hg from ingested prey (Monteiro, Granadeiro and Furness, 1998). Through analysing both blood and feather samples, an understanding of both short-term dietary uptake and long-term accumulation can be achieved.

Variation in Hg levels among seabird species has been attributed to various factors such as trophic level, migration, body size, and lifespan (Monteiro and Furness, 1995), however, the strongest determinant of Hg concentration in seabird tissues is diet (Braune and Gaskin, 1987; Lewis and Furness, 1991; Bocher *et al.*, 2003; Becker *et al.*, 2016). Species feeding predominantly on crustaceans have consistently lower Hg levels than those targeting fish and squid (Braune and Gaskin, 1987; Honda *et al.*, 1990; Stewart *et al.*, 1999). This should therefore result in seabirds foraging at higher trophic levels (higher $\delta^{15}N$) experiencing greater concentrations of Hg. However, to avoid potentially erroneous conclusions about the relationship between isotopic niche and Hg concentrations, studies also need to consider the disparity in integration time for contaminants and isotopes in feathers (Bond, 2010). Nitrogen isotopes reflect diet at the time of tissue synthesis (a snapshot; Hobson and Clark, 1992), whereas Hg is accumulated over the protracted intermoult period and excreted in bulk through new feather growth (Rumbold *et al.*, 2001; Bond and Diamond, 2009; Bond, 2010). Alternatively, in blood samples, accumulation of isotopes and Hg from diet are much more comparable, both reflecting dietary uptake during the sampling period.

The Whenua Hou diving petrel (*Pelecanoides whenuahouensis*, hereafter WHDP) is a nationally critical seabird with a population of only ~200 individuals breeding in a single colony on Whenua Hou (Robertson *et al.*, 2017; Fischer *et al.*, 2020). With such a small population size, it is important to investigate potential threats to individual survival and population growth. Given chapter 2 indicates WHDPs likely forage on high trophic level prey, such as fish and cephalopods, they are at risk of exposure to high levels of Hg (Braune and Gaskin, 1987; Honda *et al.*, 1990; Stewart *et al.*, 1999). It is therefore important to investigate the factors contributing to potentially high concentrations of Hg in WHDPs to understand which individuals are most at risk. The WHDPs share their breeding ground with a population of common diving petrels (*Pelecanoides urinatrix*, hereafter CDP; Fischer *et al.*,

2018), who in chapter 2 are shown to forage at a slightly lower trophic level during the breeding season. Therefore, CDPs are expected to have lower concentrations of Hg both in blood, from recent dietary sources, as well as in feathers, from accumulated Hg over long periods of time (Honda *et al.*, 1990; Monteiro, Granadeiro and Furness, 1998).

In this chapter, I aimed to investigate the concentration of Hg in blood and feather samples taken from WHDPs over three consecutive years (2017-2019). I constructed linear mixed-effect models to explore factors contributing to the intraspecific variation seen in Hg levels of WHDP. I used results from both blood and feather samples to examine different temporal periods of Hg acquisition; i.e. recent levels of Hg ingestion as well as the concentration accumulated over the previous intermoult period. By analysing the same samples collected from CDPs, I aimed to investigate whether the trophic pattern seen between WHDPs and CDPs is reflected in their relative Hg exposure. Based on results discussed in chapter 2, I hypothesise that: 1) male WHDPs will have a higher concentration of Hg than females as they appear to forage on higher trophic level prey, 2) the interannual variation in Hg concentration will follow the trophic fluctuations observed (i.e., the highest $\delta^{15}N$ in 2018 corresponding to the highest Hg concentration for blood samples), and 3) WHDPs will exhibit higher concentrations of Hg than CDPs, as WHDPs forage at a higher trophic level than CDPs during the breeding season.

4.3 Methods

A description of the study system can be found in section 1.4.2, along with the ethical approval for this research (section 1.4.3). Collection and preparation of the blood and feather samples followed the same protocol outlined in sections 2.3.3 and 2.3.4.

4.3.1 Sample analysis

Mercury concentrations (total Hg) were measured for samples of whole blood and feathers from WHDPs and CDPs by the Littoral Environnement et Societes laboratory (LIENSs, Université de La Rochelle, France). Homogenised samples were weighed out to ~3 mg and analysed using an advanced Hg analyser spectrophotometer (Altec AMA 254; Blévin *et al.*, 2013). There was only enough sample remaining for single analyses on the blood samples, however feather samples were repeated to check for homogenisation within each sample (standard deviation for the two samples were <10%; Fort *et al.*, 2014). The mean mercury concentrations for the repeated samples were used for the statistical analysis. To ensure measurement accuracy, a certified reference material (Lobster Hepatopancreas Tort-2; NRC, Canada; Hg concentration of 0.27 ± 0.06 μg/g dry weight) was measured every 10 samples (Fort *et al*., 2014). Additionally, blanks were run at the start of each sample set. The detection limit of this method was 0.005 μg/g dry weight (Fort *et al*., 2014).

4.3.2 Data analysis

Linear mixed-effect models (LMMs) were constructed to analyse the factors contributing to the variation seen in the WHDP whole blood and feather Hg concentration. Values for Hg concentration were z-transformed so the beta values for each variable were comparable. The fixed effects in these models were year (2017, 2018, or 2019) and sex (male or female). Year was a categorical variable, with the effect expressed as 2018 or 2019 relative to the reference state of 2017, and sex was a categorical variable, with the effect expressed as female Hg concentration relative to male. A random individual effect (ID) was also included in each model as some individuals were sampled across multiple years. For both the blood and feather datasets, all possible combinations of variables (excluding interactions), a 'null' model (where only ID was considered), and a 'full' model combining all variables were

generated. Models were run using the lme4 package in R (Bates, 2007; R Development Core Team, 2020). The Akaike Information Criterion corrected for small sample sizes (AIC_C) was used to rank models and identify the relative importance of variables affecting the Hg concentration in blood and feather tissue from WHDPs (Burnham and Anderson, 2002). For each model, the AIC_C, the difference in AIC_C values relative to the best fit model (ΔAIC_C), and the weight of each model (ω) were calculated. Models with $\Delta AIC_C < 2.0$ were considered to have substantial support from the data (Burnham and Anderson, 2002).

From the model output, the resulting beta values (slopes) for each variable were averaged across the models. The relative variable importance (RVI) for each factor was calculated by summing the ω values to discern which factors had the strongest effect on the variability on Hg concentration. Variables were considered to have a strong effect on Hg concentration if $\beta \pm 2$ SE did not intersect zero.

To compare the Hg concentrations in the blood and feathers of CDPs and WHDPs, an LMM was created combining the effects of species and ID. All analyses were performed using R 4.0.2 (R Development Core Team, 2020).

