From fish to fowl: a comparison using stable isotope analysis of the little penguin [*Eudyptula minor*] in captive and wild populations

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Submitted for Masters of Science in Ecology

University of Canterbury

School of Biological Sciences

2016

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Acknowledgements

My everlasting thanks to my husband Brian, as always, for his understanding and unending support. My parents, Rick and Elaine, for showing me what education can help you achieve in life and their assistance in my own journey. Many thanks to my sister Kara for her editing suggestions and wonderful maps. My work mates and fellow penguin keepers, this would not have been at all possible without your support and assistance in sample collection and cheerleading. To my penguins, for their feathers and eggs, but not for the bites received while in my care as a penguin keeper. My everlasting gratitude to Tim Coleman for giving me a job as a cleaner at the Antarctic Centre way back when; where I got to have so much fun and work up through the ranks, receptionist, ticketing chick and guide, driving Hagglunds, and finally, becoming a penguin keeper; thus developing the love for the birds under my care and fostering an addiction to penguin related information.

To my fellow grad student Della, your moral support has been invaluable when it came to the statistics!

I am forever in your debt, Terry and Nicola, for your pioneering the 'adopt a homeless grad-student' program.

Further thanks to my supervisors Prof. James Briskie, Dr. Richard Holdaway and Dr. Travis Horton for the moral support and editing suggestions. Most especially to Richard who was dedicated to helping keep my hand in while I was working at real life.

Further thanks to those out on the front line collecting feathers: Philippa Agnew of the Oamaru Penguin Colony, Reuben Lane of the West Coast Penguin Trust, Shireen Helps of Pohatu Penguins in Flea Bay, Banks Peninsula, and Chris Challias Harris Bay.

This work is dedicated to my father, who without his support this thesis would have never been started.

Richard Lewis Bushman

1947-2015

Abstract

I collected moulted feathers from a captive group of little penguins [*Eudyptula minor*] over the course of two years for stable isotope analysis [SIA]. Diet was also sampled during this time, and I was able to connect the isotopic signatures of the food sources to that of the moulted feathers formed and thereby ascertain enrichment differences between diet and feathers. Feathers were also collected from three colonies of wild little penguins on the east coast of the South Island and from several sub-colonies on the West Coast. I used SIA of $\delta^{13}C$ and $\delta^{15}N$ of the feathers to determine whether trophic level depended on the geographic location of each colony. Relationships between the $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures of penguins from the wild colonies were then compared to the captive colony which comprised of individuals from around New Zealand. I next examined whether the "white-flippered penguin" [*Eudyptula minor albosignata*], a colour morph or subspecifies confined largely to Banks Peninsula, differed in stable isotope ratios from other populations. Finally, I also explored variation in isotopic signatures based on gender and geographic origin of the birds within the feathers produced by birds being fed on each diet/fish lot. I found that while the isotopic signatures of penguins fed on different lots of sprats were not significantly different in $\delta^{15}N$ signatures, feathers produced on diet/fish lot 1 were different from other feather lots, and different from feathers produced from other diet/fish lots. There were no significant differences in either $\delta^{13}C$ or $\delta^{15}N$ isotopic compositions between penguins on the basis of their geographic origin in the captive population. Male and female birds also did not significantly vary in $\delta^{13}C$ or $\delta^{15}N$ when fed on an identical diet. Using $\delta^{15}N$, I found penguins on the West Coast occupied a lower trophic level than the east coast birds. Similar differences in the $\delta^{13}C$ ratios also confirmed the birds on the two coasts were feeding on different prey species and were occupying different trophic levels. Surprisingly, a colony of the white-flippered morphs at Harris Bay was more similar in both $\delta^{13}C$ and $\delta^{15}N$ compositions to the more distant Oamaru population than to another, geographically closer, colony of the white-flippered morph in Flea Bay. My study confirms that SIA can be used to provide a general estimate of diet and analysis of feathers from wild populations could provide information on the diet [and trophic level] of free-living penguins. There was no difference between birds based on colony of origin when fed an identical diet, but there was variation between wild colonies, indicating that while diet influences the composition of the feathers, morphological differences do not. My study highlights the value of using SIA as a proxy for diet studies of wild seabirds.

Glossary

δ symbol – used to express isotope values, it denotes a measurement of difference made relative to standards during sample analysis.

‰ symbol – stands for parts per thousand or 'permil'.

$\delta^{13}C$ – delineates a measure of the ratio of the stable isotope of carbon. The international standard used is Vienna PeeDee Belemnite.

$\delta^{15}N$ – delineates a measure of the ratio of the stable isotope of nitrogen. The international standard used is Air.

All information for the definitions has been taken from Fry (2006).

Abbreviations

SIA – Stable Isotope Analysis

IAC – International Antarctic Centre

Chapter 1 Literature review

1.1 Introduction

Seabirds generally occupy roles as the top predator in most marine ecosystems. As a group, they are wide-ranging, occur in a variety of habitats, and possess a wide variety of foraging strategies (Schreiber & Burger 2001). Some species can spend up to 80% of their time at sea feeding (Bost & Maho 1993). As a consequence of their diversity and range across the world's oceans, seabirds have often been used as indicator species of the marine environment (Cairns 1987; Bost & Maho 1993; Diamond & Devlin 2003). Recent concern over global climate change, including changes in ocean temperatures and weather patterns (Hoegh-Guldberg et al. 2007; Wilson et al. 2014), has led to concerns over the potential effects on food availability and foraging patterns of seabirds (Edwards & Richardson 2004). For any species, understanding how fluctuations in food availability and trophic level might affect the dynamics of a population will first require basic information on the role of each species in a local ecosystem and how that varies both spatially and over time.

Marine ecosystems generally operate on a large scale and can be quite complicated, so a candidate indicator species needs to be one that responds to change and can act as an early warning signal. Because food sources are closely linked with the environmental condition of seabird foraging areas, they have been long used as indicators of ecological "health" (Cairns 1987; Piatt et al. 2007) and various species have been used for bio-monitoring and as bio-indicators (Furness & Camphuysen 1997). Changes in the physical environment of an ecosystem can influence prey availability, and in turn, regulate the number of predators. This suggests that top marine predators, such as seabirds, can be suitable indicators of long-term variability and change within the ecosystem in question (Durant et al. 2009). Because seabirds are dependent on the oceanographic conditions under which they carry out their lives (Diamond & Devlin 2003), and their populations likely reflect spatial and temporal variability in their prey, they can reflect the status of that environment (Trathan et al. 2015). The key to using seabirds to monitor changes in the marine environment is to choose a species that reflects various aspects that have an impact on the whole environment in a negative fashion and to select suitable methods for assessing how a species fits into a given marine food web.

A first step to understanding the role of a species in a marine ecosystem is to determine its diet and how it fits into the local food web. The diets of seabirds have traditionally been investigated from an examination of stomach contents (Flemming et al. 2013) and through faecal analysis (Deagle et al. 2010), but such methods do not reveal the diet of a species over the long term and

variation in digestion rates of prey are also not taken into account by such "snapshot" approaches (Kelly 2000). An alternative approach is to use longer-term measures of diet as revealed through stable isotopic ratio analysis. Isotope analyses can provide both a short and long-term assessment of a species' diet [if measurements are also made of potential dietary items], possible trophic level in the marine food web, and how this might be affected by environmental changes (Cherel et al. 2005a; Hebert & Wassenaar 2005; Jaeger et al. 2010; Polito et al. 2011), although baselines must be set with which to compare changes within a species and between species (Mizutani et al. 1992). Stable isotope ratios can be used to map trophic levels within an ecological system and further mapping of food webs provides a wealth of information on the surrounding ecosystem (Graham et al. 2010). From the ratios between trophic levels in one species, a *ratio map*, with added layers of complexity, can be developed for different mixes of prey species (Bowen et al. 2010). Stable isotope analysis [SIA] has been used to investigate the feeding biology of both living and prehistoric populations (Holdaway et al. 2013), and can provide information on diet from within a range of hours up to the lifetime of the animal, depending on the tissue sampled. Accordingly this method can be used in a wide variety of situations and studies.

1.2 Stable isotopic analysis [SIA]

Stable isotopes are a small percentage of elements that do not undergo radioactive decay (Inger & Bearhop 2008), but are subtly different because of one more neutron present which makes the atom ever so slightly heavier. For example, the rarer isotope of nitrogen has one more neutron and a heavier weight than its more common form. This small subset of heavier atoms can be tracked because they behave in predictable ways. SIA is the examination of a physical mass-dependent phenomenon in which the lighter isotope of an element is preferentially used in metabolic processes over the heavier isotope. The discrimination against the heavier atom is predictable and can be tracked (Peterson & Fry 1987). The use of stable isotope ratios of carbon and nitrogen is based on the premise that the relative amounts of each isotope in animal tissues change [i.e., fractionate and undergo the process of isotopic discrimination] as metabolic processes preferentially incorporate lighter atoms during the assimilation of nutrients from the diet (DeNiro & Epstein 1976; DeNiro & Epstein 1978, 1981). When the stable isotopes of carbon and nitrogen are fractionated, the lighter atoms are used first as the nutrient source materials move through biological systems of either organisms or wider ecological systems. Measuring the magnitude in the shifts of relative abundance of the light and heavy isotopes can be used to track changes in temporal and spatial variability between populations in different

areas. The stable isotopes of [H] hydrogen, [C] carbon, [N] nitrogen, [O] oxygen, and [S] sulphur are considered the most useful as they have been well studied (Fry 2006). Isotope values are reported in parts per thousand, denoted by the symbol ‰ and the standard symbols for isotopic ratios, $\delta^{13}C$ and $\delta^{15}N$, are used throughout this thesis (Coplen 2011). The δ/delta value is used to denote the relative difference of the ratio of the heavy isotope to the light isotope of the element (Fry 2006).

1.2.1 *Using SIA to monitor the marine environment*

Monitoring can be defined as "the process of gathering information about system state variables at different points in time for the purpose of assessing system states and drawing inferences about change in state over time" (Yoccoz et al. 2001). How can seabirds be used as indicators of productivity and marine ecosystems? Direct measurements of body mass and clutch numbers, for example, have been used as evidence for changes in the marine environment, but these measurements are only taken on land when the birds are accessible on the nest. More information is generally needed during the foraging periods and during the non-breeding season, but it can be difficult and expensive to track an individual bird's behaviour at sea. If direct observation is not ideal for long-term collection of data, then one must be able to use other materials at hand. The collection of feathers, excrement and other metabolic products are valuable for SIA as these can provide a means of assessing different trophic connections and diet during breeding, differences between the sexes, and in some species, differences during non-breeding and breeding periods (Barrett et al. 2007).

SIA has been in use for several decades by geologists (Kelly 2000; Sharp 2007) but only relatively recently was it discovered to be an ideal way to track chemical processes in biology and ecology (Chisholm et al. 1983; Swerhone et al. 1991; Tuross et al. 2008). Its use in ornithology and in marine ecosystems has grown rapidly over the last 25 years (Hobson 1991; Cherel et al. 2002; Cherel & Hobson 2007; Kojadinovic et al. 2008; Polito et al. 2011; Trueman et al. 2012). Carbon stable isotope ratios can reflect different foraging areas and reveal possible dietary information (Rubenstein et al. 2002; Rubenstein & Hobson 2004). Nitrogen isotope ratios show trophic level equivalency, and these can become more enriched as trophic level increases as more of the heavier isotope of the element is present as the lighter isotope has been preferentially used (Minagawa & Wada 1984; Hobson & Welch 1992; Hobson et al. 1994a; Forero et al. 2005; Moody et al. 2012).

Hobson (2011) outlined the three main applications of SIA in ornithology: delineation of diets or trophic relationships and sources of nutrients to individuals or populations, assessment of the relative contributions of endogenously and exogenously derived nutrients to reproduction in birds that travel to breed and, the assignment of origin to migratory individuals. SIA can be performed on various metabolic products that can be sampled either non-destructively, such as beak and claw samples, feathers, eggs, and blood, or by destructive sampling, such as bone collagen and organs (Hobson & Clark 1992c; Bearhop et al. 2003; Kojadinovic et al. 2008; Hipfner et al. 2010). Dietary information that spans different time scales can be obtained depending on the type of tissue chosen (Hobson & Clark 1992c).

Periods of synthesis vary between different tissues of the consumer [i.e. the subject of the investigation]. Short-term changes in isotopes can be tracked using feathers, or blood (Cherel et al. 2005b), or eggs (Emslie & Patterson 2007), while medium to long-term changes have been determined using samples of either internal organs or bone collagen (Hobson & Clark 1992b; Bearhop et al. 2002). Nevertheless, the method is not without problems because confounding factors in tissue production could alter the isotopic ratios presented, if a process were nutrient-limited and the organism mobilised stored dietary components, or if the isotopic discrimination in metabolic processes changed. Processes such as growth, migration, starvation, and moulting have been known to change the speed and energy sources of metabolic activity (Bearhop et al. 2002). There are limitations to any one method, and an awareness is needed of other factors, in addition to diet, that may influence the isotopic reading in seabird tissues (Bond & Jones 2009). These factors can include body condition (Hobson et al. 1993), metabolic rate (Podlesak & McWilliams 2006), and foraging area (Quillfeldt et al. 2008).

1.2.2 *Trophic level and diet reconstruction using SIA*

SIA can be used as a representation of diet, in that it depicts metabolic processes, and also to identify the trophic level(s) of individual species (Jacob et al. 2005; Cherel et al. 2007a). Changes in stable isotope ratios can indicate a trophic level shift (Chiaradia et al. 2010), in either a seasonal fashion (Awkerman et al. 2007; Jaeger et al. 2010), or to reveal a shift in prey availability because of environmental change (Croxall et al. 1999; Edwards & Richardson 2004).

Isotopic variation and gradations are present in the nutrient cycling of oceanic basins, both between, and also within each basin, spanning the base of the food web, from primary producers to the top predators/consumers; as a result such differences can produce geographically distinct

gradients in nitrogen and carbon isotopes (Kelly 2000; Graham et al. 2010). The variability of both $\delta^{15}N$ and $\delta^{13}C$ derives from differences in the isotopic ratios of carbon and nitrogen that are available for nutritional uptake by organisms at the base of a food web, and from there, on the variable expression of isotopic discrimination during the uptake process (Post 2002).

Finding the source(s) of variation in isotopic signatures of different marine species, and understanding the degree and cause of the differences between the isotopic signatures of consumers and the resource, form the foundation of SIA in ecology (Boecklen et al. 2011). The underlying principle is that stable isotope deviations in consumers will reflect that of their prey as they become enriched in a predictable fashion (Kojadinovic et al. 2008). This is done with $\delta^{15}N$, which is a stable isotope of the element nitrogen (Fry 2006). The ratio of nitrogen isotopes can exhibit a stepwise enrichment and this separation is caused by the selective retention of the heavy isotope and the excretion of the lighter isotope (Kelly 2000). To understand and trace pathways of isotopic discrimination, one must first determine the isotopic shift between consumer and diet, and have an awareness of how processing methods can affect resulting data (McCutchan et al. 2003).

The isotope of carbon [denoted by $\delta^{13}C$] is used in a similar fashion, but it has been shown to be useful as more of an indicator of geographical latitude, or nutrient location of origin of dietary carbon, rather than for analysis of trophic level from $\delta^{15}N$ (Post 2002; Kojadinovic et al. 2008). Carbon does not show the same differential function as nitrogen because it passes through an animal's tissues. This is because the ratio of $\delta^{15}N$ to $\delta^{13}C$ has a linear covariant relationship (Kelly 2000). Primary producers have different $\delta^{13}C$ values that vary predictably. The assumption is that if an individual has a similar isotopic ratio as the local baseline, then that individual can be considered a resident of that area of primary producers. If the individual has a different isotopic ratio, then it can be considered to be a resident from another isotopically distinct region (Graham et al. 2010).

There are a number of alternative methods by which the diet and identity of prey species can be ascertained, including direct foraging observations (Kelly 2000), stomach flushing (Flemming et al. 2013), and DNA analysis of excrement (Deagle et al. 2010; Chiaradia et al. 2014), but each of these methods have their own set of biases. SIA can aid in diet reconstruction (Sydeman et al. 1997; Chiaradia et al. 2014). Bayesian mixing models can help to identify the contribution of different food macronutrients that comprise the total dietary signal found in the consumer (Fernandes et al. 2014) and can help quantify the degree of certainty over the origin of the food/prey (Inger & Bearhop 2008). With a complementary approach, it has been shown that

creating a model where the DNA analysis of prey and the digestion rates of prey were combined with stable isotope mixing models has provided better estimates of actual diet in top marine predators (Chiaradia et al. 2014).

SIA has been used to identify dietary shifts, and for linking reproductive failure as a result of dietary shift (Kowalczyk et al. 2014). Changes in prey species availability, to a lower nutritional and lower trophic level, have been linked to population declines (Karnovsky et al. 2012). Aspects of diet composition are complex and finding links that could contribute to reproductive failure could be related to both the availability and nutritional composition of each prey type available. If the main and/or desirable prey species types are reduced, then alternative sources must be used. Conversely, the less preferred source could also be scarce or nutritionally inferior to the preferred prey species. For example, Kowalczyk et al. (2014) found the adverse effect of reduced anchovy [*Engraulis australis*] abundance on breeding success in little penguins [*Eudyptula minor*] was further exacerbated by scarcity of alternate prey species. The ability to adjust to prey type availability is regulated by resource diversity and abundance; these are components of ecosystem status that affect more than just seabirds.

Monitoring of trophic dimension and annual reproductive success of a generalist seabird species can bestow further awareness of resource diversity and abundance in localised foraging areas and prey type (Kowalczyk et al. 2014), but more than prey availability contributes to overall breeding success. Habitat availability and disturbance (Weerheim et al. 2003), predation by introduced predators (King 1974), and sea surface temperature all contribute (Chiaradia & Kerry 1999; Cullen et al. 2009). Sea surface temperature can influence which fish species are abundant, because temperature can affect several ocean processes (Chambers et al. 2014; Carroll et al. 2016) as well as the ideal conditions for fish hatching and growth (Peck et al. 2012). An awareness of the whole picture is required to quantify appropriately the information found.

The study of isotopic ratios can now provide more information about different ecological and biological processes on the metabolic level of individuals and on the larger level of whole ecosystems. However, unless there is an appropriate baseline for comparison, the isotopic signature of a consumer is not sufficient to infer trophic position or carbon source (Post 2002). A baseline must be developed that, ideally, integrates isotopic movement at a similar time scale to that of the consumer in question, and one that covers the same time period, and captures the spatial variability that comes with complicated systems (Post 2002). Post (2002) also states that to obtain the resolution required to discern complex trophic interactions, stable isotopes must be used in conjunction with other information, such as direct diet analyses. Separating isotopic

signals of similar prey from coinciding trophic positions and allocating trophic niche variation when investigating and developing food web studies can be difficult (Iverson et al. 2007; Lorrain et al. 2009; Jaeger et al. 2010). Any one method, when used on its own, cannot provide a complete portrayal of food web function and structure. Only by applying a variety of research methods and by having an understanding of the life history and species interactions will a comprehensive view of the ecosystem be obtained (Layman et al. 2007). SIA is a powerful tool, but one to be used with other methods in a complementary fashion to form the most comprehensive picture. In some instances, information from captive populations can supplement our understanding of how isotopic ratios relate to changes in diet.

1.2.3 *SIA studies in captive species*

There are various considerations when carrying out observation and sampling of captive animals, including different energy requirements and metabolic rates (Hobson & Clark 1992b; Bearhop et al. 2002). The average age of the subjects can be skewed because of the longer life spans experienced in captivity. For example, the average life span for a wild little penguin is 6.5 years (Reilly & Cullen 1979) but the average age of the captive colony used in this study is approximately 10.3 years.

Captive animal studies are invaluable for the development of an isotopic baseline for comparison (Mizutani et al. 1992) so that results from similar samples of wild populations are comparable. A number of studies on horses, pigs, and seals have been undertaken in which the isotopically distinctive diet of the captive animals was changed in an effort to find the time line of isotopic discrimination, or fractionation, in different tissue samples (Hobson et al. 1996; Kurle 2002; Ayliffe et al. 2004; Tuross et al. 2008). A study using blood and feathers of captive king penguin [*Aptenodytes patagonicus*] and rockhopper penguins [*Eudyptes chrysocome*] by Cherel et al. (2005b) found that nutritional status and quality and type of diet can influence isotopic discrimination. They also found that these two species of penguin were isotopically segregated even when they fed on the same diet. Stable isotope work on captive animals must be used with caution, because they are generally well fed on a fairly homogenous diet and can have a lower metabolic rate (Hobson & Clark 1992c) as a result of not spending their day in activities similar to that of their wild counterparts.

1.2.4 *Use of feathers in SIA*

Feathers are inert once they have finished growing, and show the window of development and growth in the stable isotope ratios of the keratin (Cherel et al. 2005b). Because the penguin moults all at once, instead of throughout the year like most other birds, feathers grown during this time can represent the general isotopic composition of the diet from an intensive pre-moult foraging period (Cherel et al. 2005c) undertaken before the birds arrive on land to fast during their moult (Gales et al. 1988). Feather production in some penguins has been shown to start while the birds are pre-moult foraging at sea (Cherel et al. 1994).

The collection of feathers is non-invasive, non-destructive, and results in no final harm to the birds. Feathers are easy and inexpensive to gather, especially if colonies are monitored on a regular basis, particularly during the moult season. Conversely, feathers can present a somewhat skewed picture because it, as noted by Hobson et al. (1993) during fasting, that variations in the rate of $\delta^{15}N$ excreted have been observed because of the additional stress of going without food (Hobson et al. 1993). This has been shown to present as a moderate increase in the trophic level of the penguins (Cherel et al. 2005c) due to the source of the amino acids used for keratin production derived initially from dietary resources, and during the fasting phase, from endogenous protein reserves (Cherel et al. 1994).

