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FORAGING STRATEGY PLASTICITY IN FIORDLAND PENGUINS (EUDYPTES PACHYRHYNCHUS): A STABLE ISOTOPE APPROACH

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FORAGING STRATEGY PLASTICITY IN FIORDLAND PENGUINS (*EUDYPTES PACHYRHYNCHUS)*: A STABLE ISOTOPE APPROACH

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APPROVAL OF THESIS

We, the faculty supervising the work of Jeffrey Wayne White, affirm that the thesis, *Foraging strategy plasticity in Fiordland Penguins (Eudyptes pachyrhynchus): A stable isotope approach*, meets the high academic standards for original scholarship and creative work established by the Biology Department and the College of Arts and Sciences. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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ABSTRACT

Foraging ecology in the crested penguins (genus: *Eudyptes)* is an active area of research, with new techniques rapidly increasing our understanding of these charismatic species. The most common techniques to assess foraging ecology include stomach content analysis, fecal DNA analysis, stable isotope analysis, tracking, and video loggers. Here we review dietary research on all 8 taxa within the genus *Eudyptes* to identify gaps in our current knowledge. However, foraging studies that assess dietary segregation require a method for quickly and accurately sexing penguins in the field. Obvious sexual dimorphism in plumage is largely absent in penguins leaving behavioral cues for sex determination. We identified morphological characters that could be easily measured in the field to predict sex in the Fiordland penguin (tawaki; *Eudyptes pachyrhynchus)* by assessing the correlation between five morphological metrics and individual sex as determined by a PCR-based molecular approach. We found that a combination of foot length and either bill length or bill depth was the most accurate morphological approach to determine individual sex in the field. Finally, to identify differences in foraging strategies in tawaki among marine habitat types (pelagic, continental shelf, or fjord), we analyzed stable isotope ratios of carbon ($^{13}C/^{12}C$, expressed as $\delta^{13}C$ values) and nitrogen ($^{15}N/^{14}N$, expressed as $\delta^{15}N$ values) in penguin blood and feathers. We found that both $\delta^{13}C$ and $\delta^{15}N$ values differed significantly between tissues. During incubation (blood), $\delta^{15}N$ values were highest in the fjord and $\delta^{13}C$ values differed significantly between years. In the pre-molt period (feathers), $\delta^{13}C$ values were significantly different among sites, between sexes, and between certain years. $\delta^{15}N$ values were only different between certain years. Monitoring the foraging ecology of tawaki and other crested penguins is critical for understanding population responses to changing prey distributions in a warming ocean.

CHAPTER 1

REVIEW OF *EUDYPTES* PENGUIN DIET AND FORAGING ECOLOGY

Introduction

Pelagic seabirds are highly mobile during the non-breeding season; however, they shift to central-place foraging strategies while rearing chicks (Wakefield et al., 2009). They depend on reliable access to prey within an accessible radius of a breeding site with suitable nesting habitat for breeding success. Preferred prey species, however, are not homogenously distributed within a foraging zone. While prey distribution is a strong driver for seabird ranges, the risk of depredation by seabirds and other predators is in turn a tradeoff for prey species that are constrained by their own suite of environmental factors such as marine bathymetry, oceanic currents, sea temperature, and marine productivity (Ballance et al., 2006; Fauchald, 2009). In the face of global climate change, prey distributions will likely shift and seabirds will be forced to either change their range or increase the length of foraging trips (Grémillet & Boulinier, 2009). Longer foraging trips for flightless seabirds such as penguins has been shown to significantly reduce reproductive success (Boersma & Rebstock, 2009).

Penguins are arguably the most charismatic and iconic of seabirds, yet we know surprisingly little about many species. In particular, the crested penguins (genus: *Eudyptes)* have significantly less published research than the other penguin genera. Mattern and Wilson (2018) reviewed the status of research and current knowledge of penguin species in New Zealand, a penguin fauna dominated by *Eudyptes.* They showed that the bulk of published penguin research was on the king penguins (*Aptenodytes patagonicus*), little penguins (*Eudyptula minor),* and African penguins (*Spheniscus demersus)*. Emperor penguins (*Aptenodytes forsteri), Spheniscus*

(except the Galápagos penguin *Spheniscus mendiculus)* and *Pygoscelis* penguins all have more published research than any *Eudyptes* penguin species.

Crested penguins are primarily sub-Antarctic and temperate breeders. Members of this genus are found in Argentina and Chile (southern rockhopper), New Zealand (Fiordland and Snares penguins), sub-Antarctic islands (southern/eastern rockhopper, erect-crested, macaroni, and royal penguins), and islands around the sub-tropical front in the Atlantic and Indian Oceans (northern rockhopper) (Davis & Darby, 2012). For the purposes of this review, I consider these eight species within these three geographic species clusters to highlight current knowledge of these distinct regions while recognizing some taxonomic revision is likely needed for some taxa (Banks et al., 2006; Pan et al., 2019; Frugone et al. 2019).

Diet and foraging studies are crucial for understanding how individuals interact with their environment, the life history differences among populations, and how anthropogenic changes in these ecosystems will impact penguin conservation and management. Most crested penguin species are declining (Mattern & Wilson, 2018), but the exact causes of many of these declines are not well known. Changes in prey availability likely play a significant role in declining colonies. However, for many crested penguins, relatively little is known about their diet beyond the breeding season. Here, I assess the extent of foraging ecology research published on each of the eight crested penguin taxa described and discuss the most commonly used methods for each.

State of Foraging Ecology Research

Southern rockhopper penguin (*Eudyptes chrysocome chrysocome)*

The western subspecies of the southern rockhopper penguin is distributed primarily around the southern tip of South America, the Falklands/Malvinas, and southwestern Atlantic Ocean islands (Borboroglu & Boersma, 2015). Together with the eastern subspecies rockhopper,

the southern rockhopper is currently classified as vulnerable (BirdLife Int., 2017). The southern rockhopper is perhaps the most iconic crested penguin and indeed much of the foraging research on crested penguins has been conducted on this species. The bulk of research on southern rockhoppers focuses on colonies in the Falklands/Malvinas and Isla de los Estados, Argentina. These colonies are among the largest for this species and are relatively accessible for long-term studies.

The most common method to assess dietary composition is through stomach content analysis by stomach flushing/water offloading. This process involves passing a flexible tube directly into the stomach and to lavage with water to induce emesis (Duffy & Jackson, 2006). The researcher is then able to sort the food items that are expelled and quantify species abundance associated with particular foraging trips. Because penguins swallow prey whole, this procedure often provides prey samples that are readily identifiable down to the species level (Gales, 1987). Stomach content analyses in southern rockhoppers indicate a reliance on cephalopods at Isla de los Estados (Raya Rey & Schiavini, 2005). There is also variation in the proportion of dietary components based on period of the annual cycle. Thompson (1989) described a shift from a crustacean dominated diet during the breeding season to a predominantly cephalopod diet during the pre-molt period in the Falklands/Malvinas. Stomach content analysis has been the most common method to assess diet in seabirds, and indeed for southern rockhoppers, most of the current knowledge of their foraging ecology comes from this type of assessment (Appendix B).

Dietary preferences during extended periods at sea have been much more difficult to assess. Stable isotope analysis has opened a window into these periods (and others) to understand the trophic foraging level of many species (Newsome et al., 2007). This technique capitalizes on

the incorporation of stable isotopes from prey tissue into the tissues of the consumer (Post, 2002). The two most commonly used isotopes in dietary studies for marine predators, including penguins, are the isotope ratios of carbon ($^{13}C/^{12}C$ expressed as $\delta^{13}C$ values) and nitrogen ($^{15}N/^{14}N$ expressed as $\delta^{15}N$ values). In general, $\delta^{13}C$ identifies the foraging locations of individual penguins as it shows a predictable pattern across latitudinal, inshore/offshore, and benthic/pelagic scales (Cherel & Hobson, 2007; Rosciano et al., 2016). However, $\delta^{15}N$ values differ significantly between prey and consumer tissue (Inger & Bearhop, 2008). Therefore, $\delta^{15}N$ is used as a major indicator of trophic position and trophic foraging level of consumers (Hobson et al., 1994).

Stable isotope analysis has been used to describe the trophic niche of southern rockhoppers primarily in the Falklands/Malvinas and Isla de los Estados. Multiple studies at these locations have indicated both sexual and colonial segregation in foraging areas (Dehnhard, et al., 2011; Rosciano et al., 2016). The bimodal distribution of $\delta^{13}C$ signatures also indicate foraging over both the shallow Patagonian Shelf and deeper pelagic Atlantic waters (Hilton et al., 2006). Overall, this indicates that southern rockhoppers exhibit a highly variable foraging niche.

In recent years, advances in tracking technology have allowed the use of global positioning systems (GPS) and global location sensors (GLS) devices to follow the movement of individual penguins at sea. GPS devices are typically affixed to the lower back using waterproof tape anchored by feather shafts (Mattern et al., 2005). In most cases, accelerometers are also added to measure dive parameters. GLS systems have lower spatial resolution than GPS devices, but are typically much smaller and can be attached to the lower leg much like a standard leg band (Ratcliffe et al., 2014).

Most of the tracking studies on southern rockhoppers have been carried out in the Falklands/Malvinas (Pütz et al., 2003), Isla de los Estados (Rosciano et al., 2016), and Isla Noir, Chile (Oehler et al., 2018). Southern rockhoppers in the Falklands/Malvinas have shown spatial segregation in foraging zones with females foraging in more near-shore zones and males making longer trips during incubation (Ludynia et al., 2013). At Isla de los Estados, southern rockhoppers nesting near Magellanic penguins (*Spheniscus magellanicus)* were tracked during the breeding season to understand competition and foraging zones (Rosciano et al., 2016). Although southern rockhopper foraging and diet varies temporally, they found strong evidence of interspecific segregation in foraging niche.

The recent technological advances in satellite tag technology has created compact and light weight devices that are able to send data back remotely whenever the satellite passes over a tagged penguin (Hart & Hyrenbach, 2009). This allows remote access to data without having to wait to recover the device itself. This technology has been used on southern rockhoppers in the Falklands/Malvinas to track foraging males during incubation (Pütz et al., 2003). They were able to show spatial segregation into two foraging regions of the Patagonian Shelf across multiple years.

Eastern rockhopper penguin (*Eudyptes chrysocome filholi)*

The eastern subspecies of the southern rockhopper penguin is found primarily on sub-Antarctic islands in the southern Indian and south-western Pacific Oceans (Borboroglu & Boersma, 2015). The eastern rockhopper is currently listed as a subspecies of the southern rockhopper (Jouventin, et al., 2006), and is listed as vulnerable and declining (BirdLife Int., 2017). The conditions faced by these two populations are very different and the eastern rockhopper has experienced rapid declines in recent decades (Mattern & Wilson, 2018).

Stomach content analyses of eastern rockhoppers from the southern Indian Ocean have shown a high reliance on crustaceans as compared to southern rockhoppers. For example, dietary composition in eastern rockhoppers from Marion Island was shown to be between 91-100% euphausiid krill (Brown & Klages, 1987). Other colonies had similarly high krill composition ranging from 60-70% on Macquarie Island (Horne, 1985; Hindell, 1988; Hull, 1999) to 97% on Kerguelen Island (Tremblay & Cherel, 2003). Interestingly, Marchant & Higgins (1990) found eastern rockhoppers from Campbell Island relied mainly on small fish (no percentages given). These studies show cephalopods composed between 1-13% of the diet. This is in stark contrast to the highly varied diet found in the southern rockhoppers and indicates a shift in trophic niche of this taxon.

Stable isotope analysis in eastern rockhoppers has been limited compared to the southern rockhopper. The few published studies on eastern rockhoppers have focused on Crozet, Prince Edward, and Campbell Islands (Whitehead et al., 2017; Xavier et al., 2018). These showed subtle spatial segregation between sexes as well as between eastern rockhoppers and macaroni penguins. The eastern rockhopper occupied an overall lower trophic niche than macaroni penguins, which is expected from the heavy reliance on krill. They also showed that male eastern rockhopper occupied a higher tropic niche than females during the periods studied (primarily breeding season). GPS and GLS tracking corroborated the spatial segregation indicated by $\delta^{13}C$ values (Whitehead et al., 2017). Tracking has that eastern rockhoppers from Crozet forage close to the sub-Antarctic Front during the breeding and pre-molt periods (Ratcliffe et al., 2014).

Northern rockhopper penguin (*Eudyptes moseleyi)*

The northern rockhopper penguin is found on the Tristan da Cunha group and Gough Island in the Atlantic Ocean as well as Amsterdam and St. Paul Islands in the Indian Ocean

(Borboroglu & Boersma, 2015). It is listed as endangered and declining and relatively few dietary studies have been undertaken on this species (BirdLife Int., 2017). At Gough Island stomach content analysis indicated a reliance on euphausiid krill (90%) followed by primarily fish (Klages et al., 1989). Amsterdam and St. Paul Islands show an overall higher reliance on cephalopods (50%) and krill (40%) than on fish (10%) (Cooper et al., 1990). However, in these populations, the diet composition varies seasonally with a shift to a fish dominated diet (64%) in the late stages of chick rearing (Tremblay et al., 1997).

Stable isotope analysis has revealed resource partitioning between parents and chicks in northern rockhoppers in the Tristan da Cunha group. Adult northern rockhoppers fed chicks a lower trophic level prey (krill) than they kept for themselves (squid) during most of the chick rearing period indicating a prioritization of future reproduction over the current chick. This shifted, however, in late stages of chick rearing when fish became a higher component of the diet of both adults and chicks (Booth & McQuaid, 2013). This supports the previous findings of a mixture of lower and higher trophic level prey in stomach flushes (Cooper et al., 1990).

In addition to the trophic information provided by stable isotopes, GPS and GLS tracking has provided spatial information for the northern rockhopper during the breeding season. From Amsterdam Island, females were tracked on daily foraging trips that lasted an average of 12 hours with a high percentage (69%) of the dives occurring during the day (Tremblay et al., 1997).

Macaroni penguin (*Eudyptes chrysolophus)*

The macaroni penguin has a circumpolar distribution from the coast of Chile, the Falklands/Malvinas, the Antarctic Peninsula, and sub-Antarctic islands of the Atlantic and Indian Oceans (Borboroglu & Boersma, 2015). Although the macaroni penguin has a wide distribution,

it is listed as vulnerable and declining (BirdLife Int., 2017). Across this broad range, macaroni penguins feed primarily on euphausiid krill and myctophid fish. Penguins breeding on South Georgia and elsewhere in the southwestern Atlantic rely heavily (up to 98% by wet mass) on Antarctic krill (*Euphausia superba)*, while populations in the Indian Ocean target *Euphausia vallentini* and *Thysanoessa gregaria* (Crawford et al., 2003; Ratcliffe et al., 2014). The proportion of fish is often higher in the Indian Ocean populations than in the Atlantic.

In addition to prey abundance in stomach content analyses, macaroni penguin diets have also been examined for prey presence through fecal DNA analysis. Fecal samples are used to identify prey consumed based on the presence of prey DNA sequences. Deagle et al. (2007) used this approach to identify prey DNA in the fecal samples of macaroni penguins on Heard Island in the Indian Ocean. PCR techniques were used to amplify and sequence prey DNA based on selected primers for five targeted prey species. DNA sequences derived from the fecal samples may also be compared to a sequence library to identify other prey species. Their results supported the reliance on *E. vallentini* and *T. gregaria* as well as fluctuating proportions with myctophid fish in late chick rearing periods. Overall, fecal DNA offers a non-invasive tool to identify prey species consumed and to highlight shifts in dietary trends over time.

Macaroni penguin foraging niche has also been studied through stable isotope analysis in populations breeding on South Georgia, Marion, and Heard Islands. Male and female macaroni penguins do not occupy a significantly different trophic niche, as expected based on the high reliance of euphausiid krill in their diet (Bearhop et al., 2006). In general, a high degree of foraging niche fidelity has been described in macaroni penguins across their range (Cherel & Hobson, 2007; Cherel et al., 2007).

During the pre-molt forage period, Thiebot et al. (2014) combined stable isotopes with GLS tracking and found population level segregation in foraging area during this critical period. They show that females spent more time in colder water than males and that there was minimal overlap in foraging zones between colonies and between early and late molting penguins. Such a strategy reduces intraspecific competition for euphausiid krill.

The first application of GLS trackers to follow the winter dispersal of a penguin species was on Macaroni penguins from Kerguelen Island (Bost et al., 2009). This study was groundbreaking as it showed that although they initially dispersed over a wide range, they converged on a previously unknown narrow band of the southern Indian Ocean corresponding to the Polar Front for most of the winter (Bost et al. 2009).

Royal penguin (*Eudyptes schlegeli)*

The royal penguin breeds only on Macquarie and Bishop Islands to the south of New Zealand and is listed as near threatened and stable (BirdLife Int., 2017). Compared to macaroni penguins, relatively little is known about the foraging ecology of royal penguins. The current knowledge on the foraging ecology of royal penguins stems from stomach content analyses from Macquarie Island population. These have shown a higher reliance on myctophid fish (59%) than on euphausiid krill at (37%) (Hindell, 1988; Hull et al., 1997; Goldsworthy et al., 2001). This is a sharp contrast to macaroni penguins' preference for krill. To date, there is no published research on royal penguin foraging ecology employing any of the other methods described.

Fiordland penguin (*Eudyptes pachyrhynchus)*

The Fiordland penguin is the only crested penguin that breeds on mainland New Zealand and they are listed as vulnerable and decreasing (Warham, 1974; BirdLife Int., 2017). Until recently, this species was very poorly represented in the literature with a limited number of

dietary studies published. Stomach content analyses from Martins Bay indicates a diet of 85% cephalopods (*Nototodarus sloani*) followed by krill such as *Nyctiphanes australis* (Van Heezik, 1989). Stable isotope analysis from the early breeding season at the same colony supports this finding and suggests that winter spawning arrow squid may be a driving factor for the early breeding cycle of Fiordland penguins (Poupart et al., 2019).

In contrast to the findings at Martins Bay, stomach contents from Codfish Island indicated predominantly (85%) larval and juvenile fish (ahuru, *Auchenoceros punctuates,* and blue cod, *Pseudophycis bacchus*) (Van Heezik, 1990). Fecal DNA analyzed from Jackson Head also suggested fish as the dominant prey type for Fiordland penguins (McInnes et al., unpublished data).

The bulk of the research into foraging ecology of Fiordland penguins in recent years has focused on GPS, GLS, and satellite tracking. The marine ecosystem adjacent to the breeding colony has a significant impact on the distances the breeding females travel to forage. GPS devices show that females breeding at sites along the continental shelf in the north of their range travel up to 100 km away, while those in Milford Sound remain within the fjord itself and travel an average of 4 km from the colony (Mattern & Ellenberg, 2018).

GLS devices were deployed on Fiordland penguins departing on the pre-molt forage from Gorge River. It was previously assumed that this species foraged along the Sub-Tropical Front in the lower Tasman Sea, however GLS tracks indicated that Fiordland penguins segregated into two foraging zones, one along the Sub-Tropical Front and the other along the Sub-Antarctic Front. This study revealed Fiordland penguins made up to a 6,800km round trip during this 6-8 week period (Mattern, et al., 2018b). Satellite tags have also recently been deployed on Fiordland

penguins to track their winter movements and they indicate foraging much closer to Antarctic waters than previously thought (Mattern et al., unpublished data).