4.4 Results

4.4.1 Mercury concentration in blood samples

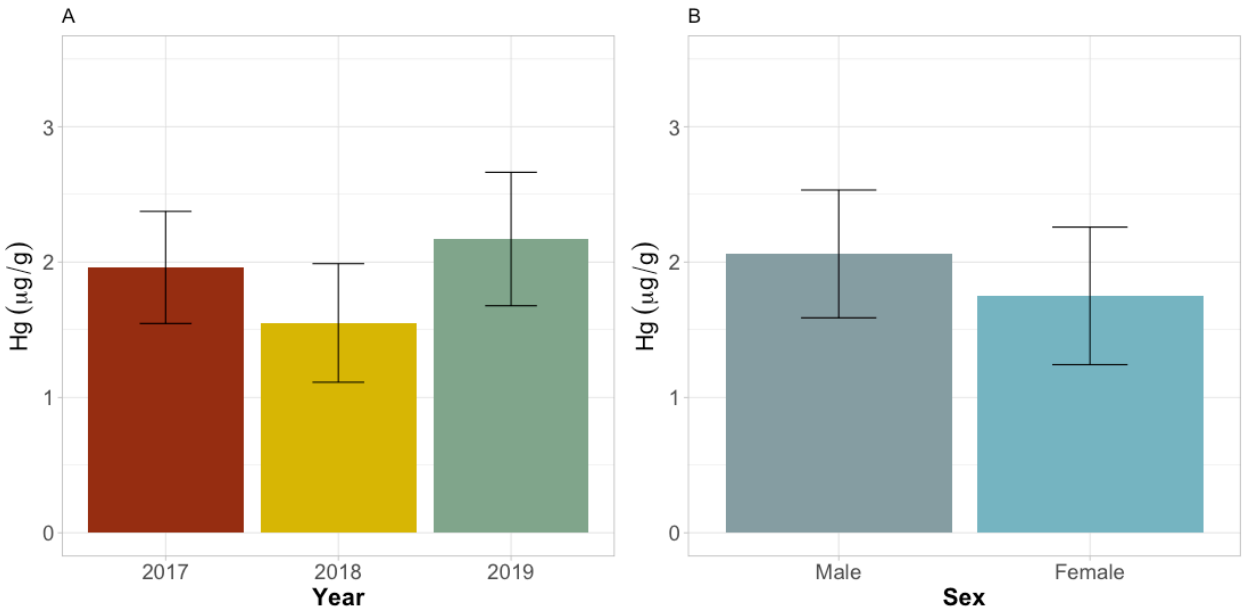

Figure 4.1: The variation of WHDP Hg concentration (mean ± SD) in blood (A) among years and (B) between sexes.

The average Hg concentration in WHDP blood samples was 1.91 µg/g, ranging from 0.79 to 3.14 µg/g (dry weight). Year and sex had a strong effect on the variation in Hg concentration in blood samples (Table 4.1). The reference state for the effect of year was 2017, with the beta values showing the model-averaged effect of samples collected in 2018 or 2019 relative to 2017. The results showed that Hg concentration was lower in 2018 (1.55 ± 0.44 µg/g) than in 2017 (1.96 ± 0.41 µg/g) and was the highest in 2019 (2.17 ± 0.49 µg/g; Figure 4.1A). The reference state for the effect of sex was male, and the results showed female blood (1.75 ± 0.51 µg/g) had a lower concentration of Hg than males (2.06 ± 0.47 µg/g).

Table 4.1: The model-averaged beta and RVI values for each fixed effect from the linear mixed-effect models attempting to describe the variation seen in blood Hg concentration for the WHDPs on Whenua Hou, New Zealand. Results are expressed as β ± SE. (Indicates that β ± 2×SE does not intersect 0).*

Blood Hg	$\beta_{Average}$	**RVI**
Year	*2018 -0.73 ± 0.18 2019 0.24 ± 0.18	1.00
Sex	*-0.48 ± 0.20	0.83

Based on AIC, the top model was the full model, which combined the effects of year, sex and ID. This model had an AIC weight of 0.83.

4.4.2 Mercury concentration in feather samples

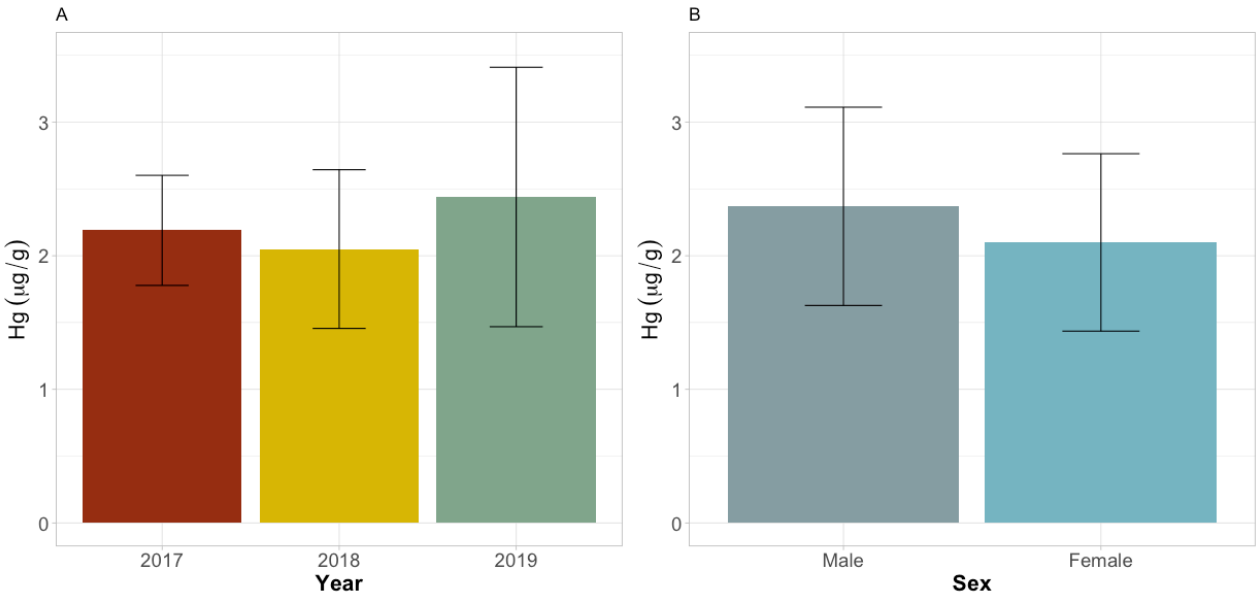

Figure 4.2: The variation of WHDP Hg concentration (mean ± SD) in feathers (A) among years and (B) between sexes.

The average Hg concentration in WHDP feather samples was 2.24 µg/g, ranging from 1.03 to 4.80 µg/g (dry weight). Year had a strong effect on the variation observed in Hg concentration in WHDP feathers (Table 4.2). The effect of year was strong between 2017 (2.19 ± 0.41 µg/g) and 2019 (2.44 ± 0.97 µg/g), with 2019 samples containing a higher concentration of Hg, but was not strong for 2018 (2.05 ± 0.59 µg/g) relative to 2017. There was no strong effect of sex on feather Hg concentration.

Table 4.2: The model-averaged beta and RVI values for each fixed effect from the linear mixed-effect models attempting to describe the variation seen in feather Hg concentration for the WHDPs on Whenua Hou, New Zealand. Results are expressed as β ± SE. (Indicates that β ± 2×SE does not intersect 0).*

Feather Hg	$\beta_{Average}$	**RVI**
Year	2018 -0.01 ± 0.16 *2019 0.40 ± 0.18	0.70
Sex	-0.34 ± 0.24	0.46

The top model combined the effects of year and ID on feather Hg concentration, with an AIC weight of 0.38 (Table S4.2). However, there is uncertainty associated with which is the best model as all four models had a ΔAIC < 2.0.

4.4.3 Comparison with CDPs

WHDPs had higher Hg concentration in both tissues, and the models showed species (WHDP relative to CDP) had a strong effect on Hg concentration in both blood (Intercept = -0.79 ± 0.09, β = 1.51 ± 0.12) and feathers (Intercept = -0.84 ± 0.09, β = 1.61 ± 0.11).