Comparing feather and prey isotope signatures between different seabird species has shown that differences in foraging areas exist, but when stable isotope data is unavailable for some species, other isotopic data of similar species has been substituted (Cherel et al. 2005b). This can be fraught, it has been shown that isotopic discrimination can vary within a species on different diets (Becker et al. 2007), let alone using a different species as proxy for another. Differences must be considered when the information used for comparison was derived from experiments that used different methods for sample preparation, such as the removal of lipids and the use of whole prey versus prey muscle. These subtle differences can affect the end isotopic reading (Cherel et al. 2005b; Post et al. 2007). For this reason, care must be taken when interpreting and comparing results.

The knowledge of patterns of movement by wild animals is vital to ongoing conservation as well as a thorough understanding of feeding ecology and life history (Cherel & Hobson 2007b). Studies that undertake the comparison of different geographically located populations of the same species are invaluable towards this understanding of patterns of movement. Louzao et al. (2011) used blood samples to look for spatial connections between breeding and foraging grounds of an endangered European seabird and results suggest that each different population

was using a geographically separate foraging area. In another study, blood values of $\delta^{13}C$ allowed Cherel and Hobson (2007b) to differentiate between sub-populations of penguin species that were found to be foraging in different areas within the same archipelago. If the isotopic range between geographically separated populations of the little penguin is known, then we could judge whether or not each somewhat isolated population [aside from other factors such as mammalian predation] were thriving, failing, or maintaining when compared to the overall population.

1.3 Penguins as marine predators

Penguins are a unique family of flightless seabirds, capable of living in extreme environments and found around most of the Southern Hemisphere, and in some cases right up to the equator (Gales & Stahel 1989). New Zealand is home to several species of penguin (Warham et al. 1986). Penguins are predators and primarily obtain their prey by diving in subsurface marine waters (Croxall & Davis 1999). As a consequence, they are dependent on areas of high ocean productivity where they can find prey (Boersma 2008). High density penguin populations occur near upwellings, areas of cold nutrient-rich ocean currents rising to replace surface waters, and convergences, which are areas of ocean where different water masses join (Gales & Stahel 1989; Shealer 2001). These areas tend to possess characteristics that result in areas of high marine productivity and support extensive food webs/ecosystems, all of which contain creatures such as krill, fish, and squid that are eaten by penguins (Gales & Stahel 1989). These areas of high marine productivity are both ecologically and economically important.

There are currently 16 accepted species of penguin (Boersma 2008). Some species, such as the Magellanic penguin [*Spheniscus magellanicus*], are migratory and visit their colonies only for breeding (Stokes et al. 2014), while other species are year-round residents, such as the little penguin and yellow-eyed penguin [*Megadyptes antipodes*] in New Zealand (Croxall & Davis 1999). In general, sedentary penguin species are inshore feeders, whereas the migratory species typically feed offshore; this difference is most noticeable during the incubation period (Croxall & Davis 1999).

Boersma (2008) found that the distribution and abundance of Magellanic penguins was closely linked to changes in prey. She observed over the course of several years that Magellanic penguins were traveling further and taking longer on foraging and incubation trips. By the end of her study, breeding penguins were found to be foraging 60 km further from the coast then they

did a decade previously, evidently to meet the migration of key prey species. She observed that because of the increased duration of the foraging trips, chicks were starving in the nest as the foraging parent was away for longer periods of time. Boersma (2008) cautions that climate warming is likely to shift prey species, which could reduce productivity of the birds if they have to expend more energy on travel. Where the foraging patterns of penguins have been linked to patterns of oceanographic production, changes in the diet of penguins could be a useful marine indicator of production (Boersma et al. 2009).

Another example of penguins being used as a marine sentinel is a study by Croxall et al. (1999), who examined the availability of Antarctic krill and the way its abundance affected productivity responses of different seabirds. They found that during a decline in the krill numbers, the black-browed albatross [*Diomedea melanophris*] maintained meal size at the expense of trip duration, because they had to fly to search for more food, but in contrast gentoo penguins [*Pygoscelis papua*] maintained trip duration, but with no increase in time spent foraging, and therefore suffered a decrease in meal size as a result. This sort of information on the relationship between predator performance and prey availability is important for an indicator species because it helps in the assessment of the potential consequences of competition between animals and humans for natural resources and further change in environmental cycles (Croxall et al. 1999).

1.4 Climate change, prey availability and trophic mismatch in penguins

Changes in prey availability can change the trophic level at which prey is taken (Chiaradia et al. 2003; Emslie & Patterson 2007; Chiaradia et al. 2010). Depending on the nutritional value to the hunting bird, this change in trophic level can negatively affect breeding success (Gutowsky et al. 2009; Morrison & Thompson 2014), and depending on how long a period this trophic misstep lasts, the feeding population could negatively suffer over a long enough period that it may not be able to sufficiently recover (Furness & Monaghan 1987). Kowalczyk et al. (2014) addressed concerns about the lack of high trophic and nutritional prey where they linked poor breeding success to low dietary diversity, and a good year for reproductive success, with the availability of high value prey, such as the anchovy. The little penguin was one species judged to be quite vulnerable to fluctuations in food availability (Gales & Green 1990).

Mass mortality events, such as the 1995 and 1998 pilchard [*Sardinops sagax*] die off in the waters around southern Australia (Griffin et al. 1997; Whittington et al. 2008), and the resulting changes to consumer populations have been well studied. Surveys were carried out on the Phillip

Island population of little penguin during this time. Prey availability of pilchard was low and studies, though somewhat contrasting, found correlations to breeding success (Dann et al. 2000; Chiaradia et al. 2003). Dann et al. (2000) observed that of the 1,926 dead penguins found during the die off, 131 were from Philip Island and of those 86% were adult birds; only 14% were first year birds. Of the autopsies carried out, 90% showed death from starvation and mild to severe intestinal parasite presence (Dann et al. 2000). Chiaradia et al. (2003) pointed out that the little penguin, in the absence of the availability of pilchards, had shifted to other types of prey, including barrocouta [*Thyrsites atun*] and red cod [*Pseudophycis bachus*], and suggested that the absence of pilchards was not necessarily enough to reduce breeding success even after an initial bad year [December of 1995] of 0.3 chicks raised per pair. The next year's breeding success was quite high [when compared to the average annual mean of 1.0 chicks raised per pair] at 1.3 chicks raised per pair (Chiaradia, Costalunga et al. 2003), though the chicks produced were lighter at fledging than normal (Chiaradia et al. 2010). Although the diet had shifted to include other species, two years after the first pilchard crash in 1995, breeding was again low (Chiaradia et al. 2010). After a second crash of the pilchard in 1998, breeding success of the little penguin in Phillip Island was back within the overall average, and it is suggested that the penguins were still feeding on a varied diet without pilchards and avoided a repetition of the poor breeding season of 1995 (Chiaradia et al. 2010).

Widespread populations are thought to be less susceptible to localised environmental changes, meaning that one portion of the population can escape dealing with negative changes in another area. Changes in the oceanic conditions, diet segregation and nesting sites, if over a large enough area, could tip more than one population of penguins too far past the point of recovery (Furness & Monaghan 1987; Chiaradia et al. 2012). But first there needs to be an understanding of what a "relative range of normal" is in different areas with different environmental pressures (Mizutani et al. 1992; Inger & Bearhop 2008). A knowledge of the current average isotopic signature of key species could help to understand the effects that any changes could have on local populations.

1.5 The little penguin

The little penguin [*Eudyptula minor*] [Maori: Korora, (Taylor 2000)], is found around the coast of New Zealand. It is also commonly referred to as the little blue penguin, and as the fairy penguin in Australia, where it is found on the southern coast (Gales & Stahel 1989). The International Union for the Conservation of Nature [IUCN] Red List classifies this species as

decreasing but of “least concern” (BirdLife International 2012). The species has been classified as “at risk”, and declining in New Zealand (Miskelly et al. 2008; Robertson et al. 2013).

The little penguin is an ideal study subject for understanding how birds interact with their environment and human impacts on this environment. Because of their widespread distribution throughout the coastlines of New Zealand, comparisons can be made between colonies that differ in local environmental conditions. Geographic variability between colonies on both the North Island and South Island also provides an opportunity to investigate possible differences between the colonies, using a variety of comparisons within the SIA arsenal. These differences could be dietary, physiological, or within ecological interactions, as well as male to female differences in foraging habits and differences in preferred prey among individuals within colonies.

Introduced predators in both New Zealand and Australia are currently reducing the numbers and distribution of little penguins. Introduced predators of little penguins in New Zealand include stoats, ferrets (Hocken 2000), cats (Reilly & Cullen 1979; Gales & Stahel 1989; Reilly 1994), and dogs (Wilson et al. 2012). In addition to predation on the birds themselves, rats have been recorded preying on eggs (Gales & Stahel 1989). Further threats include direct conflict with human activity while on land [e.g. road kills], oil spills while at sea (White & Conayne 2012), and the indirect impact of human-mediated habitat modification (Heber et al. 2008; Wilson et al. 2012), and death from rubbish in the bird’s environment (Dann 1991). Extensive mammalian predator trapping in some areas has had a documented positive effect on little penguin numbers, indicating the detrimental effects of introduced species (Allen et al. 2011).

The little penguin, in the past, included several subspecies, but the limits of each have been the subject of much debate in the literature. Kinksy and Falla (1976) first addressed little penguin taxonomy by examining museum specimens and live birds. They proposed six subspecies of little penguin, consisting of five subspecies in New Zealand and the sixth located on the southern shores of Australia. This was undertaken as a preliminary study and was later found to be inconsistent with some basic parameters, such as seasonal variability and time of the year in body weight and pre-moult feather condition (Meredith 1984; Meredith & Sin 1988b). A genetic analysis, however, of four populations of the little penguin (Meredith 1984) suggested clines be used to describe geographically different groups instead of different populations being classified as different subspecies. Further support of the use of clines was suggested by Meredith and Sin (1988a), although they only sampled little penguin populations from the northern part of the South Island. More recently, Banks et al. (2008) compared mitochondrial gene regions between various Australian populations, and confirmed the existence of two main clades between

Australia and New Zealand but found little in the way of genetic separation of different Australian penguin populations. An Australian study, also using mitochondrial genes, established that there were some genetic differences between geographic colonies that progressed in a weak graded fashion, but this was only significant when the population of one Australian coast was compared to the opposite coast (Overeem et al. 2008). One study on the genetic differences of the little penguin suggested the existence of two clades; the Otago /Australian populations and the rest of the little penguin population found throughout New Zealand (Banks et al. 2002). The latest work using ancient-DNA by Grosser et al. (2016) has concluded that the current group of Otago birds have originated as the result of a recent colonisation from Australia.

Despite the controversy over the subspecific status of little penguins, one population of this species has long been recognised as morphologically unique. The white-flippered penguin [in the past referred to as *Eudyptula minor albosignata*], as its name suggests, tends to possess flippers with a white strip around the outside of the entire flipper. These birds also tend to be a lighter grey blue and weigh on average 0.3 kg more than other little penguins, which range between 800 g and 1 kg (Allen et al. 2011). The white-flippered penguin has been classed as an endemic subspecies, with an IUCN rating as endangered in Taylor (2000) and as nationally vulnerable in the Conservation status of New Zealand Birds document published by the Department of Conservation (Robertson et al. 2013). Banks et al. (2002), however, did not find any significant difference in mitochondrial sequences between this and other populations of little penguin. The white-flippered penguin is mostly confined to the Banks Peninsula area on the east coast of the South Island, although there are records from Oamaru, some 300 Km further south (Hocken 1997). Surveys undertaken in 2000-2001 and 2001-2002 breeding seasons by Challies and Burleigh (2004) estimated the total population of white-flippered penguins at 10,460 birds. Evidence of predation by ferrets and stoats was noted during the course of the survey. They estimated that the current population of the white flippered penguin is much reduced when compared to the time of European settlement (Challies and Burleigh 2004).

There are some who argue that the white flippered penguin should be classified as a separate species (Baker et al. 2006). On the other flipper, some have suggested that the *albosignata* subgroup is merely a colour morph (Sitar-Gonzales & Parsons 2012). Personal observation at the Antarctic Centre showed two individuals in a relationship of which one was a white-flippered female and the other a male from the West Coast of the South Island. They have been “together” for several years and have parented laid [real] and incubated [false] eggs as a couple so the possibility exists that the populations would be quite compatible should they be exposed to individuals from other areas.

1.5.1 *Little penguin ecology and life history*

The little penguin is classed as polyphasic, as it can be active both during the day and also at night (Gales & Stahel 1989). On average, sleep duration measures four to eight minutes at a time, undertaken throughout the day and the night (Stahel et al. 1984), and as I have observed, these birds sleep while in the water or on land. If they have spent the night on land, they will then head out to sea at dawn and spend the day hunting for small prey (Gales & Pemberton 1990). Time whilst at sea can vary, from a few hours to several days at a time, depending on the time of the year (Gales & Green 1990).

Penguins are quite agile and dive in pursuit of prey. The foraging range of the little penguin can, again depending on the season, range up to several hundred kilometres from their colony (Weavers 1991; Collins et al. 1999). Because of the limitations of studying behaviour at sea, there is little information about their foraging techniques, although this species has been shown to engage in short and shallow dives (Ropert-Coudert et al. 2003). Diving studies have found that little penguins dive between 10 m to 20 m (Ropert-Coudert et al. 2006b), though dives of up to 27 m (Bethge et al. 1997) and a maximum of 66.7 m (Ropert-Coudert et al. 2006a) have been recorded.

Pursuit diving is used when penguins are foraging during the day (Shealer 2001) and on return trips to their colonies in the evening. They hunt during the day, though foraging trips at sea can last several days to a number of weeks depending on the season (Ropert-Coudert et al. 2006b; McCutcheon et al. 2011). During the breeding season, adults are restricted to the nesting area as they feed their young. Because the guard period for chicks needs to be short, foraging trips that are only within 20 km of the nest (Hoskins et al. 2008) are required before the bird returns to the nest, (Weavers 1991; Deagle et al. 2010; McCutcheon et al. 2011) so that one parent can relieve the other in nest-minding duties. As in other species, this is known as central place foraging; however, after the breeding season finishes, penguins are no longer bound to one place, so the potential exists for them to shift from being central place foragers and to therefore possibly widen the trophic niche in which they are able to hunt (Cherel et al. 2007a; Labbé et al. 2013; Chiaradia et al. 2016).

Stomach sampling undertaken by several studies (Montague & Cullen 1988; Fraser & Lalas 2004; Flemming 2012) has indicated that the little penguin tends to be a generalist forager, consuming small inshore species but also able to switch between different species depending on availability (Gales & Pemberton 1990; Flemming et al. 2013). In a stomach sampling study undertaken in Tasmania, Gales and Pemberton (1990) concluded that prey species can vary

significantly with season, year, and also across different locations. Small schooling fish appear to be the primary prey type (Ropert-Coudert et al. 2006b).

A range of prey types have been recorded in the South Island at different colonies, with the result that there are distinct dietary differences in birds in each colony (Flemming et al. 2013). For example, whereas little penguins in the Oamaru colony had Graham's gudgeon [*Grahamichthys radiata*] as the main prey mass, the Banks Peninsula population primarily prey on arrow squid [*Nototodarus sloanii*], slender sprat [*Sprattus antipodum*] and ahuru [*Auchenoceros punctatus*], and, in contrast, the Stewart Island populations of penguins mainly subsisted on arrow squid. These differences were based only on a survey of 69 stomach samples taken in late November to early December, and thus represents only one portion of a year, and in only one year.

The little penguin is a burrow or crevice nester (Nelson 1980) and has been shown to travel up to a kilometre inland to get to their nesting site (Dann & Norman 2006). Breeding populations of the little penguin are found along the coasts of both the North Island and South Island of New Zealand and also on the southern shore of Australia (Meredith & Sin 1988a; Dann 1994; Heber et al. 2008; Chiaradia et al. 2012). Most adult penguins seem to stay within range of their original colony if they have survived their first year at sea (Fortescue 1995), but newly fledged young adults have been known to disperse much further afield (Furness & Monaghan 1987), and birds banded in Australia have been recovered up to 1,000 km away from the original banding site (Reilly & Cullen 1982; Gales & Stahel 1989).

Breeding activities generally start in late September and continue to early January as shown in Figure 1.1. The timing of breeding shows considerable variation across years (Nelson 1980) and the start of the breeding season has been recorded as early as May in Australia (Reilly & Cullen 1981; Robinson et al. 2005) and as late as September [refer Figure 1.1]. Breeding has also been documented as early as May and as late as September in some locations in New Zealand (Agnew et al. 2014), although late spring and start of summer is generally considered the beginning of breeding season (Gales & Stahel 1989).

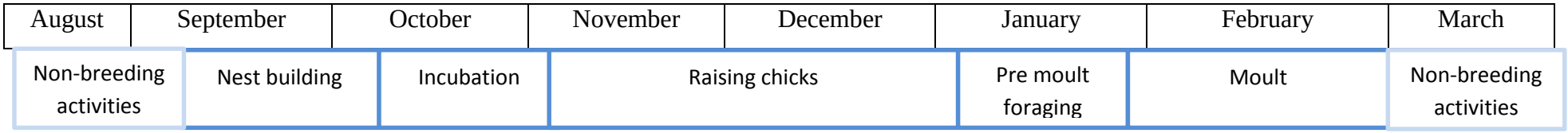


Figure 1.1 The average annual cycle of the little penguin.

Penguins appear to mate for life, but "widowed" and "divorced" penguins tend to nest, on average, three weeks earlier, before other, more established pairs (Reilly & Cullen 1981). Ocean temperature (Cullen et al. 2009), or sea surface temperature (Reilly 1994), and body condition (Robinson et al. 2005) are also possible factors affecting the start of the breeding cycle.

Little penguins breed at two to three years of age (Agnew et al. 2014) and tend to lay two eggs, generally within three days of each other, and will incubate both eggs for approximately 35 days (Kemp & Dann 2001). In years of early egg laying double broods have been recorded and in a year of high food supply, a second set of double brooded chicks can be successfully fledged as well (Reilly 1994; Agnew et al. 2014).

The two eggs are white and of roughly identical volume, weighing around 55 g (Gales & Stahel 1989). I have, however, observed eggs from a white flippered penguin weighing up to 63 g. Hatching success of the eggs is generally around 60%, though years of low and high hatching success have been recorded and have been attributed to annual variations in prey availability (Kemp & Dann 2001). Once hatched, parents take turns guarding the chick(s) until about 5 weeks of age, and then both parents forage at the same time to feed themselves and the rapidly growing chick(s) as they start to require more food (Reilly & Cullen 1981; Agnew et al. 2014).

Development of the young happens over a fledging period of 56-70 days (Nelson 1980); they then spend the majority of their first year at sea (Reilly & Cullen 1981) and it is estimated that 69 % do not survive that first year (Reilly 1994). The parents leave the nest to start their moult process and the newly fledged chick is left to learn how to swim and hunt.

Moulting in penguins is unique, because no other bird undergoes this process in the same way; the moult is concentrated into three to four weeks and during this time the birds are unable to swim and therefore are unable to maintain a constant body temperature (Nelson 1980). The feathers of penguins are evenly packed all over their body, not in tracts like other bird types, but rather in a dense fashion of up to 30 to 40 feathers per square centimetre in some species (Dawson et al. 1999). February and March are when most little penguins tend to moult, though some moulting birds have been found as early as January and as late as June (Gales & Stahel 1989). Because they are unable to swim for food during this period these birds will attempt to gain enough weight, nearly double of what they normally weigh, prior to starting the moult process (Gales et al. 1988). The high metabolic demands to sustain feather replacement results in a decrease of approximately 50 g of weight per day during the moulting process (Gales & Stahel 1989) and therefore the birds must lay down enough fat to survive the fasting period (Gales et al. 1988). In theory, the feathers are grown from fat stores gained just before the moulting period

starts, so new feathers reflect, isotopically, the combined signature of the prey consumed (Cherel et al. 2000; Jaeger et al. 2009). However, the process of the moult does not happen under normal metabolic conditions. Because of the fasting nature of the process for this family of birds, nutrients can be derived from diet initially, but as the moult continues, the source of keratin production comes from some fat and protein reserves, which have been ingested and stored prior to the pre-moult foraging period, the presence of which might alter the typical excretion rate of the heavier isotope of nitrogen [$\delta^{15}N$] (Cherel et al. 2005c).

1.6 Outline of thesis

Using the SIA of carbon and nitrogen in tissues such as feathers, it is possible to track, trace, and record changes in the feeding behaviour of a bird species over time. This may help predict what could occur, especially in different circumstances, such as changes in seasonal food availability, or in different parts of the range of a species. Documenting the pattern and scale of changes in carbon and nitrogen isotope ratios in feathers thus has the potential to provide a better understanding of the place of a species in its food web, and sets a baseline for measuring any further response to environmental pressures. For these reasons, in this thesis, I use isotope analyses to determine the trophic position with nitrogen isotopes and dietary carbon isotope signatures of little penguins in captivity and then examine other colonies in the wider South Island marine food web for the way in which this might vary both geographically and temporally.