Snares penguin (*Eudyptes robustus)*

The Snares penguin is another New Zealand endemic and is currently listed as vulnerable and stable (BirdLife Int., 2017). They are found exclusively in the Snares Archipelago to the south of mainland New Zealand (Borboroglu & Boersma, 2015). To date, very few studies have focused on the Snares penguin. Stomach content analyses have all been conducted during the chick rearing period (Cooper et al., 1990; Marchant & Higgins, 1990; Mattern et al., 2009). Adult Snares penguins target primarily pelagic fish (redbait, *Emmelichthys nitidus,* and juvenile red cod, *Pseudophycis bacchus*) and cephalopods. Snares penguin chicks, however, were fed a diet of 60% krill (*N. australis)*, and 30% fish (Mattern et al., 2009). This shows a segregation in trophic foraging level between chick provisioning and adult diet.

Mattern et al. (2009) compared stable isotope values of $\delta^{13}C$ and $\delta^{15}N$ in Snares penguin feathers collected from live penguins and museum specimens representing a temporal range between 1880 and 2004. This study found that the trophic position this species has no significant temporal change over that time period, suggesting limited fluctuations in diet and marine productivity.

Finally, GPS tracking has shown that while females are incubating, males travel up to 200 km from the colony to forage along the Sub-Tropical Front (Mattern, 2007). This foraging trip coincides with spring blooming phytoplankton and increasing productivity along the frontal zone. Once the males returned to complete incubation, females made much shorter foraging trips into regions surrounding the Snares Archipelago (Mattern, 2007).

Erect-crested penguin (*Eudyptes sclateri)*

The erect-crested penguin is New Zealand's third endemic crested penguin species. They are found exclusively on the Bounty and Antipodes Islands (Borboroglu & Boersma, 2015). Erect-crested penguins have been identified as the least studied penguin species in the world (Mattern & Wilson, 2018). They are listed as endangered and declining, but there is a significant lack of research to support any claims about this species (BirdLife Int., 2017). At the time of this review, no research has been published on the foraging ecology or diet of the erect-crested penguin. They are assumed to forage on a mixture of krill, cephalopods, and fish but the exact composition and species are not known. The presumed diet and foraging behavior of this species is based on other New Zealand crested penguins, particularly the Snares and eastern rockhoppers. However, the diet of crested penguins, even within a single species, can be highly variable and efforts should be made to understand the foraging ecology of this species.

Considerations for Assessing Foraging Ecology

Stomach Content Analysis

Perhaps the most direct method for quantifying diet in most species is to analyze the contents of the stomach. In fact, this method has been used in 7 of the 8 crested penguin taxa to understand dietary composition (Appendix B). This method is time sensitive, as prolonged time in the stomach will inevitably break down prey. This can potentially obscure the presence of soft bodied prey while indicating a higher abundance of harder body parts such as fish otoliths, squid beaks and crustacean carapaces (Barrett et al., 2007). Nevertheless, this method is widely used and is an important tool for understanding not only the specific species of prey consumed but also their abundance in the diet.

There is some concern that this method may have negative impacts on the sampled individuals due to stress induced by the procedure, complications from passing sharp bones or fins, or by removing a large volume of prey intended for growing chicks. Goldsworthy et al. (2016) assessed the impacts of stomach flushing on the endangered yellow-eyed penguin (*Megadyptes antipodes)*. They used long term data to assess the overall survival rates between flushed and non-flushed birds and concluded that there was not a significant difference in long term survival (except in years with poor food availability). They also showed that there were comparable hatching and fledging rates, but chick weights were lower in nests where parents were flushed more than four times in a season (Goldsworthy et al., 2016). Overall, stomach flushing is a viable option for assessing diet composition, but protocols should be implemented to account for environmental conditions to reduce potential impacts.

Another method for analyzing stomach contents is through dissection of the stomach, gizzard, and intestines of dead penguins (Barrett et al., 2007). Lethal collection of wild penguins for such a study is not feasible but deceased penguins found dead on beaches or euthanized due to severe injuries or disease may provide an opportunity to learn more about their diet. However, the causes of death should be considered. Penguins sampled in this method may not be representative of normal foraging if they died of starvation or disease. This is particularly important for understanding the impact that marine plastic pollution may have on penguins (Colabuono et al., 2009). Parents may mistake plastic bags for jellies or other natural prey and then pass that along to their chicks (Pierce et al., 2004; Brandão et al., 2011; Savoca et al., 2016).

Fecal DNA Analysis

This method has several advantages including a reduced or no handling of the penguins, and the ability to collect multiple samples throughout the season to monitor fluctuations in diet

without compromising survival of adults or chicks (Oehm et al., 2011). Fecal DNA is not constrained by the digestion process in the same way that stomach content analyses are. Species with hard and soft body parts will be represented without bias. However, species identifications are limited by the available reference library of prey sequences (Oehm et al., 2011). However, with next generation sequencing techniques, it is possible to identify more of the diet. DNA metabarcoding markers targeting plant, vertebrate and invertebrate components of the diet of brown bears have been shown to allow identification down to the genus or species level for over 60% of taxa found in the fecal samples (De Barba et al., 2014).

While this tool allows identification of prey species consumed, it is not able to quantify their abundance within the diet. Nevertheless, it is likely that this method will be a prominent feature in future research for other crested penguins (Appendix C). While fecal DNA analysis can provide a detailed account of prey diversity, it is still limited to reflecting only prey consumed during a very short window while penguins are accessible on land (usually breeding and molt). While these seasons are vitally important to penguin survival, they only represent a small proportion of their annual cycle, and therefore should be combined with other techniques that have broader temporal spans.

Stable Isotope Analysis

When considering stable isotopes for foraging studies, it is vital to choose tissues that reflect the targeted period of interest. Isotope values present in penguin whole blood reflect prey consumed roughly 20 days previous (Barquete et al., 2013), while those in feathers always reflect the diet that fueled feather growth during the molt (i.e. the pre-molt forage) (Cherel et al., 2005). Based on the predictability of the incorporation rates into the penguin's tissues, researchers can then select the correct tissue type that corresponds to the time period of interest.

Another factor that influences the interpretation of stable isotope data is differences in isotope values between prey and penguin tissues (Cherel et al., 2005). These discrimination factors vary depending on the prey type (fish, cephalopod, crustacean), the prey tissue (whole prey, muscle), and the penguin tissue (feather, blood, toenails) (Cherel et al., 2005; Barquete et al., 2013). It is therefore critical to correct isotope data to reflect these circumstances in order to accurately interpret the results (Cherel et al., 2005). This information is then used to construct trophic webs to understand the level of prey targeted by penguins during the period of interest (Layman et al., 2012).

A benefit of stable isotope analysis is the capacity to compare individual dietary strategies within a colony as well as between colonies, populations, and species (Appendix D). This method provides a means to determine segregation of foraging niche between species (Rosciano et al., 2016), within species (Cherel & Hobson, 2007), and between sexes (Forero et al., 2002) as well as resource partitioning between parents and chicks (Booth & McQuaid, 2013). It is an especially powerful tool to assess historical diets and marine conditions through museum specimens (Mattern et al., 2009).

Although there are many advantages to using stable isotope analysis to understand the trophic level of targeted prey species during periods that might otherwise be inaccessible, there is a trade off with identifying specific species in the diet. Stable isotopes provide general classes of prey consumed based on the available information on the baseline isotope values of the ecosystem but is unable to positively identify prey species. It is therefore recommended to combine stable isotope analysis first with more traditional sampling techniques such as stomach flushing to contextualize the isotopic values collected. In a growing number of studies, stable

isotopes are being paired with tracking technologies to more accurately understand not only the marine zones where foraging occurs, but also to identify important foraging areas at sea.

Tracking

Global Location Sensors (GLS)

Another tracking device that is often used is GLS or global location sensors. This method has lower spatial resolution than GPS devices, but what these systems lack in spatial resolution, they make up for with temporal data. GLS devices can track movements over the course of an entire year (Carey et al., 2009). This has provided unprecedented information on the at sea movements of many species (Appendix E).

Global Positioning System (GPS)

Although tracking is not a method for directly assessing diet, it is nevertheless an important tool for understanding foraging ecology. Most crested penguins travel substantial distances from their breeding colony during the chick rearing period, so it is important to identify critical foraging areas. The information collected from GPS devices has revealed spatial overlap and segregation in foraging behavior based on species, sex, colony, season, and marine conditions (Appendix E). GPS tracking devices are an important tool but are limited by the weight of the device, battery life, and ability to acquire positional information only when the penguin surfaces long enough to communicate with the satellites (Mattern et al., 2005).

Satellite Tags

One drawback to both GPS and GLS systems is the device must be recovered to download the data. Satellite tags send data back remotely whenever the satellite passes over the penguin (Hart & Hyrenbach, 2009). This allows remote access to data without having to wait to recover the device itself. Although this is an exciting method to track penguins throughout the

year, very few studies have been published on crested penguins at this time (Appendix E). As with all tracking methods, satellite tags do not reflect the prey species selected. Any tracking method should therefore ideally be combined with other techniques, such as stable isotope analysis, to corroborate the two sides of the story that each method provides.

Video Loggers

A relatively new and exciting method to visualize prey preference is through body camera deployment. This involves attaching high-definition video loggers to the back of the penguin to see exactly what prey species are being targeted (Mattern et al., 2018a). This is a relatively new method and has to date only been published on a few penguin species (Appendix E). Most notably, it has been shown in the yellow-eyed penguin (Mattern et al., 2018a), the Adélie penguin (*Pygoscelis adeliea)*, Magellanic penguin, and little blue penguin (*Eudyptula minor)* (Thiebot et al., 2017). In each of these studies, the camera footage has shown foraging behaviors that were otherwise unknown. For example, the yellow-eyed penguins were recorded exhibiting different hunting strategies based on the prey type they were targeting (Mattern et al., 2018a). In all four species, jellies were shown either directly as prey or as a resource for collecting small fish and crustaceans hiding under the bell (Thiebot et al., 2017).

Although they are also limited by battery life and attachment duration, video loggers show tremendous potential for uncovering behaviors, foraging strategies, and prey species that other techniques are not able to depict. This technology has yet to be published on any crested penguin species; however, studies employing video loggers are currently underway in at least the Fiordland penguin (Mattern et al., unpublished data).

Discussion

Penguins are experiencing significant population declines across many speices with 10 out of 18 species globally threatened (Trathan et al., 2015; BirdLife International, 2017). One factor that contributes to this is a lack of reliable prey resources. Protecting these prime foraging areas is key for protecting declining penguin species. As feeding grounds are over exploited by human activity, penguins are forced to find new locations to forage which may increase risk of becoming bycatch or result in a shift in foraging strategy to include available prey (Crawford et al., 2017). Without a concrete understanding of the foraging requirements of each crested penguin species, it is difficult to develop and implement conservation plans that protect not only resources for penguins but also foraging zones and corridors between them.

However, interactions with fisheries are not the only human induced factor that stands to alter the foraging ability of crested penguins. Global climate change has the potential to pose a serious threat to penguins and other sea birds over the next century (Trathan et al., 2015). Changing oceanic conditions inevitably cause shifts in prey distribution (Murphy et al., 2007). Such regime shifts in mid-trophic level prey communities will likely alter the distribution of penguins. In most cases, such shifts in penguin distribution are constrained by other environmental factors and may lead to declines in penguin populations (Boersma & Rebstock, 2009). However, in some species (gentoo and Adéile penguins) such shifts may be beneficial at least in the short term (Lynch et al., 2012; La Rue et al., 2013). Conservation plans for marine reserves and fisheries should consider the impacts of climate and how penguin distributions and foraging requirements may shift.

The foraging ecology of crested penguins is a dynamic and ever evolving field with new techniques increasing our understanding of this charismatic group of penguins. The most

common tools for assessing diet and foraging include stomach content analysis, fecal DNA analysis, stable isotope analysis, tracking methods (GPS, GLS, and satellite tags), and most recently high-resolution video loggers. Although some species (macaroni and southern rockhopper) have had significant research into their foraging ecology, others have had virtually none (erect-crested, Snares). Even for comparatively well studied species, our knowledge is largely limited to a few populations.

Future work on crested penguin foraging ecology should broadly focus on two key areas. The first is undertaking large scale projects implementing multiple methods to resolve the gaps in the knowledge of the diet and foraging behavior of the lesser studied crested penguin species (primarily the New Zealand species). There is virtually no information on the erect-crested penguin, and the Snares, Fiordland, and royal penguins are still poorly studied when compared to other crested penguins. These species are all endemic to no more than a handful of islands and their non-breeding periods largely remain a mystery. Competition with fisheries and accidental bycatch in gill nets are major threats to penguins (Crawford et al., 2017), so understanding the foraging ecology of these species is vital to mitigating impacts from anthropogenic factors.

The second major direction is to evaluate the foraging trends over time for all crested penguins. Penguin colonies fluctuate over time (Oehler et al., 2007; 2008), but we are entering an unprecedented phase of global oceanic changes (Grémillet & Boulinier, 2009). Some penguin colony declines and disappearances in Antarctica have been attributed to climate change (Trathan, et al., 2011), but not all species are anticipated to have the same response (Forcada et al., 2006). Ecological niche theory predicts that generalist species will likely adapt to changes in their ecosystem while specialists will be more sensitive to change (Leibold, 1995). This has been shown in Antarctic species where generalist gentoo penguins are able to shift their diet in line

with changing prey composition while krill specialist chinstraps are not (McMahon et al., 2019). For crested penguins, more research should focus on understanding the foraging parameters that affect colony occupancy to better model how climate change will affect these species.

Although there has been significant research into the diet and foraging behavior or crested penguins, it is largely focused on two species (southern rockhopper and macaroni). It is vital to create a more complete picture of the foraging ecology of all crested penguin species. It is only by combining multiple methods that we can understand how diet and foraging behavior change across both temporal (within the annual cycle, across years, historically), spatial (segregation within and among foraging areas), and biological (between sexes, age classes, breeder/non-breeders) scales that then inform conservation and management efforts for this iconic group.

CHAPTER 2

IDENTIFYING FIELD SEXING PROTOCOLS FOR FIORDLAND PENGUINS

Introduction

The unique dual lifestyle that characterizes the annual cycle of penguins presents a complication for researchers. Most penguin species spend most of the year at sea foraging vast and inaccessible expanses of ocean and resume a terrestrial lifestyle only to breed and molt. Sex is likely a key factor in the chronology and extent of these shifts between marine and terrestrial habitats. Males and females exhibit differences in behavior that may expose them to unequal risk of predation, energy expenditure, or access to resources (Donald, 2007; González-Solís et al., 2000; Morrison et al., 2017). However, understanding differences in ecology and behavior between sexes, as well as accurately assessing the demographics of declining species is confounded by the lack of clear sexually dimorphic traits.

In many penguin species, males and females occupy predictable roles during the breeding season that dictate both the duration and time of foraging periods (Warham, 1974; Williams & Croxall, 1991). *Eudyptes* penguins exhibit strict division of incubation and chick rearing duties that require extended fasting periods in males and increased energy expenditure for foraging trips for females. These and other behavioral observations have been traditionally used to determine the sex of *Eudyptes* penguins in the field (Kriesell et al., 2018).

Fiordland penguins (*Eudyptes pachyrhynchus,* hereafter referred to by their Māori name tawaki*)*, typical of penguins, lack any obvious external sexual dimorphism (Warham, 1974). Although past studies have used behavioral cues to determine sex, a more immediate and accessible method that would allow reliable sexing of both breeding and non-breeding tawaki at

any point of the annual cycle is critical for understanding the demography and ecology of this highly elusive species.

The use of morphometrics to sex penguin species in the field has employed multiple techniques; however, the same metrics are not always reliable as a sexing citerion for all penguin species. Some, such as vent measurements (Boersma & Davies, 1987) and cloacal examinations, (Clarke et al., 1998) can be performed in the field, but require significant expertise. Morphological measurements must be obtained quickly to avoid excessive handling times and the accompanying stress. Common metrics assessed for multiple penguin species include body mass, bill length and depth, head length, wing length, total foot length, and tarsus length. These have been used in southern rockhopper penguins (*Eudyptes chrysochome;* Poisbleau et al., 2011), northern rockhopper penguins *(Eudyptes moseleyi;* Steinfurth et al., 2019), little penguins (*Eudyptula minor;* Overeem et al., 2006), and yellow-eyed penguins (*Megadyptes antipodes;* Setiawan et al., 2004), but have not been tested in tawaki in conjunction with a molecular genetic approach.

Behavioral cues have been the primary method of determining the sex of breeding tawaki, although morphometrics have also been assessed (Warham, 1974). Metrics including bill length, bill depth, culmen width, head length, foot length, and flipper length have been considered for sexing adult tawaki. In all but flipper length, males were shown to have significantly higher values than females. Overall, the bill shape index [(length *x* width *x* height)/10] was chosen as the most indicative of sex (Warham, 1974).

Although Warham (1974) identified the overall bill shape and size to be the most distinguishable metric, this technique was supported only by behavioral cues. Tawaki engage in reliably sex-specific behavioral patterns during the breeding season; however, the sexes are more

similar in their behavior at other times of the year (i.e., post-guard and molt) or among non-breeding and vagrant individuals (Warham, 1974). Here we use morphometrics and DNA-based sexing to identify those morphological measurements that are both consistently variable between sexes and are also able to be obtained quickly in the field.

Methods

Study Area

We sampled tawaki at three sites in southern New Zealand. Each site is associated with one of the three main marine environments exploited by tawaki during the breeding season. They are also representative of the overall breeding range of this species from near the northern limit of the breeding range to the southern. The first study site is in south Westland at Jackson Head (-43.963°, 168.611°) near the village of Jackson Bay. The Harrison Cove colony (-44.624°, 167.913°) sits near the mouth of the Harrison River in Milford Sound/Piopiotahi. The fjord is protected both by Fiordland National Park and the Piopiotahi Marine Reserve. The Whenua Hou colony (-46.760°, 167.641°) is located on Sealer's Bay on the north-eastern coast of Codfish Island/Whenua Hou. Codfish Island/Whenua Hou sits off the north-west coast of Stewart Island/Rakiura in the Foveaux Strait (Mattern & Ellenberg, 2018).

Capture and Measurement

We captured adult penguins at all three sites by targeting accessible nests. Sampling began September 19, 2018 and continued through October 5, 2018. Like other *Eudyptes* penguins, each sex follows a predictable pattern during incubation and chick rearing. During late incubation and the guard stage, males remain at the nest while females forage during the day. We captured males by hand or with a leg crook (deeper nests) at their nests. Females were primarily

intercepted on the beach as they returned at dusk, but any that evaded capture upon arrival were allowed to feed the chick and rest before being caught at the nest.

Each penguin was implanted with a subcutaneous microchip (Allflex TIRIS 23 mm transponder) in the back of the neck for individual identification. Measurements taken included total mass (kg), foot length (mm), head length (mm), culmen length (mm), and bill depth (mm) (Warham, 1972; Murie et al., 1991). We weighed each using a Pesola 5 kg spring balance to the nearest 10 g. The total foot length was measured to the nearest 1mm from the heel to the distal tip of the last pad of the central toe using an osteometric board. The total head length was also measured to the nearest 1mm from the post occipital crest to the tip of the culmen with an osteometric board. Culmen length and bill depth were assessed using digital calipers (Jobmate J701-2702) to the nearest 1 mm. Following Warham (1972), bill (culmen) length included the exposed portion of the culmen while bill depth was measured perpendicular to the point of the inter-ramal feather patch.