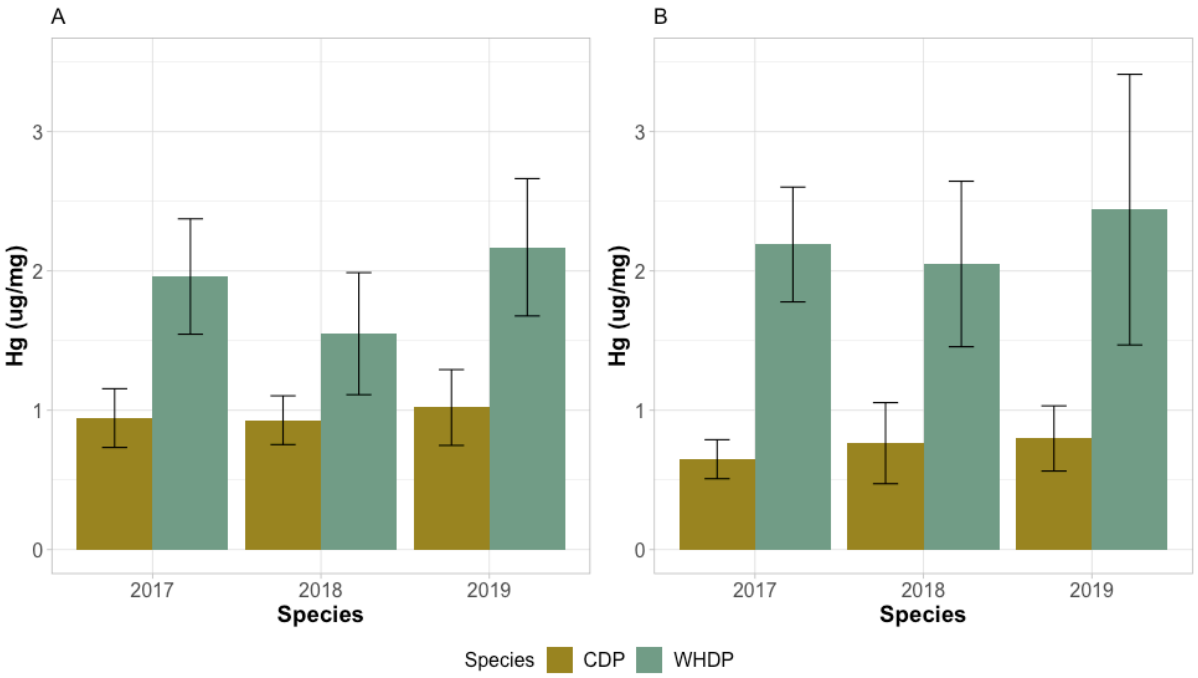

Figure 4.3: The differences in Hg concentration (mean ± SD) in (A) blood and (B) feather tissues sampled from WHDPs and CDPs on Whenua Hou during the breeding seasons of 2017. 2018 and 2019.

4.5 Discussion

4.5.1 Mercury concentration in blood and feathers of WHDPs and CDPs

My first hypothesis was supported by my results, as male WHDPs had higher levels of Hg in their tissues than females. This could be attributable to males targeting prey of higher trophic values than females during their breeding season, as seen in chapter 2, or to the additional excretion avenue females have by excreting Hg through the egg (Lewis, Becker and Furness, 1993; Monteiro, Granadeiro and Furness, 1998).

My second hypothesis, that Hg levels would follow the same interannual pattern seen in the WHDP trophic dynamics for blood samples, was not supported by my results. In chapter 2, WHDPs are shown to forage at the highest trophic level in the breeding season of 2018, which is unexpected considering this was an El Niño year and marine food webs generally

experience reduced food supply during this climatic phase (Boersma, 1978). In 2018, when WHDP blood samples indicated foraging at a higher trophic level, these samples unexpectedly show a lower Hg concentration (Figure 4.1). Previous studies have demonstrated that variation in Hg concentration in the tissues of a seabird is largely determined by diet (Lewis and Furness, 1991; Monteiro, Granadeiro and Furness, 1998). Species targeting higher trophic level prey, such as fish and squid, are exposed to a higher level of Hg (Braune and Gaskin, 1987; Honda *et al.*, 1990; Bustamante *et al.*, 2006), however this is not seen in the blood Hg concentrations for WHDPs. This might be explained by the influence of the El Niño Southern Oscillation on Hg concentrations in the biosphere. A study has shown the highest levels of Hg in the atmosphere to lag ~ 8-10 months behind an El Niño year (Slemr *et al.*, 2016). As the breeding season in 2018 was an El Niño phase, WHDPs should be exposed to high levels of Hg during the breeding season in the following year. This is a potential explanation for the high concentration of Hg in the blood samples of WHDPs in 2019 relative to 2017 and 2018.

My final hypothesis, that WHDPs would have higher concentrations of Hg in their blood and feathers than CDPs, was supported by my results. Hg concentrations in the tissues of WHDPs were consistently higher than those in CDP tissues (Figure 4.3). This coincides with the pattern of trophic segregation seen between WHDPs and CDPs during the breeding season, where WHDPs appear to be targeting higher trophic level prey, such as fish and cephalopods, than CDPs (Braune and Gaskin, 1987; Honda *et al.*, 1990). These higher trophic level prey may be responsible for exposing the WHDPs to higher concentrations of Hg (Braune and Gaskin, 1987; Honda *et al.*, 1990; Stewart *et al.*, 1999). It is concerning to see higher levels of Hg in the Nationally Critical WHDP given the adverse effects high levels of Hg can have on individual survival and reproductive success (Heath and Frederick, 2005; Tan, Meiller and Mahaffey, 2009; Goutte *et al.*, 2014).

The interpretation of Hg concentration in feathers in relation to stable isotope ratios is more complicated than for blood samples (Bond, 2010). Feathers represent the level of Hg accumulated over the previous intermoult period and are highly correlated to the Hg levels assimilated and stored in internal tissues (Lewis and Furness, 1991; Thompson, Hamer and Furness, 1991). This results in feathers being an effective, non-invasive sampling avenue to monitor Hg contamination in seabirds (Cherel *et al.*, 2018). A similar average concentration of Hg in feathers was observed across the years for WHDPs, with a slight increase in 2019 (Figure 4.2). This could indicate an increase of higher trophic level prey (i.e. fish and cephalopods) in the WHDP diet during the previous intermoult period (i.e., the 2018 breeding season; Braune and Gaskin, 1987). The increase in Hg concentration in feathers sampled in 2019 correlates with the higher $\delta^{15}N$ observed in the 2018 blood samples, indicating that WHDPs were targeting higher trophic level prey during the breeding season (intermoult period) of 2018 (Braune and Gaskin, 1987; Lewis and Furness, 1991; Bocher *et al.*, 2003).

The results from modelling the intraspecific variation seen in WHDP blood samples show that year and sex have a strong effect on Hg concentration. Using AIC to compare the models has shown that the full model, combining the effects of year and sex, had substantial support from the data as it was the only model with a $\Delta AIC < 2.0$ (Burnham and Anderson, 2002). The results for modelling the feather Hg variation show that year had a strong effect on Hg concentration, however, none of my models stood out from the rest as all four models had $\Delta AIC < 2.0$ and relatively small Akaike weights to support them (Table S4.2; Burnham and Anderson, 2002). This suggests that other biotic or abiotic variables not included in this study were influencing the Hg concentration in WHDP feathers.