My first objective was to test the assumption that the stable isotope ratios of carbon and nitrogen can provide information on the approximate trophic position of the little penguin. To test the link between diet and isotope ratio, in a situation in which diet was known, I first measured the stable carbon and nitrogen isotope compositions of little penguin feathers and their diet in a captive colony housed at the International Antarctic Centre, in Christchurch, New Zealand. The annual moult feathers were analysed for stable isotope carbon and nitrogen ratios, and because the penguins had been fed a known diet, the isotope ratios of the feathers could then be compared with the ratios from the fish fed to the penguins. The captive group was composed of penguins rescued from the North Island, and two South Island colonies: from the West Coast, Dunedin, and from Flea Bay on Banks Peninsula. The colony also included several birds exhibiting characters of the formerly recognised “white-flippered penguin” from a large colony in Flea Bay, Banks Peninsula. The range of birds from different locations allowed further comparisons based on origin. This captive colony is formed of penguins who have sustained injuries in the wild that

prevent them from returning to their native environment once rehabilitation is complete. A calibration study group is essential to determine the type of isotopic discrimination with wild little penguins by testing the assumption that diet can be inferred from stable isotope ratios. The results of this calibration study are presented in Chapter 2.

I then compared the isotope ratios of feathers collected from little penguins from three wild colonies around the South Island: Oamaru, Otago; Flea Bay, Banks Peninsula; and the West Coast. My objective was to test the hypothesis that different geographic populations of little penguins differ in their isotopic signatures. The utility of using the stable isotope method depends on the presence of significant isotopic differences in different areas (Hobson et al. 2001) and thus I tested whether there were different isotopic signatures between birds in the captive colony and their counterparts in wild colonies that live around the coast of New Zealand, and the way in which this varies temporally. I also tested the hypothesis that individual penguins, bearing different characteristics that are sometimes recognised taxonomically as a separate race or species [such as the so-called white-flippered penguin], differ in their position in the food chain from other populations of little penguins, and that they have different isotopic signatures when fed the same diet in captivity. The results of this work are given in Chapter 3.

Finally, in Chapter 4, I review the findings, present the conclusions of this study, and suggest avenues for future research.

Although the methods of this study are interlinked, each chapter is written to stand alone because the separate chapters are in preparation for submission for publication in refereed journals.

1.7 Ethical considerations

Because the collection of feathers is non-invasive and hence non-fatal to the bird, the ethical considerations for this study were limited. If addled eggs are present after the breeding season is finished, then again, no permanent harm would befall the birds, nor affect overall breeding success, if these eggs are inspected. Disturbance of the birds would be a concern, there is variation between different species reactions and would depend on time of year at point of collection, if this is during or after the moult, the damage to their psyche would, hopefully, be limited.

This study was carried out under permit authorisation number: 39867-FAU, issued by the Department of Conservation and with full permission from the University of Canterbury Animal Ethics Board.

Chapter 2 Calibration of captive colony to diet

Abstract

I collected moulted feathers from a captive group of little penguins [*Eudyptula minor*] over the course of two years for stable isotope analysis [SIA]. Because diet was sampled during this time, I was able to connect the isotopic signatures of the food source to that of the moulted feathers formed and thereby to ascertain the enrichment differences between diet and feathers; however the variation between different feathers produced on various diets was inconclusive. I found that while the isotopic signatures of penguins fed on different lots of sprats were not significantly different on $\delta^{15}N$ signatures, feathers produced on diet/fish lot 1 were different from other feather lots, and different from feathers produced on variable types of fish from other diet/fish lots. I also explored diet and feather variation in isotopic signatures based on gender and geographic origin of the birds, within individual diet/fish lot produced feathers. There did not appear to be any significant differences in either $\delta^{13}C$ or $\delta^{15}N$ isotopic composition between penguins on the basis of their geographic origin. Male and female feathers also did not significantly vary in $\delta^{13}C$ or $\delta^{15}N$ isotopic compositions when fed on an identical diet. My study confirms that SIA can be used to provide a reliable general estimate of diet location and analysis of feathers from wild populations could provide some information on the diet [and trophic level] of free-living penguins.

2.1 Introduction

Studies on animals in controlled environments can provide a useful framework for further research and as a prelude to similar studies on wild populations of the same species. This is especially true in situations with studies of diet using stable isotopic analyses [SIA], where there is a need to confirm a direct relationship between the diet of the animal and the isotopic signature of the tissue to be sampled. As a result, a number of studies have now examined stable isotopes in captive pigs, horses, seals, as well as several species of birds (Hobson & Clark 1992b; Kurle & Worthy 2002; Ayliffe et al. 2004; Tuross et al. 2008). For example, using a known diet, Kurle and Worthy (2002) confirmed that the diet of captive northern fur seals [*Callorhinus arsinus*] reflects isotopic enrichment in a predictable fashion, but also that enrichment levels can fluctuate depending on which type of tissue sampled. They also found that different tissues from the same individual are seldom uniform in their isotopic response

despite a consistent diet (Kurle & Worthy 2002). A similar study on horses concluded that different caches of nutrients within the body are not used uniformly but instead are dispensed within varying time frames, thereby leading to different isotopic signatures in different tissues (Ayliffe et al. 2004). As a result of these types of findings, Tuross et al. (2008) stressed that understanding the innate biological variability that exists under structured environmental conditions can provide the background needed for similar studies in less controlled environments, such as those that attempt to use SIA to assess the diet of free-living animals.

Studying the diet of penguins and other seabirds is typically quite difficult, due to their wide ranging foraging trips out at sea, and potential variation in diet resulting from geographic and seasonal effects. SIA thus provides an attractive alternative to assess the diet of penguins with ease and at a larger scale than would otherwise be the case using more direct measures such as stomach or faecal analyses. There have now been a number of stable isotope studies on different captive populations of penguins (Mizutani et al. 1992; Cherel et al. 2005b; Polito et al. 2011a). In a fairly recent stable isotope study carried out on both wild and captive individuals of the little penguin [*Eudyptula minor*], McKenzie (2011) sampled blood and feathers in six little penguins over a period of two months to capture changes in isotopic signatures over one moult period. However, this study was based on a small number of birds and it is not clear how isotopic signatures may vary over a longer period of feather collection, or whether there are innate differences in the signatures of captive birds that are caused by factors other than diet, such as sex or geographic origin of the birds.

For the research in this chapter, I used a captive group of little penguins at the International Antarctic Centre [IAC] in Christchurch, New Zealand as a means of calibrating the isotopic signatures of feathers to that of a known diet. The use of captive penguins in a controlled environment is an ideal way to test how the isotopic signature of an individual can react to known changes in diet. By feeding birds a known diet and then examining the isotope ratios in the moulted feathers produced on said diet, I tested if, and how, different batches of the same fish species affected the stable isotope reading of the feathers that were produced while birds were on that diet. Because the captive population of little penguins that I used also included individuals derived from geographically separated areas, including birds who in the past qualified as a subspecies, I tested whether individuals of the same species displayed a similar or dissimilar isotopic signature in response to a similar diet. Finally, isotopic

signatures could vary between the sexes, due to their differing physiologies and behaviours. By comparing males and females fed on the same diet, I was also able to assess variation in isotopic signatures that may be due to sexual differences that are not directly related to diet. The results of this research allowed me to test the assumption that the isotopic signatures of feathers from penguins reflect their diet, as well as estimating the amount of variation in those signatures that may be result either from the geographic origin, or from the gender of the birds.

2.2 Methods

2.2.1 International Antarctic Centre penguin colony

Penguins were housed at the International Antarctic Centre in Christchurch, New Zealand. The birds in this captive population are deemed unable to survive in the wild as a result of injuries, as well as two birds that hatched in captivity and these are also housed with the wild hatched birds. At the time of this study, the population included birds from around New Zealand: one from Otago, two from the West Coast, eight from the North Island, and nine birds from Banks Peninsula. The latter are currently classified as the white-flippered penguin [*Eudyptula minor albosignata*], and there is some debate on whether it is considered a subspecies (Banks et al. 2002), or a colour morph (Sitar-Gonzales & Parsons 2012) of the little penguin. At present the New Zealand check list does not recognise any subspecies of the New Zealand little penguin (Checklist Committee 2010).

The penguin enclosure can house a maximum of 26 little penguins although the most at any one time has been 24. The enclosure is open but fitted with a predator net over the top, thus the birds are exposed to all weather and daylight changes. Wooden nest boxes are provided on the beach area, and plastic retreat boxes are fitted to the shelving unit on one side which are accessible to the birds via tunnels. A freshwater pool [~ 80,000 litres] in the enclosure is filtered to prevent oil build up on the water. The water for the pool is supplied by the municipal system and the temperature, depending on the season, ranges between 14°C and 23°C. A picture of the enclosure is shown in Appendix 3.

2.2.2 Moult timing of captive colony

Little penguins in the wild moult after breeding, generally in January to February [Figure 1.1]. The pre-moult foraging period is vital to support fasting during the period of feather replacement, which must occur on land, as moulting birds are not waterproof and are unable to fish. This means the feathers must be formed from nutrients stored in pre-moult foraging period and it is expected that the feathers would reflect the isotopic composition of the prey population consumed by the bird during this period.

During the period in which I collected feathers, several individuals were found to be moulting six to eight months apart. Records have been kept for several years on moulting dates [Figure 2.1]. From 2010 to 2011, 23 birds were present in the colony, and all moulted in a span of five months, from one bird in October to six birds moulting in February. Each bird only moulted once in this time frame. The summer season of 2011 to 2012 was similar with the exception of a female from the West Coast, which moulted in October 2011 and then six months later in April 2012. In the 2012 to 2013 summer period, the period of moult increased to span the period from July 2012 to May 2013. For example, one white-flippered male started to moult more often even though previously he had moulted only once a year [Note: he moulted four times from September 2013 to April 2015]. From July 2013 to June 2014, at least one bird every month [with the exception of June] was found moulting. It is not clear why moulting appears less regular in the captive population than observed in conspecifics in the wild, but this may be due the feeding arrangement [they are fed until satiation] and were not subject to a long fast during moulting as is experienced by wild birds.

2.3 Stable Isotope Analysis [SIA]

2.3.1 Food types, supplements and timing

The captive penguins were fed on a variety of fish during this study, some obtained locally and some imported from Europe. The fish caught in European waters were provided frozen to the Antarctic Centre in batches, most which lasted several months. This consisted of small, whole European sprats [*Sprattus sprattus*] which originated from Major Fishing Area 027, in the Atlantic Northeast (Fisheries and Aquaculture Department 2015). The European sprat is a pelagic small schooling fish; it is zooplanktivorous and distributed from the North Sea and

Baltic Sea to the Black Sea and Mediterranean Sea (Peck et al. 2012). A total of 4 different lots of sprat were obtained during the course of this study, and as they may have different isotopic signatures, they were treated and analysed separately [and referred to as sprat lots or diet/fish lots 1 to diet/fish lots 4]. Later in my study, a shortage of sprats in lot 3 had to be supplemented with New Zealand caught salmon [*Oncorhynchus tshawytscha*], pilchard [*Sardinops neopilchardus*], and then with mackerel [three species found in New Zealand but managed as one; *Trachurus declivis T. novaezelandiae*, and *T. symmetricus murphyi* (Bird 2011)] and anchovies [*Engraulis mordox*]. Sprats were preferred for health reasons (Veterinarian, Pauline Howard, *pers. comm.*), but the daily feedings were supplemented with anchovies, the most successful trialled fish, in addition to the sprats. The anchovy stocks were purchased from United Fisheries [Christchurch] and were apparently caught originally in the Pacific Ocean off the Northern California coast. Sprat lot 4 was supplemented with just anchovies. All information for labels of each fish/diet lot and type are listed in Appendix 2.

These different types of fish were trialled on the whole colony, at various feeding times, not for the purposes of this study but in an attempt to stretch the supply of sprats available until more could arrive from Europe. The change in diet did however provide me with the opportunity to assess how the different types of fish might affect the isotope ratios found in the feathers grown at the same time. Samples of the various prey types were collected and analysed against the possibility that any birds who might have consumed them would have altered isotopic signatures in their feathers. The overall diet/fish lots can be considered more homogenous than found in the wild; however, because the time line for these diets was known, they could then be related to the isotopic signatures later obtained from the feathers that developed on each diet/fish lot. The time line of the change in diet/fish lots of the captive penguins is given in Figure 2.1.

The penguins were fed twice a day, at 10:30 am and 3:30 pm. The birds were only fed whole fish, damaged or partial fish were discarded. The fish were rinsed in a sea salt water solution once thawed for use. The sea salt mineral composition list can be found in Appendix 1.

Mazuri tablets were used to supplement the diet of the captive penguins. Two tablets were hidden in a fish, and attempts were made to get one vitamin-spiked fish into each penguin every day. Some of the birds regularly snagged a second vitamin fish because of the inexact

nature of the delivery method. The nutritional content for Mazuri tablets are listed in Appendix 1. The Mazuri tablets are produced in the USA and imported to New Zealand.

Three penguins, out of the 20 captive individuals, were also given 1/4 of a 1000 mg Nutri-Zing tablet daily, a nutritional joint supplement that contains Green-Lipped Mussel, registered to United Fisheries in Christchurch, New Zealand and produced from local ingredients. The nutritional content is listed in Appendix 1. Both types of nutritional supplement were powdered and weighed into tin cups between 0.4 μg and 0.6 μg for sampling in the elemental analyser.

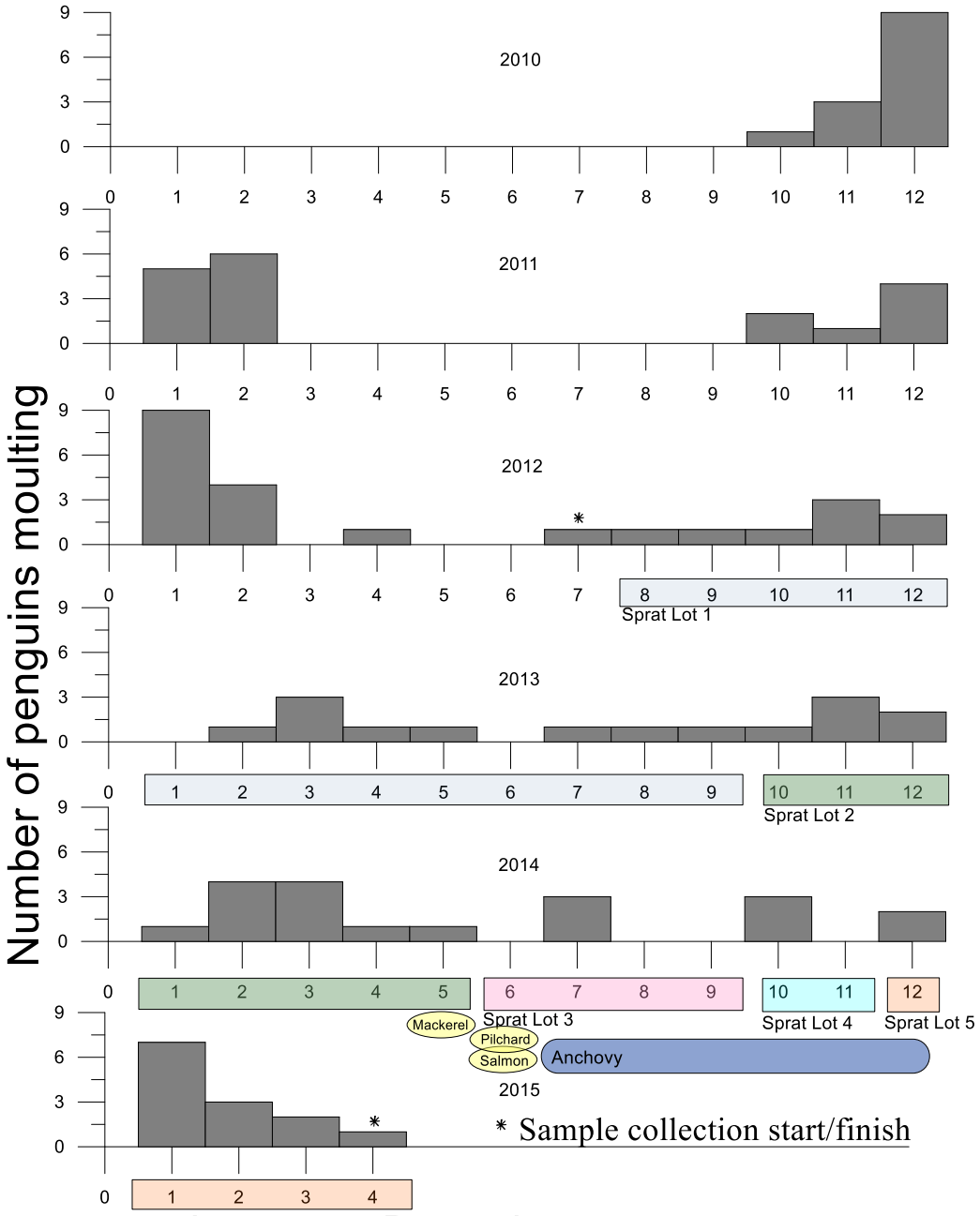


Figure 2.1: Time line for moult and changes in the diet of little penguins in a captive population at the International Antarctic Centre. Feather collection commenced in August of 2012 [indicated by *]. The periods when birds were fed each of the different diets are indicated by the bars enclosed the month numbers [January as 1, December as 12] and labelled with the type of fish, though the different fish lots were started when the previous lot was finished, and not at the beginning or end of the month. Each lot of sprat represents a different shipment and was analysed separately; lots of sprat are numbered in order they were fed to the birds. The mackerel were fed to the birds during the changeover between sprat lots 2 and 3 but have been included in lot 3 as the one bird moulting at the end of sprat lot 2 was already in process and not eating when the mackerel was introduced at the end of lot 2. As no feathers were collected that could have been produced on sprat lot 5, but no comparison between lot 5 fish is included in this study.

2.3.2 Collection and preparation of fish for stable isotope analysis

As each box of either sprat or other types of fish was opened for use at the IAC, one fish was removed, labelled, and kept frozen for later stable isotope analysis. Because there was initially one box of each potential new food type, several mackerel, salmon, and pilchards were collected from each box. Towards the end of the study, anchovies were included as a permanent addition to the diet [see Figure 2.1], and so I removed one fish for analysis from each box opened. All samples were stored frozen prior to processing and were freeze dried while frozen. Whole fish were freeze dried in two batches and stored in individual bags. The first batch of fish was freeze dried whole over four days. The second batch of fish was freeze dried for six days because one of the fish lots was extremely oily and I wanted to ensure there was no remaining water content. Each fish was percussively homogenised with a rolling pin until powdered. This powder was then weighed into tin cups to between 0.4 µg and 0.6 µg for sampling in the elemental analyser. Whole fish were used in this process as is recommended by both Cherel et al. (2005b) and Polito et al. (2009) when estimating the diets of wild penguins, even though the diet for this colony is known.

2.3.3 Collection and preparation of feathers for stable isotope analysis

Feathers from each bird were collected during each moult and were sealed in individual plastic bags labelled with the bird's name and date of collection. If more than one bird was moulting at the same time in the captive colony, feathers were collected directly from individual birds. If only one bird was in moult, then feathers were collected from nesting material in their nest box. Because of the nature of this study, the delay from the feeding of fish to the penguins to sampling of feathers could be six months to a year, depending on the time of moult. The feathers were collected once the birds had their next moult, so one must keep in mind that the feathers had been "in use" and exposed to the elements.

The feathers were first cleaned using the methods described in Greer et al. (2015), that is, a plastic tray with c. 2 L of distilled water and one small drop of Home Brand dish detergent was used. Feathers were washed individually, gentle brushing with a toothbrush, to remove dirt and faeces. After washing, they were rinsed in three separate beakers and agitated in 250 mL - 500 mL of microfoil water per beaker. The washing solution and rinse water was changed after at least every 25 feathers to prevent oil build up. Individual feathers, generally

three per sampled moult, were then placed into one half of a glass petri dish, covered with a porous nappy liner held in place by rubber bands and placed into a fume hood for at least 24 hours to dry.

Once the feathers were dry, they were placed into small sealed universal bottles filled with c. 20 ml of 2:1 chloroform/methanol solution for 24 hours. No more than six feathers were placed into each bottle at a time, and most samples consisted of three feathers. After 24 hours, feathers were rinsed and agitated in two separate beakers which contained a 2:1 chloroform/methanol solution of c. 20 mL. The 2:1 chloroform/methanol rinse solution was changed at least every 24 feathers. To dry the feathers, each sample was again placed into a glass petri dish, covered with a nappy liner and rubber bands and left in the fume hood for at least 48 hours. Once dry, the feathers were removed and placed into new labelled plastic bags until used for SIA.

Prior to analysis, each feather was cut into small fragments using stainless steel scissors. Only the vane part of the feather was used. Feather pieces were then placed into small tin cups until a total of 0.4 μg and 0.6 μg was obtained [using a Mettler Toledo UMX2 microbalance]. Each cup was folded into a small ball and placed into a nunc tray to be processed by the elemental analyser. Because static electricity complicated the use of small size feather pieces during sample preparation, an anti-static bracelet was used. It was found that if the feathers were placed on the metal plate part of the bracelet, instead of the bracelet being worn, the static electricity was minimised.

All work surfaces, with the exception of the scale [to avoid damage], were wiped down with 70% ethanol to maintain sterility and prevent cross contamination of samples during processing.

2.3.4 Mass Spectrometry

Samples were analysed for $\delta^{13}C$, $\delta^{15}N$, %C and %N using a Costech Elemental Combustion System [ECS] 4010 [Costech Analytical Technologies, California, USA] connected to a Delta V Plus Isotope Ratio Mass Spectrometer [IRMS; Thermo Fisher Scientific, Bremen, Germany] via a Finnigan Conflow III [Thermo Fischer Scientific]. All samples were loaded into a ZeroBlank autosampler with an isolation valve [Costech Analytical Technologies] and

were individually combusted at 900°C under a continuous flow [c. 110 mL min $^{-1}$] of ultra-high-purity helium [>99.999%]. Molecular N_2 and CO_2 were separated using a gas chromatography column housed in the ECS and held at a static 45°C. IRMS fast peak jumps were calibrated at least daily, and reference gas linearity tests were performed at the start of every other analytical sequence. Internal precision [the standard deviation across ten reference gas analyses, i.e., zero-enrichment test] was determined prior to every analytical sequence and was always <±0.06‰ for both $\delta^{13}C$ and $\delta^{15}N$. Data were normalised to international standards, Vienna PeeDee Belemnite for $\delta^{13}C$ and Air for $\delta^{15}N$, using a stretch-and-shift 2-point normalisation based on replicate analyses of certified reference materials within individual analytical sequences. External precision [the standard deviation of replicate analyses of certified reference materials and internal laboratory check standards over the course of the sampling] was <±0.20‰ for both $\delta^{13}C$ and $\delta^{15}N$.