Molecular Sexing

Whole blood (0.1 - 0.5 mL) was collected from the brachial vein using a new 25-gauge needle and 1.0 mL tuberculin syringe. Samples were stored in 70 % ethanol until field work was completed and extraction procedures began.

Total genomic DNA was extracted from each using standard phenol-chloroform protocols. We performed a polymerase chain reaction (PCR) using the primers SEX1 (5′-CTCC-CAAGGATGAGAAACTGTGCAAAACAGGTA-3′) and SEX2 (5′-CCTTCACTT CCATT-AAAGCTGATCTGGAATTTC-3′) designed to match conserved exon flanking regions of an intron in the chromo-helicase-DNA binding protein (CHD) gene on the Z (CHD-Z) and W (CHD-W) sex chromosomes in birds (Wang & Zhang, 2009). Length variations between CHD-Z

and CHD-W alleles allowed for discrimination between the heterogametic (females) and homogametic sex (males). We chose these primers and protocol based on previous success sexing northern rockhopper penguins (Steinfurth et al., 2019) as well as southern rockhopper, macaroni, and little penguins (White, unpublished data). PCR reactions were run in 20 μL volumes with 1 μM of each primer (SEX1 and SEX2), 1X Accuris™ Hot Start Taq Master Mix, approximately 25 ng of genomic DNA on an Applied Biosystems Veriti™ Thermal Cycler at 95°C for 5 minutes, followed by 30 cycles of 95°C (1 min), 55°C (1 min), and 72°C (2 min), and a final extension at 72°C for10 minutes.

Amplicons were separated by size by loading the entire 20 μL of each reaction on a 3 % agarose TBE gel stained with ethidium bromide. Electrophoresis proceeded for 200 volt-hours and bands were analyzed using a BioRad Molecular Imager®. The two similar sized alleles associated with the Z chromosome in the homogametic sex (males) migrate on the gel as a single band while the smaller W allele together with the larger Z allele in the heterogametic sex (females) appear as two distinct bands.

Data Analysis

Data were analyzed in R (RStudio Team 2006-2018, Version 1.1.442). We employed the Lilliefors (Kolmgorov-Smirnov) test to assess all variables for normality. T-tests were applied to compare sexes for each of the variables studied. An exploratory principle components analysis (PCA) was conducted (JMP®, Version 14. SAS Institute Inc., Cary, NC, 1989-2019) to visualize the parameters most associated with determining sex. MANOVA was performed on all variables. A recursive partitioning tree was generated using the R package "*rpart*" along with a linear discriminant analysis on the data using the R package "*MASS.*" Data were scaled to have

an equal variance of 1 using the R scale function for both linear discriminate analyses and recursive partitioning (Dykstra et al., 2012).

Results

We collected blood and measurements from 34 adult tawaki with 8 from Harrison Cove (4 male & 4 female), 20 from Jackson Head (9 male & 11 female), and 6 from Whenua Hou (1 male & 5 female). Two individuals (1 male & 1 female) had missing weights and were excluded, leaving a total of 32 individuals in the final dataset. The sex for each penguin was confirmed by molecular analysis.

All morphological characters measured (foot length, head length, bill length, bill depth, and mass) differed significantly between males and females against a Bonferoni corrected α=0.01 (MANOVA, $F = 29.396$, Wilks $\lambda = 0.19587$, $p < 0.001$; Table 1). A principle components analysis (PCA) indicated a clear separation between males and females when all variables were considered (Figure 1). PC1 reflects overall size and explained 63.2% of the variation. Overall, males from all sites were larger than females in each measurement (Figure 2). The recursive partitioning indicated cutoff values to classify male and female tawaki (Table 2) and the resulting decision tree identified foot length as the most distinguishing variable (Figure 2). A linear discriminate analysis indicated the morphological parameters correctly classified 94% of the penguins sampled (93% males, 95% females).

Measurement	Male Mean ± SD (n=14)	Female Mean ± SD (n=20)	F	P (< 0.01)
Mass (kg)	3.12 ± 0.40	2.69 ± 0.29	12.056	< 0.01
Bill Length (mm)	48 ± 2	43 ± 2	45.937	< 0.001
Bill Depth (mm)	28 ± 3	23 ± 3	38.955	< 0.001
Head Length (mm)	125 ± 4	116 ± 6	26.396	< 0.001
Foot Length (mm)	117 ± 6	108 ± 3	31.016	< 0.001

Table 1. Morphological parameters assessed in tawaki. Mean values, standard deviation, and statistics for each metric assessed. All were found to be significant following MANOVA and Bonferoni correction of $\alpha = 0.01$. All metrics other than mass were significant at $p < 0.001$.

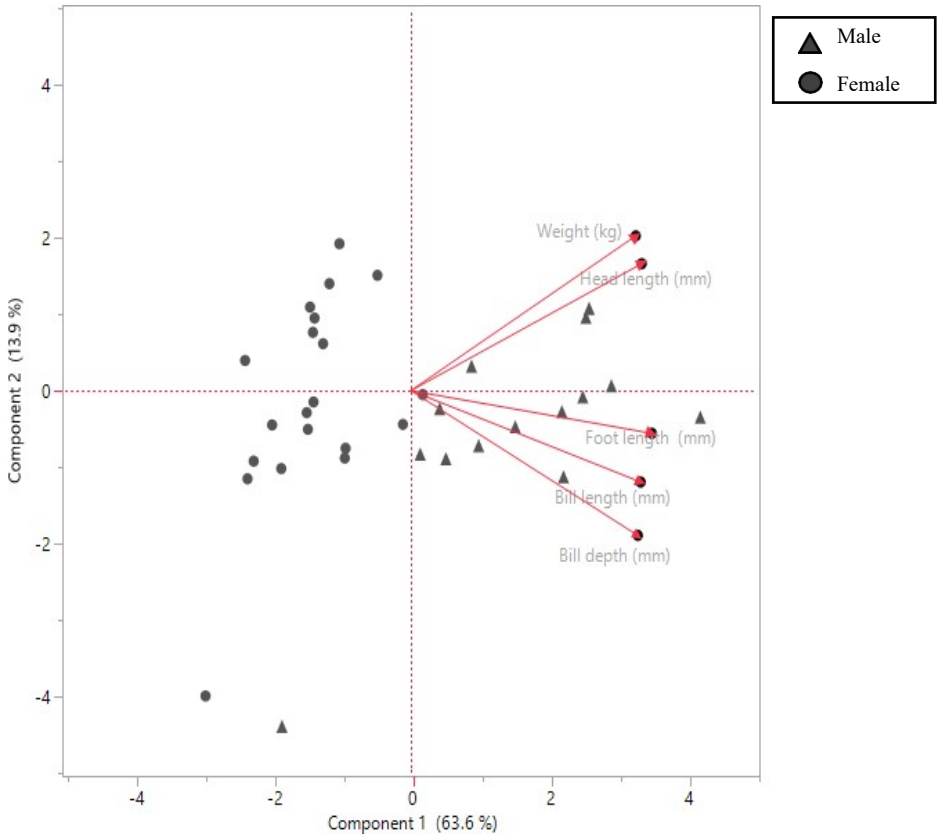

Figure 1. Principle components analysis of morphological parameters. Bill length and bill depth are highly correlated, and all measurements indicate discreet separation of males (triangle) and females (circle). Arrows represent the eigenvectors for each metric. As each of these measurements increase, the individual is more likely a male. One male and one female fell within the range of the opposite sex.

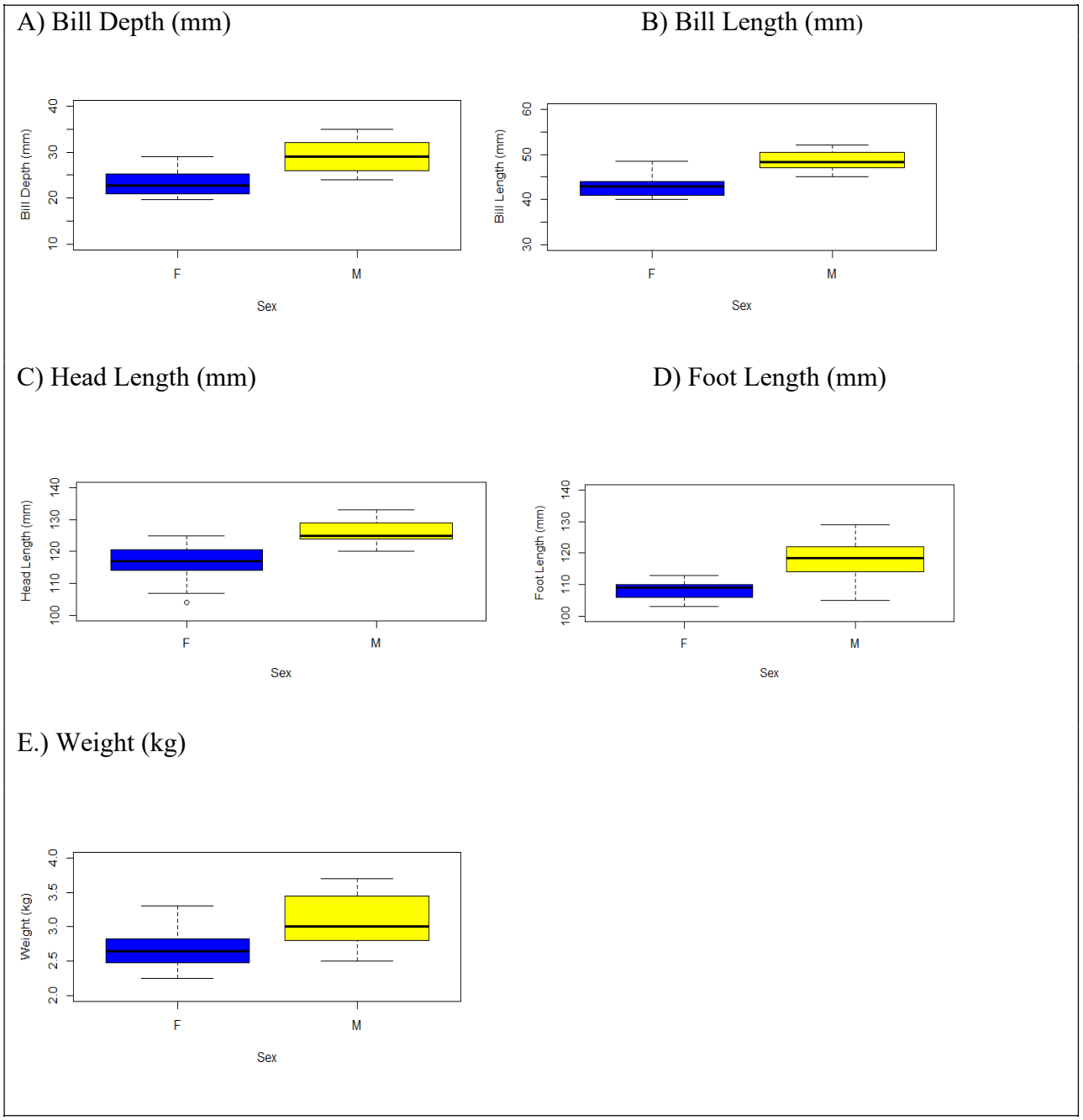

Figure 2. Boxplots of morphological parameters measured. While males were generally larger than females in all measurements, bill depth (A), bill length (B), head length (C) and foot length (D) showed the least overlap.

Measurement	Cutoff Value	Variable Importance
Foot Length (mm)	113.5	37
Bill Depth (mm)	25.5	20
Bill Length (mm)	44.5	17
Head Length (mm)	121.5	13
Mass (kg)	2.87	13

Table 2. Cutoff values and variable importance for morphological parameters. Females fall below the cutoff values while males fall above.

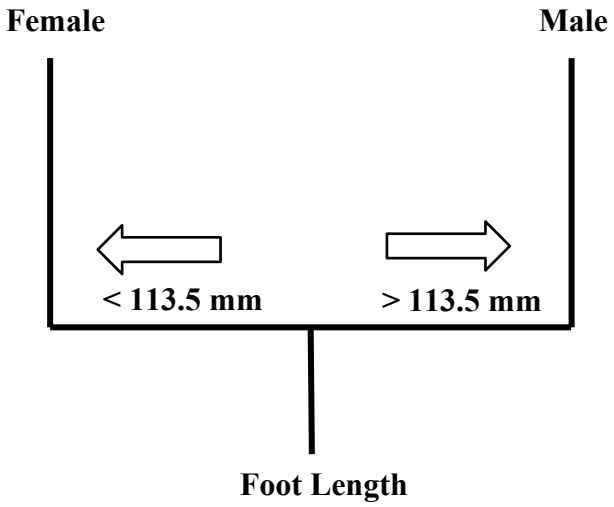

Figure 3. The recursive partitioning tree of foot length. 94% of tawaki were correctly classified by this metric alone.

Discussion

Accurately assigning sex to tawaki in the field is integral to ecological, behavioral, and demographic research and conservation efforts. Like most other penguins, tawaki are not sexually dimorphic which makes field sexing even more challenging. During the breeding season, behavioral cues are used to predict the sex of individuals; however, this can lead to erroneous classifications, particularly among non-breeding individuals. Typically, males and

females follow predictable patterns during incubation and the guard stage, but during post-guard it can be difficult to use behavior alone as tawaki may leave their nest sites while chicks form crèches in the forest. Non-breeding individuals also appear in breeding colonies and use nests as temporary shelters (White, personal observations) which can lead to misidentification when sexing based on behavioral clues alone.

Here we analyzed various morphological parameters that have been used as reliable indicators of sex in other *Eudyptes* penguins (Poisbleau et al., 2011; Steinfurth et al., 2019) and others that have been suggested for tawaki in the past (Warham, 1972). Males are larger overall than females in all parameters measured. Such sexual size dimorphism has been proposed to be attributed to marine productivity (female-biased in less productive tropics) and to body size (male-biased in larger bodied species) (Fairbairn & Shine, 1993). The pattern of male-biased sexual size dimorphism is common across penguin species (Croxall, 1995).

We favored parameters that can be measured quickly in conjunction with other sampling procedures. All measurements tested separated males and females, but weight was the least significant ($p < 0.01$, variable importance = 13) which was expected given the life stage examined in the study period. From late incubation through the guard stage male tawaki remain at the nest and fast. Females, however, are foraging daily to feed themselves and the growing chicks. This added nutrition in comparison to the fasting males potentially reduces the disparity in mass compared to other periods of the year when both are actively feeding. Therefore, we do not recommend using mass as a deciding factor in sex determination as it is dependent on period of the year as well as overall condition.

Linear measurements of skeletal size exhibited greater variation between the sexes ($p < 0.01$ for all) compared to mass. Warham (1972) proposed the use of the beak shape index as a measure of the overall size of the bill to be the most significant variable. We also found that

measurements associated with bill and head size (bill length, bill depth, and head length) all were significantly larger in males than in females. However, when faced with young males or older females, bill shape and size are more likely to overlap. Older female tawaki may develop thickened bills comparable to younger males requiring a second factor to positively determine sex. We suggest using foot length in conjunction with bill depth as the most repeatable and reliable metrics to identify sex in the field.

While the results presented here show a clear indication of overall larger size in males than in females, more sampling and work should continue. Mass alone is not a sufficient indicator of sex as it is a factor of overall body condition as well as influenced by the period of the annual cycle (Croxall, 1995). The ontology of sexual size dimorphism in tawaki and other *Eudyptes* penguins is unknown. Future work should collect morphological data on chicks and juveniles of known sex to determine the trajectory of growth between males and females and to assess the reliability of morphological measures in sexing individuals of different age classes. The ability to accurately sex tawaki is vital for assessing population structure and trends and will further the ability of ecological studies to recommend specific conservation efforts to protect this enigmatic species.

CHAPTER 3

STABLE ISOTOPE ECOLOGY IN FIORDLAND PENGUINS

Introduction

Seabird species worldwide are under threat due to changing climate, over-exploitation of resources, pollution, and the introduction of terrestrial mammalian predators (Croxall et al., 2012). The penguins of New Zealand are not immune to these threats. The New Zealand region is home to diverse marine habitats and six (of 18) species of penguins (Mattern & Wilson, 2018). The survival of penguins is dependent on reliable access to resources and nesting sites. New Zealand's penguins are threatened by rapidly changing marine environments affecting prey abundance, conflict with fisheries, invasive predators, and habitat loss (Wilson & Long, 2016). In order to better predict how increased threats may impact these sensitive penguin populations, it is vital to understand their dietary preferences and foraging behavior.

Changing oceanic conditions inevitably cause shifts in prey distribution (Murphy et al., 2007). Such regime shifts in mid-trophic level prey communities will likely alter the distribution of penguins. In most cases, such shifts in penguin distribution are constrained by other environmental factors and foraging shifts to environments that may be less favorable in terms of these other physical and ecological variables may lead to declines in penguin populations (Boersma & Rebstock, 2009). In some species (gentoo and Adéile penguins), such shifts may be beneficial at least in the short term (Lynch et al., 2012; La Rue et al., 2013). Conservation plans for marine reserves and fisheries should consider the impacts of climate and how penguin distributions and foraging requirements may shift.

Fiordland penguins (*Eudyptes pachyrhynchus,* hereafter referred to by their Māori name tawaki) are small crested penguins endemic to the South Island of New Zealand from

Haretaniwha Point in the north to Port Pegasus, Stewart Island/Rakiura in the south (Mattern & Wilson, 2018). Tawaki breed at sites associated with a wide range of marine habitats across the west coast of New Zealand's South Island (Warham 1974). Breeding colonies are often found in protected bays, fjords, or along coastal bluffs with nests hidden in rock fall caves, rainforest underbrush, coastal shrubland, and sea caves (Ellenberg et al., 2015). Marine conditions vary from shallow continental shelf, shallow coastal seas, and fjords. This distribution across diverse marine habitats suggests that tawaki may be able to tailor their foraging strategy to local resources and target a wider variety of prey sources than other penguin species.

Tawaki are late-winter breeders that arrive in early July to form breeding colonies and their chicks typically fledge by the end of November (Warham 1974). Like other *Eudyptes* penguins, males and females adhere to predictable roles during the breeding season (Warham, 1974; Williams & Croxall, 1991). Male and female tawaki alternate incubation duties. Females begin the process and males return from a short foraging trip during the final days before hatching. During chick rearing, male tawaki fast at the nest and guard the chick while females forage. Once the chicks reach the crèche phase both parents forage to feed the rapidly maturing chick. This division of incubation and chick rearing duties requires extended fasting periods in males and potentially long foraging trips for females.

During the incubation and guard periods, female tawaki from Jackson Head and Whenua Hou travel up to 60-80 km from the colony to forage while female foraging ranges from Harrison Cove in Milford Sound rarely exceed 4 km (Mattern & Ellenberg, 2018). These GPS tracked foraging ranges place tawaki into three distinct marine habitats: continental shelf, pelagic, and fjord (Mattern & Ellenberg, 2018).