4.5.2 Implications for WHDP conservation

As top predators, seabirds are exposed to high levels of Hg through their diet given Hg bioaccumulates in prey tissues and bioamplifies up the food web (Bryan, 1979; Monteiro and Furness, 1995; Driscoll *et al.*, 2013). Exposure to even low levels of the highly toxic form, MeHg, has been shown to damage neural cells and inhibit embryo development (Ceccatelli, Daré and Moors, 2010; Dietz *et al.*, 2013). Chronic exposure to Hg can compromise survival and reproductive success, contributing to population decline (Goutte *et al.*, 2014). The lay rate of WHDPs follows the trend in blood Hg concentration, where the lowest concentration seen in 2018 corresponds to the highest rate of egg production (89% of monitored nests; n = 64) and the highest Hg concentration in 2019 corresponds with the lowest lay rate (78% of monitored nests; n = 78; Fischer J.H., unpublished data). High levels of Hg exposure in WHDPs compared to sympatric CDPs may be contributing to the slow population recovery of WHDPs through interfering with reproductive processes (Heath and Frederick, 2005; Tan, Meiller and Mahaffey, 2009; Fischer *et al.*, 2020). Over the sampling period from 2017 to 2019, there was a 64-70% incidence of infertility in eggs that failed to hatch (n = 24; Fischer J.H., unpublished data). As WHDPs breed once a year and only lay a single egg, understanding the factors contributing to infertility is key to ensuring survival of the population. Measuring the concentration of Hg present in these infertile or unsuccessful eggs may contribute to our understanding of the restricted population growth of the WHDPs (Fischer *et al.*, 2020).

An accepted threshold of Hg concentration in feathers for adverse effects to appear in seabirds has been suggested to be 5.0 µg/g (Burger and Gochfeld, 1997), however this appears to be largely species specific. Previous studies have shown seabird species to have much higher concentrations of Hg in feathers without exhibiting adverse effects, such as the Bulwer's petrel (*Bulweria bulwerii*) with an average feather concentration of 22.3 µg/g

(Monteiro, Granadeiro and Furness, 1998) and the wandering albatross (*Diomedea exulans*) with an average ranging between 16.59 and 27.43 µg/g (Carravieri *et al.*, 2014; Cherel *et al.*, 2018). At the other end of the spectrum, reproductive impairment through endocrine disruption has been demonstrated in kittiwakes in the Arctic with low Hg concentrations (~3 µg/g; Tartu *et al.*, 2013). This demonstrates how response to Hg contamination is likely species specific, and further research into the physiological effects of Hg on WHDPs (with a maximum Hg concentration in feathers of 4.80 µg/g) is required to understand how they are affected by Hg pollution.

4.5.3 Limitations of this study

In this study, I only analysed the total Hg (THg) rather than analysing both MeHg and THg to understand the proportion of the more toxic MeHg in the tissue samples (Dietz *et al.*, 2013). Previous studies have shown that the chemical form of Hg in seabird feathers is predominantly MeHg (Braune and Gaskin, 1987; Thompson and Furness, 1989; Renedo *et al.*, 2017), but this may vary between species. Through analysing the proportion of MeHg in the blood and feather samples, I would have a better understanding of the potential toxic effects experienced by the WHDPs (Driscoll *et al.*, 2013).

Studies have also shown there to be less variation in THg concentration in body feathers than wing or tail feathers due to the gradual depletion of Hg accumulated throughout the moult sequence (Furness, Muirhead and Woodburn, 1986; Thompson *et al.*, 1998). Feathers are also heterogenous, with different concentrations of Hg incorporated into the feather throughout its growth (Bond, 2010). To account for these limitations, I sampled the same feather from each wing of each individual and homogenised the feather samples before analysis. However, sampling body feathers instead of wing feathers may provide a more

consistent comparison of Hg across species with different moult patterns (Carravieri *et al.*, 2014).

4.5.4 Conclusions and future directions

In this study, I have characterised some of the variation in Hg concentration of WHDP blood and feather samples collected over three consecutive breeding seasons. The blood samples provided an indication of recent ingestion levels at the breeding colony and the feathers represented Hg levels accumulated over the long intermoult period (Furness, Muirhead and Woodburn, 1986; Monteiro, Granadeiro and Furness, 1998). My results have demonstrated patterns of interannual variation in blood Hg levels within the WHDP population which, interestingly, do not align with the trophic dynamics demonstrated in chapter 2. This may be due to environmental fluctuations in Hg levels having a stronger effect on Hg concentrations in blood than trophic dynamics. When I compared the WHDP Hg levels to those of the sympatric CDPs, I found that WHDPs consistently had higher Hg concentration across all three sampling years. This is supported by the trophic segregation shown in chapter 2, where WHDPs appear to forage on prey of a higher trophic value, and inherently a higher Hg concentration (Honda *et al.*, 1990; Stewart *et al.*, 1999).

My results highlighted several potential conservation concerns, for which future investigations are required to understand the potential responses of the WHDP population. The Hg levels in WHDPs were higher than those of CDPs, however they did not exceed the currently accepted 'threshold' of 5.0 μg/g (Burger and Gochfeld, 1997). Characterising the physiological state of individuals with high Hg levels will reveal whether WHDPs are facing detrimental effects from Hg exposure. It would also be beneficial to measure the selenium (Se) concentration in WHDPs, as Se interacts with Hg to form the nontoxic Hg-Se complex, thereby acting as a form of protection against MeHg toxicity (Satoh, Yasuda and Shimai,

1985; Cuvin-Aralar and Furness, 1991). Finally, as maternal transfer of Hg into the egg has proven to be detrimental to embryo development (Kenow *et al.*, 2011; Goutte *et al.*, 2014), it would be valuable to measure the Hg concentration in eggs that failed to hatch. Studies such as these will improve our understanding of the threat Hg exposure poses to this vulnerable species.

CHAPTER 5

General Discussion

The purpose of my research was to characterise the foraging ecology of the Whenua Hou diving petrel (*Pelecanoides whenuahouensis*, hereafter WHDP), investigate the degree of interspecific competition over prey with the sympatric common diving petrel (*Pelecanoides urinatrix*, hereafter CDP), and examine the level of mercury exposure for these two species. In chapter 2, I used stable isotope analysis of blood and feather samples to investigate the trophic dynamics of the WHDPs and CDPs on Whenua Hou. My results showed that the isotopic niche of WHDPs during the breeding season was influenced by the effects of year and sex. The analysis of carbon isotopes showed an enrichment of the heavier ^{13}C isotope in WHDPs with each consecutive year. It also showed that male WHDPs had a higher $\delta^{13}C$ than female WHDPs. The nitrogen analysis revealed that males had a higher $\delta^{15}N$ than females, and that in 2018, WHDPs had an enrichment of ^{15}N compared to 2017 and 2019. During the non-breeding season, the isotopic niche of WHDPs was only influenced by year, with 2019 having an enrichment of ^{13}C and ^{15}N compared to the previous two years. My results showed an isotopic niche shift in $\delta^{13}C$ of ~4 ‰ and in $\delta^{15}N$ of ~3 ‰ between the breeding and non-breeding season. I used kernel utilisation density estimates to calculate the overlap of isotopic niches between WHDPs and the sympatric CDPs. The core isotopic niches of WHDPs and CDPs overlapped < 10% during the breeding season and between 16-48% during the non-breeding season. In chapter 3, I designed a multiplex-PCR protocol to identify specific prey taxa present in faecal samples from WHDPs and CDPs. The initial phase was aimed at a broad level of taxonomic resolution, to identify the presence of amphipods, decapods,