2.4 Data analyses

Feather samples were not included in analyses of the different fish lots if the moult/feather formation period occurred during or just after a diet change in which one fish lot was finished and a new one started. This was done in an attempt to only include feathers that were formed by a bird which had been on the same diet for at least one month before moulting commenced. Two birds that arrived into the captive colony have also had their first "wild" moult data removed from any data analyses of the captive colony as a whole because these feathers had been grown while the birds were still free-living and feeding in the marine environment on an unknown diet.

Because there are more than two sets of samples to compare between the colonies sampled in this study, I used both the Shapiro-Wilk and the Bartletts tests to check for normality and homogeneity of variance to compare between the diet/fish lots and between the feathers produced on each diet lot. The data had non equal variance and a non-parametric Kruskal-Wallis rank sum test was therefore used to test significant statistical difference. I then used TukeyHSD to show where the differences existed between samples. These tests were done using the R software package (R Development Core Team 2005).

Excel® was used to carry out F-tests and *t*-tests, depending on variance, on the gender of the colony as a whole and within diet/fish lot 2. In analysing gender, diet/fish lot 1 moulted

penguins contained only one male, lot 3 contained only three individuals in total who moulted, and lot 4 contained included only one bird producing moult feathers. As a result, these birds were not included in the gender and origin analysis due to lack of samples within the last two batches of feathers produced.

The enrichment values were found using $\delta_{tissue} - \delta_{diet}$ (Germain et al. 2012).

2.5 Results

2.5.1 Diet

The isotopic composition of the diet consumed compared to the feathers that were produced on those diets are shown in Table 2.1 and the scatter plot [Figure 2.2] and the differences in enrichment between fish to feathers in the whisker box plot Figure 2.3 [nitrogen] and Figure 2.4 [carbon].

2.5.2 Nutritional supplements

The isotopic ratios of the Mazuri tablets were substantially different from some of the fish types with the mean of $\delta^{13}C$ at –22.88 ‰ ± 0.78 [range –23.91 to –22.21] and $\delta^{15}N$ at –3.09 ‰ ± 0.66 [range –3.72 to –2.27]. Those that were statistically different for $\delta^{13}C$ were anchovy [Kruskal Wallis: H=12.36, df=9, TukeyHSD P<0.001], lot 1 sprats [TukeyHSD P=0.022], lot 3 sprats [TukeyHSD P=0.014] and the pilchards [TukeyHSD P=0.0037]. The $\delta^{15}N$ signatures that were significantly different included anchovy again [Kruskal Wallis: H=12.76, df=9, TukeyHSD P=0.020], lot 2 and lot 3 sprats [TukeyHSD P<0.001], salmon [TukeyHSD P=0.014] and mackerel [TukeyHSD P=0.026]. However, the expected impact on dietary feather readings was likely to be minimal as this supplement was only a small percentage of the diet consumed.

The green-lipped mussel supplement was different from some of the fish samples. The $\delta^{13}C$ signatures mean was –22.93 ‰ ± 0.25 [range –23.14 to –22.66] and $\delta^{15}N$ at 6.60 ‰ ± 0.57 [range 6.24 to 7.26]. The mussel pill was significantly different in $\delta^{13}C$ from anchovies [Kruskal Wallis: H=12.36, df=9, TukeyHSD P<0.001], lot 1 sprats [TukeyHSD P=0.022], lot 3 sprats [TukeyHSD P=0.032] and the pilchards [TukeyHSD P=0.0057]. However, both lot 2

and lot 3 sprats [H=12.76, df=9, TukeyHSD P<0.001] were the only samples significantly different in their $\delta^{15}N$ from the mussel pill. This supplement contributes a small percentage to diet consumed because it was only given daily to three birds. It therefore has not been included in the whisker box plots because it does not apply to all the birds sampled in this study [Figure 2.3 and Figure 2.4].

Diet					Feathers				
	δ ^{13}C [‰]		δ ^{15}N [‰]			δ ^{13}C [‰]		δ ^{15}N [‰]	
	Mean ± SD	Range [‰]	Mean ± SD	Range [‰]		Mean ± SD	Range [‰]	Mean ± SD	Range [‰]
Sprat Lot 1 n=10	–20.54 ± 1.06	–21.98 to –18.62	12.86 ± 0.59	12.01 to 14.12	Feathers Lot 1 n=12	–18.33 ± 0.45	–18.99 to –17.38	16.41 ± 0.51	15.37 to 17.15
Sprat Lot 2 n=10	–20.81 ± 1.33	–22.91 to –18.20	14.02 ± 1.18	12.50 to 16.74	Feathers Lot 2 n=16	–18.19 ± 0.26	–18.55 to –17.71	17.01 ± 0.70	14.88 to 17.61
Sprat Lot 3 n=13	–20.56 ± 1.06	–23.41 to –19.53	13.76 ± 0.77	12.00 to 14.95	Feathers Lot 3 n=3	–18.10 ± 0.34	–18.39 to –17.73	17.39 ± 0.41	16.93 to 17.69
Anchovy n=10	–18.09 ± 0.70	–19.12 to –16.94	12.21 ± 1.01	10.68 to 13.76					
Salmon n=3	–20.53 ± 1.84	–22.64 to –19.28	12.43 ± 2.33	9.89 to 14.49					
Pilchard n=2	–19.41 ± 0.21	–19.56 to –19.26	7.73 ± 0.75	7.20 to 8.27					
Mackerel n=2	–20.06 ± 0.09	–20.12 to –19.99	13.10 ± 0.42	12.80 to 13.39					
Sprat Lot 4 n=5	–22.69 ± 0.88	–23.95 to –21.94	10.59 ± 1.09	8.82 to 11.58	Feathers Lot 4 n=1	–18.07		17.95	
Anchovy	As above								
Mazuri Tab n=4	–22.88 ± 0.78	–23.91 to –22.21	–3.09 ± 0.66	–3.72 to –2.27					
Mussel Pill n=3	–22.93 ± 0.25	–23.14 to –22.66	6.60 ± 0.57	6.24 to 7.26					

Table 2.1 Results showing mean, standard deviation [SD] and range of data of the stable isotope ratios of both diet items and feathers, produced on each diet/fish lot, of the little penguins from the captive colony of at the International Antarctic Centre.

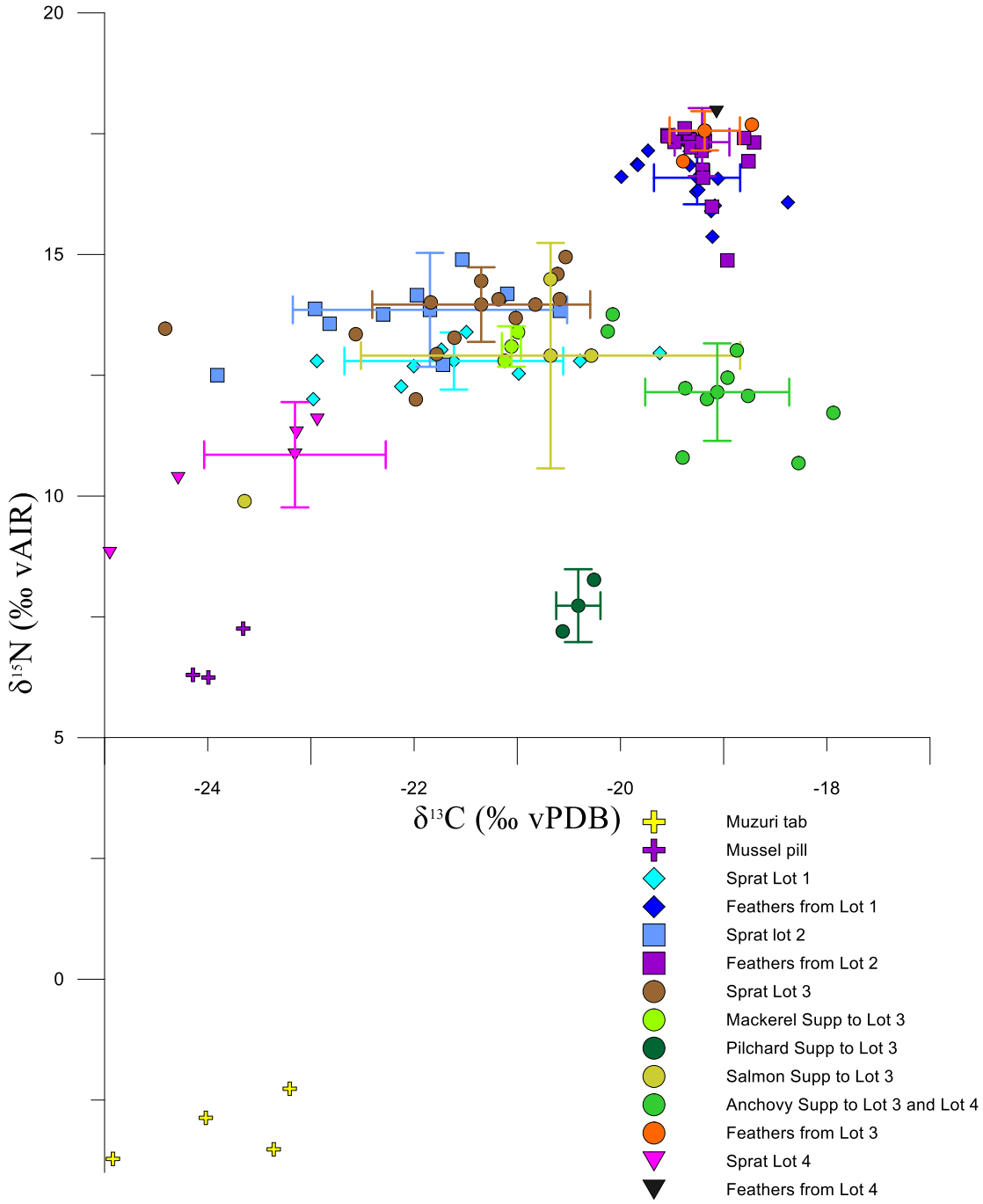


Figure 2.2

This graph shows the C and N isotope signatures of each diet prey type [of all diet/fish lots] in relation to feathers produced on each of the diet/fish lots. This graph encompasses all of the recorded diet compared to all of the feathers produced that could be linked to specific diets/fish lots. The “diet/fish lot” term is used interchangeably with “sprat lot” as sprat lot 1 and sprat lot 2 were only comprised of sprats, but lot 3 and lot 4 had other species of fish in addition to the sprats. The standard deviation of each group is indicated by error bars.

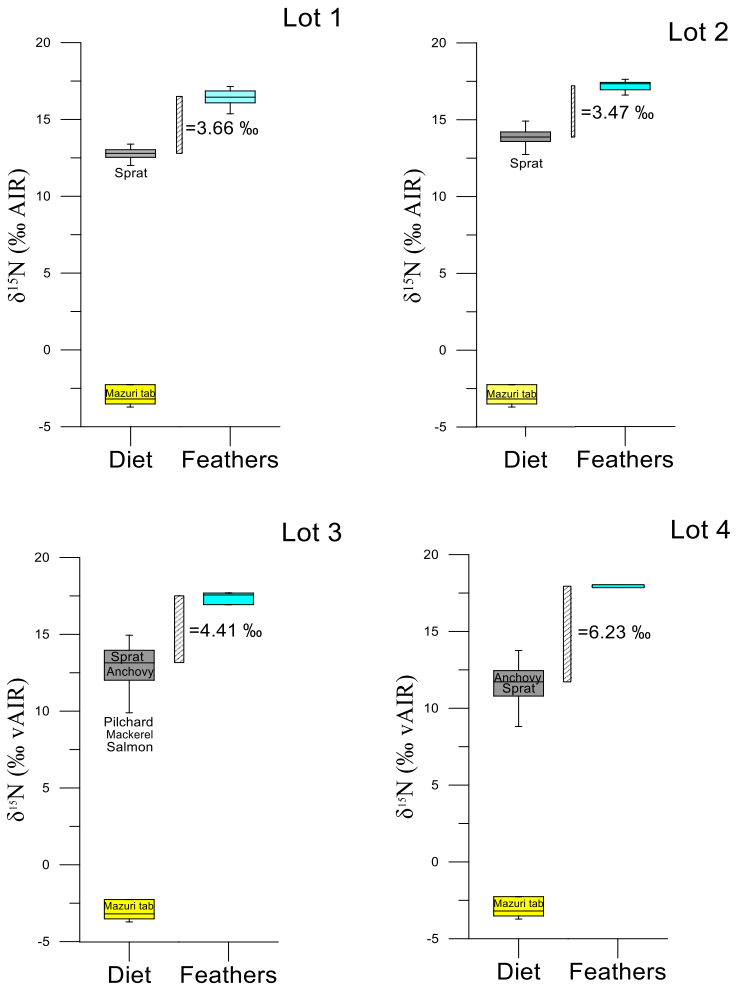


Figure 2.3 Whisker box plots showing the isotopic difference in $\delta^{15}N$ between the total diet and feathers produced whilst consuming different diet/fish lots.

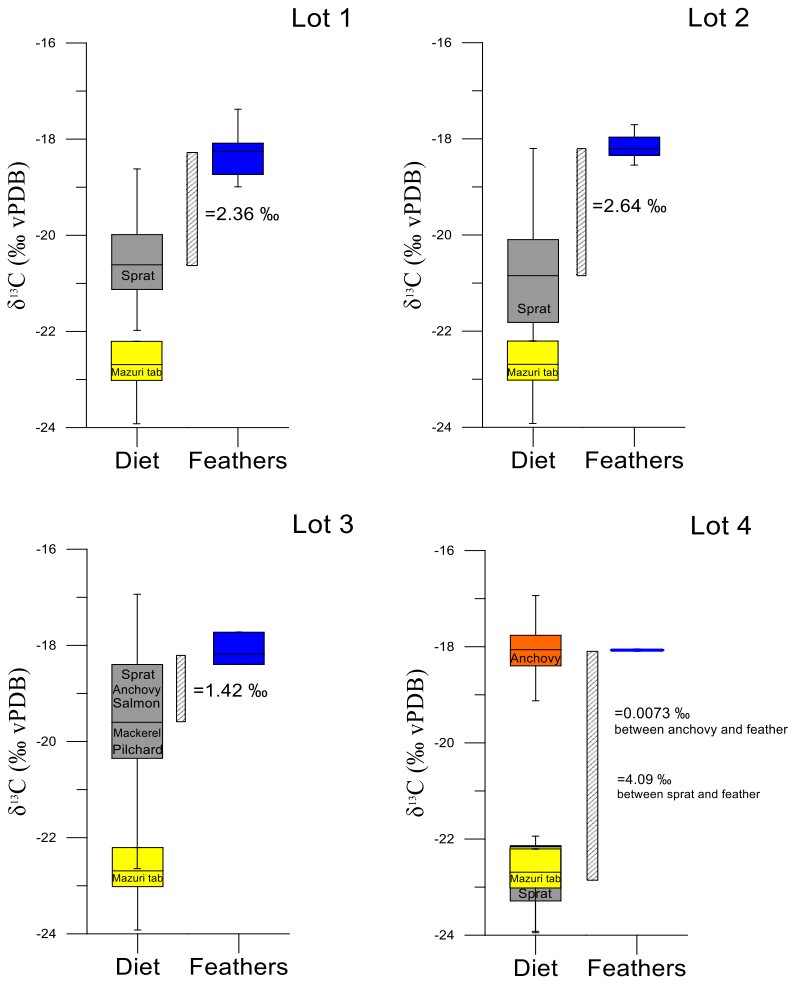


Figure 2.4 Whisker box plots showing the isotopic difference in $\delta^{13}C$ between the total diet and feathers produced while consuming different diet/fish lots.

2.5.3 Diet to feathers in Lots 1 and 2

Birds that were fed on sprat lot 1 and sprat lot 2 received no other types of fish. All sprat lots sampled in this study were caught over a two-year period. The mean of sprats from lot 1 for $\delta^{13}C$ is at –20.54 ‰ ± 1.06 [range –21.98 to –18.62] and $\delta^{15}N$ at 12.86 ‰ ± 0.59 [range 12.01 to 14.12]. Lot 1 sprats were not significantly different from any other sprat lots in either $\delta^{13}C$ [Kruskal Wallis: H=10.41, df=7, lot 2, TukeyHSD P=0.99, lot 3, TukeyHSD P=0.99 and lot 4, TukeyHSD P=0.055] or $\delta^{15}N$ [Kruskal Wallis: H=8.35, df=7, lot 2, TukeyHSD P=0.096, lot 3, TukeyHSD P=0.12, and lot 4, TukeyHSD P=0.080].

The feathers produced on sprat lot 1 [n=12] showed a mean $\delta^{13}C$ signature of –18.33 ‰ ± 0.45 [range –18.99 to –17.38] and $\delta^{15}N$ at 16.41 ‰ ± 0.51 [range 15.37 to 17.15]. Lot 1 produced $\delta^{13}C$ feather signatures that were not significantly different from any other feathers produced during this study [Kruskal Wallis: H=0.48, df=3, lot 2, TukeyHSD P=0.93, lot 3, TukeyHSD P=0.90 and lot 4, TukeyHSD P=0.73]. The $\delta^{15}N$ signatures in the feathers produced on lot 1 were significantly different from the rest [Kruskal Wallis: H=7.60, df=3, lot 2, TukeyHSD P=0.0077, lot 3, TukeyHSD P=0.0091, and lot 4 feathers at TukeyHSD P=0.029].

The enrichment of diet to feathers in lot 1 for carbon was 2.36 ‰ and for nitrogen was 3.66 ‰ [Figure 2.3 and Figure 2.4].

The sprat lot 2 mean signature for $\delta^{13}C$ was –20.81 ‰ ± 1.33 [range –22.91 to –18.20] and $\delta^{15}N$ at 14.02 ‰ ± 1.18 [range 12.50 to 16.74], respectively. The $\delta^{13}C$ of sprat lot 2 was not significantly different from any of the other sprat lots in $\delta^{13}C$ [Kruskal Wallis: H=10.41, df=7, lot 3, TukeyHSD P=0.99 and lot 4, TukeyHSD P=0.14] but the $\delta^{15}N$ was significantly different between lot 4 and lot 2 [Kruskal Wallis: H=8.35, df=7, TukeyHSD P<0.001] but not between lot 2 and lot 3 [TukeyHSD P=0.99].

The lot 2 feather [n=16] $\delta^{13}C$ mean signature was –18.19 ‰ ± 0.26 [range –18.55 to –17.71] and $\delta^{15}N$ at 17.01 ‰ ± 0.70 [range 14.88 to 17.61]. Feathers produced on lot 2 were not significantly different in their $\delta^{13}C$ signatures [Kruskal Wallis: H=0.48, df=3, lot 3 feathers TukeyHSD P=0.98 and lot 4 TukeyHSD P=0.85] or in $\delta^{15}N$ for lot 3 and lot 4 [Kruskal Wallis: H=7.60, df=3, lot 3 TukeyHSD P=0.51, and lot 4 feathers TukeyHSD P=0.35].

The enrichment from diet to feathers from lot 2 was 2.64 ‰ for carbon and 3.47‰ for nitrogen [Figure 2.3 and Figure 2.4].

2.5.4 Diet to feathers in lot 3 sprats and supplemental fish

The mean $\delta^{13}C$ signature of sprat lot 3 was –20.56 ‰ ± 1.06 [range –23.41 to –19.53] and $\delta^{15}N$ was 13.76 ‰ ± 0.77 [range 12.00 to 14.95]. Sprat lot 3 was significantly different in $\delta^{13}C$ from sprat lot 1 [see above] and from sprat lot 4 [Kruskal Wallis: H=10.41, df=7, TukeyHSD P=0.028]. The $\delta^{15}N$ of sprat lot 3 was different from that of lot 4 [Kruskal Wallis: H=8.35, df=7, TukeyHSD P<0.001].

Sprat lot 3 was not significantly different in $\delta^{13}C$ signatures from the mackerel [Kruskal Wallis: H=10.41, df=7, TukeyHSD P=0.99], pilchards [TukeyHSD P=0.54] or the salmon [TukeyHSD P=0.99] but was different from the anchovies [TukeyHSD P<0.001]. The $\delta^{15}N$ of sprat lot 3 was different from the pilchards [Kruskal Wallis: H=8.35, df=7, TukeyHSD P=0.0018] and anchovies [TukeyHSD P=0.002] and not different from either the mackerel [TukeyHSD P=0.94] or the salmon [TukeyHSD P=0.71].

Anchovies were used to supplement sprat stocks [lot 3 and lot 4] from 16 June 2014 to 7 December 2014. The isotope ratios of the anchovies were a fairly distinct group from the sprat lots, with their mean at –18.09 ‰ ± 0.70 [range –19.12 to –16.94] for $\delta^{13}C$ and 12.21 ‰ ± 1.01 [range 10.68 to 13.76] for $\delta^{15}N$. However, the anchovies were not significantly different in either the $\delta^{13}C$ signatures, with mackerel at [Kruskal Wallis: H=10.41, df=7, TukeyHSD P=0.32], pilchards [TukeyHSD P=0.96] or the salmon [TukeyHSD P=0.054], or $\delta^{15}N$ signatures of the mackerel [Kruskal Wallis: H=8.35, df=7, TukeyHSD P=0.95], the pilchards [TukeyHSD P=0.51] and the salmon [TukeyHSD P=0.96].