Once the chicks have fledged, adults disperse on the pre-molt foraging trip into the lower Tasman Sea (Mattern et al. 2018b). Until recently, their foraging grounds and routes were unknown. During the pre-molt, tawaki from the breeding colony at Gorge River were tracked along a 6,800 km round trip over the course of 4-6 weeks (Mattern et al., 2018b). Tawaki were segregated along either the Sub-Tropical Front or the Sub-Antarctic Front south of Tasmania. This journey along the Sub-Antarctic Front constitutes the longest pre-molt foraging trip of any crested penguin species (Mattern et al., 2018b).

A limited number of studies have investigated tawaki dietary preferences. Van Heezik (1989) assessed prey in stomach contents at Martin's Bay and found that arrow squid (*Nototodarus sloanii)* comprised the majority (85%) of their diet. Stomach contents from Codfish Island/Whenua Hou showed 80% of sampled tawaki consumed 9 taxa representing 7 families, including juvenile red cod (*Pseudophyscis bachus),* ahuru (*Auchenoceros punctatus)*, arrow squid, and octopus (*Octopus maorum)* (Van Heezik, 1990). Based on the predominance of juvenile and post-larval stage fish (85%) in stomach contents during chick rearing, tawaki are likely feeding on masses of pelagic macro-zooplankton such as schools of larval fish, krill, and cephalopods, and are less likely to be selecting specific prey species within this size class (Van Heezik, 1989; 1990). Stable isotope values derived from blood samples collected at Taumaka/Open Bay Island suggest a reliance on winter-spawning squid (Poupart et al., 2019).

Traditionally, seabird diet has been assessed by retrieving gut contents (Gales, 1987; Goldsworthy et al., 2016). Although this method provides a snap shot of the most recent meal consumed, it also has the potential to overestimate reliance on species with hard body parts (e.g., squid beaks, fish bones) that will be digested more slowly than soft-bodied prey (e.g., jellyfish) (Duffy & Jackson, 1986). While valuable, gut content analysis is often best when complimented

by the use of other techniques such as fatty acid analysis (Karnovsky et al., 2012), fecal DNA (Deagle et al., 2007), or stable isotope analysis (SIA; Polito et al., 2011).

SIA is commonly used to quantify the importance (i.e., % contribution) of the digestible diet of individuals (Quillfeldt et al., 2005; Caron-Beaudoin et al., 2013), and to investigate the trophic interactions among species (Jaeger et al., 2013). For example, SIA has been used to elucidate the trophic niche of southern rockhopper penguins (*Eudyptes chyrsocome)* and Magellanic penguins (*Spheniscus magellanicus)* in Argentina (Rosciano et al., 2016), gentoo penguins (*Pygoscelis papua)* on South Georgia (Ratcliffe et al., 2018), and emperor penguins (*Aptenodytes forsteri)* and Adélie penguins (*Pygoscelis adeliae)* in Antarctica (Cherel, 2008). Tissues derived from particulate organic matter (POM) are enriched in ^{13}C along a latitudinal gradient, providing a metric for reconstructing seabird foraging and migration routes (Trull & Armand, 2001). Additionally, $\delta^{13}C$ reflects inshore/offshore and pelagic/benthic input to the diet of seabirds (Hobson et al., 1994; Cherel & Hobson, 2007).

Although $\delta^{13}C$ variation can be low among consumers and their prey, nitrogen stable isotope ratios ($^{15}N/^{14}N$ expressed as $\delta^{15}N$ values) increase with trophic level. Primary producers are the baseline of the trophic web so the $\delta^{15}N$ values derived from their tissues reflect environmental $\delta^{15}N$. All consumers show a stepwise enrichment in $\delta^{15}N$ values compared to their prey (Minagawa & Wada, 1984). For example, $\delta^{15}N$ values measured in the blood and feathers of captive southern rockhopper penguins fed a controlled diet of capelin (*Mallotus villosus)* were higher ($\Delta^{15}N$: 1.9 ‰ – 4.4 ‰) than whole fish or fish muscle (Cherel et al., 2005).

Tissues incorporate isotopes from diet at varying rates, so choosing the correct tissue for the time period of interest is vital for understanding trophic interactions across a temporal scale. Whole blood in penguins reflects dietary isotopes integrated over roughly 20 days (Barquete et

al., 2013). Feathers, however, only incorporate isotopes from the diet prior to feather growth (Cherel et al., 2005; Hobson et al., 1994). Like all penguins, tawaki undergo a single catastrophic molt per year in which all feathers are shed and replaced. During the molt, tawaki are restricted to land. Therefore, the stable isotope values measured in tawaki feathers reflect only the diet consumed during the pre-molt period where feather synthesis began (Cherel et al., 1994).

In this study, we used stable isotope values (δ^{13}C and δ^{15}N) derived from penguin tissues to investigate the diets of tawaki that foraged along the continental shelf (Jackson Head & Gorge River), shallow coastal seas (Whenua Hou), and fjord (Harrison Cove) habitat types. Our objective was to determine if penguins foraged differently in these habitat types and between sexes during two periods of the annual cycle when tawaki have increased demand for reliable resources (late incubation and the pre-molt). Characterizing how tawaki forage during these two critical periods of the annual cycle allows a better understanding of their resource requirements at a time when this species appears to be faring better than many other penguin species.

Methods

Study Area

We sampled tawaki at three sites across their range in southern New Zealand (Fig. 4). Each location is representative of one of the three major marine habitat types occupied during the breeding season. The Jackson Head colony (-43.962°, 168.611°) is located near the village of Jackson Bay in southern Westland. Nests are hidden in dense stands of Kiekie (*Freycinetia banksia)* and small rock overhangs atop steep coastal bluffs. GPS tracked females from Jackson Head show typical daily foraging distances of 40-80 km from shore (Mattern & Ellenberg, 2018).

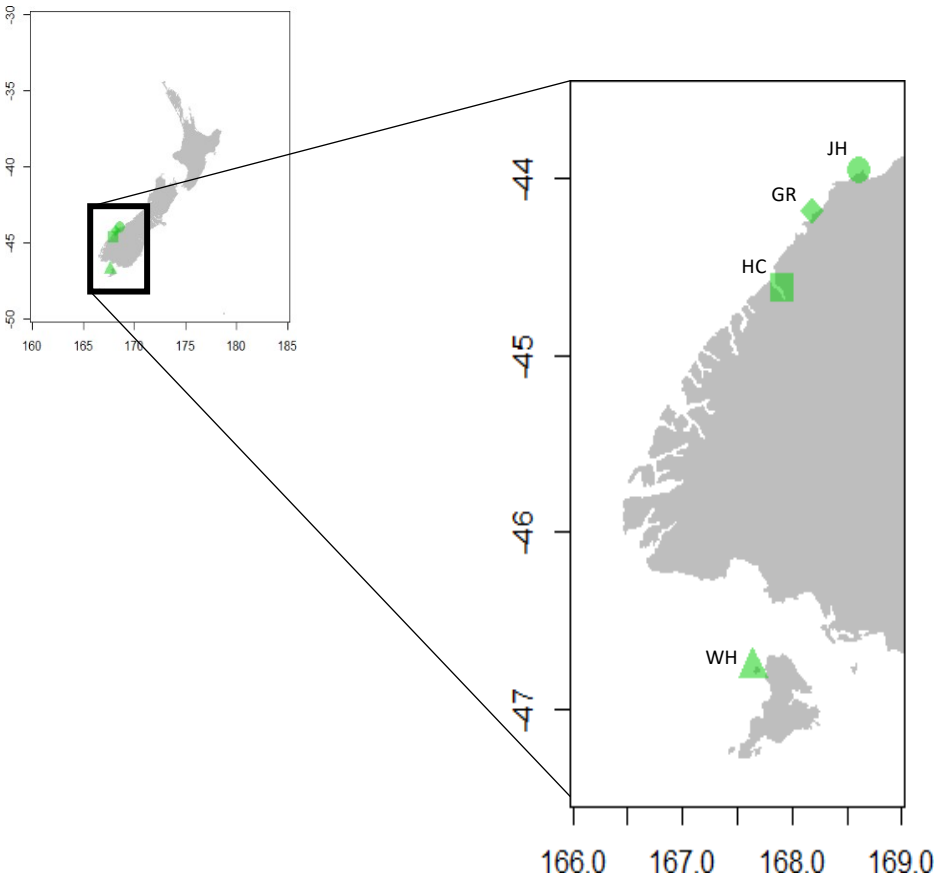

Figure 4. Map of field sites on the South Island. Jackson Head (circle) and Gorge River (diamond) are associated with continental shelf foraging, Harrison Cove (square) with fjord, and Whenua Hou (triangle) with the Foveaux Strait

The second study site, Whenua Hou, is on Sealer's Bay on the north-eastern coast of Codfish Island/Whenua Hou (-46.760°, 167.640°). It is characterized by soft tree ferns as well as abundant understory fern species. Nests are found under vegetation and rock shelters. Codfish Island/Whenua Hou sits off the north-west coast of Stewart Island/Rakiura in the Foveaux Strait at the interface of the shallow continental shelf and the deeper Solander Trough. Tawaki have been shown to exploit both ecosystems within 50 km from the nest (Mattern & Ellenberg, 2018).

Finally, the Harrison Cove colony (-44.623°, 167.912°) is located within Fiordland National Park and the Piopiotahi Marine Reserve near the mouth of the Harrison River in

Milford Sound/Piopiotahi, Fiordland. This site is dominated by dense temperate rainforest of silver beech (*Lophozonia menziesii)*, tree fuchsia (*Fuchsia excorticate*), and soft tree fern (*Cyathea smithi).* Nests are located under rock overhangs and in rockfall caves within 20 m from the shore. The colony is located 10 km from the mouth of the fjord, but GPS tracked females forage exclusively within the fjord, rarely more than 5 km from the colony (Mattern & Ellenberg, 2018).

Sample Collection

We captured adult penguins at all three sites by targeting the most accessible nests. During the guard stage, males remain with the chicks at the nest while females forage. Males were captured on the nest by hand or using a leg crook. Most females were intercepted on the beach. Those that evaded capture were allowed to feed their chick and rest before being captured and sampled at their nest.

In September and October 2017 and 2018 (late incubation into the guard stage; Fig. 5) we collected whole blood (0.1 - 0.5 mL) from the brachial vein of tawaki using a new 25-gauge needle and 1.0 mL tuberculin syringe (n = 70). Stable isotope values derived from whole blood reflect diet consumed roughly 20 days prior to sampling, equating to the latter half of the incubation stage (late August - September) (Barquete et al., 2013).

We also cut feathers (n = 165) from the lower back during the guard stage of 2017 and 2018 (n = 98) at Whenua Hou (n = 26), Jackson Head (n = 41), and Harrison Cove (n = 36) while blood samples were being collected. We also included feathers collected in 2010 at Jackson Head (n = 20) and Munro Beach (n = 2), in 2015 at Jackson Head (n = 2), and Harrison Cove (n = 11), and in 2016 at Gorge River (n = 13) and Whenua Hou (n = 14) by the Tawaki Project.

Feather derived stable isotope values reflect the diet during the pre-molt period alone (Fig. 5). We preserved all samples in 70 % ethanol and stored at -20°C (Hobson et al., 1994).

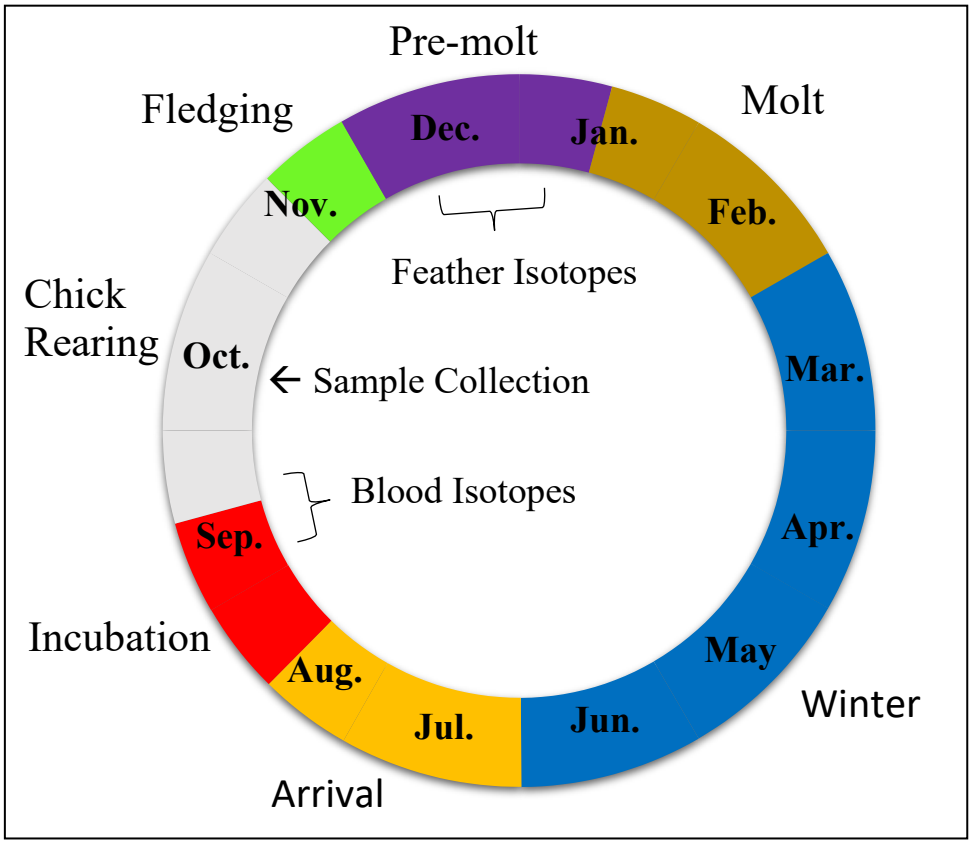

Figure 5. Tawaki annual cycle. Sample collection time, blood isotope period, and feather isotope periods are indicated.

Prey Samples

Reconstructing the trophic web for each of the primary marine ecosystems occupied by tawaki is critical for interpreting trophic interactions between penguins and their prey. As existing isotopic data is limited for this region of New Zealand, we aimed to collect samples from each site to test primary producers (baseline) through putative tawaki prey species. New Zealand green-lipped mussels (*Perna canaliculus*) and goose barnacles (*Hexelasma nolearia*) were collected from piers and buoys as proxies for the pelagic baseline. Snails (*Lunella sp.*)

acted as proxies for the benthic baseline. These species feed on primary producers, so isotope values derived from their muscles will reflect the first step in the trophic cascade.

Prey species identified by Van Heezik (1989; 1990) such as krill, sprat (*Sprattus muelleri*) (*Nyctiphanes australis*), arrow squid (*Nototodarus sloanii*) were collected via dip nets (krill), opportunistically from spillage at nests (krill & sprat), purchased from local fishermen (arrow squid), or provided by the Tawaki Project (Snares Islands samples). Shrimp (*Palaemon affinis*), salps (*Thalia sp.*), short-finned eel (glass eels; *Anguilla australis*), and larval *Galaxis* fish were collected opportunistically either at nests or found on shore. Soft tissues were collected from mussels, snails and goose barnacles. Muscle biopsies were taken from larger squids and sprats. All other species were collected whole.

Molecular Sexing Protocol

The sex of each penguin was determined in the field based on behavioral and morphological cues (Warham, 1974). In order to verify correct sex assignment of study individuals, we genetically sexed a subsample (n = 58) of tawaki using whole blood. We extracted total genomic DNA using standard phenol-chloroform protocols. A polymerase chain reaction (PCR) was performed using the CHD-W and CHD-Z specific primers SEX1 (5′-CTCC-CAAGGATGAGAAACTGTGCAAAACAGGTA-3′) and SEX2 (5′-CCTTCACTT CCATT-AAAGCTGATCTGGAATTTC-3′) following Wang and Zhang (2009). These primers and protocol were chosen based on previous success sexing northern rockhopper penguins (Steinfurth et al., 2019) and other penguin species (White, et al., unpublished data). In each sample well, 15.0 µL of a 240x solution of PCR water (375.0 µL), primers (120.0 µL each), Accuris™ Hot Start Taq Master Mix (1200 µL) was added to 5.0 µL of extracted DNA (25 ng). The thermal cycler (Applied Biosystems Veriti™ Thermal Cycler) was set on ST55 which

included an initial cycle at 95°C (5 minutes), thirty cycles of 95°C (1 minute), 55°C (1 minute), and 72°C (2 minutes), and a final cycle at 72°C (10 minutes).

Following completion of PCR, 20 µL of product and 3.0 µL of BioLabs® Gel Loading Dye (Purple 6x) were pipetted onto a 3 % agarose gel stained with ethidium bromide. Electrophoresis proceeded for 200 Volt-hours and bands were analyzed using a BioRad Molecular Imager®. Samples exhibiting a single band were classified as male and those with two bands as female (Miyaki et al. 1998).

Stable Isotope Analysis

We removed feather samples from 70% ethanol and rinsed with deionized water. Next, we sonicated the feathers in a 2:1 methanol:chloroform solution for 30 minutes to remove lipids and debris. We then rinsed each tissue with deionized water every 10 minutes and followed with a final rinse (Paritte & Kelly, 2009). We did not pre-treat whole blood.

Once appropriately cleaned, all samples were oven dried at 60° C for 48 hours. Dried blood was homogenized using a mortar and pestle, whereas feathers were finely chopped using stainless steel scissors. Only the distal barbs (excluding the rachis) of the feathers were included. Growing feathers emerge from the shaft such that the more distal portions represent the earliest growth of the feather likely before a switch to endogenous protein sources (Cherel et al., 1988). Aliquots of 0.40 mg were weighed into 5x8 mm tin capsules. The University of Cincinnati Stable Isotope and Biogeochemistry Laboratory measured stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes in all samples using a Thermo Scientific Delta V IRMS Delta V Advantage isotope ratio mass spectrometer (IRMS; Bremen, Germany) and a Costech 4010 Elemental Analyzer and Conflo IV interface (Valencia California, USA).

The lab corrected data for linearity and drift using caffeine, and for scale using caffeine and USGS 41. We assessed accuracy based on independent measurements of glycine (carbon) and gelatin (nitrogen) and precision based on measurements of glycine, gelatin, caffeine, and USGS 41 (Table 3). The mean isotopic difference for 27 samples ran in duplicate was 0.05 ‰ for $\delta^{13}C$ and 0.02 ‰ for $\delta^{15}N$.

SIRM	Range $\delta^{13}C$ (‰)	Mean $\delta^{13}C$ (‰)	SD $\delta^{13}C$ (‰)	Range $\delta^{15}N$ (‰)	$\delta^{15}N$ (‰)	SD $\delta^{15}N$ (‰)
Caffeine	-38.6 to -37.6	-38.2	± 0.6	1.5 to 2.9	1.6	± 0.1
Glycine	-33.1 to -32.1	-32.6	± 1.1	5.6 to 6.0	5.7	± 0.1
Gelatin	-14.0 to -14.8	-14.4	± 0.6	5.6 to 5.8	5.6	± 0.1
USGS 41	37.4 ro 37.7	37.6	± 0.7	47.4 to 47.8	47.6	± 0.2

Table 3. Secondary isotopic reference materials (SIRMs). Accuracy and precision were assessed using caffeine, glycine, gelatin, and USGS 41. For 14 runs, the within run range of means, all run mean, and standard deviations of $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) for each SIRM are given.