euphausiids, maxillopods, cephalopods, and fish in their diets. I successfully designed a set of primers to identify the presence of cephalopods DNA, however the myriad of obstacles faced in the development of this method demonstrated that, with additional resources, a metabarcoding approach would be more suitable to detect the prey present in the faecal samples collected (Kress *et al.*, 2015). In chapter 4, I analysed the mercury concentration in blood and feather samples from WHDPs and CDPs. My results showed that the concentration of mercury in WHDP blood was lower in 2018 than 2017 and 2019, and lower for females than males. The mercury level in WHDP feathers was higher in 2019 than the previous two years. Comparing the mercury concentration in the tissues of WHDPs and CDPs showed that WHDP blood and feathers have higher concentrations of mercury. Given the detrimental effects of mercury on the survival and reproductive success of a species (Goutte *et al.*, 2014), this may be of concern for the conservation of the 'Nationally Critical' WHDP population (Robertson *et al.*, 2017).

5.1 Intraspecific trophic dynamics for the WHDPs

My results from the stable isotope analysis revealed differences in the foraging ecology between males and females. Despite the lack of obvious sexual dimorphism within WHDPs (Fischer *et al.*, 2018), the nitrogen stable isotope results indicated that males forage on prey of higher trophic value than females (Table 2.2; Minagawa and Wada, 1984). As the carbon isotope results indicate females may also be foraging further from shore than males (Peterson and Fry, 1987), it is possible that their increased energy demands from a longer flight are not being met with energy rich prey (Dean *et al.*, 2013). Previous studies have demonstrated that the stress of travelling further to forage for prey can contribute to higher levels of stress hormones (corticosterone; Crossin *et al.*, 2012; Dunphy *et al.*, 2020), which in turn can have a negative impact on reproductive success (Kitaysky, Piatt and Wingfield, 2007). If female

WHDPs are experiencing high levels of stress during the breeding period due to the energetic demands of foraging further from the colony than males, this could be a contributing factor to the slow population recovery through compromising reproductive success.

Previous studies have demonstrated that species foraging on prey of higher trophic value have higher concentrations of mercury accumulating in their tissues (Honda *et al.*, 1990; Stewart *et al.*, 1999). The results from analysing mercury concentration in WHDP blood and feathers correlate with the trophic dynamics between males and females as males had higher concentrations of mercury in their tissues than females. As mercury has been shown to disrupt endocrine functioning and reproductive success (Heath and Frederick, 2005; Frederick and Jayasena, 2011), high levels of mercury could be contributing to the high rates of infertility seen in WHDP eggs that failed to hatch (Fischer J.H., unpublished data).

5.2 Niche partitioning between the WHDPs and CDPs

My stable isotope results revealed a degree of trophic segregation between WHDPs and CDPs during the breeding season, as the core area of their isotopic niches overlapped less than 10% for all three years (2017-2019). The WHDPs are shown to forage at a slightly higher trophic level than the CDPs, indicating they are targeting different prey during the breeding season (Post, 2002; Quillfeldt, McGill and Furness, 2005). This suggests that, during the breeding season, there is a low risk of interspecific competition from the CDP population compromising food availability for the WHDP population during the breeding season (Navarro *et al.*, 2013; Ravache *et al.*, 2020).

The results in chapter 4 are supported by the pattern of trophic segregation above, as by foraging on higher trophic level prey, WHDPs were exposed to higher levels of mercury through their diet (Lewis and Furness, 1991; Stewart *et al.*, 1999). WHDPs consistently had higher concentrations of mercury in both their blood and feather tissues across all three years.

This relatively high exposure to mercury through their diet has the potential to limit population growth by compromising individual survival and reproductive success (Tartu *et al.*, 2013; Goutte *et al.*, 2014).

5.3 Temporal variation

By analysing both blood and feathers from WHDPs, I revealed a seasonal shift in isotopic niche (Figure 2.3). I showed that the WHDPs are foraging an entire trophic level higher in the breeding season than the non-breeding season (Minagawa and Wada, 1984), suggesting they have adapted to the increased energy demands of a central-place forager providing for both themselves and their offspring (Zimmer *et al.*, 2011; Booth *et al.*, 2018).

The foraging plasticity observed, both seasonally and interannually, suggests that WHDPs may respond well to changing climatic conditions (Peers *et al.*, 2014). Isotopic analysis revealed that during the breeding season of 2018, WHDPs appeared to be foraging at a slightly higher trophic level than in 2017 and 2019, despite 2018 being an El Niño year when food supply should have been reduced (Boersma, 1978). This indicates that the changing ocean conditions and food supply resulting from climate change may not pose a direct threat to the survival of the WHDP population (Grémillet *et al.*, 2012).

5.4 Future directions

My research provides a baseline and highlights opportunities for many future research avenues to better inform the conservation management of the 'Nationally Critical' WHDP. The stable isotope analyses highlighted the potential for female WHDPs to be under additional stress as a result of foraging further from the breeding colony than males (Kitaysky, Piatt and Wingfield, 2007; Crossin *et al.*, 2012; Dunphy *et al.*, 2020). Therefore, a study into the stress hormone levels (i.e. corticosterone) of both males and females during the

breeding season may reveal whether females are under additional stress that could be compromising the breeding success of the WHDPs (Kitaysky, Piatt and Wingfield, 2007). The WHDPs have been tracked using GLS technology to understand their breeding and non-breeding distribution (Fischer J.H., unpublished data), however there is no distribution data for the sympatric population of CDPs. Populations of CDP from the north of New Zealand have been shown to migrate east during the non-breeding season (Rayner *et al.*, 2017). Tracking the Whenua Hou CDP population will reveal whether they too migrate east or whether they migrate west with the WHDPs. Therefore, by using GLS technology to study the distribution of the sympatric CDPs, the potential for interspecific competition between WHDPs and CDPs for prey during the non-breeding season can be better understood. Specific details on the dietary components of WHDPs and CDPs can be further elucidated using a DNA metabarcoding approach to detect the prey species present in the faecal samples collected (Kress *et al.*, 2015).

My results showed that WHDPs had higher concentrations of mercury in their tissues than CDPs. As mercury has several detrimental effects on seabird physiology (Hoffman, Spalding and Frederick, 2005; Tan, Meiller and Mahaffey, 2009; Ceccatelli, Daré and Moors, 2010; Goutte *et al.*, 2014), it would be beneficial to find a relatively non-invasive way to monitor the physiological wellbeing of the WHDPs. Given that previous studies have demonstrated that maternal transfer of mercury into eggs can be detrimental for embryo development (Kenow *et al.*, 2011; Goutte *et al.*, 2014), it would be valuable to analyse the mercury concentrations in eggs that failed to hatch. As approximately 64-70% of the WHDP eggs that failed to hatch over the study period were infertile (Fischer J.H., unpublished data), investigating the connection between infertility and mercury concentration will contribute to our understanding of the slow population growth for the WHDPs (Fischer *et al.*, 2020). It would also be beneficial to analyse the levels of selenium in WHDPs as selenium forms a

non-toxic complex with mercury, thereby acting as protection against methylmercury toxicity (Satoh, Yasuda and Shimai, 1985; Cuvin-Aralar and Furness, 1991).