The supplemental fish included mackerel, which was trialled on the population during feeding times occasionally between May and June 2014, pilchards, trialled over 9 days in June 2014 and salmon, which were trialled during three feedings, two days in June 2014 and one day in August 2014. The mean isotopic signatures for these fish are given in Table 2.1.

Because only three birds underwent their pre-moult weight gain over the period of fish trialling, the supplemental fish is counted as a small percentage of the possible diet consumed by the three birds. The mean of the feathers [n=3 birds] produced on sprat lot 3 [with probable supplemental fish addition to the diet of these three birds] is, for $\delta^{13}C$, –18.10 ‰ ± 0.34 [range –18.39 to –17.73] and $\delta^{15}N$ at 17.39 ‰ ± 0.41 [range 16.93 to 17.69]. The $\delta^{13}C$ signatures of lot 3 feathers were not significantly different from the other lots: lot 1 [Kruskal Wallis: H=0.48, df=3, TukeyHSD P=0.89], lot 2 [TukeyHSD P=0.98] and lot 4 [TukeyHSD P=0.94]. However, lot 3 produced $\delta^{15}N$ feather signatures that were significantly different from lot 1 [Kruskal Wallis: H=6.39, df=3, TukeyHSD P=0.021] but not different from either lot 2 [TukeyHSD P=0.58] or lot 4 [TukeyHSD P=0.88].

The enrichment of the feathers produced on the lot 3 diet had an increase of 4.41 ‰ for the nitrogen and 1.42 ‰ for the carbon [Figure 2.2 and Figure 2.3].

2.5.5 Diet to feathers in lot 4

Sprats in lot 4 had a mean of –22.69 ‰ ± 0.88 [range –23.95 to –21.94] for $\delta^{13}C$, and for $\delta^{15}N$ 10.59 ‰ ± 1.09 [range 8.82 to 11.58]. The $\delta^{13}C$ for lot 4 are significantly different from lot 3 [Kruskal Wallis: H=10.41, df=7, TukeyHSD P=0.029] while the $\delta^{15}N$ signatures are significantly different from lot 2 [Kruskal Wallis: H=8.35, df=7, TukeyHSD P<0.001] and from lot 3 [TukeyHSD P<0.001]. Anchovies were also used to supplement feeding the captive colony during the use of sprat lot 4.

The feathers of one bird were collected at the end of this study. The $\delta^{13}C$ signature for this individual was –18.07 ‰ and the $\delta^{15}N$ signature was 17.95 ‰. There was no significant difference found between this feather sample produced by lot 4 fish for the $\delta^{13}C$ signatures of the other feather lots, but there was a significant difference in the $\delta^{15}N$ between lot 1 feathers [Kruskal Wallis: H=7.60, df=3, TukeyHSD P=0.029] and lot 4.

The difference between the sprat signature of lot 4 and this individual's feathers are curious in the whisker plot figures [Figure 2.3 and Figure 2.4]. There was a 6.23 ‰ difference between the diet and feathers for nitrogen, and although the nitrogen signatures for both of the fish types were quite close, the carbon signatures were quite

different. The carbon difference between sprat and feathers was 4.09 ‰, again much higher than expected, though only 0.0073 ‰ difference between the anchovy and feather signatures, but because this was based on only one individual, this result should be treated with caution.

2.5.6 Origin of birds and isotopic signature

In an effort to investigate further the possible existence of physiological effects, based on area of origin and possible subspecific distinctions, on the isotopic composition of the little penguin, statistical comparisons were done. If there were differences present between birds originating from various areas, then this would preclude the use of stable isotopes as proxies for diet because the physiology of the birds, and not the environment, would be the controlling factor.

If the origin of each of the birds is analysed, within the context of comparing all feather signatures [Table 2.2], there was no significant difference between the subgroups in either the $\delta^{13}C$ [Kruskal Wallis: H=2.082, df=4, TukeyHSD all comparisons P=0.26 or higher] or the $\delta^{15}N$ [Kruskal Wallis: H=2.544, df=4, TukeyHSD all comparisons P=0.1 or higher].

	δ ^{13}C (‰) Mean ± SD	Range	δ ^{15}N (‰) Mean ± SD	Range
West Coast n=7	–18.45 ± 0.30	–18.87 to –18.16	17.18 ± 0.35	16.57 to 17.56
North Island n=20	–18.23 ± 0.57	–19.35 to –17.27	16.68 ± 0.63	14.88 to 17.39
White-flippered n=32	–18.12 ± 0.37	–18.99 to –17.29	16.70 ± 0.62	15.37 to 17.95
Canterbury n=2	–18.64 ± 0.14	–18.73 to –18.54	17.30 ± 0.21	17.15 to 17.44
Otago n=2	–17.94 ± 0.37	–18.20 to –17.67	15.59 ± 1.41	14.59 to 16.59
Male n=18	–18.36 ± 0.46	–19.24 to –17.48	17.10 ± 0.51	16.08 to 17.95
Female n=45	–18.14 ± 0.43	–19.35 to –17.27	16.58 ± 0.66	14.59 to 17.75

Table 2.2 Isotopic results for all feather samples from captive colony based on origin and gender.

To control for any effect of diet, I only used the results of feathers from birds fed on lot 1 and lot 2. Figure 2.5 shows origin of the birds based on each diet/fish lot. Lot 1 feathers [n=12] had no significant differences in the $\delta^{13}C$ [Kruskal Wallis: H=0.853, df=3, TukeyHSD P=0.52 or higher] and lot 1 feathers $\delta^{15}N$ also had no significant difference [Kruskal Wallis: H=2.05, df=3, TukeyHSD P=0.27 or higher].

In the lot 2 feathers [n=16] there were no significant differences in the $\delta^{13}C$ signatures of any of the birds by origin [Kruskal Wallis: H=1.19, df=4, TukeyHSD P=0.52 or higher]. The $\delta^{15}N$ signatures were also non-significantly different between any of the sub groups based on origin [Kruskal Wallis: H=1.05, df=4, TukeyHSD P=0.63 or higher] with the difference between the West Coast group and the white-flippered group not significant [TukeyHSD P=0.99].

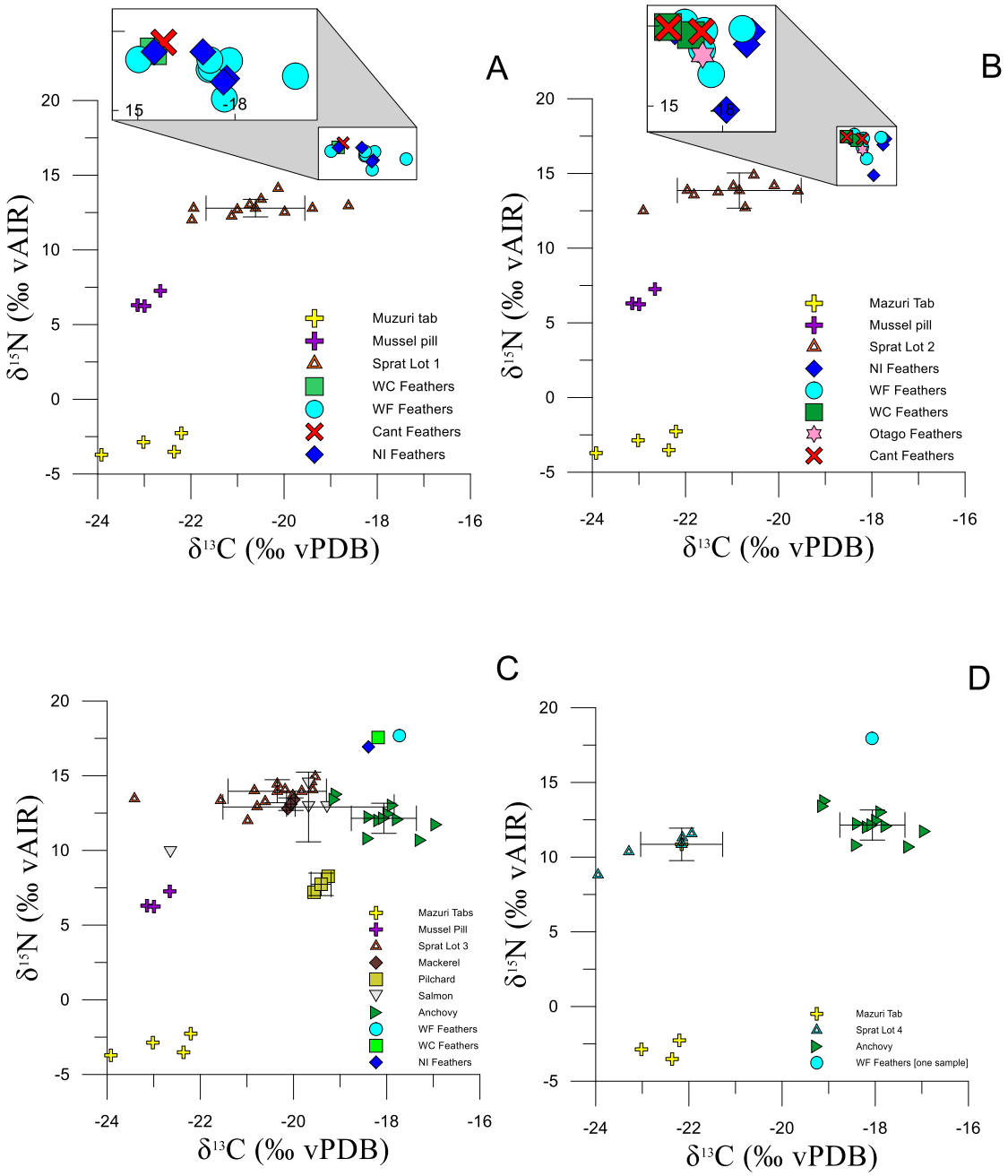


Figure 2.5

Feathers produced on each diet/fish lot by origin, graph A denotes lot 1, graph B represents lot 2, graph C denotes lot 3, and graph D represents lot 4. WC stands for the West Coast, WF for white-flippered birds, NI as North Island and Cant as Canterbury. Standard deviation is shown by the error bars for each group.

2.5.7 Gender and isotopic signature

When the isotopic signatures of the feathers of the whole colony were analysed [also see scatterplot Figure 2.6 graph B], there was a significant difference found between the male and female in the $\delta^{15}N$ [*t*=-2.99, df=61, P=0.0040] but not for $\delta^{13}C$ [*t*=-1.81, df=61, P=0.076]. To better control for diet, I compared only feathers from birds fed on lot 2 [female n=10 and male n=6; the lot 1 grouping only had one male moult]. The $\delta^{15}N$ signatures of lot 2 approached significance [*t*=-2.07, df=10, P=0.066], which suggests some effect of gender on the isotopic signatures even when males and females were on the same diet. The $\delta^{13}C$ signatures were, again, not signifficanly different [*t*=-1.018, df=14, P=0.33].

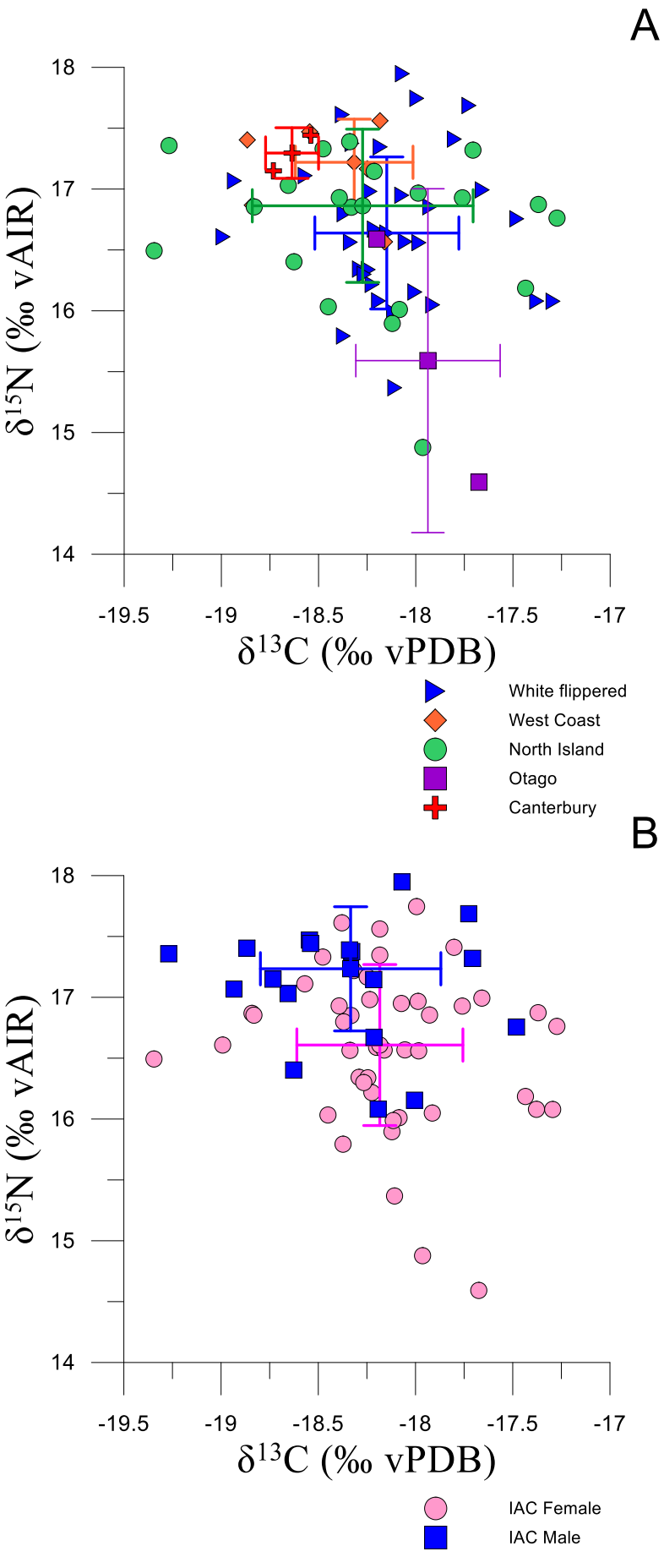


Figure 2.6

Graph A: All of the collected feather data from the International Antarctic Centre [IAC] colony divided into colony of origin. The two birds new to the colony had their first moult in captivity discarded from this data set. Standard deviation is shown by the error bars for each group.

White-flippered birds were originally from Banks Peninsula [Flea Bay area] and the Canterbury bird was found on New Brighton Beach in Christchurch.

Details about the Otago moults are further discussed in Appendix 4.

Graph B: Captive colony male to female comparison of all the collected feather data from the IAC population. The lack of foraging activity in this colony, ideally, removes the bias of gender specific foraging behaviour. There is also a gender imbalance in numbers for this colony, favouring the female side. The two birds new to the colony had their first moult in captivity discarded from this data set. Standard deviation is shown by the error bars for each group.

2.6 Discussion

My objectives for this portion of the study were, first, to measure the enrichment values between diet and feathers, and then to investigate if and how diet/fish lots differed, and if they did differ, how those differences might have affected the feathers produced on each diet. I found significant differences in the isotopic signatures between the later lots of fish, but sprat lot 1 was not significantly different from any of the other sprat lots used, even though the feathers produced on sprat lot 1 were significantly different from feathers produced on lot 2, lot 3, and lot 4. On the other hand, lot 2, lot 3, and lot 4 did not produce feathers that were statistically different from each other, despite the addition of different types of fish in lot 3 and the continued use of anchovy to supplement sprat lot 4. This suggests that although there were differences in isotopic signatures between the different types of fish fed to the captive penguins, these did not necessarily lead to similar differences in the isotopic signatures of the feathers produced at the time.

One potential explanation for the lack of differences in isotopic signatures of feathers despite the birds feeding on a diet that was known to differ isotopically is that they did not consume a substantial enough portion of the different types of fish in lot 3 or lot 4 to influence their resulting feather isotopic signatures. Furthermore, birds fed on diet/fish lot 3 and lot 4 only produced three moults and one moult, respectively, and this small sample size limited my ability to detect anything but the largest difference.

My second and third objectives were to investigate variation within the captive colony based on gender and origin of the captive birds in the wild. When I investigated differences in isotopic signatures in relation to gender using all of the feather data gained from the wild colony, I found a statistically significant difference between the male and female birds in their $\delta^{15}N$ signatures, but when controlling for diet and examining gender within food lot 2, the values between the genders were not different enough to be significant [although they did move in the direction predicted]. There were no significant differences in the $\delta^{13}C$ signatures when analysed for gender. There were also no significant differences found in either $\delta^{13}C$ or $\delta^{15}N$ based on origin when the entire colony was compared or within diet/fish lot 1 or lot 2 when controlling for diet.

2.6.1 Diet

Because sprat lot 1 is not significantly different from the other sprats, but that lot 1 produced feathers that are different in their $\delta^{15}N$ values [but not in $\delta^{13}C$] from the other feather lots could result from a number of factors. Sprat lot 1 was particularly long running, just over 12 months of being fed to the captive birds, so a larger amount of fish was provided to be fed to the captive population. The assumption that this lot was all caught at the same time in the same general area might not apply because of the larger amount of fish. Because sprat lot 1 is not different to sprat lot 2, sprat lot 3, or sprat lot 4 in either $\delta^{13}C$ or $\delta^{15}N$, but sprat lot 2 is different from lot 4 in $\delta^{15}N$ [but not $\delta^{13}C$] but not to lot 3, and lot 3 and lot 4 are both quite different in both $\delta^{13}C$ and $\delta^{15}N$, this would suggest that sprat lot 1 came from a variety of locations. It is a possibility that such a variation in locations of sprat lot 1 could have caused the variation in lot 1 produced feathers that were significantly different from all the other diet/fish lot produced feathers. This does not necessarily explain the lack of difference of feathers produced on lot 2, lot 3 or lot 4.

Other possible causes of the variation of lot 1 feathers to the rest include the production of feathers from unequal use of internal nutrient reserves (Ayliffe et al. 2004). Furthermore, only female birds were initially sampled in the beginning of feather collection until it was decided that a larger project could be derived from the captive birds. Other influences to consider might be human error in feather collection, feather colour, or penguins which previously underwent incomplete moults. Feather colour has been shown to cause variation in isotopic signatures between black and white parts of feathers (Michalik et al. 2010) and both breast [white] feathers and body [blue] feathers were sampled in this study. Some of the captive birds at the IAC have undergone only partial moults, generally of either of the head and neck area, or of the breast area. Although this happened rarely (personal observation), it is possible that there may have been feathers collected after a full moult which contained feathers produced on two different diets. Further research would be useful because it could be used to investigate what might cause the bird to only partially moult, especially because all penguin species are assumed to undergo a full moult every time.

Ultimately, the fish in this study can really only be indicative purely to a general area and the overall trophic levels of creatures inhabiting that region. It is difficult to be specific to location when the samples [fish] have the ability to swim around. At least the birds breeding colonies do not shift in location overnight. The combination of SIA with stomach sampling would be the ideal for in depth further research (Chiaradia et al. 2012) of diet types.

2.6.2 Origin and gender

The fact that these birds have all been fed on the same diet for some time helps to remove outside factors that can influence isotopic reactions in terms of sexually dimorphic foraging behaviour (Awkerman et al. 2007), possible subspecific physiological differences and dietary influences of colony of origin (Hocken 1997; Allen et al. 2011; Flemming et al. 2013). That there are differences between the isotopic signatures of various colonies has been established [see chapter 3], but, less so the exact nature of how these differences come about. The objectives for this portion of the study were to investigate the variation present within subgroups, such as male versus female, and to explore the white-flippered morph versus the rest of the little penguins, and to see if birds from different areas of New Zealand reacted in a similar fashion when the local diet is replaced with a consistent one.

There does appear to be some segregation between the male and female moult data when all the feather data from the whole colony is examined [Figure 2.6B] but none that was significant when feathers produced on a diet/fish lot, in an attempt to control for diet, were investigated. Lot 2 feathers, produced from feeding on sprat lot 2, did not show any statistically significant differences, in either $\delta^{13}C$ or $\delta^{15}N$, between male and female little penguins. I was not able to examine gender in sprat lot 1 produced feathers because there was only one male moult collected during sprat lot 1. This is because the project initially had been to investigate only female feathers and egg shell differences but then it was extended to the parameters of the current study.

This lack of significant differences could be that although there have been some trophic level differences between male and female little penguins in the wild due to the variation in foraging behaviour, the homogenous diet of fish combined with the lack of

foraging activity in captivity would have led to the same efforts on the part of both male and female captive birds. More research is needed to further confirm this line of inquiry for this species.

Because there were no significant differences in either the $\delta^{13}C$ or the $\delta^{15}N$ signatures, either within diet/fish lot 1 or lot 2, or within all feather data derived from the captive colony [Figure 2.6A], the origin of the birds do not contribute to variation of the overall feather isotopic signatures. The white-flippered penguin, once regarded as a subspecies of the little penguin, did not display subtle metabolic differences in feather production based on taxonomic position or area of origin.