Data Analysis

We compared stable isotopes between sexes and tissues, and among sites and years. Stable isotope values from all groups were first assessed for normality and homoscedasticity using a Shapiro-Wilkes and Levene's test, respectively. If pair-wise groups were normally distributed, we used t-tests and ANOVA for comparison; otherwise, we used Wilcoxon-Mann Whitney or Kruskall-Wallis tests. For variables found to have significant differences, suggesting variation in diet, we employed the Steel-Dwass test for pairwise comparisons. We used the program JMP (JMP®, Version 14. SAS Institute Inc., Cary, NC, 1989 - 2019) to conduct all data analyses.

Results

Incubation

We collected 70 whole blood samples in 2017 (n = 27) and 2018 (n = 43). Of these, 3 (2 from 2017 and 1 from 2018) were unable to be analyzed due to insufficient sample volume and were excluded from the study (n = 67). We analyzed 14 samples from Whenua Hou, 25 from Harrison Cove, and 28 from Jackson Head (Fig. 6; Appendix F)

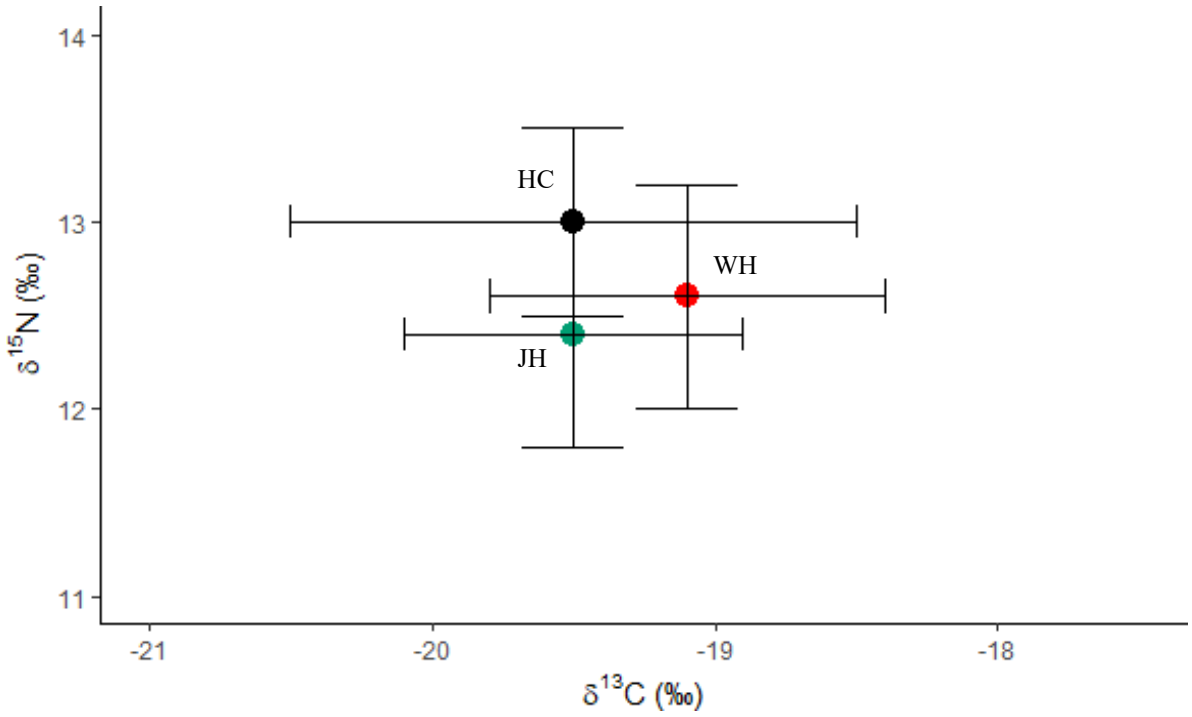

Figure 6. Mean tawaki blood isotope values by colony. Samples collected at Jackson Head (green), Whenua Hou (red), and Harrison Cove (black), during the late incubation and guard stage of 2017 and 2018 breeding seasons. This shows a clustering of isotope values during the incubation period with δ^{13}C between -20 ‰ to – 18.5 ‰ and δ^{15}N between 11.5 ‰ to 13.5 ‰.

We found no significant differences in $\delta^{13}C$ values among Harrison Cove, Jackson Head, or Whenua Hou (Kruskal Wallis, $X^2 = 5.2452$, df = 2, p = 0.073). $\delta^{15}N$ values were higher from Harrison Cove (Kruskal Wallis, $X^2 = 14.1483$, df = 2, $p < 0.001$) than from both Jackson Head (Steel-Dwass, $p < 0.001$) and Whenua Hou (Steel-Dwass, p = 0.007; Fig. 7). We also found that $\delta^{13}C$ and $\delta^{15}N$ values were not significantly different between sexes ($\delta^{13}C$: t = 0.8011, p = 0.106; $\delta^{15}N$: t = 0.2168, p = 0.067; Figure 8). Lastly, when we compared years (2017 and 2018), we found stable isotope differences between $\delta^{13}C$ (t = 1.972, p = 0.019) but not $\delta^{15}N$ (t = 1.874, p = 0.067; Figure 9).

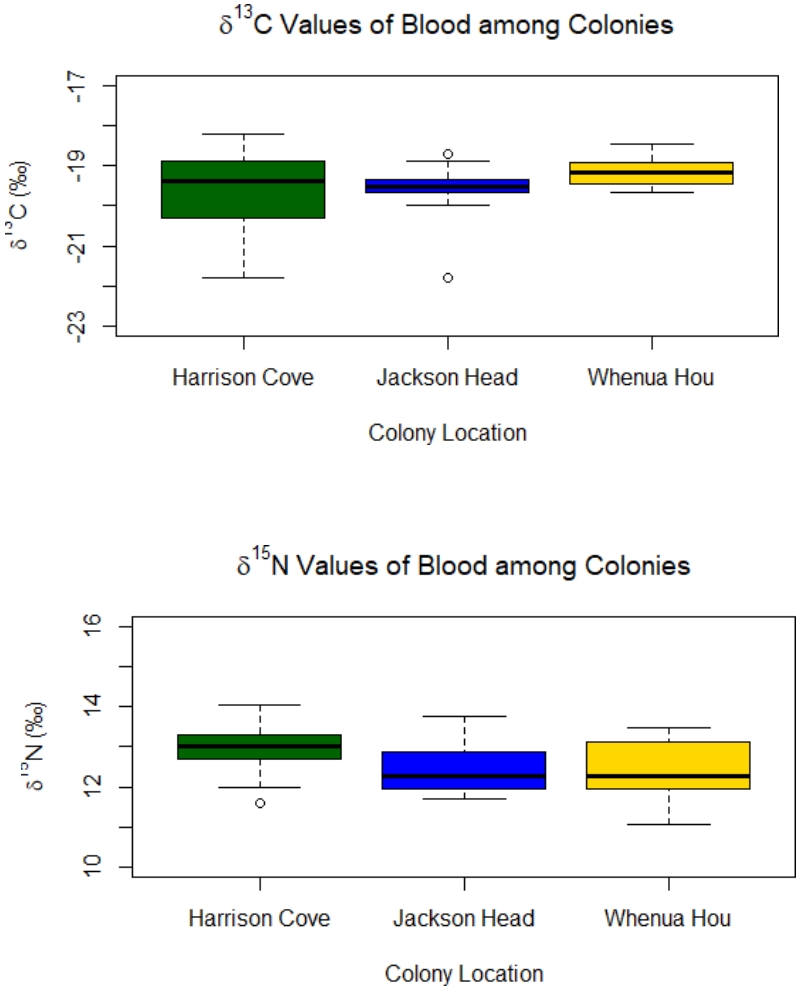

Figure 7. Boxplots of $\delta^{13}C$ and $\delta^{15}N$ values derived from whole blood during incubation by colony.

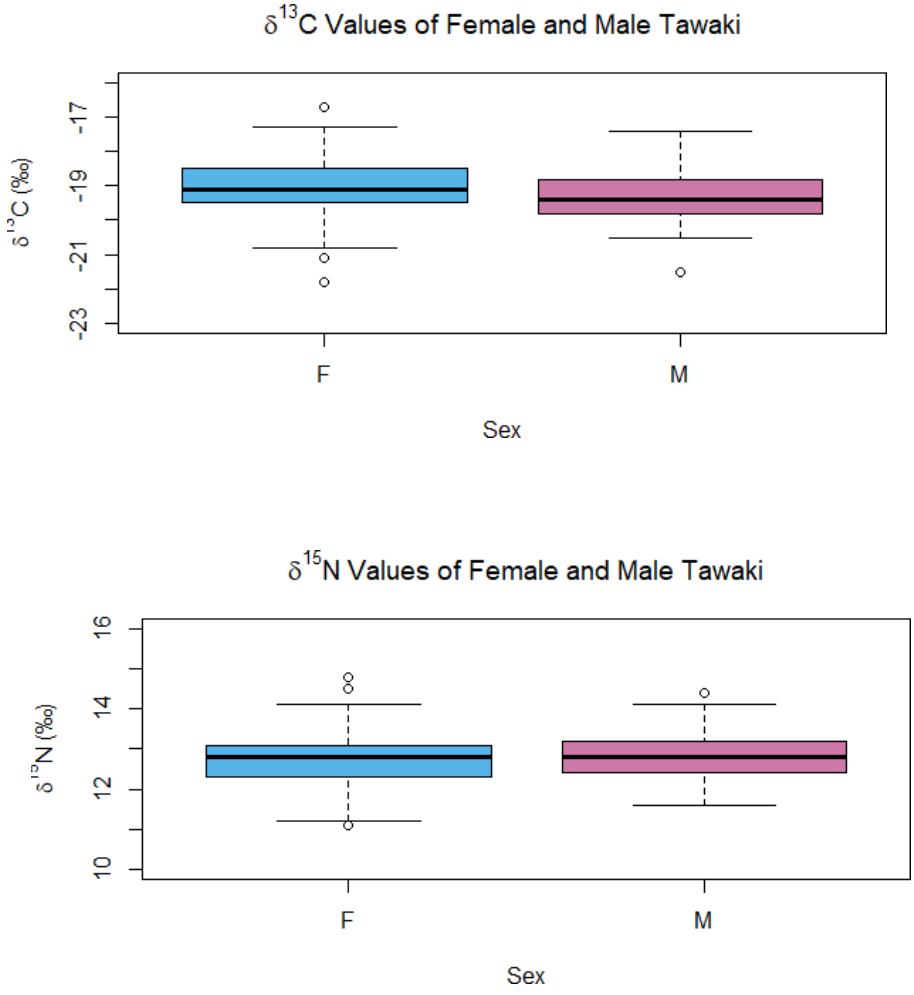

Figure 8. Boxplots of δ^{13}C and δ^{15}N values derived from whole blood between sexes during the incubation period

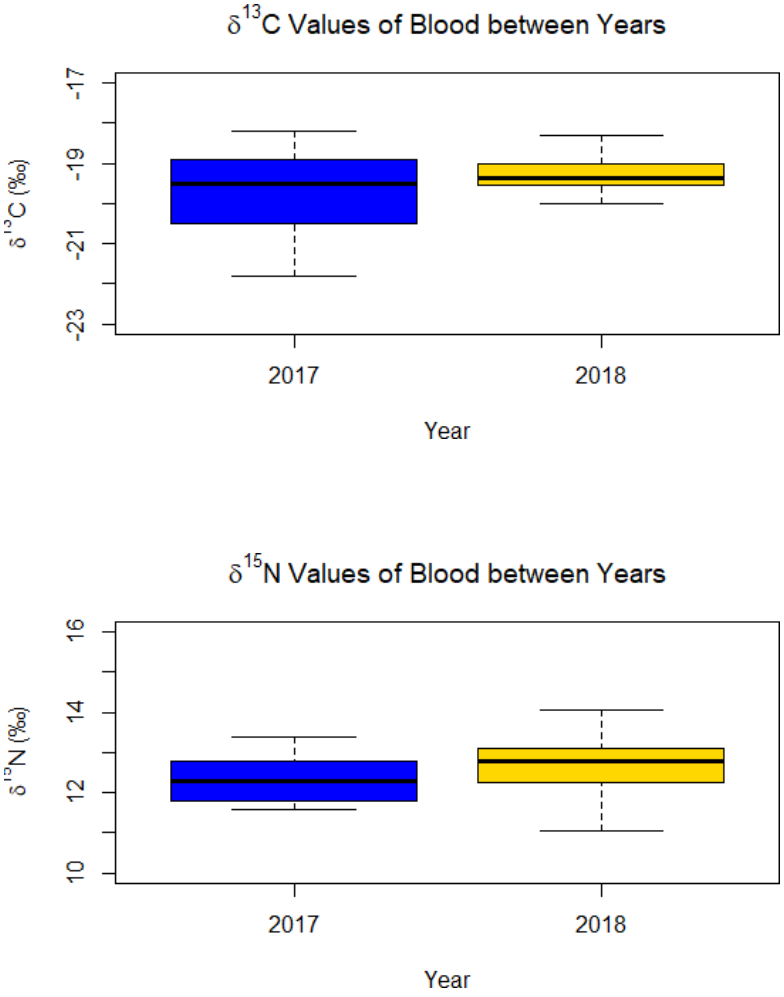

Figure 9. Boxplots of $\delta^{13}C$ and $\delta^{15}N$ values derived from whole blood between years (2017 - 2018) during the incubation period.

Pre-molt

In total, 174 feather samples were analyzed in this study (Fig. 10). Due to low sample size and proximity to Jackson Head, Munro Beach samples were considered as Jackson Head for analysis. The mean $\delta^{13}C$ value was -19.0 ± 0.7 ‰ while the mean $\delta^{15}N$ value was 12.8 ± 0.6 ‰ (Appendix G).

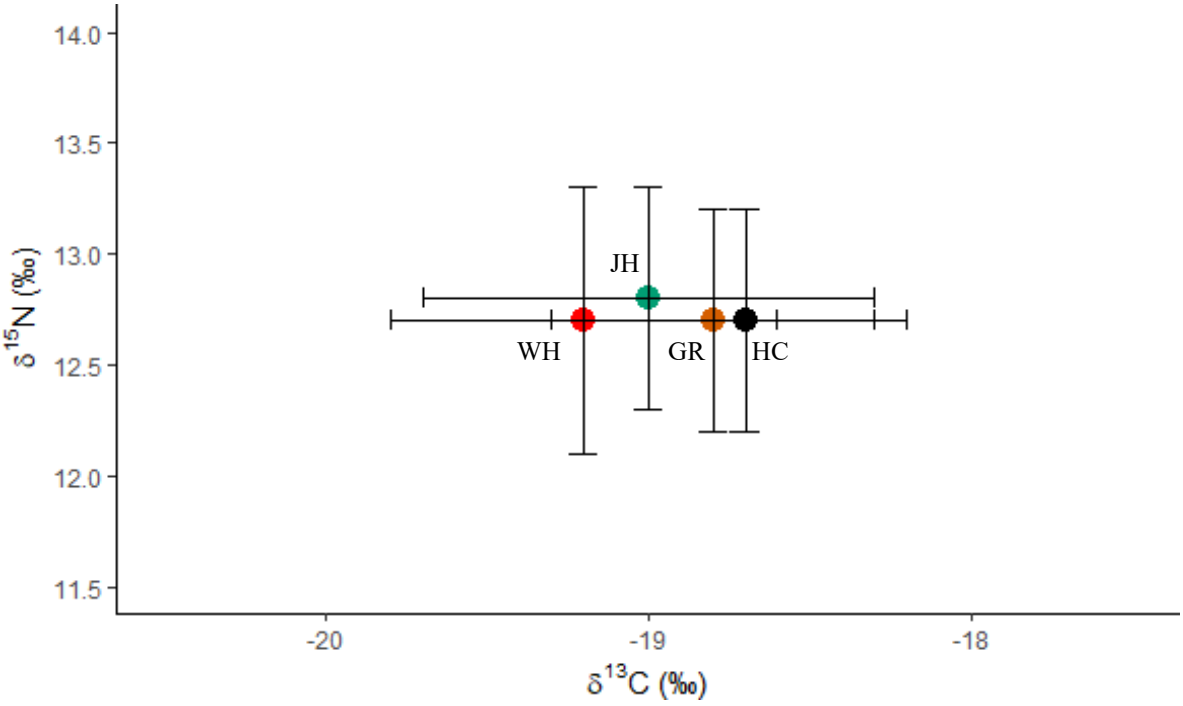

Figure 10. Mean tawaki feather isotope values by colony. Samples collected at Gorge River (orange), Harrison Cove (black), Jackson Head (green), and Whenua Hou (red). This shows a range of δ^{13}C values from around -20.0 ‰ to -18.0 ‰ while δ^{15}N clustered between 12.0 ‰ to 13.5 ‰.

We compared the mean δ^{13}C and δ^{15}N values between colonies. Only the Harrison Cove and Whenua Hou colonies were isotopically different in δ^{13}C values (Kruskal Wallis, $X^2 = 10.525$, df = 2, p = 0.033; Steel-Dwass, p = 0.047). δ^{15}N values were not significantly different among sites (Kruskal Wallis, $X^2 = 6.435$, df = 2, p = 0.169; Fig. 11).

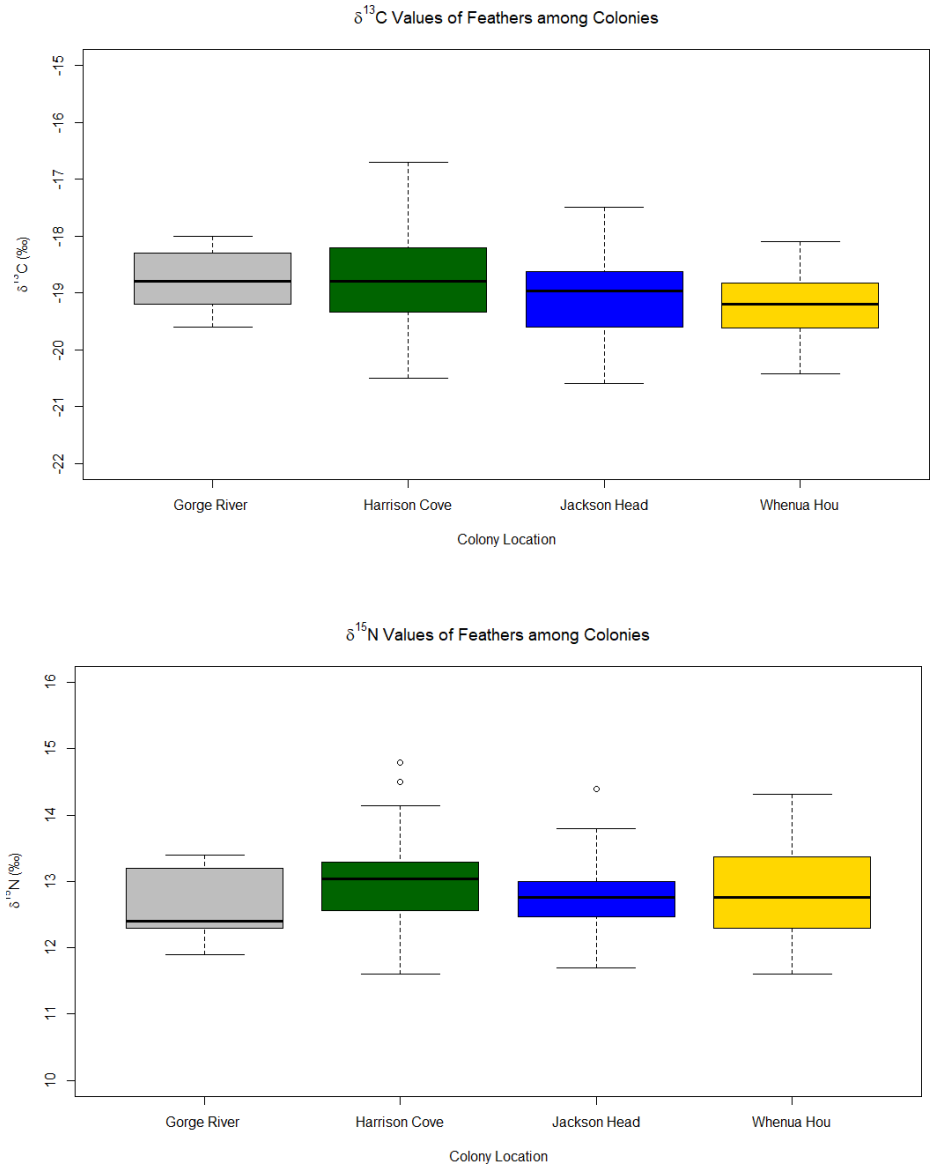

Figure 11. Boxplots of $\delta^{13}C$ and $\delta^{15}N$ values derived from feathers among colonies during the pre-molt period.