Finally, given the substantial threat plastic pollution poses to seabird populations (Cole *et al.*, 2013; Eriksen *et al.*, 2014; Wilcox *et al.*, 2015), it would be valuable to the conservation of WHDPs to assess the amount of plastic they ingest. A method has been developed which measures plastic exposure non-invasively by analysing the oil secreted from the preen gland at the base of a seabird's tail (Hardesty *et al.*, 2015; Yamashita *et al.*, 2018). During this study period, I also collected three years of preen gland samples from each individual that was sampled for blood and feathers. However, due to the travel constraints associated with the covid-19 global pandemic, I was unable to travel to Japan to take the samples to one of the few labs that are currently performing this analysis. When possible, the analysis of these samples will reveal whether plastic ingestion is a potential threat to WHDPs and whether this needs to be included in the conservation management plan for this species.

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SUPPLEMENTARY MATERIAL

Table S2.1: Full AIC table for the linear mixed-effect models describing the sources of variability in WHDP carbon isotope ratios during the breeding season on Whenua Hou. Results are expressed as $\beta \pm$ SE. (Estimated variance component does not indicate that ID is a contributing factor).

Breeding Season $\delta^{13}C$	***k***	**Δ AIC**	**ω**	**Intercept**	β_{Year}	β_{Sex}	$\beta_{B.\ Stage}$	**Estimated variance component for ID ± SD**
Year + Sex + ID	6	0.00	0.57	-0.26 ± 0.19	2018 0.79 ± 0.19 2019 0.86 ± 0.20	-0.55 ± 0.21		0.42 ± 0.65
Year + Sex + Breeding Stage + ID	8	1.27	0.30	0.03 ± 0.33	2018 0.78 ± 0.19 2019 0.88 ± 0.20	-0.52 ± 0.21	-0.33 ± 0.31	0.44 ± 0.66
Year + ID	4	4.18	0.07	-0.54 ± 0.16	2018 0.78 ± 0.19 2019 0.87 ± 0.20			0.51 ± 0.72
Year + Breeding Stage + ID	6	4.72	0.06	-0.15 ± 0.33	2018 0.77 ± 0.19 2019 0.89 ± 0.20		-0.43 ± 0.32	0.52 ± 0.72
Sex + ID	3	15.15	0.00	0.28 ± 0.16		-0.57 ± 0.22		0.30 ± 0.54
Sex + Breeding Stage + ID	5	16.80	0.00	0.52 ± 0.34		-0.54 ± 0.22	-0.28 ± 0.35	0.31 ± 0.56
ID (Null model)	1	19.05	0.00	0.00 ± 0.12				0.44 ± 0.66
Breeding Stage + ID	3	20.08	0.00	0.36 ± 0.35			-0.39 ± 0.36	0.43 ± 0.66

Table S2.2: Full AIC table for the linear mixed-effect models describing the sources of variability in WHDP nitrogen isotope ratios during the breeding season on Whenua Hou. Results are expressed as $\beta \pm SE$. (Estimated variance component does not indicate that ID is a contributing factor).

Breeding Season $\delta^{15}N$	***k***	**Δ AIC**	**ω**	**Intercept**	β_{Year}	β_{Sex}	$\beta_{B.\ Stage}$	**Estimated variance component for ID ± SD**
Year + Sex + ID	6	0.00	0.52	0.05 ± 0.19	2018 0.63 ± 0.20 2019 -0.05 ± 0.21	-0.48 ± 0.22		0.47 ± 0.69
Year + Sex + Breeding Stage + ID	8	1.65	0.23	-0.19 ± 0.35	2018 0.64 ± 0.20 2019 -0.06 ± 0.21	-0.50 ± 0.22	0.29 ± 0.33	0.45 ± 0.67
Year + ID	4	2.28	0.17	-0.20 ± 0.16	2018 0.62 ± 0.20 2019 -0.03 ± 0.21			0.54 ± 0.73
Year + Breeding Stage + ID	6	4.30	0.06	-0.36 ± 0.35	2018 0.63 ± 0.20 2019 -0.04 ± 0.21		0.19 ± 0.34	0.53 ± 0.73
Sex + ID	3	7.34	0.01	0.21 ± 0.16		-0.46 ± 0.23		0.39 ± 0.62
ID (Null model)	1	9.17	0.01	-0.03 ± 0.12				0.46 ± 0.68
Sex + Breeding Stage + ID	5	9.34	0.00	0.05 ± 0.35		-0.48 ± 0.23	0.18 ± 0.36	0.38 ± 0.61
Breeding Stage + ID	3	11.32	0.00	-0.10 ± 0.35			0.08 ± 0.36	0.46 ± 0.67

Table S2.3: Full AIC table for the linear mixed-effect models describing the sources of variability in WHDP carbon isotope ratios during the non-breeding season on Whenua Hou. Results are expressed as $\beta \pm SE$. (Estimated variance component does not indicate that ID is a contributing factor).

Non-Breeding Season $\delta^{13}C$	***k***	**Δ AIC**	**ω**	**Intercept**	**β_{Year}**	**β_{Sex}**	**Estimated variance component for ID ± SD**
Year + Sex + ID	6	0.00	0.46	-0.35 ± 0.20	2018 -0.09 ± 0.22 2019 0.50 ± 0.22	0.37 ± 0.22	0.33 ± 0.58
Year + ID	4	0.64	0.33	-0.16 ± 0.17	2018 -0.08 ± 0.23 2019 0.49 ± 0.23		0.34 ± 0.59
Sex + ID	3	2.78	0.11	-0.19 ± 0.16		0.36 ± 0.23	0.38 ± 0.61
ID (Null model)	1	3.03	0.10	-0.01 ± 0.11			0.39 ± 0.62

Table S2.4: Full AIC table for the linear mixed-effect models describing the sources of variability in WHDP nitrogen isotope ratios during the non-breeding season on Whenua Hou. Results are expressed as $\beta \pm SE$. (Estimated variance component does not indicate that ID is a contributing factor).

Non-Breeding Season $\delta^{15}N$	***k***	**Δ AIC**	**ω**	**Intercept**	**β_{Year}**	**β_{Sex}**	**Estimated variance component for ID ± SD**
Year + Sex + ID	6	0.00	0.49	0.07 ± 0.20	2018 -0.07 ± 0.23 2019 0.49 ± 0.23	-0.42 ± 0.21	0.25 ± 0.50
Year + ID	4	1.50	0.23	-0.14 ± 0.17	2018 -0.09 ± 0.23 2019 0.48 ± 0.23		0.31 ± 0.56
Sex + ID	3	1.91	0.19	0.24 ± 0.16		-0.44 ± 0.23	0.37 ± 0.61
ID (Null model)	1	3.37	0.09	0.02 ± 0.12			0.43 ± 0.65

Table S3.1: Results from the literature search on the components of diving petrel diets and their relative abundances (%) if available.