This captive group has provided a range of samples over a sufficient period of time to show that the stable isotopic ratios can vary between individual birds despite continued consumption of the same diet. The IAC started with a pre-formed population of North Island-derived birds in 2006, but did not start accepting rescued white-flippered birds until sometime later, in late 2007, so the age of the North Island birds, on average, could be significantly higher than the white-flippered birds that had been rescued after the IAC colony had formed. This study was not able to address any questions about age nor the possibility of change in isotopic readings due to metabolic processes undergoing adjustment because of ageing in the birds. It must also be taken into account that the average age of a wild little penguin is less than that of a captive bird at the IAC. It is possible that isotopic ratios due to age could respond in various ways; however, exact age can be difficult to acquire for animals in the wild (Mizutani et al. 1992). Further research is needed to explore and to quantify the way in which differences in increasing age can have an impact on the stable isotope results. Salt water exposure on wild penguin feathers must also be considered as a variable when comparing a wild population to this group of captive birds, who swam only in fresh water.

There are limitations to be considered when using captive animals for stable isotope analysis especially when the subjects are on a restricted diet, which possibly causes physiological stresses that might alter the isotopic discrimination and therefore final results (Hobson & Clark 1992b). Factors that also might affect the results include average age of the captive colony, level of physical activity [low because the birds used in this study were fed twice a day and did not hunt for their fish] and as a result, a

possible difference in metabolic rate. Metabolic rates in captive birds are lower and this could affect the isotopic turnover rate (Hobson & Clark 1992c). The birds in captivity have very little choice when it comes to their diet and in the wild have more flexibility/preferences in their foraging behaviour (Davies et al. 2009), and as a consequence individual bird choice/prey availability could also influence wild isotopic results and could not be accounted for in a captive trial.

Chapter 3 Geographic variation in the stable isotope composition of little penguin feathers in New Zealand

Abstract

My objective in this chapter was to compare the stable isotopic composition of feathers between several little penguin [*Eudyptula minor*] colonies. Feathers were collected from three colonies on the east coast of the South Island and in several sub-colonies on the West Coast. I used stable isotope analysis [SIA] of $\delta^{13}C$ and $\delta^{15}N$ of feathers to determine whether trophic level depended on the geographic location of each colony. Correlations between the $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures of penguins from the wild colonies were then compared to a captive colony at the International Antarctic Centre comprised of individuals from around New Zealand. I also examined whether the "white-flippered penguin" [*Eudyptula minor albosignata*], a colour morph formerly recognised as a subspecies confined largely to Banks Peninsula, differed in stable isotope ratios from other populations. Based on $\delta^{15}N$ ratios, I found penguins on the West Coast showed a lower trophic level than the east coast birds. Similar differences in the $\delta^{13}C$ ratios also confirmed the birds on the two coasts were feeding on different prey species and thus were occupying different trophic levels. Surprisingly, a colony of the white-flippered morphs at Harris Bay was more similar in both carbon and nitrogen compositions to the geographically more distant Oamaru population than to another, geographically closer, colony of the white-flippered morph in Flea Bay. This suggests that the carbon isotope signatures reflect the location of the foraging area and the approximate trophic level of the birds during their pre-moult foraging period, and are not due to sub-specific differences. Isotopic compositions of captive penguins, which were fed primarily on fish sourced from Europe, were quite different from the compositions of all wild populations, confirming the link between differences in diet and differences in isotopic ratios.

3.1 Introduction

The stable isotopic compositions in feathers of carbon and nitrogen are well established proxies for conditions connected with geographic location of diet and trophic position (Cherel & Hobson 2007; Cherel et al. 2007). Differences in location should be reflected in the stable isotope compositions because carbon has been shown to correlate with origin of nutrients in food webs while nitrogen can reflect differences in trophic level (Cherel & Hobson 2007). A number of researchers have used stable isotope analysis [SIA] to identify the foraging areas used by marine predators, such as several penguin species foraging in the Southern Ocean (Cherel & Hobson 2007), and the prey availability of key species, such as the Antarctic krill [*Euphausia superba*] consumed by four different top Antarctic predators (Croxall et al. 1999). SIA can be a valuable tool in studies of foraging behaviour, because it can provide an overall picture of the foraging habits of a species (Jaeger et al. 2009; Flemming & van Heezik 2014), especially when used to identify foraging areas of seabirds while at sea, which by their nature, are difficult to follow effectively without further impacting on normal behaviour.

Knowing the general diet and trophic levels of seabirds would give us a benchmark to measure future changes against. Establishing an initial array of data of stable isotopic compositions, of various tissues, across a number of seabird species could assist in future investigations in response to change in environmental conditions [e.g., climate change], prey species availability [e.g., overfishing], and the ability of food webs to support higher trophic level marine predators. For example, Hilton et al. (2006) investigated the causes of decline in the sub-Antarctic rockhopper penguin [*Eudyptes chrysocome*] using SIA of feathers from museum collections. Using museum skins in addition to collection of contemporary feather samples from several sites to cover a period of 148 years, they found evidence of decreased primary productivity in $\delta^{13}C$, and using $\delta^{15}N$, a long-term decline in trophic level, which were then correlated with sea surface temperatures. Together, this indicated a shift in diet over time, especially in warmer years, to prey that occupied a lower trophic level and suggesting declines were due to ongoing habitat change (Hilton et al. 2006).

The little penguin [*Eudyptula minor*] is a good model with which to examine variation in stable isotope ratios as they occupy a high trophic level and are generalist feeders,

but with some evidence for variability in diet between colonies and over different seasons and this flexibility of diet is then reflected in their trophic interactions (Chiaradia et al. 2012; Flemming 2012; Flemming et al. 2013). In both New Zealand and Australia, little penguins have been found to feed on several different species of fish, either small near shore schooling fish or pelagic juveniles, as well as some cephalopods, although the exact species and range of species in the diet varies geographically across their distribution (Klomp & Wooller 1988; Fraser & Lalas 2004; Flemming et al. 2013). Overall, fish comprise more than 95% of the diet and they are caught primarily by pursuit-diving (Marchant & Higgins 1990).

In this chapter, I take advantage of this foraging plasticity to examine variation in stable isotope ratios among a variety of little penguin colonies around the coast of New Zealand. I also compared the isotope ratios of birds from the wild with individuals in a captive colony that was fed on fish imported from Europe. I hypothesized that each colony would have varying carbon values, though perhaps tempered by geographical proximity, as the prey in each region are likely to be more similar. In contrast, I expected little penguins to have fairly similar nitrogen values across all colonies because they should, in theory, still be foraging at a comparable trophic level, even if prey species differed slightly. In comparison, birds from the captive colony should differ from wild birds given the distinctly different origin of prey consumed. Finally, I also compare the isotopic compositions of little penguins with the white-flippered morph of the little penguin, which was formerly recognised as a distinct subspecies restricted to the Banks Peninsula area of the South Island. If this morph differs in trophic level and diet from its congeners, then such a difference may be detected in its isotopic signatures. Together, the isotopic compositions will also provide a benchmark for future use, if a changing climate affects the prey base and thus the trophic level of little penguins over the coming decades.

3.2 Methods

The location of the wild little penguin colonies sampled for this study are shown in Figure 3.1. The Oamaru site is one of the three situated on the east coast of the South Island and has a current population of 120 breeding pairs. Both the Flea Bay [1304

breeding pairs/nests] and Harris Bay [35 breeding pairs] penguin colonies are located on Banks Peninsula and are comprised entirely of the white-flippered penguin morph. Penguins were also sampled from a range of sites on the West Coast of the South Island and these sites include the Punakaiki River Road Reserve [2-6 breeding pairs], Bullock Creek Road Reserve [5-6 pairs], Joyce Bay Road Reserve Colony [21-35 pairs], Nile River Road Reserve Colony [22-32 pairs], Rahui Colony [9-16 pairs] and White Horse Creek [17 -21 pairs]. Locations of these colonies are shown in Figure 3.1B.

Feather collection was carried out by the local rangers or caretakers in each area. In Oamaru, feathers were collected during weekly nest-box checks. Between two and four feathers were collected per bird and stored in plastic screw top vials. All penguins sampled in Oamaru were female. Samples from Flea Bay penguins were collected during February 2015; feathers were identified as coming from each box, though two feather samples were from individual moulting birds identified by bands, and initially stored in paper envelopes. Harris Bay penguin feathers were collected from banded individuals and placed in individual plastic Ziploc bags and were collected in February 2015. The West Coast penguin feathers were either collected from road-killed birds or from nest boxes, and were also stored in plastic bags, labelled either as a road killed bird or from nest box number. The road-kill birds are referred to as the accidental death samples and were collected from 2009 until January 2012 from a variety of locations. The burrow/nest box feathers collected were mostly collected during the moult season of 2013, but there were some samples from some of the same locations collected in late 2012.

For comparison, I also analysed the stable isotope ratios of captive little penguins held in the Antarctic Centre [see chapter 2]. Feathers were collected and analysed for a total of 20 captive penguins. Nine of the penguins in the captive colony were classified as "white-flippered" penguins and originated from the Flea Bay colony on Banks Peninsula.

3.2.1 Stable Isotope Analysis [SIA]

The methods used to process feathers and measure the stable isotopes of both C and N are described in detail in Chapter 2. Briefly, the feather processing involved washing

the feathers with microfoil/distilled water and a small amount of detergent. Feathers were washed individually, with gentle brushing with a toothbrush, to remove dirt and faeces, and then rinsed in microfoil water. The washing solution and rinse water were changed after at least every 25 feathers to prevent oil build up. Individual feathers, generally three per sampled moult, were then placed into a one half of a glass petri dish, covered with a porous nappy liner held in place by rubber bands and placed into a fume-hood for at least 24 hours to dry, then into small sealed universal bottles filled with approximately 20 mL of 2:1 chloroform/methanol solution for 24 hours. After 24 hours, feathers were removed from the solution, rinsed and agitated in two separate beakers containing the 2:1 chloroform/methanol solution, of c. 20 mL, and then placed in petri dishes to dry for at least 48 hours in a fume hood.

Once dry, each feather was individually cut into small fragments; only the feather barbs on one side of the pennaceous part of the vane, not the rachis or the philoplume [as shown in Appendix 3],was used for analysis as this was generally sufficient for sampling purposes. These pieces of feather were placed into small tin cups, individually tared on a 7 decimal point scale [Mettler Toledo UMX2 microbalance]. Once within the required weight range, each cup was folded into a small ball and placed into a nunc tray to be processed by the elemental analyser. Samples were analysed for $\delta^{13}C$, $\delta^{15}N$, %C and %N using a Costech Elemental Combustion System [ECS] 4010 [Costech Analytical Technologies, California, USA] connected to a Delta V Plus Isotope Ratio Mass Spectrometer [IRMS; Thermo Fisher Scientific, Bremen, Germany] via a Finnigan Conflow III [Thermo Fischer Scientific].

3.2.2 Comparison of potential prey isotopic signatures with penguin signatures

Although I was unable to sample and measure the isotopic compositions of potential prey items of little penguins in each of my study sites, I was able to make one such isotopic comparison with the penguins feathers on the east coast, as the isotopic signatures of the plankton and fish in this region have been measured as part of another project (D. G. Bennet, *pers. comm.*). Prey items were sampled between one and seven km off of the Kaikoura coastline; these plankton and fish samples were collected in January and February, during the same year in which the pre moult foraging period

occurred for the penguin colonies sampled on the east coast of the South Island. I then compared the fish and plankton isotope signatures to that of the three wild little penguin colonies on the east coast of the South Island that were sampled in this study.

3.3 Data analysis

I used Shapiro-Wilk and Bartletts tests were used to check for normality and homogeneity of variance, respectively. Because the data had non-equal variance, a non-parametric Kruskal-Wallis rank sum test was used to test significant statistical difference, and this was followed by Tukey HSD tests to show where significant differences between colonies existed. These tests were done using the R software package (R Development Core Team 2005).

For samples collected from West Coast penguins, two subgroups had equal variance as shown by F-test and so I then compared using *t*-tests in Excel®. All West Coast data was combined for comparisons between colonies, but was then divided by subgroup for analysis within just the West Coast area. Comparisons between the captive birds originating from the West Coast and from the white-flippered morph colonies were done also using F-test and *t*-test functions in Excel®.

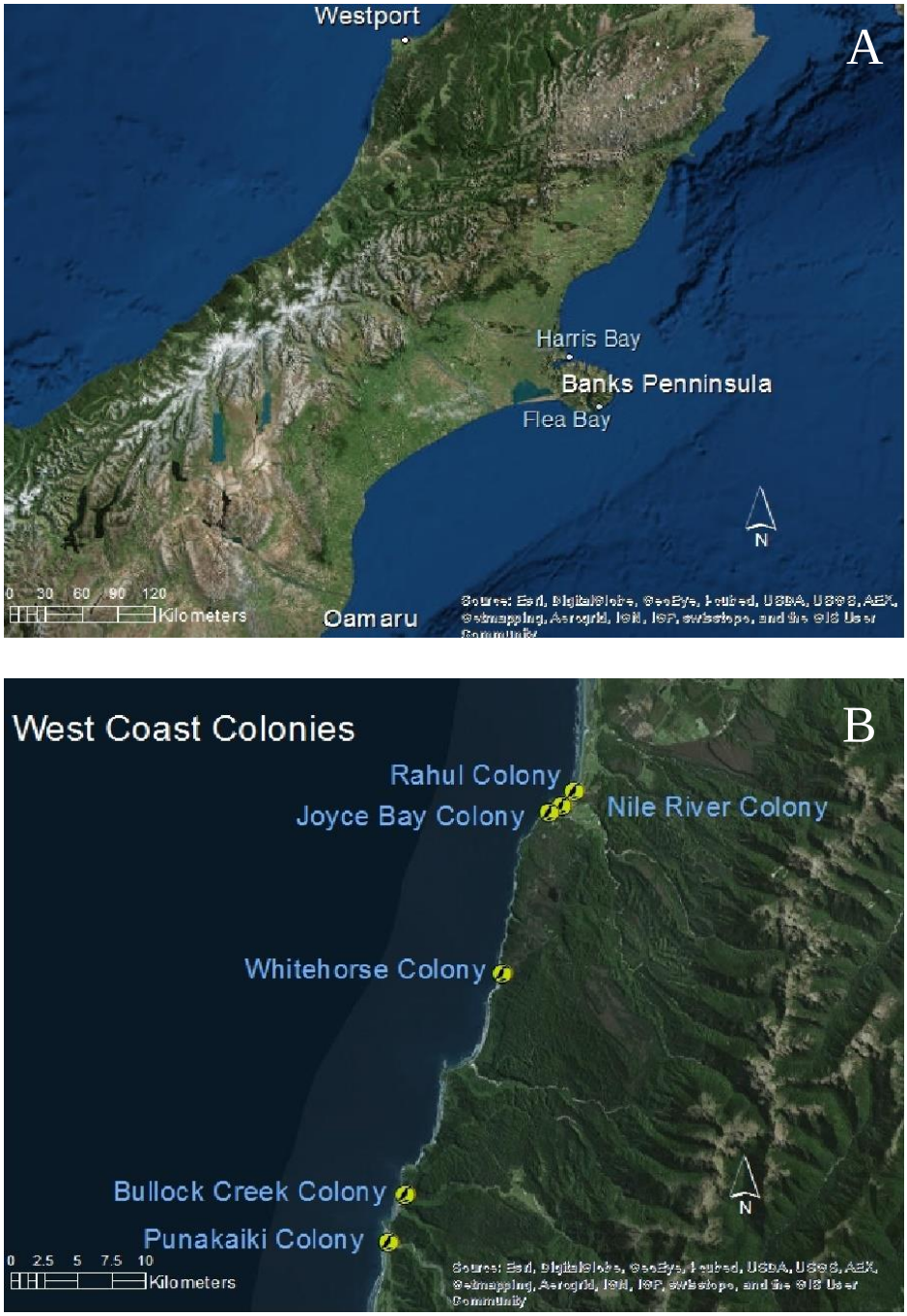


Figure 3.1

[A] Map of the South Island showing the little penguin colonies that were sampled during the course of this study.

[B] Details of colonies around Westport on the West Coast.

Maps were produced on ArcGIS 10.4 for Desktop – ArcMap Product version 10.4.0.5524

3.4 Results

3.4.1 Isotopic compositions of little penguins in wild colonies

The average $\delta^{13}C$ and $\delta^{15}N$ isotopic compositions of little penguins across all the wild colonies are given in Table 3.1 and plotted in Figure 3.2. The $\delta^{13}C$ signatures of the feathers differed significantly among penguin colonies [Kruskal Wallis: H=104.7, df = 3, Tukey HSD P<0.001], however, there was no significant difference between the Oamaru and Harris Bay penguin colonies [Tukey HSD: P=0.80]. This pattern was echoed with the $\delta^{15}N$ signatures, with a significant difference among the colonies [Kruskal Wallis: H=59.92, df = 3, Tukey HSD P<0.001], but again with no difference between penguins from Harris Bay and Oamaru [Tukey HSD: P=0.61]. The $\delta^{15}N$ signatures between Harris Bay and Flea Bay [Tukey HSD: P=0.0080] show a significant difference between the two white-flippered colonies.

Table 3.1 Mean, standard deviation [SD] and the range of $\delta^{13}C$ and $\delta^{15}N$ for feathers of little penguins sampled at all wild colonies. All West Coast data was combined in this table for the comparison between colonies.

Colony location	$\delta^{13}C$ [‰] Mean ± SD	Range [‰]	$\delta^{15}N$ [‰] Mean ± SD	Range [‰]
Harris Bay n=10	–20.12 ± 0.25	–20.43 to –19.72	15.93 ± 0.43	14.93 to 16.43
Flea Bay n=48	–19.41 ± 0.33	–19.90 to –18.49	15.58 ± 0.44	14.31 to 16.32
West Coast n=26	–18.49 ± 0.70	–19.59 to –16.80	14.72 ± 0.66	13.36 to 15.80
Oamaru n=12	–19.99 ± 0.26	–20.40 to –19.65	16.15 ± 0.47	15.35 to 17.03

Comparison of samples collected from the birds in the accidental death series with that of the burrow-collected moult feathers [Table 3.2], showed the two groups were quite different. Statistically significant differences were found between the accidental death series and the burrow collected feathers in both $\delta^{13}C$ [t=3.18, df=25, P=0.0039] and $\delta^{15}N$ [t=2.44, df=25, P=0.017].

Table 3.2 Mean, standard deviation [SD] and the range of $\delta^{13}C$ and $\delta^{15}N$ for feathers of little penguins sampled on the West Coast. Data from the West Coast colonies is divided into burrow collected feathers and accidental death feathers for analysis within West Coast area as shown below. Accidental refers to samples collected from road kill birds year round [2009 to early 2012, though some of the samples did not have a collection date noted] while the burrow feathers were collected from nest boxes at the end of the moulting season [2012-2013].

West Coast	$\delta^{13}C$ [‰] Mean ± SD	Range [‰]	$\delta^{15}N$ [‰] Mean ± SD	Range [‰]
Accidental n=13	–18.12 ± 0.71	–19.39 to –16.80	15.03 ± 0.65	13.49 to 15.80
Burrow n=14	–18.84 ± 0.48	–19.59 to –18.02	14.43 ± 0.55	13.36 to 14.42

3.4.2 Isotopic compositions of captive vs wild birds

The captive white-flippered penguins were significantly different for both $\delta^{13}C$ [t=13.96, df=54, P<0.001] and $\delta^{15}N$ [t=7.65, df=55, p<0.001] from the white-flippered morph penguins at Flea Bay, and in the $\delta^{13}C$ [t=19.75, df=23, P<0.001] and $\delta^{15}N$ [t=4.44, df=22, P<0.001] from the white-flippered penguins at Harris Bay. Thus, the isotopic signatures of the wild birds showed no similarities with those of the captive birds that originated from the same geographic area but were fed on a diet of European origin [Figure 3.3A]. A similar result was found when comparing the captive birds sourced from the West Coast with their wild counterparts; there was a significant difference between the two groups for $\delta^{15}N$ [t=–13.55, df=18, P<0.001] but not for $\delta^{13}C$ [t=–0.17, df=33, P=0.86; see Figure 3.3B].

3.4.3 Relationship between isotopic signatures of little penguins and their potential prey

When the isotopic compositions of birds from the east coast colonies were compared to those of the Kaikoura fish samples, the mean difference between possible diet and feathers was 5.04 ‰ for nitrogen and 1.34 ‰ for carbon [Table 3.2; Figure 3.4]. In contrast, these do not match the mean of the captive colony enrichment factors of diet to feathers. Overall, the captive colony, as shown in Chapter 2, had a mean of difference between diet and feather of 3.57 ‰ ± 0.13 in nitrogen and 2.5 ‰ ± 0.20 for

the carbon [only using first two diet lots as the last two lots only had n=3 and n=1 feather samples].The West Coast contrasts between Kaikoura prey signatures to feathers are 3.88 ‰ for nitrogen and 3.00 ‰ for carbon. The plankton to fish nitrogen is 1.42 ‰ and carbon 0.83 ‰, showing the expected enrichment of trophic level and similarity of location.

A linear regression is shown for the $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures for possible prey from the Kaikoura area more closely intersects for the isotopic signatures for penguins on the east coast than the West Coast colonies [Figure 3.4], suggesting that despite the prey not being sampled in the immediate area of the east coast penguin colonies, the possible diet and trophic level are likely to be similar to that found in Kaikoura. The data of the plankton and fish [Table 3.3] that have been sampled could be considered to represent a much generalized overview of the isotopic signatures of the east coast of the South Island, but not specific enough to represent the areas in which the sampled east coast colonies would have been carrying out foraging activities [shown in Figure 3.4].