We then compared $\delta^{13}C$ and $\delta^{15}N$ values between sexes during the pre-molt period. We found a significant difference in $\delta^{13}C$ (t = -2.384, p = 0.019) with mean $\delta^{13}C$ values of -19.1 ‰ and -18.8 ‰ for males and females respectively. $\delta^{15}N$ values were not isotopically different between sexes (t = 0.7642, p = 0.4465; Fig. 12).

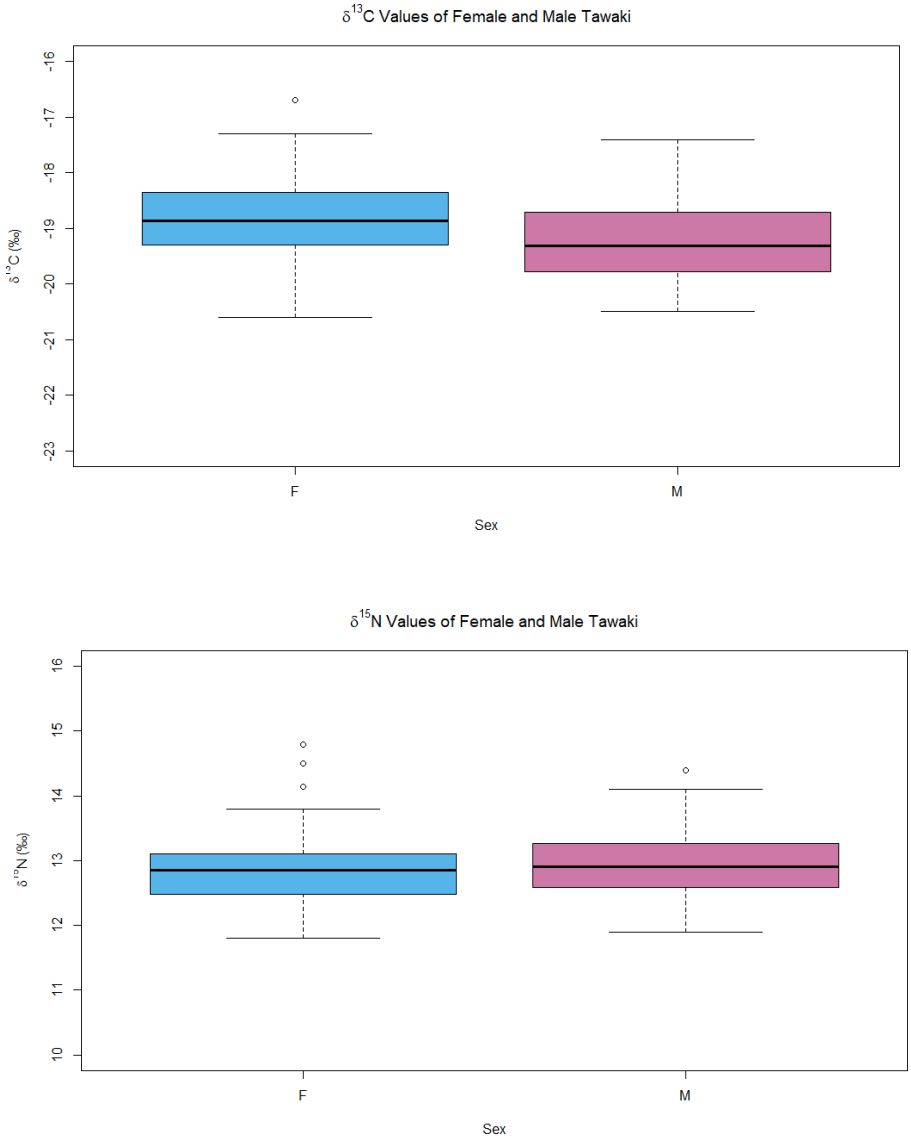

Figure 12. Boxplots of $\delta^{13}C$ and $\delta^{15}N$ values derived from feathers between sexes during the pre-molt period.

We next compared stable isotope values among years and found significant differences in both $\delta^{13}C$ (Kruskal Wallis, $X^2 = 14.1438$, df = 4, $p = 0.007$) and $\delta^{15}N$ (Kruskal Wallis, $X^2 = 40.6391$, df = 4, $p < 0.0001$; Fig. 13). The year 2015 stands out as isotopically different from 2010 ($\delta^{15}N$: Steel-Dwass, $p = 0.004$), 2016 ($\delta^{13}C$: Steel-Dwass, $p = 0.018$), 2017 ($\delta^{15}N$: Steel- Dwass, $p = 0.0001$), and 2018 ($\delta^{13}C$: Steel-Dwass, $p = 0.014$; $\delta^{15}N$: Steel-Dwass, $p < 0.001$;

Appendix H). We also found the 2016 was significantly different from 2017 (δ^{15}N: Steel-Dwass, $p < 0.001$) and 2018 (δ^{15}N: Steel-Dwass, $p < 0.001$; Appendix H). No other pair-wise combinations were significant.

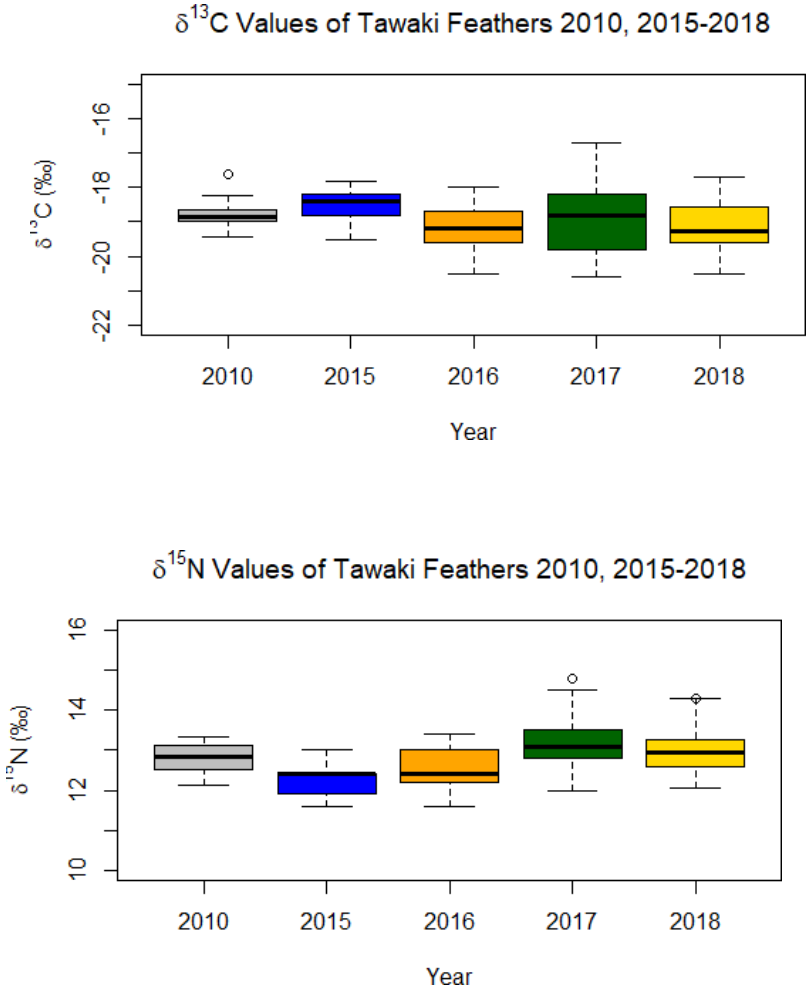

Figure 13. Boxplots of δ^{13}C and δ^{15}N values derived from feathers among years during the pre-molt period.

Finally, we compared δ^{13}C and δ^{15}N values between blood and feather samples. These tissues reflect distinct periods of the tawaki annual cycle (whole blood = incubation; feathers = pre-molt) so isotopic variation between these tissues will reflect differences in diet at a temporal

scale. We found significant differences in both $\delta^{13}C$ values ($t = 4.5662$, $p < 0.0001$) and $\delta^{15}N$ values ($t = 2.7703$, $p = 0.004$; Fig. 14)

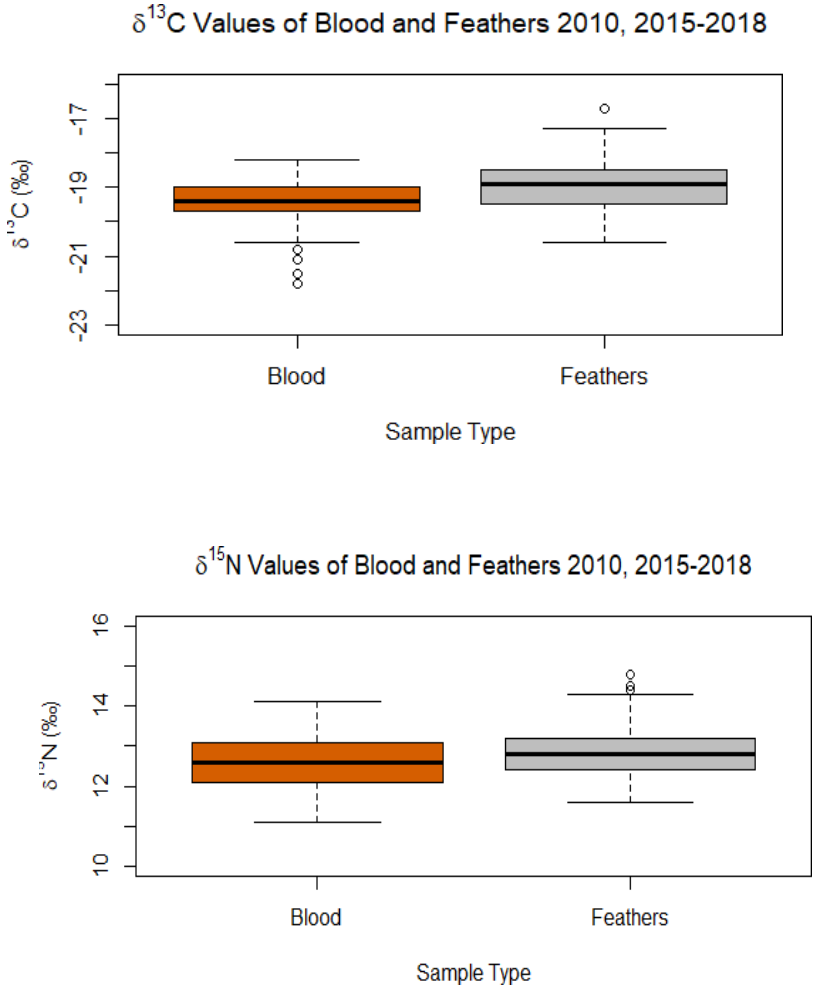

Figure 14. Boxplots of $\delta^{13}C$ and $\delta^{15}N$ values derived from both whole blood and feathers to compare incubation and pre-molt periods.

Prey

We analyzed two size classes of small marine fish (adult sprat n = 13 and larval n = 6); krill (n = 7), arrow squid (n = 5), short-finned eels (n = 6), salps (n = 4), and green-lipped mussels (n = 20) were also collected and analyzed to better understand the marine food web in each of the habitat types. We were also supplied with adult sprat (n = 6), krill (n = 5), and arrow

squid (n = 4) from the Snares Islands. Previous studies have indicated small schooling fish, arrow squid, and, to a lesser extent, krill as dietary components prominent in tawaki stomach contents (Van Heezik, 1989;1990; Poupart et al., 2019). These samples are not included in these analyses due to high carbon-nitrogen ratios (C:N; indicating incomplete lipid removal from the samples during processing) leading to erroneously elevated δ^{13}C and δ^{15}N values in some samples and low sample size. However, preliminary analysis of fish, squid, and krill which were within acceptable C:N indicate clear isotopic separation of these prey.

Discussion

Tawaki follow a complex yet predictable annual cycle that distributes them across a wide variety of marine ecosystems. Even within the breeding season, tawaki occupy diverse habitats each of which has unique resources and challenges. This habitat plasticity may be an advantage in the face of changing marine conditions if tawaki can exploit a wide variety of food sources. Past dietary studies at Whenua Hou/Codfish Island (Van Heezik, 1990) and Taumaka/Open Bay Island (Poupart et al. 2019), indicated a reliance on small and larval stage fish as well as winter spawning squid during incubation and post-guard. However, very little information exists for dietary preferences during other periods of the annual cycle (pre-molt forage and winter dispersals in particular).

***δ*^{13}C**

Across marine systems, δ^{13}C values vary by latitude as well as zone (i.e., pelagic/benthic and inshore/offshore). δ^{13}C values, therefore, provide a method of estimating foraging area of marine predators based on these latitudinal and zonal gradients. Tawaki blood samples taken during the 2017 and 2018 breeding seasons indicated no significant differences among sites or between sexes, suggesting a similar diet during this period. This lack of variation is consistent with the relatively

small latitudinal distribution of tawaki during breeding; however, we expected tawaki breeding the fjord (Harrison Cove) to be higher. We found significant differences in δ^{13}C values between years sampled; however, GPS tracking by Mattern et al. (2018b) does not support major differences in foraging areas between these two particular years. Instead, variation may be due to interannual fluctuations in baseline δ^{13}C.

Feathers exhibited significant differences in δ^{13}C values between sexes. This variation suggests that during the pre-molt, male and female may differ more in their foraging range while at sea in the non-breeding season than during the breeding season. Interestingly, variation in δ^{13}C among years (particularly 2015) reflects responses in the foraging to shifting marine conditions.

Mean δ^{13}C values did not differ significantly among all sites. Only Whenua Hou and Harrison Cove had significantly different δ^{13}C values. The range δ^{13}C fell between -17.61 ‰ and -20.43 ‰ (a difference of 2.82 ‰). In gentoo penguins, a δ^{13}C variation of 4.1 ‰ indicated a separation into two subpopulations based on foraging zone (Cherel & Hobson, 2007). One group foraged in inshore open seas while the other was foraging in inshore closed seas. A δ^{13}C value variation of 5.3 ‰ existed between northern rockhoppers (Sub-Tropical Front foraging) and emperor penguins (Antarctic foraging) (Cherel & Hobson, 2007). With the small difference between δ^{13}C values found in this study, it is unlikely to represent segregation across a large latitudinal scale.

Tawaki fitted with GLS leg bands from the Gorge River colony during the 2016 pre-molt were shown to segregate into two foraging groups along either the Sub-Tropical Front or the Sub-Antarctic Front (Mattern et al., 2018b). The same GLS banded penguins were included here as the Gorge River colony and did not show significantly different variation in δ^{13}C values than the other colonies assessed. It is possible that tawaki are foraging along these separate

frontal zones to replenish weight lost during breeding, but then enter a period of hyperphagia to fuel feather growth closer to the New Zealand mainland on the return journey. This scenario would allow tawaki to take advantage of the high marine productivity near the mainland.

Indeed, Mattern et al. (2018b) noted that returning tawaki follow more similar return routes than outgoing routes which may explain the ability to return to molt in prime condition (Warham, 1974) despite the 6,800 km round trip journey. However, more work is needed to fully understand this spatial segregation during the pre-molt period. Pairing spatial data such as satellite and GPS tags with isotopic data obtained from blood collected upon return to land to molt will greatly increase our understanding of their foraging behavior.

δ^{15}N

Another critical factor when assessing the adaptability of tawaki is to identify their trophic position within local food webs. Unlike δ^{13}C, δ^{15}N is reflective of the trophic level of the organism (Hobson et al., 1994). In marine ecosystems, phytoplankton makes up the base of the food web and δ^{15}N values increase with trophic level. We found no significant differences in δ^{15}N values between sexes for blood or feathers, indicating that male and female tawaki may not forage for different trophic levels of prey, similar to many species of seabirds (Bearhop et al., 2006), eastern rockhopper penguins (*Eudyptes chrysocome filholi*) (Morrison et al., 2014), and southern rockhopper penguins (Dehnhard et al., 2011).

We did find a significant difference in δ^{15}N values of blood between Harrison Cove and all other colonies sampled. Harrison Cove δ^{15}N values averaged 13.0 ‰ while both Whenua Hou and Jackson Head averaged 12.4 ‰. While extensive prey and baseline sampling is needed in all sites, it is likely that the difference lies in baseline δ^{15}N values in fjord ecosystems versus open ocean and continental shelf environments. Fjord ecosystems have been shown to be

enriched in δ^{15}N due to high terrestrial influx of nutrients (Nilsen et al, 2008; Renaud et al., 2011). It is therefore possible that tawaki in Harrison Cove are not foraging at a higher trophic level, but that the baseline δ^{15}N values in the fjord are higher than those on the continental shelf or Foveaux Strait.

Interestingly, the variation in feather δ^{15}N values among years reflects differences in diet particularly in 2015 and 2016 as compared to the other years sampled. In these two years, the mean δ^{15}N value was 12.2 ‰ (2015) and 12.5 ‰ (2016). The mean δ^{15}N value for the other three years was 13.1 ‰. This shift in δ^{15}N values is associated with the 2015 - 2016 El Niño Southern Oscillation (ENSO) that occurred in the eastern Pacific Ocean. ENSO warms ocean temperatures and reduces primary productivity (Barber et al., 1996). Such changes in productivity can shift prey, and subsequently, seabird ranges in search of more nutrient rich regions (Tershy et al., 1991). This change in δ^{15}N values during the pre-molt periods associated with an ENSO event suggests that tawaki adapt to different prey sources while at sea.

However, the same may not be true for all colonies during the breeding season when penguins are forced into a central place foraging strategy. We did not have blood samples for 2015 or 2016; however, tawaki breeding at Jackson Head were noted to have experienced high chick mortality during the ENSO event, suggesting a shift in prey distribution outside the acceptable foraging range from the colony (Mattern & Ellenberg, 2015).