Group	Order	Family	Species	Which closely related species eat these?	Abundance in WHDP diet (%)	Abundance in CDP diet (%)	Sources
Crustaceans	Calanoida	Calanidae	*Calanoides acutus*	CDP, SGDP	77.8	47.7	Reid et al. 1997
Crustaceans	Calanoida	Rhincalanidae	*Rhincalanus gigas*	CDP, SGDP	3.4	23.4	Reid et al. 1997
Crustaceans	Amphipoda	Hyperiidae	*Themisto gaudichaudii*	CDP, SGDP			Reid et al. 1997
Crustaceans	Amphipoda	Phrosinidae	*Primno macropa*	CDP, SGDP			Reid et al. 1997
Crustaceans	Euphausiacea	Euphausiidae	*Euphausia superba*	CDP, SGDP			Reid et al. 1997
Crustaceans	Euphausiacea	Euphausiidae	*Thysanoessa sp.*	CDP, SGDP			Reid et al. 1997
Crustaceans	Calanoida	Calanidae	*Calanus propinquus*	CDP, SGDP	18.3	19.9	Reid et al. 1997
Crustaceans	Calanoida	Calanidae	*Calanus simillimus*	CDP, SGDP	0.5	2.4	Reid et al. 1997
Crustaceans	Calanoida	Euchaetidae	*Euchaeta sp.*	CDP, SGDP			Reid et al. 1997
Crustaceans	Calanoida	Clausocalanidae	*Drepanopus sp.*	CDP, SGDP			Reid et al. 1997
Crustaceans	Amphipoda	Hyperiidae	*Hyperiella sp.*	CDP, SGDP			Reid et al. 1997
Crustaceans	Amphipoda	Hyperiidae	*Hyperoche sp.*	CDP, SGDP			Reid et al. 1997
Crustaceans	Amphipoda	Vibilidae	*Cyllopus sp.*	CDP, SGDP			Reid et al. 1997
Cephalopoda	Octopoda	Argonautidae	*Argonauta sp.*	WHDP			Imber & Nilsson 1980
Cephalopoda	Oegopsida	Histioteuthidae	*Histioteuthis atlantica*	WHDP			Imber & Nilsson 1980
Cephalopoda	Teuthida	Cranchiidae	*Teuthowenia sp.*	WHDP			Imber & Nilsson 1980
Cephalopoda	Oegopsida	Chiroteuthidae	*Chiroteuthis sp.*	WHDP			Imber & Nilsson 1980
Crustaceans				CDP, SGDP	98	100	Bocher et al. 2003
Fish				CDP, SGDP	2	<0.1	Bocher et al. 2003
Crustaceans	Euphausiacea	Euphausiidae	*Euphausia vallentini*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Euphausiacea	Euphausiidae	*Euphausia sp.*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Euphausiacea	Euphausiidae	*Thysanoessa macrura/vicina*	SGDP	50.3		Bocher et al. 2000
Crustaceans	Amphipoda	Tryphosidae	*Orchomenopsis sp.*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Amphipoda	Hyperiidae	*Hyperoche leutkenides*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Amphipoda	Hyperiidae	*Themisto gaudichaudii*	SGDP	1.5		Bocher et al. 2000

Crustaceans	Amphipoda	Phrosinidae	*Primno macropa*	SGDP	1.4		Bocher et al. 2000
Crustaceans	Calanoida	Calanidae	*Calanus simillimus*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Calanoida	Calanidae	*Calanoides acutus*	SGDP	44.5		Bocher et al. 2000
Crustaceans	Calanoida	Rhincalanidae	*Rhincalanus gigas*	SGDP	1.8		Bocher et al. 2000
Crustaceans	Calanoida	Clausocalanidae	*Drepanopus pectinatus*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Calanoida	Euchaetidae	*Paraeuchaeta antarctica*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Pedunculata	Lepadidae	*Lepas australis (cypris larvae)*	SGDP	0.1		Bocher et al. 2000
Fish	Myctophiformes	Myctophidae	*Krefftichthys anderssoni*	SGDP	<0.1		Bocher et al. 2000
Fish	Perciformes	Nototheniidae	*Notothenia rossii (pelagic stage)*	SGDP	<0.1		Bocher et al. 2000
Crustaceans	Euphausiacea	Euphausiidae	*Euphausia sp.*	CDP		<0.1	Bocher et al. 2000
Crustaceans	Decapoda	Hymenosomatidae	*Halicarcinus planatus (zoeal larvae)*	CDP		11.4	Bocher et al. 2000
Crustaceans	Mysida	Mysidae	*Mysidetes morbihanensis*	CDP		<0.1	Bocher et al. 2000
Crustaceans	Amphipoda	Hyperiidae	*Themisto gaudichaudii*	CDP		60.6	Bocher et al. 2000
Crustaceans	Calanoida	Calanidae	*Calanus simillimus*	CDP		2.5	Bocher et al. 2000
Crustaceans	Calanoida	Calanidae	*Calanoides acutus*	CDP		0.1	Bocher et al. 2000
Crustaceans	Calanoida	Clausocalanidae	*Drepanopus pectinatus*	CDP		3.7	Bocher et al. 2000
Crustaceans	Calanoida	Euchaetidae	*Paraeuchaeta antarctica*	CDP		21.3	Bocher et al. 2000
Fish			Unidentified	CDP		<0.1	Bocher et al. 2000
Cephalopoda			Unidentified squid	Peruvian Diving Petrel	<0.1		Jahncke et al. 1999
Crustaceans	Euphausiacea	Euphausiidae	*Euphausia mucronata*	Peruvian Diving Petrel	28.6		Jahncke et al. 1999
Crustaceans	Decapoda		*Caridea (megalopod)*	Peruvian Diving Petrel	<0.1		Jahncke et al. 1999
Crustaceans	Decapoda	Callianassidae	*Callianassa sp.*	Peruvian Diving Petrel	<0.1		Jahncke et al. 1999
Crustaceans	Decapoda	Munididae	*Pleuroncodes monodon*	Peruvian Diving Petrel	18.9		Jahncke et al. 1999
Crustaceans	Decapoda	Porcellanidae	*Porcellanidae (megalopod)*	Peruvian Diving Petrel	<0.1		Jahncke et al. 1999
Crustaceans	Decapoda	Hippidae	*Emerita analoga (zoea)*	Peruvian Diving Petrel	0.1		Jahncke et al. 1999
Crustaceans	Decapoda	Hippidae	*Emerita analoga (megalopod)*	Peruvian Diving Petrel	<0.1		Jahncke et al. 1999
Crustaceans	Decapoda	Xanthidae	*Xanthidae (megalopod)*	Peruvian Diving Petrel	0.1		Jahncke et al. 1999
Crustaceans	Amphipoda	Phronimidae	*Phronima sp.*	Peruvian Diving Petrel	<0.1		Jahncke et al. 1999
Fish	Beloniformes	Scomberesocidae	*Scomberesox saurus*	Peruvian Diving Petrel	1.7		Jahncke et al. 1999