Table 3.3 Mean, standard deviation [SD] and the range of $\delta^{13}C$ and $\delta^{15}N$ for plankton/fish data. Plankton and fish were collected in January and February of 2015, and from two to seven km off the coast of Kaikoura [north of Banks Peninsula on the east coast of the South Island of New Zealand] (Bennet *pers. comm.*).

east coast	$\delta^{13}C$ [‰] Mean ± SD	Range [‰]	$\delta^{15}N$ [‰] Mean ± SD	Range [‰]
Plankton n=262	–22.01 ± 1.78	–26.04 to –13.56	9.42 ± 1.56	3.74 to 15.56
Fish n=77	–21.18 ± 1.04	–24.97 to –20.40	10.84 ± 1.57	8.25 to 14.01

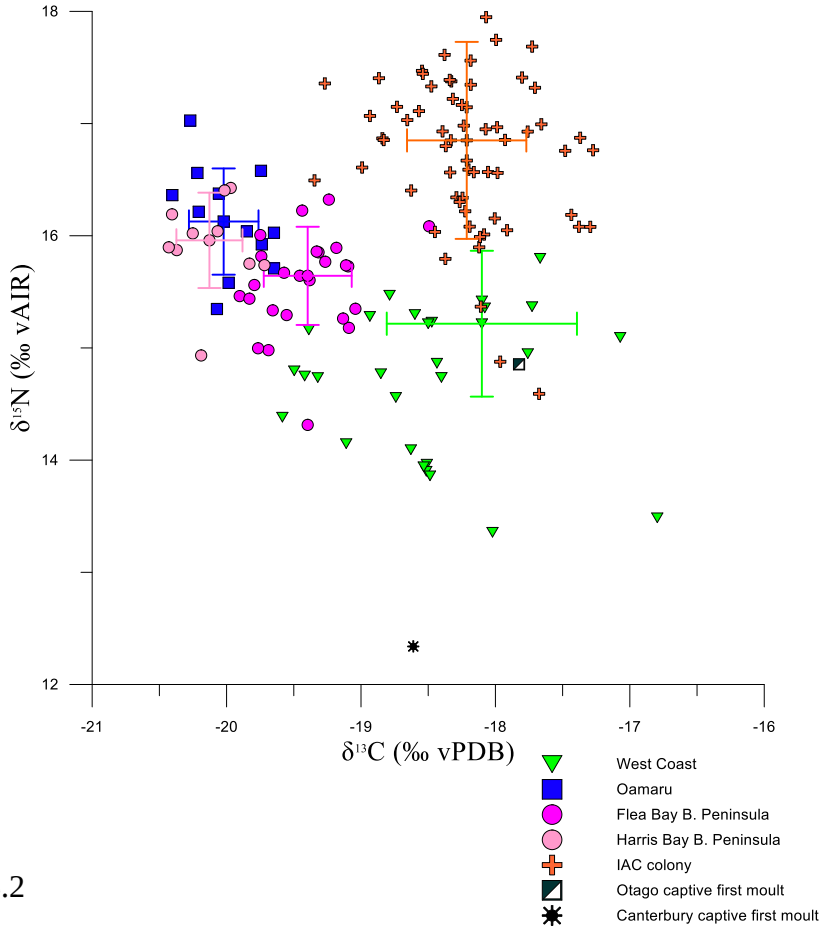


Figure 3.2

Isotopic feather signatures from all little penguins used in this study, including wild birds from both the West Coast and three colonies on the east coast of the South Island, as well as captive birds. Note that birds from Banks Peninsula are comprised of the "white-flippered" morph. IAC = International Antarctic Centre. The east coast signatures group together but differ to both the feather signatures from West Coast birds and captive birds. Note the feathers from a bird introduced into the captive colony from Dunedin as a newly-fledged adult are indicative of Otago coastline signatures. This bird's next moult was not in line with the rest of the captive colony until undergoing her third moult [Appendix 4]. Another new bird to the captive colony, found on New Brighton beach, underwent a moult in recovery and diet was unknown, on his next moult he was in line with the rest of the captive birds. Figures also show, using error bars, mean ± SD.

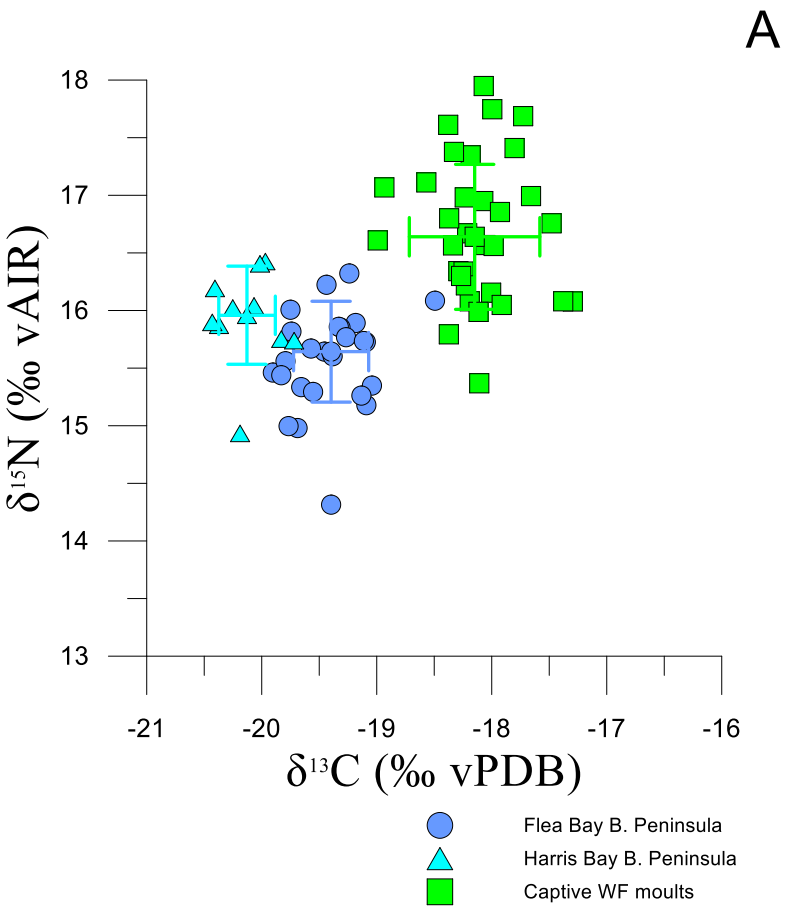
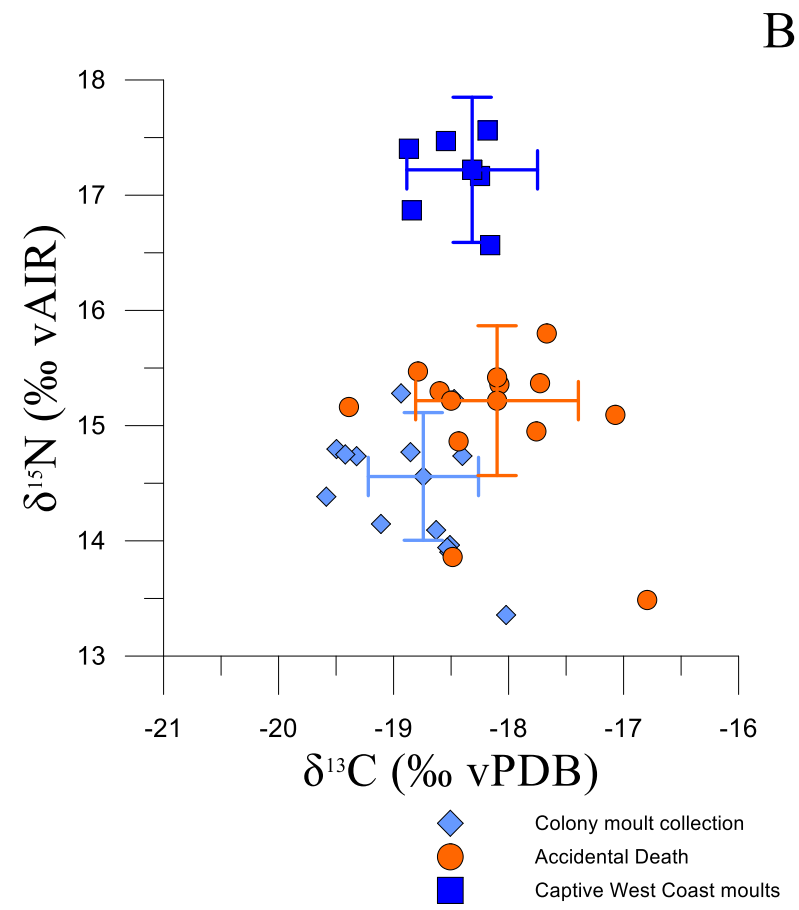


Figure 3.3:

A: Comparison of $\delta^{13}C$ and $\delta^{15}N$ ratios between two colonies of "white-flippered" penguin on Banks Peninsula. Ratios of feathers moulted from captive individuals originating from the Flea Bay [Captive WF moults] colony are also plotted to show the significant difference of the captive birds on a European fish based diet. Figures also show, using error bars, mean ± SD.



B: Comparison of $\delta^{13}C$ and $\delta^{15}N$ ratios between penguins sampled on the West Coast and captive penguins originating from the coast. Wild samples were collected from either burrows or directly sampled from road-killed birds [Accidental Death]. There is a significant difference between the wild and captive birds and then between the accidental death feathers and the burrow collected moult feathers. Figures also show, using error bars, mean ± SD.

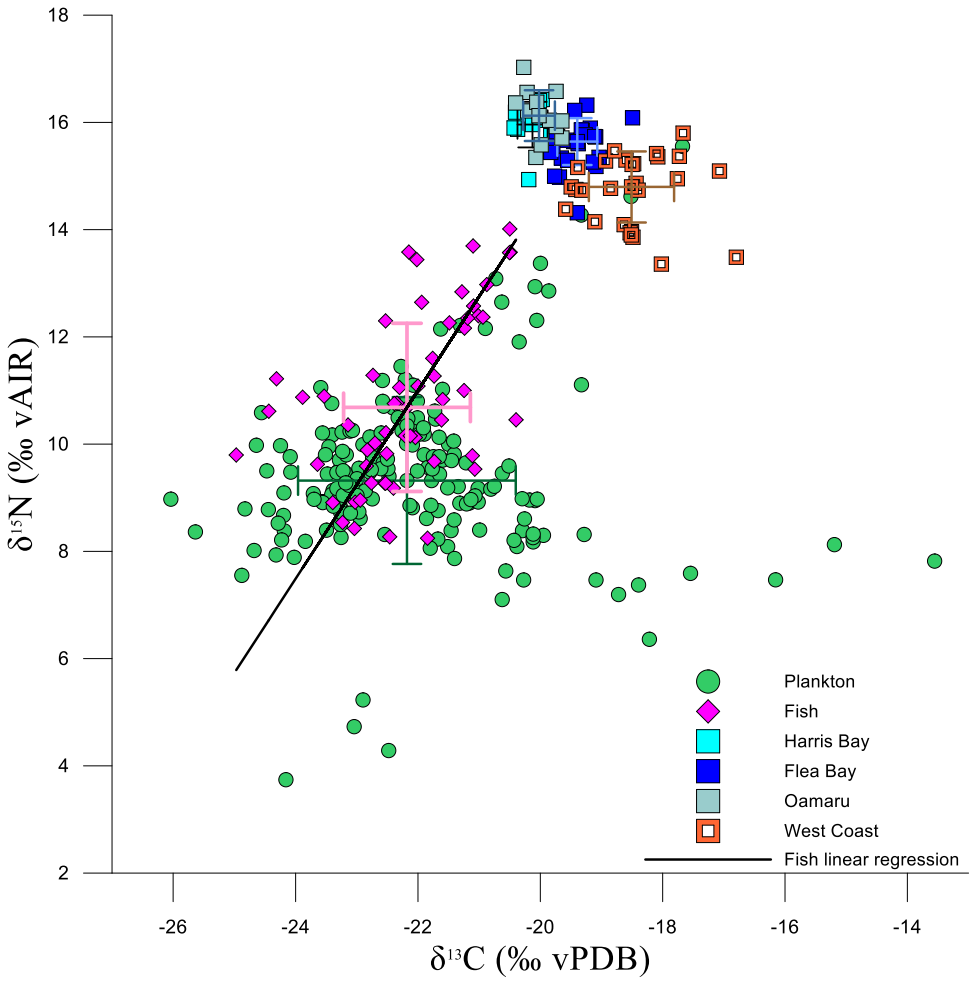


Figure 3.4

Isotopic signatures for east coast plankton and fish samples collected in 2014 in comparison to isotopic signatures from wild penguins. This shows the linear relationship between nitrogen enrichment levels as trophic level increases for the colonies located on the east coast. However, as the prey data was collected further north of the wild colonies, this is only a general relationship reflecting a possible indication of east coast prey signatures rather than specific to the foraging area of the sampled wild colonies. West Coast feather signatures are put in for comparison but note those samples were collected from 2009 to 2013. Figures also show, using error bars, mean ± SD.

3.5 Discussion

In comparing the isotopic results of each wild colony, I found that, with the exception of the Oamaru and Harris Bay colonies being quite similar, the West Coast was different from all other colonies, as was Flea Bay. My original hypothesis was that the Flea Bay and Harris Bay colonies would have been quite similar due to their relative close geographic positioning and that the two locations are both predominantly composed of white-flippered penguin morph. However, this was not the case, suggesting that the geographic location of the colony had a bigger bearing on the isotopic signature [and thus diet and trophic level] than any potential taxonomic ranking. I also found that captive penguins, fed on a diet sourced largely from Europe, had a different isotopic signature than any of the wild populations sampled, supporting my conclusion that the differences among wild populations were likely due to differences in their diet.

Little penguins are known to feed on a variety of prey species, and across their range they are considered to be generalist feeders (Flemming et al. 2013). Each colony appears to exploit geographically separated foraging areas, such as has been found in other studies, for example, the Balearic shearwater [*Puffinus mauretanicus*] (Louzao et al. 2011), and from a variable range of prey species showing flexibility in foraging habits (Weavers 1991; Chiaradia et al. 2016). The similarities in isotopic signatures I found between the Oamaru and Harris Bay colonies could therefore be predominantly attributed to remarkably similar prey species types or different prey species operating at a parallel trophic level in terms of $\delta^{15}N$.

It is remarkable that the $\delta^{13}C$ signatures were also quite similar between the Oamaru and Harris Bay colonies, especially since Flea Bay is located between Oamaru and Harris Bay and the values from there were quite different from those at Oamaru and Harris Bay. What is different about how penguins in these two colonies of white-flippered birds forage? It would be interesting to see if the Oamaru and Harris Bay colonies remain isotopically similar over several different moult seasons, and whether the Flea Bay and Harris Bay colonies shift to become more similar than observed in this study. The differences between these two fairly closely located colonies show the need for more information on isotopic variation based on each locale, including isotopic studies on their prey base. More research is needed before generalizations can

be made about diet in one area versus another, and further collection of feather samples from different colonies of the white flippered morph located around Banks Peninsula would be ideal for future research.

My conclusion that the white-flippered morph penguins do not display an isotopic signature that distinguishes them from birds in other little penguin colonies [i.e., those without white flippers] supports the removal of the subspecific status for birds with that pattern of plumage. My results instead suggest that any variation in the isotopic signatures of white-flippered morph penguins results is more likely to be based solely on location/diet of the colony and not on any underlying taxonomically-based difference in how they produce feathers, confirming, in this instance at least, that these birds are not different from other little penguins. This result supports the genetic evidence that the white-flippered morph penguin is not separable from other little penguins (Banks et al. 2008; Peucker et al. 2009), or at the very least, that differences in diet and trophic level cannot be used as features that distinguish this morph from other little penguins.

The signatures of West Coast penguins differed between the accidental death series and the burrow-collected samples. I do not have an explanation for this difference, but it may be due to the time span over which the accidental death series feathers were collected, ranging from 2009 to early 2012, while the burrow-collected samples were only sampled during the moult season spanning late 2012 to early 2013. Annual differences in prey available could affect the isotopic signatures of feathers [even on the same individual] but this was a factor I was unable to study. The difference could also be due to differences in degrees of feather degradation between the two groups. Because the feathers were collected from burrows and nest boxes and were in storage for a year before sample processing took place, there may have been some change in the isotopic signatures of the feathers. The accidental death series birds, once collected, were placed in the freezer for some time and then feathers sampled several years later in some cases. Furthermore, the feathers collected from accidentally killed birds were ‘in use’ until the moment of death and not subject to outside forces of decomposition (Sugiura & Masuya 2015).

Ideally, it would have been valuable to have directly compared the isotopic signatures of potential prey items in each of the areas being used by each colony for foraging with

that of the feathers of birds from that colony. Apart from the fact that the exact foraging areas are not known for each of the different colonies [except in a general sense of being nearby], it was not logistically possible to sample prey items of wild penguins in the timeframe of my study. However, I was able to undertake one comparison using a series of plankton and fish isotopic data that was collected and analysed in conjunction with my project. Although the prey samples were collected slightly north of the area that penguins in the east coast colonies in my study likely foraged, the data nonetheless does provide a generalized picture of the isotopic relationships of the area.

The plankton and fish results followed the expected linear relationship, showing an increase in trophic levels, and this was consistent with the wild east coast colonies, suggesting that fit was actually quite good, especially when considering the poorer fit of West Coast colonies and the captive flock. A small population of little penguins does breed in the Kaikoura area, and it would be useful to sample the feathers for a more direct comparison with the prey isotope data. My expectation is that the Kaikoura penguins would probably show quite similar isotopic signatures to that of other east coast colonies [especially Flea Bay].

In comparing captive birds [from Chapter 2] to the wild penguin colonies, the wild feathers show isotopic signatures that could only be gained from their prey species and foraging location in the wild. The captive birds have little variation that could be attributed to their geographic origin and instead reflected only the increased trophic level and location of a European fish-based diet. This is further demonstrated by the first two moults in captivity by a female juvenile little penguin from Otago. She exhibited an isotopic signature similar to the West Coast compositions despite her eventual shift to fall within the isotopic range of the rest of the captive colony [see Appendix 4]. Similarly, an adult captive male originating from Canterbury [and thus with a signature that reflects an unknown diet] underwent a moult with the vet while recovering from surgery, before he came to the IAC, but then his isotopic signature later shifted to be within line with the rest of the captive colony. Thus, the isotopic signatures of the wild colonies appear to reflect only the diet consumed in the foraging range and not on underlying physiological differences. The isotopic signatures of captive animals generally reflect the diets consumed (Kurle 2002; Cherel et al. 2005b; Germain et al. 2012) and as the isotopic compositions of the captive penguin feathers

are different from that of the wild birds, then it must hold that the diet fed to the captive birds is different from the wild consumed diet.

The trophic level of penguins in the wild colonies sampled in this study were all higher than found in an Australian study, with their $\delta^{15}N$ signature at 13‰, though they appeared to use muscle tissue as their sample type, and gained their samples opportunistically from birds that had died of natural causes or after an oil spill (Davenport et al. 2002). Thus is it possible that differences in the sample type [muscle vs. feather] and season could be a contrasting factor to the range found in this study. Of course, the trophic level of the birds sampled could also have shifted in subsequent seasons. Chiaradia et al. (2016) found significant differences in blood $\delta^{13}C$ and $\delta^{15}N$ of little penguins among the different stages of breeding and between years. However, Kelly (2000) points out that feathers, when compared to other tissues, tended to be among the more enriched. Variation between sampled tissues and colonies over several different seasons need to be identified at the start of a temporal isotopic baseline, and ideally, for different areas.

I was unable to account for variation in isotopic signatures that could be due to seasonal or annual changes, or to age of the birds. For example, there could be seasonal shifts in the physiology and metabolism of the birds, which would in turn affect their isotopic signatures. Conversely, the results reported in Chapter 2 of this thesis might suggest that physiology is not a primary control on the isotopic composition of little penguin feathers. Nevertheless, because penguins moult and re-grow their feathers in a much shorter time period than most other seabird species, and do it at a fairly consistent time of year, this seems unlikely unless age becomes a factor. As the life span of little penguins in the wild is only six to seven years (Reilly & Cullen 1979), but up to 24 years of age in captivity (personal observation), this could be an interesting avenue of further study.

Finally, further research is needed on the methodology of using feathers in stable isotope ecology (Bortolotti 2010). How long should a feather be left in the burrow before collection if not directly sampled from the bird? Does the isotopic signature of the feather alter as it grows older [before collection or in storage] or decays? A variety of insects, bacteria, and fungi feed on and degrade feathers (Sugiura & Masuya 2015) and information is needed to find at what point does this process starts to undermine the

original isotopic signature of the feather. Colour of the feather might also contribute to variation in isotopic ratios presented (Michalik et al. 2010). More information on the building blocks of feather growth is also needed, such as knowledge of what nutrient pools the feathers are formed from and how long those various molecules are present in the penguin before “use”.

In conclusion, my study has confined that the isotopic signatures of little penguins differ geographically and that this likely reflects broad scale differences in diet and/or trophic level. However, further work is needed to detail exactly how diets might differ at a species specific level, especially when prey species can occupy similar trophic levels in different geographic areas. Although significant differences in isotopic composition were found between some of the colonies, the exceptions [i.e., the similarity of some geographically separate colonies], are intriguing and require further study. I hope that feather collection will continue, and further, to confirm if the variation found in this study in the isotopic compositions between colonies, and whether these signatures change in the future as we continue to alter and disrupt the marine environment.

Chapter 4 Conclusions

4.1 Revision of findings

This study compared little penguins [*Eudyptula minor*] in two different types of environments, that of the rehabilitated captive bird and that of the wild penguin. I used stable isotope analysis [SIA] of $\delta^{13}C$ and $\delta^{15}N$ to investigate the isotopic compositions of little penguin feathers in relation to their diet in a captive colony and then made further comparisons with, and between, four wild colonies.