We found that δ^{13}C and δ^{15}N values differed significantly between blood and feather samples, meaning that tawaki are foraging in different marine zones (either latitudinally or inshore/offshore) and on different trophic level prey (or in habitats with different baseline δ^{15}N) in the incubation and pre-molt periods. This difference based on tissue type is in line with the dual lifestyle of tawaki and other penguin species who shift between pelagic migrant and central-place foraging depending on the period of the annual cycle.

Conclusions

Tawaki spend extended periods at sea and only return to shore to breed and molt. This dual lifestyle requires conservationists to understand foraging ecology in two distinct marine regions. Tawaki breeding sites occupy a diverse range of marine habitats which suggest high habitat plasticity. The fjord ecosystem appears to provide tawaki with ample prey of the preferred trophic level eliminating the need to travel long distances in search of food. Fjord ecosystems have a high input of terrestrial nutrients from forest debris and run off (Prebble et al., 2018). This influx of nutrients along with unique hydrology (Rutger & Wing, 2006) and protection from rough seas creates a unique ecosystem that supports a vibrant community (McLeod et al., 2010).

We have shown that tawaki foraging ecology is complex and responds to environmental factors such as ENSO events, period of the annual cycle and colony location (fjord versus nob-fjord). Future research should include extensive sampling of prey and other lower trophic level organisms in fjords and other regions to determine if the higher δ^{15}N values in Harrison Cove are truly due to higher trophic niche or an artefact of higher baseline δ^{15}N values in the environment. Tawaki nesting in inner fjord colonies should be compared to those near the mouth and in other fjords to determine if the pattern of exclusive fjord foraging holds. Finally, to understand if trophic niche and foraging zones have shifted over time, museum specimens should also be assessed.

In the light of global climate change and differential impacts on marine ecosystems, it is critical to understand how tawaki and other seabirds will respond to changing conditions. If

tawaki are not able to overcome warming events such as ENSO, it is unlikely that they will adapt if warming ocean temperatures drive prey distribution away from mainland New Zealand. However, it is possible that fjord ecosystems may act as a refugia for tawaki (at least in the short term) and conservation plans to protect the fjords of the South Island could be critical for the future of this enigmatic species.

REFERENCES

Baker, A. J., Pereira, S. L., Haddrath, O. P., & Edge, K. A. (2005). Multiple gene evidence for expansion of extant penguins out of Antarctica due to global cooling. *Proceedings of the Royal Society B: Biological Sciences*, *273*(1582), 11-17.

Ballance, L. T., Pitman, R. L., & Fiedler, P. C. (2006). Oceanographic influences on seabirds and cetaceans of the eastern tropical Pacific: A review. *Progress in Oceanography*, *69*(2–4), 360–390.

Banks, J., Van Buren, A., Cherel, Y., & Whitfield, J. B. (2006). Genetic evidence for three species of rockhopper penguins, Eudyptes chrysocome. *Polar Biology*, *30*(1), 61–67. 0-3

Barber, R. T., Sanderson, M. P., Lindley, S. T., Chai, F., Newton, J., Trees, C. C., Foley, D. G. and Chavez, F. P. (1996). Primary productivity and its regulation in the equatorial Pacific during and following the 1991–1992 El Nino. *Deep Sea Research Part II: Topical Studies in Oceanography*, *43*(4-6), 933-969.

Barquete, V., Strauss, V., & Ryan, P. G. (2013). Stable isotope turnover in blood and claws: A case study in captive African Penguins. *Journal of Experimental Marine Biology and Ecology*, *448*, 121–127.

Barrett, R. T., Camphuysen, K., Anker-Nilssen, T., Chardine, J. W., Furness, R. W., Garthe, S., Hüppop, O., Leopold, M. F., Montevecchi, W. A. and Veit, R. R. (2007). Diet studies of seabirds: A review and recommendations. *ICES Journal of Marine Science*, *64*(9), 1675– 1691.

Bearhop, S., Phillips, R. A., McGill, R., Cherel, Y., Dawson, D. A., & Croxall, J. P. (2006). Stable isotopes indicate sex-specific and long-term individual foraging specialisation in diving seabirds. *Marine Ecology Progress Series*, *311*, 157-164.

BirdLife International. 2017. The IUCN Red List of Threatened Species; [cited 2019 Dec. 7].

Boersma, P. D., & Davies, E. M. (1987). Sexing monomorphic birds by vent measurements. *The Auk*, *104*(4), 779-783.

Boersma, P. D., & Rebstock, G. A. (2009). Foraging distance affects reproductive success in Magellanic penguins. *Marine Ecology Progress Series*, *375*, 263–275.

Booth, J. M., & McQuaid, C. D. (2013). Northern rockhopper penguins prioritise future reproduction over chick provisioning. *Marine Ecology Progress Series*, *486* (Southwood 1988), 289–304.

Borboroglu, P. G., & Boersma, P. D. (Eds.). (2015). *Penguins: natural history and conservation*. University of Washington Press.

Bost, C. A., Thiebot, J. B., Pinaud, D., Cherel, Y., & Trathan, P. N. (2009). Where do penguins go during the inter-breeding period? Using geolocation to track the winter dispersion of the macaroni penguin. *Biology Letters*, *5*(4), 473-476.

Brandão, M. L., Braga, K. M., & Luque, J. L. (2011). Marine debris ingestion by Magellanic penguins, Spheniscus magellanicus (Aves: Sphenisciformes), from the Brazilian coastal zone. *Marine Pollution Bulletin*, *62*(10), 2246–2249.

Brown, C. R., & Klages, N. T. (1987). Seasonal and annual variation in diets of macaroni and rockhopper penguins. *Ostrich* 57:180-84

Carey, M. J., Meathrel, C. E., & May, N. A. (2009). A new method for the long-term attachment of data-loggers to shearwaters (Procellariidae). *Emu*, *109*(4), 310–315.

Caron-Beaudoin, É., Gentes, M. L., Patenaude-Monette, M., Hélie, J. F., Giroux, J. F., & Verreault, J. (2013). Combined usage of stable isotopes and GPS-based telemetry to understand the feeding ecology of an omnivorous bird, the Ring-billed Gull (Larus

delawarensis). *Canadian journal of zoology*, *91*(10), 689-697.

Cherel, Y. (2008). Isotopic niches of emperor and Adélie penguins in Adélie Land, Antarctica. *Marine Biology*, *154*(5), 813-821.

Cherel, Y., & Hobson, K. A. (2007). Geographical variation in carbon stable isotope signatures of marine predators: A tool to investigate their foraging areas in the Southern Ocean. *Marine Ecology Progress Series*, *329*(November 2016), 281–287.

Cherel, Y., Charrassin, J. B., & Challet, E. (1994). Energy and protein requirements for molt in the king penguin Aptenodytes patagonicus. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *266*(4), R1182-R1188.

Cherel, Y., Hobson, K. A., & Hassani, S. (2005). Isotopic discrimination between food and blood and feathers of captive penguins: implications for dietary studies in the wild. *Physiological and Biochemical Zoology*, *78*(1), 106–115.

Cherel, Y., Hobson, K. A., Guinet, C., & Vanpe, C. (2007). Stable isotopes document seasonal changes in trophic niches and winter foraging individual specialization in diving predators from the Southern Ocean. *Journal of Animal Ecology*, *76*(4), 826-836.

Cherel, Y., Robin, J. P., Walch, O., Karmann, H., Netchitailo, P., & Le Maho, Y. (1988). Fasting in king penguin. Hormonal and metabolic changes during breeding. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *254*(2), R170-R177.

Clarke, J., Manly, B., Kerry, K., Gardner, H., Franchi, E., Corsolini, S., & Focardi, S. (1998). Sex differences in Adélie penguin foraging strategies. *Polar Biology*, *20*(4), 248-258.

Clarke, J. A., Olivero, E. B., & Puerta, P. (2003). Description of the earliest fossil penguin from South America and first Paleogene vertebrate locality of Tierra del Fuego, Argentina. *American Museum Novitates*, *2003*(3423), 1-18.

Colabuono, F. I., Barquete, V., Domingues, B. S., & Montone, R. C. (2009). Plastic ingestion by Procellariiformes in southern Brazil. *Marine Pollution Bulletin*, *58*(1), 93-96.

Cooper, J., Brown, C. R., Hindell, M. A., Klages, N. T. W., Moors, P. J., Pemberton, D., Ridoux, V., Thompson, K. R., & Van Heezik, Y. M. (1990). Diets and dietary segregation of crested penguins (*Eudyptes)*. In *Penguin Biology*, ed., Davis, L.S., & Darby, J.T., 131-56. San Diego, CA: Academic Press.

Crawford, R. J. M., Cooper, J., & Dyer, B. M. (2003). Population of the macaroni penguin Eudyptes chrysolophus at Marion Island, 1994/95–2002/03, with information on breeding and diet. *African Journal of Marine Science*, *25*(1), 475-486.

Crawford, R., Ellenberg, U., Frere, E., Hagen, C., Baird, K., Brewin, P., Crofts, S., Glass, J., Mattern, T., Pompert, J. and Ross, K (2017). Tangled and drowned: A global review of penguin bycatch in fisheries. *Endangered Species Research*, *34*, 373–396.

Croxall, J. P. (1995). Sexual Size Dimorphism in Seabirds. *Oikos*, *73*(3), 399.

Croxall, J. P., Prince, P. A., Baird, A., & Ward, P. (1985). The diet of the southern rockhopper penguin Eudyptes chrysocome chyrsocome at Beauchene Island, Falkland Island. *Journal of Zoology, 206*(4), 485-496

Croxall, J. P., Butchart, S. H. M., Lascelles, B. E. N., Stattersfield, A. J., Sullivan, B., Symes, A., & Taylor, P. (2012). Seabird conservation status and threats: a global assessment of priorities. *Bird Conservation International*, *22*, 1–34.

Davis, L. S., & Darby, J. T. (Eds.). (2012). *Penguin biology*. Elsevier.

De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., & Taberlet, P. (2014). DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources*, *14*(2), 306–323.

Deagle, B. E., Gales, N. J., Evans, K., Jarman, S. N., Robinson, S., Trebilco, R., & Hindell, M.

A. (2007). Studying seabird diet through genetic analysis of faeces: a case study on macaroni penguins (Eudyptes chrysolophus). *PLoS One*, *2*(9), e831.

Dehnhard, N., Voigt, C. C., Poisbleau, M., Demongin, L., & Quillfeldt, P. (2011). Stable isotopes in southern rockhopper penguins: Foraging areas and sexual differences in the non-breeding period. *Polar Biology*, *34*(11), 1763–1773.

Donald, P. F. (2007). Adult sex ratios in wild bird populations. *Ibis*, *149*(4), 671–692.

Duffy, D. C., & Jackson, S. (1986). Diet Studies of Seabirds: A Review of Methods. *Colonial Waterbirds*, *9*(1), 1.

Dykstra, C. R., Mays, H. L., Hays, J. L., Simon, M. M., & Wegman, A. R. (2012). Sexing Adult and Nestling Red-Shouldered Hawks Using Morphometrics and Molecular Techniques 1. *Journal of Raptor Research*, *46*(4), 357–364.

Ellenberg, U., Edwards, E., Mattern, T., Hiscock, J. A., Wilson, R., & Edmonds, H. (2015). Assessing the impact of nest searches on breeding birds–a case study on Fiordland crested penguins (Eudyptes pachyrhynchus). *New Zealand Journal of Ecology*, *39*(2), 231-244.

Fairbairn, J., & Shine, R. (1993). Patterns of Sexual Size Dimorphism in Seabirds of the Southern Hemisphere. *Oikos*, *68*(1), 139.

Fauchald, P. (2009). Spatial interaction between seabirds and prey: Review and synthesis. *Marine Ecology Progress Series*, *391*, 139–151.

Forcada, J., Trathan, P. N., Reid, K., Murphy, E. J., & Croxall, J. P. (2006). Contrasting population changes in sympatric penguin species in association with climate warming. *Global Change Biology*, *12*(3), 411–423.

Forero, M. G., Hobson, K. A., Bortolotti, G. R., Donázar, J. A., Bertellotti, M., & Blanco, G. (2002). Food resource utilisation by the Magellanic penguin evaluated through stable-

isotope analysis: Segregation by sex and age and influence on offspring quality. *Marine Ecology Progress Series*, *234*, 289–299.

Frugone, M. J., López, M. E., Segovia, N. I., Cole, T. L., Lowther, A., Pistorius, P., Dantas, G. P., Petry, M. V., Bonadonna, F., Trathan, P. and Polanowski, A. (2019). More than the eye can see: Genomic insights into the drivers of genetic differentiation in Royal/Macaroni penguins across the Southern Ocean. *Molecular phylogenetics and evolution*, *139*, 106563.

Gales, R. P. (1987). Validation of the stomach-flushing technique for obtaining stomach contents of penguins. *Ibis*, *129*(2), 335-343.

Goldsworthy, B., Young, M. J., Seddon, P. J., & van Heezik, Y. (2016). Stomach flushing does not affect apparent adult survival, chick hatching, or fledging success in yellow-eyed penguins (Megadyptes antipodes). *Biological Conservation*, *196*, 115-123.

Goldsworthy, S. D., He, X., Tuck, G. N., Lewis, M., & Williams, R. (2001). Trophic interactions between the Patagonian toothfish, its fishery, and seals and seabirds around Macquarie Island. *Marine Ecology Progress Series*, *218*, 283-302.

González-Solís, J., Croxall, J. P., & Wood, A. G. (2000). Sexual dimorphism and sexual segregation in foraging strategies of northern giant petrels, Macronectes halli, during incubation. *Oikos*, *90*(2), 390–398.

Grémillet, D., & Boulinier, T. (2009). Spatial ecology and conservation of seabirds facing global climate change: A review. *Marine Ecology Progress Series*, *391*(2), 121–137.

Hart, K. M., & Hyrenbach, K. D. (2009). Satellite telemetry of marine megavertebrates: the coming of age of an experimental science. *Endangered Species Research*, *10*, 9-20.

Hilton, G. M., Thompson, D. R., Sagar, P. M., Cuthbert, R. J., Cherel, Y., & Bury, S. J. (2006). A stable isotopic investigation into the causes of decline in a sub-Antarctic predator, the rockhopper penguin. *Global Change Biology*, *12*(4), 611–625.

Hindell, M. A. (1988). The diet of rockhopper penguins *Eudyptes chrysocome* at Macquarie Island. *Emu* 88:227-33.

Hobson, K. A., Piatt, J. F., & Pitocchelli, J. (1994). Using stable isotopes to determine seabird trophic relationships. *Journal of Animal Ecology*, *63*(4), 786–798.

Horne, R. S. C. (1985). Diet of royal and rockhopper penguins at Macquarie Islands. *Emu* 85:150- 56.

Hull, C. L., Hindell, M. A., & Michael, K. (1997). Foraging zones of royal penguins during the breeding season, and their association with oceanographic features. *Marine Ecology Progress Series*, *153*, 217-228.

Hull, C. L. (1999). Comparison of the diets of breeding royal (Eudyptes schlegeli) and rockhopper (Eudyptes chrysocome) penguins on Macquarie Island over three years. *Journal of Zoology*, *247*(4), 507–529.

Inger, R., & Bearhop, S. (2008). Applications of stable isotope analyses to avian ecology. *Ibis*, *150*(3), 447–461.

Jaeger, A., Jaquemet, S., Phillips, R. A., Wanless, R. M., Richard, P., & Cherel, Y. (2013). Stable isotopes document inter- and intra-specific variation in feeding ecology of nine large southern procellariiformes. *Marine Ecology Progress Series*, *490*.

Jouventin, P., Cuthbert, R. J., & Ottvall, R. (2006). Genetic isolation and divergence in sexual traits: Evidence for the northern rockhopper penguin *Eudyptes moseleyi* being a sibling species. *Molecular Ecology*, *15*(11), 3413–3423.

Karnovsky, N. J., Hobson, K. A., & Iverson, S. J. (2012). From lavage to lipids: estimating diets of seabirds. *Marine Ecology Progress Series*, *451*, 263-284.

Klages, N. T. W., Gales, R. P., & Pemberton, D. (1989). Dietary segregation of macaroni and rockhopper penguins at Heard Island. *Australian Wildlife Research* 16:599-604.

Kriesell, H. J., Aubin, T., Planas-Bielsa, V., Benoiste, M., Bonadonna, F., Gachot-Neveu, H., … Le Bohec, C. (2018). Sex identification in King Penguins Aptenodytes patagonicus through morphological and acoustic cues. *Ibis*, *160*(4), 755–768.

LaRue, M. A., Ainley, D. G., Swanson, M., Dugger, K. M., Phil, O., Lyver, B., Barton, K. and Ballard, G. (2013). Climate change winners: receding ice fields facilitate colony expansion and altered dynamics in an Adélie penguin metapopulation. *PLoS One*, *8*(4), e60568.

Layman, C. A., Araujo, M. S., Boucek, R., Hammerschlag-Peyer, C. M., Harrison, E., Jud, Z. R., Matich, P., Rosenblatt, A. E., Vaudo, J. J., Yeager, L. A. and Post, D. M. (2012). Applying stable isotopes to examine food-web structure: an overview of analytical tools, *87*, 545– 562.

Leibold, M. A. (1995). The niche concept revisited: mechanistic models and community context. *Ecology*, *76*(5), 1371-1382.

Lorrain, A., Graham, B., Ménard, F., Popp, B., Bouillon, S., Van Breugel, P., & Cherel, Y. (2009). Nitrogen and carbon isotope values of individual amino acids: a tool to study foraging ecology of penguins in the Southern Ocean. *Marine Ecology Progress Series*, *391*, 293-306.

Ludynia, K., Dehnhard, N., Poisbleau, M., Demongin, L., Masello, J. F., Voigt, C. C., & Quillfeldt, P. (2013). Sexual segregation in rockhopper penguins during incubation. *Animal behaviour*, *85*(1), 255-267.

Lynch, H. J., Fagan, W. F., Naveen, R., Trivelpiece, S. G., & Trivelpiece, W. Z. (2012). Differential advancement of breeding phenology in response to climate may alter staggered breeding among sympatric pygoscelid penguins. *Marine Ecology Progress Series*, *454*, 135-145.

Marchant, S., & Higgins, P. J. (1990). *Vol1A. Handbook of Australian, New Zealand and*

Antarctic Birds. Melbourne: Oxford University Press.

Mattern, T. (2007). Marine ecology of offshore and inshore foraging penguins: The Snares penguin Eudyptes robustus and yellow-eyed penguin Megadyptes antipodes. PhD diss., University of Otago, Dunedin, New Zealand.

Mattern, T., & Ellenberg, U. (2015). The Tawaki Project: Field Report 2015 - year 2, 16 September-02 March 2015, (September 2015)

Mattern, T,. & Ellenberg, U. (2018). The Tawaki Project: Field Report 2017 - year 4, 16 September-02 March 2015, (September 2017), 17.

Mattern, T., & Wilson, K.J. (2018). New Zealand penguins - current knowledge and research priorities. West Coast Penguin Trust. *Birds New Zealand*, (July).

Mattern, T., Houston, D. M., Lalas, C., Setiawan, A. N., & Davis, L. S. (2009). Diet composition, continuity in prey availability and marine habitat - Keystones to population stability in the Snares Penguin (*Eudyptes robustus*). *Emu*, *109*(3), 204–213.

Mattern, T., Ludynia, K., Houston, D. M., Garthe, S., & Davis, L. S. (2005). How to get the most (or anything) out of GPS loggers: a case study with Snares penguins. *2nd International Bio-Logging Science Symposium*, (November), 1–27.