Fish	Stomiiformes	Photichthyidae	*Vinciguerria lucetia*	Peruvian Diving Petrel	0.2		Jahncke et al. 1999
Fish	Myctophiformes	Myctophidae	*Mictophum nitidulum*	Peruvian Diving Petrel	3.7		Jahncke et al. 1999
Fish	Atheriniformes	Atherinopsidae	*Odontesthes regia*	Peruvian Diving Petrel	1.7		Jahncke et al. 1999
Fish	Clupeiformes	Engraulidae	*Engraulis ringens*	Peruvian Diving Petrel	9.1		Jahncke et al. 1999
Fish	Scorpaeniformes	Normanichthyidae	*Normanichthys crockeri*	Peruvian Diving Petrel	11.3		Jahncke et al. 1999
Crustaceans	Calanoida			CDP, SGDP	3.5	21	Roby 1991
Crustaceans			*Euphausia superba*	CDP, SGDP	26.3	78	Roby 1991
Crustaceans			*Thysanoessa sp.*	CDP, SGDP	63.3	0.1	Roby 1991
Cephalopoda	Teuthoidea		Unidentified	Peruvian Diving Petrel	0.41		García-Godos & Goya 2006
Crustaceans	Euphausiacea		*Euphausia mucronata*	Peruvian Diving Petrel	72.56		García-Godos & Goya 2006
Crustaceans	Copepoda		Unidentified	Peruvian Diving Petrel	0.79		García-Godos & Goya 2006
Crustaceans	Decapoda		Unidentified	Peruvian Diving Petrel	0.84		García-Godos & Goya 2006
Crustaceans	Decapoda		*Pleuroncodes monodon*	Peruvian Diving Petrel	5.08		García-Godos & Goya 2006
Crustaceans	Decapoda		*Emerita analoga*	Peruvian Diving Petrel	3.09		García-Godos & Goya 2006
Fish		Engraulidae	*Engraulis ringens*	Peruvian Diving Petrel	7.18		García-Godos & Goya 2006
Fish		Normanichthydae	*Normanichthys crockeri*	Peruvian Diving Petrel	0.53		García-Godos & Goya 2006
Fish		Atherinidae	*Odontesthes regia*	Peruvian Diving Petrel	0.04		García-Godos & Goya 2006
Fish		Photichthyidae	*Vinciguerria lucetia*	Peruvian Diving Petrel	0.01		García-Godos & Goya 2006
Crustaceans	Euphausiacea			CDP, SGDP	76	15	Payne & Prince 1979
Crustaceans	Copepoda			CDP, SGDP	20	68	Payne & Prince 1979
Crustaceans	Amphipoda			CDP, SGDP	4	17	Payne & Prince 1979
Crustaceans	Copepoda		Unidentified calanids	CDP		2.6	Ridoux 1994
Crustaceans	Cirripedia		*Lepas australis*	CDP		2.2	Ridoux 1994
Crustaceans	Euphausiacea		*Euphausia vallentini*	CDP		58.5	Ridoux 1994
Crustaceans	Euphausiacea		*Thysanoessa sp.*	CDP		7.4	Ridoux 1994
Crustaceans	Amphipoda		*Themisto gaudichaudii*	CDP		16.8	Ridoux 1994
Crustaceans	Amphipoda		*Primno macropa*	CDP		12.4	Ridoux 1994
Crustaceans	Copepoda		Unidentified calanids	SGDP	0.3		Ridoux 1994
Crustaceans	Cirripedia		*Lepas australis*	SGDP	0.2		Ridoux 1994

Crustaceans	Euphausiacea	*Euphausia sp.*	SGDP	22.3		Ridoux 1994
Crustaceans	Euphausiacea	*Thysanoessa sp.*	SGDP	70.3		Ridoux 1994
Crustaceans	Amphipoda	*Themisto gaudichaudii*	SGDP	0.5		Ridoux 1994
Crustaceans	Amphipoda	*Primno macropa*	SGDP	6.3		Ridoux 1994
Crustaceans	Euphausiacea	*Nyctiphanes australis*	CDP		87	Schumann et al. 2008
Crustaceans	Amphipoda	*Themisto australis*	CDP		12.5	Schumann et al. 2008
Crustaceans	Decapoda	*Crab megalopa*	CDP		0.1	Schumann et al. 2008
Crustaceans	Copepoda	*Calanoid copepods*	CDP		0.3	Schumann et al. 2008
Crustaceans	Stomatopoda	*Mantis shrimp*	CDP		0.01	Schumann et al. 2008

Table S3.2: Summary of all primers used for this study.

Primer Pair	**Target Gene**	**Forward Primer**	**Reverse Primer**	**Amplicon Size (bp)**	**Annealing Temperature (˚C)**	**Source**
Amph/Dec	COI	GDGTAGATATAGCTTTCCCTCGTAT	GVACCTCTATGHCCTATATWAGAAG	158	~57	This study
Euph	COI	WGCTGARTTAGGACAACCAGGWAS	AAAGCRTGRGCTGTAACWAYDAC	78	~60	This study
Maxill	COI	AGTAATATTGCCCATGCTGGRG	ATAGGGTCTCCTCCTCCACC	287	~59	This study
Ceph	COI	GACTTCTCCCTCCATCYTTAAC	GGDCCTGCATGAGATAGATTTC	116	~57	This study
Fish	COI	GCHTCATCTGGNGTWGAAGC	TGTGAAATGGCAGGAGGTTT	194	~58	This study
Chiar16S	16S	TARTYCAACATCGRGGTC	CYGTRCDAAGGTAGCATA	250-470	~55	Marquina et al. 2018
Fish16S	16S	TGCAGAAGCGGGSATAMAYWC	AGGGGATTGCGCTGTTATCCCTAG	306	~60	This study
Amph18S	18S	CGAAGGCATTGGTATTGCGG	AGGGAACGCTTTGACGGATT	187	~60	This study
Jelly16S	16S	AGTTTAGTTGGGGCGACTRYCTT	CTGTTATCCCTAARGTAGCTTTTAT	193	~58	This study
Salp18S	18S	GCCATGCAAGTGTAAGTACGAGCTC	GCGCTTCACGCATGTATTAGCTCT	136	~63	This study

Table S4.1: Full AIC table for the linear mixed-effect models describing the sources of variability in Hg concentration in WHDP blood samples collected during the breeding season on Whenua Hou. Results are expressed as $\beta \pm$ SE. (Estimated variance component does not indicate that ID is a contributing factor).

Blood Hg	***k***	**Δ AIC**	**W**	**Intercept**	β_{Year}	β_{Sex}	**Estimated variance component for ID ± SD**
Year + Sex + ID	6	0.00	0.83	0.36 ± 0.17	2018 -0.73 ± 0.18 2019 0.24 ± 0.18	-0.48 ± 0.20	0.41 ± 0.64
Year + ID	4	3.19	0.17	0.12 ± 0.14	2018 -0.73 ± 0.17 2019 0.23 ± 0.19		0.49 ± 0.70
Sex + ID	3	22.59	0.00	0.26 ± 0.16		-0.52 ± 0.23	0.49 ± 0.70
ID (Null model)	1	25.37	0.00	0.00 ± 0.12			0.58 ± 0.76

Table S4.2: Full AIC table for the linear mixed-effect models describing the sources of variability in Hg concentration in WHDP feather samples collected during the breeding season on Whenua Hou. Results are expressed as $\beta \pm SE$. (Estimated variance component does not indicate that ID is a contributing factor).

Feather Hg	***k***	**Δ AIC**	**W**	**Intercept**	β_{Year}	β_{Sex}	**Estimated variance component for ID ± SD**
Year + ID	4	0.00	0.38	-0.17 ± 0.15	2018 -0.01 ± 0.16 2019 0.40 ± 0.18		0.77 ± 0.88
Year + Sex + ID	6	0.36	0.32	0.00 ± 0.19	2018 -0.01 ± 0.16 2019 0.40 ± 0.18	-0.33 ± 0.24	0.74 ± 0.86
ID (Null model)	1	1.82	0.15	-0.03 ± 0.12			0.78 ± 0.88
Sex + ID	3	1.96	0.14	0.15 ± 0.17		-0.35 ± 0.24	0.74 ± 0.86

Diving petrel burrows on Sealer's Bay, Whenua Hou. Photo credit: Grace Tocker, 2019