The thrust of my argument has been that the stable isotopic composition of fish from a known diet would directly affect the isotopic composition of little penguin feathers, as supplied by moult feathers. These enrichment factors, once determined from the feathers of captive little penguins, could then be used to compare their conspecifics from wild colonies. I also investigated the extent to which geographic variation [i.e., colony of origin] had an effect on the possible physiological formation of the feathers, based on location in which various birds originated from, including whether this aligned with possible subspecific limits, such as the white-flippered penguin. Penguins with the characteristics of the white-flippered morph are found only on the east coast of the South Island and they have obvious morphological differences from the rest of the little penguin populations found throughout New Zealand and Australia, although are not currently considered as a subspecies (Checklist Committee 2010). I wanted to ascertain if there were any significant isotopic variance in the feathers of this once subspecies when fed on the same diet as the rest of the captive colony.

The final goal of this study was to compare feather isotope ratios from four geographically separated little penguin colonies, between the colonies themselves and then to the isotopic signatures of the wild birds to the captive birds. The feathers collected from the wild colonies also yielded useful information to contribute to the general available knowledge base for this species and for contribution to future stable isotope mapping of the oceans surrounding New Zealand. These objectives have been fulfilled. This study is, to the best of my knowledge, the first long term study of stable isotopes undertaken on little penguin feathers in a captive environment.

The first objective was to measure the enrichment factors using the isotopic signatures of captive little penguins fed on a known diet, but one that was different from that eaten

by wild birds. The results confirmed the link between diet and differences in isotopic signatures. The enrichment factors I found in the captive little penguin colony will also assist in creating the bigger picture isotopically when research is undertaken on wild little penguin colonies. Stable isotope compositions of feathers [and other tissues] have revealed the generalised feeding behaviour of animals (Mizutani et al. 1990), and controlled studies (Cherel et al. 2005b; Polito et al. 2009; Polito et al. 2011b) in which enrichment factors were found and have then been used to reconstruct dietary inputs (Polito et al. 2011a). Coupled with other dietary studies of the little penguin (Flemming et al. 2013), long term feather collection could help indicate shifts in diet and trophic level now that the species-specific enrichment factors of $\delta^{13}C$ and $\delta^{15}N$ are known for the little penguin.

The second main finding of this study was, when controlled for diet, that the captive birds showed no variation in isotopic signatures based on gender or the area of their origin in the wild. Because the captive colony contained birds that had originated from the North Island, the West Coast of the South Island, and several white-flippered individuals from Banks Peninsula, there was a variety of birds which may or may not have reacted in similar ways to the same diet; namely, possible differences in the isotopic fractionation in their metabolization of diet and subsequent feather growth. The same principle also applies to potential differences between genders when controlled for gender-specific foraging behaviours.

Subspecific status for the white-flippered penguin has been contested by genetic studies carried out on various populations of the little penguin. Subspecific categorisation has been an issue since Kinsky and Falla (1976) proposed six groups to categorise the various populations of the little penguin. Studies by Banks and colleagues (Banks et al. 2002; Banks et al. 2008) found that there were genetic differences between little penguins found in Australia and the Otago area of the South Island, and the rest of the little penguin population of New Zealand, a result later confirmed by Grosser et al. (2016). The lack of isotopic variation in the feathers between white-flippered and other little penguins that I found in this study is an important result because this suggestion confirms that the use of stable isotopes as proxies for diet research of the little penguin will not be influenced by varying metabolic responses to diet and feather formation such as that may be expected between different subspecies. Nevertheless, the genetic

differences suggest that further research on how the Otago/Australian birds respond to a controlled diet shared by other New Zealand little penguins would be worth investigating.

Because little penguins have been shown to change their foraging behaviours depending on time of year (McCutcheon et al. 2011; Chiaradia et al. 2016), and certainty of sexing birds in the field is somewhat limited (Renner & Davis 1999), my finding that captive male and female penguins do produce feathers of similar isotopic compositions while being fed an identical diet contributes to the wider understanding of gender specific foraging behaviour. Nevertheless, more research is needed as there was possible variation between the sexes, shown by the P-value approaching significance.

I then built on the findings about captive penguins, the diet and feathers in this study, and compared the isotopic compositions of feathers collected from wild little penguins. Again the results provided confirmation that the isotopic signatures appeared to be influenced, or possibly even driven by diet. There were striking significant similarities between a colony of the white-flippered morph, at Harris Bay on Banks Peninsula, and those at Oamaru, which is mostly composed of little penguins (Hocken 1997). The interesting thing is that these two colonies are 220 km apart and lie on opposite sides of Flea Bay [also located on Banks Peninsula], another colony dominated by the white-flippered morph, which did not show the same similarities in isotopic composition of feathers. This contributes to the conclusion that the Oamaru penguins and Harris Bay penguins were foraging on similar isotopic types of prey that occupied comparable trophic levels because both the $\delta^{13}C$ and $\delta^{15}N$ compositions were significantly similar. Furthermore, Flea Bay feathers were found to accord with isotopic prey data from Kaikoura, 150 km north along the east coast, on the other side of Harris Bay.

This finding further confirms the lack of a physiological difference in feather production of the white-flippered morph from that found in other little penguins, and instead suggests that the isotopic compositions of feathers are more influenced by geographic location and trophic level of diet. Confirmation of this variation is shown by the distinctive isotopic signatures of feathers from Flea Bay penguins and the West Coast colonies, indicating that the birds in each area that are occupying slightly different trophic levels or at least feeding on different types of prey in their different foraging areas.

4.2 Limitations of the study

Feathers, if they are the only sampling medium, possess potential characteristics that might affect the isotopic data. Research has shown that different colours within a feather can provide differing results (Michalik et al. 2010), but that also raises the issue of the time taken for feather growth (Bortolotti 2010) because different portions of the feather can present different signatures when an individual feeds on different food items over the course of growing its feathers.

Taking feather samples from penguins, which undergo a concentrated moult, would hopefully mitigate some of the doubts about how changes in diet affect the signatures of feathers as they develop. This is because penguins fast during their annual moult, when most other bird species do not, and thus one would not expect the $\delta^{15}N$ composition to be altered which would present a skewed trophic level derived from different prey being eaten during different periods of feather growth. There are, however, still limitations because feathers only represent one portion of the foraging activity of the penguin over the course of a year, and other tissues may need to be analysed to assess changes in diet, or trophic level, at those times of the year when feather growth is not occurring. In contrast, the annual pattern of moult does provide a consistent annual "snapshot" of diet and trophic level using SIA and thus a long-term collection and analysis of feathers may be able to provide evidence of possible dramatic changes [decade to decade] in the marine environment as compared to one or two seasons of variation.

Other variables which could also have affected the results of this study include the lack of salt water exposure to feathers from birds in the captive population. The feathers of little penguins in the wild would, of course, be exposed daily to salt water. The captive penguins, at least in the period after they entered the population, had their feathers exposed only to the fresh water provided in the pool in their enclosure. Would differences in exposure of this type, as well as other environmental factors, [preen oil or anything not removed by the sample cleaning process], explain the variation I found between captive and wild penguins? In the first instance it seems unlikely, because the feathers in the two groups did not appear physically different, but it would be worth investigating experimentally by, for example, immersing feathers from the captive penguins in salt water for a period and then comparing their isotopic signatures with

that from control [wild penguin] feathers. Further consideration should also be given how feathers are stored and the state they were collected in, and whether decomposition becomes an influencing factor.

The use of the captive population to gain an enrichment factor was useful in that it provides a baseline for future comparisons. Nevertheless, this enrichment factor reflects only one prey species [when considering the first two diet/fish lots]. Would the enrichment factor change in response to a more varied but still consistent diet? Data on blood samples would show those variations in isotopic response, such as may have happened when the captive birds were subjected to supplemental fish trials [the last two diet/fish lots sampled], but the diversity of the supplemental fish consumed may not have been consistent enough to show a marked change in feather composition overall.

4.3 Future research

The information gained from SIA can be useful to infer general details about the diet of the animal sampled. Ideally, when combined with other sampling methods, it offers a well-rounded picture of the various mechanisms and conditions under study. It would be ideal to gather blood samples from the captive little penguins used in this study at regular intervals throughout the course of a year to discover and map fluctuations on a known diet [with variation of prey species]. Furthermore, a more detailed study using SIA of blood could be used to ascertain if seasonal factors influence the isotopic results, as well as providing a comparison of isotopic responses between the different ages of the birds. The ideal would be to undertake sampling of eggshell and the contents of eggs produced by the captive birds while on the known diet to be able to compare between diet, feathers produced on diet, and eggs formed. If long-term sampling of the diet were undertaken, eventual sampling of beak and claw samples could also be linked to various food sources, in addition to providing an investigation of beak and claw growth rates of these birds in captivity.

Other prospects for study within the captive colony also include the introduction of juvenile birds [“grown” on a wild diet] into the captive population to find out at what point they no longer use internal nutrient resources gained from their parents, and instead begin to show the signatures of the captive diet. Another avenue is the

observation of some partial moults on a couple of the birds in the captive colony, of the head and neck area only, to see if such a phenomenon is a symptom of a non-varied diet, or whether it has been observed in the wild.

The methodology of SIA of feathers in ecology also requires further research, both in the criteria in the collection and suitability of feathers after a certain point of decomposition, and also in the duration or age of feathers in long term storage. Salt water exposure and other environmental factors [e.g., preen oil], as well as colour, are also avenues for further investigation as to how those factors might influence isotopic measurements.

The collection and analysis of feathers from wild little penguin colonies along various portions of the coast of the South Island would be worth continuing. In addition to the role such a collection would play in the creation of a long-term feather bank, such as was used by Hilton et al. (2006) and by Farmer and Leonard (2011), it would also be interesting to see if the Harris Bay and Oamaru little penguin populations continue to remain similar, or if the Flea Bay penguin populations and those at Harris Bay would ever align, and then if this alignment corresponds to overlap in their foraging areas. That would also require high precision [GPS] tracking of the birds at sea.

4.4 Concluding statements

Because of their relatively long life span, seabirds are ideal subjects for studies of possible environmental changes over large spatial and temporal scales (Diamond & Devlin 2003). Little penguins in particular appear to be a useful monitoring species because they would reflect different geographical areas and as a consequence different responses to their geography, and therefore a range of comparisons could be drawn between many different colonies. The use of stable isotopes would be an ideal research methodology to add to monitoring systems already in place, especially with regard to long term tracking of prey availability, trophic level variation, and species plasticity.

This study has ascertained the enrichment factor of diet to feather formation in captive little penguins and found that there is no variation between genders or between sub-populations based on origin that might be caused by physiological differences. This

knowledge contributes to our overall understanding of this species and its possible range of responses when exposed to various environmental pressures. The surprising similarity in isotopic composition of feathers from two geographically relatively distant colonies [Oamaru and Harris Bay], with a third [Flea Bay], between these sites, showing different values, and then aligning with prey species some distance away [further up the east coast at Kaikoura], was intriguing. This "bunny-hopping" of isotopic similarities along the east coast highlights the need for further investigation of differences in the behaviour and ecology of little penguins throughout their range, a project that would be supported by continued feather sampling. Results such as these provide ammunition for an argument for the need for long term feather collection to further investigate patterns and foraging behaviours of this unique bird.

Feather isotopic chemistry shows a general indication of area of diet origin and trophic level. Because it is only a general indication, long-term collection is needed to demonstrate any large shifts or changes over time. Given the relative ease of collecting and analysing feathers, the use of feathers would be an optimal use of resources and could act as a supplement to other methods, or tissues, sampled when studied colonies are undergoing annual checks. With the marine environment likely to change significantly over the next century, the use of SIA may provide a valuable tool to track how these changes affect little penguins, so that potential causes of any future decline [or increase] in their populations can be identified.

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Appendix 1

Nutritional supplements

Mazuri® Vita-Zu® Small Bird Tablet
(Available at www.mazuri.com or through a Mazuri retailer)

Formula Code – 5M25

Description
Mazuri® Vita-Zu® Bird tablets are formulated to provide supplemental water- and fat- soluble vitamins to fish-eating bird species consuming smaller prey items.

Features and Benefits
- **Contains stabilized vitamin C** - Longer shelf life.
- **Outer coating decreases instance of oxidative vitamin losses.**
- **No artificial flavors or colors.**

Product Form	**Catalog #**
Oval tablet weighing 0.19 g. • 770 g per canister (~4,000 tablets); 12 canisters per case	0007537

Guaranteed Analysis

Crude protein not less than	0.50%
Crude fat not less than	0.0%
Crude fiber not more than	5.0%
Moisture not more than	10%
Ash not more than	8.0%

Nutrient Composition (per tablet)

Vitamin A, IU	835
Vitamin E, IU	25
Vitamin C, mg	25
Thiamin, mg	24
Riboflavin, mg	1.8
Pyridoxine, mg	1.8
Pantothenic acid, mg	1.8
Folic acid, µg	60
Biotin, µg	30

Ingredients
Dried corn syrup, thiamin mononitrate, stabilized vitamin C, maltodextrin, dl-alpha tocopheryl acetate (source of vitamin E), brewers dried yeast, calcium pantothenate,riboflavin, pyridoxine hydrochloride, shellac (outer coating), diacetylated monoglyceride (outer coating), magnesium stearate (binder), silicon dioxide (tableting agent), corn oil, vitamin A acetate, folic acid, biotin.

Feeding Directions
- Feed to birds by placing tablet into the food (shellfish or fish).
- For 0.19 g tablet, feed 1 tablet per ½ lb. of food.
- Recommendation: feed out within 12 hours of pilling food.

Storage Conditions
For best results, after removal of required tablet dosage store in original container with lid securely affixed in a cool (75°F or colder), dry (approximately 50% RH) location. Use within 1 year lot date code.
Note: Ingredients cause pill to have a speckled appearance. Coating on tablets may become speckled with exposure to air - this is not indicative of any vitamin degradation.

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02/20/14

(PMI Nutritional International 2016)

Nutri-Zing®

Nutritional Profile

MacroNutrients

Protein	19.6%	Ash/Minerals	29.5%
Carbohydrate	39.6%	Moisture	8.0%
Fat	3.3%		

Amino Acids (per 100g)

Alanine	0.74g	Lysine	0.91g
Arginine	0.96g	Methinonine	0.37g
Aspartic acid	1.71g	Phenylalanine	0.57g
Cystine	0.45g	Proline	0.62g
Glutamic acid	1.84g	Serine	0.69g
Glycine	1.62g	Threonine	0.59g
Histidine	0.17g	Tryptophan	0.25g
Isoleucine	0.66g	Tyrosine	0.59g
Leucine	0.91g		

Fatty Acids (per 100g of Fat)

C 14:0	Myristic Acid	2.0g
C 16:0	Palmitic	7.2g
C 16:1	Palmitoleic	4.0g
C 18:0	Oleic	2.1g
C 18:n-6	Linoleic	3.3g
C 18:3n-3	Linolenic	2.1g
C 18:3n-6	gamma Linolenic	<0.1g
C 18:4n-3	Stearidonic	<0.1g
C 20:0	Arachidic	<0.1g
C 20:3n-3	homo-Linolenic	3.1g
C 20:4n-3	Eicosatetranoic (ETA)	0.3g
C 20:5n-3	Eicosapentanoic (EPA)	18.0g
C 22:6n-3	Docosahexanoic (DHA)	23.0g

Glucosaminoglycans (per 100g) 800mg

Minerals (per 100g)

Calcium	7290mg	Sodium	1260mg
Potassium	704mg	Sulphur	680mg
Phosphorus	327mg	Magnesium	290mg
Iron	107.5mg	Zinc	3mg
Boron	1.6mg	Iodine	1.5mg
Manganese	2.0mg	Copper	0.33mg
Chromium	0.1mg	Selenium	0.16mg

Heavy Metals (per 100g)

Lead	0.031mg	Mercury	0.02mg
Cadmium	0.026mg	Arsenic	0.28mg

Typical analysis. Subject to variation due to being from a natural source.

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Product of New Zealand

(United Fisheries 2016)

Flaky sea salt

Dominion Salt Limited

PACIFIC Salt

Below is a spectral analysis taken from a typical Marlborough Flaky Sea Salt sample. The composition may vary slightly between batches. Marlborough Flaky is very popular with chefs and gourmet food producers worldwide due to its unique flake texture which adheres to the food to create a mild yet delicious flavour. Many food enthusiasts are also looking for the best nutritional value in their ingredients which is the added benefit of this speciality salt which is laden with the following trace minerals.

Marlborough Flaky Sea Salt – Trace Mineral Content		
Mineral Description	**Result**	**Units**
Aluminium	3.4	mg/kg
Antimony	<0.02	mg/kg
Arsenic	<0.05	mg/kg
Barium	0.15	mg/kg
Beryllium	<0.02	mg/kg
Bismuth	<0.02	mg/kg
Boron	<5	mg/kg
Cadmium	<0.02	mg/kg
Calcium	320	mg/kg
Chromium	0.07	mg/kg
Cobalt	<0.02	mg/kg
Copper	<0.1	mg/kg
Caesium	<0.01	mg/kg
Mercury	<0.05	mg/kg
Iron	4.9	mg/kg
Lanthanum*	<0.05	mg/kg
Lead	<0.05	mg/kg
Lithium	<0.05	mg/kg
Magnesium	210	mg/kg
Manganese	0.24	mg/kg
Molybdenum	<0.1	mg/kg
Nickel	<0.1	mg/kg
Phosphorus	<50	mg/kg
Potassium	110	mg/kg
Rubidium	<0.3	mg/kg
Silver	<0.05	mg/kg
Tin	<0.2	mg/kg
Sodium	40.5	mg/kg
Strontium	20	mg/kg
Sulphur*	<2000	mg/kg
Thallium	<0.02	mg/kg
Uranium	<0.01	mg/kg
Vanadium	<0.1	mg/kg
Zinc	<2	mg/kg
Bromine	54	mg/kg
Iodine	0.20	mg/kg
Selenium	0.11	mg/kg
Salt (as Sodium Chloride)	97.9	mg/kg

These results have been provided from tests by an independent IANZ Accredited Laboratory.

< Means less than, > means greater than

* Indicates an analysis that is not IANZ accredited

(Dominion Salt 2014)

Appendix 2

Diet/Fish lot labels

Sprat Lot 1

10 kg boxes

Block frozen sprats

Batch:601050465G

Size: 60-65 pieces/kg

Pack date: 17-12-2011

Best before: 17-12-2013

Caught in the Northeast Atlantic, FA027

EU approval number: UK CQ 019 EC

Sprat Lot 2

20 kg boxes

Product: Whole round frozen sprat [*Sprattus sprattus*]

Temp: -20 degr C.

EU Approval no: D-MV-EFS 008

Season of catch: January 2013

Area of catch: FA027

Use by date: Jan 2014

Storage -18°

P&P PO BOX 504

2220Am Katwijk Holland

DED-MV

EFS-008

Sprat Lot 3

20 kg boxes

Product: Whole round frozen sprat [*Sprattus sprattus*]

Temp: -20 degr C.

EU Approval no: MV25003

Season of catch: September 2013

Area of catch: FA027

Use by date: Sept 2014

P&P PO BOX 504

2220Am Katwijk Holland

DED-MV

EFS-008

Supplemental fish trialled along with Sprat Lot 3:

Salmon 2 kg boxes

Oncorhynchus tshawytscha

Smolt 0-30g

Product of NZ

Lot #: 1211 34 5485

Packed by: The New Zealand King Salmon Co. Limited

10-18 Bullen St. Nelson 7011

Packed on 20-Nov-12 13:39 -18°

The mackerel were supplied by Independent Fisheries in Christchurch. The pilchards and anchovies did not come with labels. After enquiring with United Fisheries, the anchovies were found to be wild caught (off the coast of California) lot #64877, however no paperwork was supplied.

Sprat Lot 4

10 Kg boxes

Product: Whole round frozen sprat [*Sprattus sprattus*]

Temp: -20 degr C.

EU Approval no: UK CQ019 EC

Season of catch: September 2013 [24th]

Area of catch: FA027 [Northeast Atlantic]

Use by date: Sept. 14

Produced in the UK by:

Falfish Ltd. Cardrew

Industrial Estate, Redruth

Cornwall, TRI51SS, UK

Appendix 3

Photos relevant to study

A little penguin feather collected during the course of this study showing the parts of the feather used in SIA.

Feeding time at the International Antarctic Centre. First part of the feeding was fish being thrown into the pool, then the second portion on the beach for birds unable to swim and eat at the same time due to their injuries.

Appendix 4

Wild birds integrated into the captive colony

When a new male, found on a beach in Canterbury, was brought to the IAC, his first set of moulted feathers, grown while in recovery from an injury sustained in the wild, replaced with feathers from a pre-moult diet supplied at the Antarctic Centre, show the isotopic signature of his recovery diet. His recovery diet is largely unknown though he was fed some anchovy during his recovery, spanning approximately six months; it is not known if this was during the pre-moult weight gain period. The next moult for this bird was well within the captive colony range fed on the diet provided.

Another arrival in December of 2012, a juvenile female, was recovered from Dunedin with a paralyzed left flipper; the vet contracted to the IAC determined that she had hatched with a birth defect in the nerves that control the left flipper. This bird was fed, from Otago waters, by her parents until fledged and she would have only been out in the water on her own for a couple of days since she had not yet starved. Her results, towards the bottom of Figure 3.2 can thus be cautiously considered representative of the prey consumed by her parents around Dunedin. Unlike the Cantabrian male, this Otago female's second moult, feathers produced while consuming the captive diet, was not close to captive feather signatures of the rest, and it was only her third moult in captivity that her feathers fell within the average range with the rest of the captive colony. Unfortunately, these results are only comprised of one bird from so cannot be considered fully indicative of those coastal isotopic signatures for the Otago female, without further research into those areas.

The delay of the juvenile bird in coming into line with the rest of the captive colony, but the adult male not evidencing the same delay, was intriguing and would benefit from further study.