Mattern, T., McPherson, M. D., Ellenberg, U., Van Heezik, Y., & Seddon, P. J. (2018a). High definition video loggers provide new insights into behaviour, physiology, and the oceanic habitat of a marine predator, the yellow-eyed penguin. *PeerJ*, *2018*(9).

Mattern, T., Pütz, K., Garcia-Borboroglu, P., Ellenberg, U., Houston, D.M., Long, R., Lüthi, B. and Seddon, P.J. (2018b). Marathon penguins–Reasons and consequences of long-range dispersal in Fiordland penguins/Tawaki during the pre-moult period. *PLoS ONE*, *13*(8), 1.

McLeod, R. J., Wing, S. R., & Skilton, J. E. (2010). High incidence of invertebrate-chemoautotroph symbioses in benthic communities of the New Zealand fjords. *Limnology*

and Oceanography, *55*(5), 2097-2106.

McMahon, K. W., Michelson, C. I., Hart, T., McCarthy, M. D., Patterson, W. P., & Polito, M. J. (2019). Divergent trophic responses of sympatric penguin species to historic anthropogenic exploitation and recent climate change. *Proceedings of the National Academy of Sciences*.

Minagawa, M., & Wada, E. (1984). Stepwise enrichment of 15N along food chains: further evidence and the relation between δ15N and animal age. *Geochimica et cosmochimica acta*, *48*(5), 1135-1140.

Miyaki, C. Y., Griffiths, R., Orr, K., Nahum, L. A., Pereira, S. L., & Wajntal, A. (1998). Sex identification of parrots, toucans, and curassows by PCR: perspective for wild and captive population studies. *Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association, 17*(5), 415-423.

Morrison, K. W., Armstrong, D. P., Battley, P. F., Jamieson, S. E., & Thompson, D. R. (2017). Predation by New Zealand sea lions and Brown Skuas is causing the continued decline of an Eastern Rockhopper Penguin colony on Campbell Island. *Polar Biology*, *40*(4), 735–751.

Morrison, K. W., Bury, S. J., & Thompson, D. R. (2014). Higher trophic level prey does not represent a higher quality diet in a threatened seabird: implications for relating population dynamics to diet shifts inferred from stable isotopes. *Marine Biology*, (October), 2243–2255.

Murie, J. O., Davis, L. S., & McLean, I. G. (1991). Identifying the Sex of Fiordland crested penguins by using morphometric characters. *Notornis*, *38*(July), 233–238.

Murphy, E. J., Trathan, P. N., Watkins, J. L., Reid, K., Meredith, M. P., Forcada, J., Thorpe, S. E., Johnston, N. M. and Rothery, P. (2007). Climatically driven fluctuations in Southern Ocean ecosystems. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1629), 3057- 3067.

Newsome, S. D., Rio, Martinez del, C., Bearhop, S., & Phillips, D. L. (2007). A Niche for Isotope Ecology. *Frontiers in Ecology and the Environment*, *5*(8), 429–436.

Nilsen, M., Pedersen, T., Nilssen, E. M., & Fredriksen, S. (2008). Trophic studies in a high-latitude fjord ecosystem—a comparison of stable isotope analyses (δ13C and δ15N) and trophic-level estimates from a mass-balance model. *Canadian Journal of Fisheries and Aquatic Sciences*, *65*(12), 2791-2806.

Oehler, D. A., Fry, W. R., Weakley, L. A., & Marin, M. (2007). Rockhopper and Macaroni Penguin Colonies Absent from Isla Recalada, Chile. *The Wilson Journal of Ornithology*, *119*(3), 502–506.

Oehler, D. A., Pelikan, S., Fry, W. R., Weakley, L., Kusch, A., & Marin, M. (2008). Status of Crested Penguin (*Eudyptes* spp.) populations on three islands in Southern Chile. *The Wilson Journal of Ornithology*, *120*(3), 575–581.

Oehler, D. A., Marin, M., Kusch, A., Danielle, L., Weakley, L. A., & Fry, W. R. (2018). Foraging ranges in southern rockhopper penguins (Eudyptes chrysocome chyrsocome) on Isla Noir, Chile. *Int J Avian & Wildlife Biol*, *3*(4), 320-325.

Oehm, J., Juen, A., Nagiller, K., Neuhauser, S., & Traugott, M. (2011). Molecular scatology: How to improve prey DNA detection success in avian faeces? *Molecular Ecology Resources*, *11*(4), 620–628.

Overeem, R., Wallis, R., & Salzman, S. (2006). Sexing Little Penguins (Eudyptula minor) Using Bill Measurements, 1–14.

Pan, H., Cole, T. L., Bi, X., Fang, M., Zhou, C., Yang, Z., … Zhang, G. (2019). High-coverage genomes to elucidate the evolution of penguins. *GigaScience*, *8*(9), 1–17.

Paritte, J. M., & Kelly, J. F. (2009). Effect of cleaning regime on stable-isotope ratios of feathers in Japanese Quail (Coturnix japonica). *The Auk*, *126*(1), 165-174.

Pierce, K. E., Harris, R. J., Larned, L. S., & Pokras, M. A. (2004). Obstruction and starvation associated with plastic ingestion in a Northern Gannet Morus bassanus and a Greater Shearwater Puffinus gravis. *Marine Ornithology*, *32*(2), 187–189.

Poisbleau, M., Demongin, L., Noordwijk, H. J. van, Strange, I. J., & Quillfeldt, P. (2011). Sexual Dimorphism and use of Morphological Measurements to Sex Adults, Immatures and Chicks of Rockhopper Penguins. *Ardea*, *98*(2), 217–224.

Polito, M. J., Trivelpiece, W. Z., Karnovsky, N. J., Ng, E., Patterson, W. P., & Emslie, S. D. (2011). Integrating stomach content and stable isotope analyses to quantify the diets of pygoscelid penguins. *PloS one*, *6*(10), e26642.

Post, D. A. (2002). Using stable isotopes to estimate trophic position: models, methods, and assumptions. *83*(3), 703–718.

Poupart, T., Waugh, S., Bost, C., Kato, A., Miskelly, C., Rogers, K., & Arnould, J. (2019). Foraging ecology of a winter breeder, the Fiordland penguin. *Marine Ecology Progress Series*, *614*, 183–197.

Prebble, J. G., Hinojosa, J. L., & Moy, C. M. (2018). Palynofacies assemblages reflect sources of organic matter in New Zealand fjords. *Continental Shelf Research*, *154*, 19-25.

Pütz, K., Smith, J. G., Ingham, R. J., & Lüthi, B. H. (2003). Satellite tracking of male rockhopper penguins Eudyptes chrysocome during the incubation period at the Falkland Islands. *Journal of Avian Biology*, *34*(2), 139–144.

Quillfeldt, P., McGill, R. A., & Furness, R. W. (2005). Diet and foraging areas of Southern Ocean seabirds and their prey inferred from stable isotopes: review and case study of Wilson's storm-petrel. *Marine Ecology Progress Series*, *295*, 295-304.

Ratcliffe, N., Adlard, S., Stowasser, G., & Mcgill, R. (2018). Dietary divergence is associated with increased intra-specific competition in a marine predator. *Scientific Reports*, (April),

1–10.

Ratcliffe, N., Takahashi, A., Oulton, C., Fukuda, M., Fry, B., Crofts, S., Brown, R., Adlard, S., Dunn, M.J. and Trathan, P.N. (2014). A leg-band for mounting geolocator tags on penguins. *Marine Ornithology*, *42*(1), 23–26.

Raya Rey, A., & Schiavini, A. (2005). Inter-annual variation in the diet of female southern rockhopper penguin (Eudyptes chrysocome chrysocome) at Tierra del Fuego. *Polar Biology*, *28*(2), 132-141.

Raya Rey, A., Trathan, P., & Schiavini, A. (2007). Inter-annual variation in provisioning behaviour of Southern Rockhopper Penguins Eudyptes chrysocome chrysocome at Staten Island, Argentina. *Ibis*, *149*(4), 826-835.

Renaud, P. E., Tessmann, M., Evenset, A., & Christensen, G. N. (2011). Benthic food-web structure of an Arctic fjord (Kongsfjorden, Svalbard). *Marine Biology Research*, *7*(1), 13-26.

Rosciano, N. G., Polito, M. J., & Raya Rey, A. (2016). Do penguins share? Evidence of foraging niche segregation between but not within two sympatric, central-place foragers. *Marine Ecology Progress Series*, *548*(April), 249–262.

Rutger, S. M., & Wing, S. R. (2006). Effects of freshwater input on shallow-water infaunal communities in Doubtful Sound, New Zealand. *Marine Ecology Progress Series*, *314*, 35-47.

Savoca, M. S., Wohlfeil, M. E., Ebeler, S. E., & Nevitt, G. A. (2016). Marine plastic debris emits a keystone infochemical for olfactory foraging seabirds. *Science*, 1–9.

Setiawan, A. N., Darby, J. T., Lambert, D. M. (2004). The Use of Morphometric Measurements to Sex Yellow-eyed Penguins. *Waterbirds*, *27*(4), 411–423.

Steinfurth, A., Booth, J., White, J., Bond, A., & McQuaid, C. (2019). Sexual and geographic dimorphism in northern rockhopper penguins breeding in the South Atlantic Ocean.

Endangered Species Research, *39*, 293–302.

Tershy, B. R., Breese, D., & Alvarez-Borrego, S. (1991). Increase in cetacean and seabird numbers in the Canal de Ballenas during an El Niño-Southern Oscillation event. *Marine Ecology Progress Series*, 299-302.

Thiebot, J. B., Cherel, Y., Acqueberge, M., Prudor, A., Trathan, P. N., & Bost, C. A. (2014). Adjustment of pre-moult foraging strategies in Macaroni Penguins *Eudyptes chrysolophus* according to locality, sex and breeding status. *Ibis*, *156*(3), 511-522.

Thiebot, J. B., Arnould, J. P., Gómez-Laich, A., Ito, K., Kato, A., Mattern, T., Mitamura, H., Noda, T., Poupart, T., Quintana, F. and Raclot, T. (2017). Jellyfish and other gelata as food for four penguin species – insights from predator-borne videos. *Frontiers in Ecology and the Environment*, *15*(8), 437–441.

Thompson, K. R. (1989). An assessment of the potential for competition between seabirds and fisheries in the Falkland Islands. Falklands Islands Foundation Project Report. Stanley, Falkland Islands. 94 pp.

Trathan, P. N., García-Borboroglu, P., Boersma, D., Bost, C. A., Crawford, R. J. M., Crossin, G. T., Cuthbert, R. J., Dann, P., Davis, L. S., De La Puente, S., Wienecke, B. (2015). Pollution, habitat loss, fishing, and climate change as critical threats to penguins. *Conservation Biology*, *29*(1), 31–41.

Trathan, P. N., Fretwell, P. T., & Stonehouse, B. (2011). First recorded loss of an emperor penguin colony in the recent period of Antarctic regional warming: Implications for other colonies. *PLoS ONE*, *6*(2).

Tremblay, Y., & Cherel, Y. (2003). Geographic variation in the foraging behaviour, diet and chick growth of rockhopper penguins. *Marine ecology progress series*, *251*, 279-297.

Tremblay, Y., Guinard, E., & Cherel, Y. (1997). Maximum diving depths of northern rockhopper

penguins (*Eudyptes chrysocome moseleyi)* at Amsterdam Island. *Polar Biology.* 17:199-22.

Trull, T. W., & Armand, L. (2001). Insights into Southern Ocean carbon export from the δ13C of particles and dissolved inorganic carbon during the SOIREE iron release experiment. *Deep Sea Research Part II: Topical Studies in Oceanography*, *48*(11-12), 2655-2680.

Van Heezik, Y. (1989). Diet of Fiordland crested penguins during the post guard phase of chick growth. *Notornis* 36:151-56.

Van Heezik, Y. (1990). Diets of yellow-eyed, Fiordland crested, and little blue penguins breeding sympatrically on Codfish Island, New Zealand. *New Zealand journal of zoology*, *17*(4), 543-548.

Wakefield, E. D., Phillips, R. A., & Matthiopoulos, J. (2009). Quantifying habitat use and preferences of pelagic seabirds using individual movement data: A review. *Marine Ecology Progress Series*, *391*, 165–182.

Wang, N., & Zhang, Z. W. (2009). The novel primers for sex identification in the brown eared-pheasant and their application to other species. *Molecular ecology resources*, *9*(1), 186-188.

Warham, J. (1972). Breeding seasons and sexual dimorphism in Rockhopper Penguins. *The Auk*, *89*(1), 86-105.

Warham, J. (1974). The Fiordland Crested Penguin. *Ibis*, *116*(1), 1–27.

Whitehead, T. O., Connan, M., Ropert-Coudert, Y., & Ryan, P. G. (2017). Subtle but significant segregation in the feeding ecology of sympatric penguins during the critical pre-moult period, *Marine Ecology Progress Series, 565*(1), 227–236.

Williams, T. D., & Croxall, J. P. (1991). Annual variation in breeding biology of macaroni penguins, Eudyptes chrysolophus, at Bird Island, South Georgia. *Journal of Zoology*, *223*(2), 189–202.

Wilson, K., & Long, R. (2016). Tawaki / Fiordland Crested Penguin Conservation Management; Predator control – three year report, 2014-2016.

Xavier, J. C., Cherel, Y., Ceia, F. R., Queirós, J. P., Guimarães, B., Rosa, R., Cunningham, D. M., Moors, P. J. and Thompson, D. R., (2018). Eastern rockhopper penguins Eudyptes filholi as biological samplers of juvenile and sub-adult cephalopods around Campbell Island, New Zealand. *Polar Biology*, *41*(10), 1937-1949.

APPENDIX A

APPROVAL LETTER FROM THE OFFICE OF RESEARCH INTEGRITY

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

June 10, 2019

Jeff White
1007 Oakmont Road
Charleston, WV 25314

Dear Mr. White:

This letter is in response to the submitted thesis abstract entitled *"Foraging strategy plasticity in Fiordland Crested Penguins (Eudyptes pachyrhynchus): A stable isotopic approach."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the study under protocol #686. The applicable human and animal federal regulations have set forth the criteria utilized in making this determination. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B
REVIEW OF STOMACH CONTENT ANALYSIS RESEARCH

Taxon	Stomach Content Analysis	
	Locations Studied	**Publications**
Southern Rockhopper	Isla de los Estados, Falklands/Malvinas, Steeple Jason	(Raya Rey & Schiavini, 2005; Raya Rey et al., 2007; Thompson, 1989; Croxall et al., 1985; Pütz et al., 2003)
Eastern Rockhopper	Marion, Crozet, Kerguelen, Heard, Macquarie, Campbell	(Brown & Klages, 1987; Tremblay & Cherel, 2003; Klages et al., 1989; Horne, 1985; Hindell, 1988; Hull, 1999; Marchant & Higgins, 1990)
Northern Rockhopper	Gough, Amsterdam, St. Paul	(Klages et al., 1989; Cooper et al., 1990; Tremblay et al., 1997)
Macaroni	South Georgia, SW Atlantic islands, Marion, Heard	(Crawford et al., 2003; Ratcliffe et al., 2014; Brown & Klages, 1987; Deagle et al., 2007)
Royal	Macquarie	(Hindell, 1988; Hull et al., 1997; Goldsworthy et al., 2001)
Fiordland	Codfish, Martin's Bay	(Van Heezik, 1989, 1990)
Snares	Snares	(Cooper et al., 1990; Marchant & Higgins, 1990; Mattern et al., 2009)
Erect-crested	*none*	*none*

Appendix B. Review of stomach content analyses in *Eudyptes* penguins.

APPENDIX C
REVIEW OF FECAL DNA RESEARCH

Taxon	Fecal DNA	
	Locations Studied	**Publications**
Southern Rockhopper	*none*	*none*
Eastern Rockhopper	*none*	*none*
Northern Rockhopper	*none*	*none*
Macaroni	Heard	(Deagle et al., 2007)
Royal	*none*	*none*
Fiordland	Jackson Head, Milford Sound	(McInnes et al., unpublished data)
Snares	none	*none*
Erect-crested	none	*none*

Appendix C. Review of fecal DNA analysis studies in *Eudyptes* penguins.

APPENDIX D
REVIEW OF STABLE ISOTOPE RESEARCH

Taxon	Stable Isotope Analysis	
	Locations Studied	**Publications**
Southern Rockhopper	Isla de los Estados, Falklands/Malvinas	(Hilton et al., 2006; Dehnhard et al., 2011; Rosciano et al., 2016)
Eastern Rockhopper	Crozet, Prince Edward, Campbell	(Whitehead et al., 2017; Xavier et al., 2018; Morrison et al., 2014)
Northern Rockhopper	Amsterdam, Gough	(Lorrain et al., 2009; Jaeger et al., 2013; Booth & McQuaid, 2013)
Macaroni	Marion, Heard, South Georgia	(Bearhop et al, 2006; Cherel et al., 2007; Cherel & Hobson, 2007; Thiebot et al., 2014)
Royal	*none*	*none*
Fiordland	Codfish, Milford Sound, Jackson Head, Open Bay	(Poupart et al., 2019)
Snares	Snares	(Mattern et al., 2009)
Erect-crested	*none*	*none*

Appendix D. Review of stable isotope analysis studies in *Eudyptes* penguins.

APPENDIX E
REVIEW OF TRACKING RESEARCH

Taxon	Tracking (GPS, GLS, Satellite)	
	Locations Studied	**Publications**
Southern Rockhopper	Isla de los Estados, Falklands/Malvinas, Isla Pingüno, Isla Noir	(Pütz et al, 2003; Raya Rey et al., 2007; Rosciano et al., 2016)
Eastern Rockhopper	Crozet, Kerguelen	(Ratcliffe et al., 2014; Whitehead et al., 2017)
Northern Rockhopper	Amtsterdam, St. Paul, Gough, Tristan da Cunha, Nightingale	(Tremblay et al, 1997)
Macaroni	Macquarie	(Hull et al., 1997)
Royal	*none*	*none*
Fiordland	Codfish, Milford Sound, Jackson Head, Open Bay	(Mattern et al., 2018b; Poupart et al., 2019)
Snares	Snares	(Mattern, 2007)
Erect-crested	*none*	*none*

Appendix E. Review of tracking (GLS, GPS, and satellite tag) studies in *Eudyptes* penguins.

APPENDIX F
FIORDLAND PENGUIN
BLOOD ISOTOPE VALUES

Tawaki Blood δ^{13}C and δ^{15}N Values Across Sites					
Colony	**δ^{13}C (‰)**		**δ^{15}N (‰)**		**Mean C:N Ratio**
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	
Whenua Hou	-19.1 ± 0.4	-19.7 to -18.5	12.4 ± 0.8	11.1 to 13.5	3.3
Jackson Head	-19.5 ± 0.6	-21.8 to -18.7	12.4 ± 0.6	11.7 to 13.8	3.4
Harrison Cove	-19.5 ± 1.0	-21.8 to -18.2	13.0 ± 0.5	11.6 to 14.1	3.3
All Tawaki	**-19.5 ± 0.7**	**-21.8 to -18.2**	**12.6 ± 0.6**	**11.1 to 14.1**	**3.3**

Appendix F. Tawaki blood δ^{13}C and δ^{15}N mean values and ranges from all sites studied.

APPENDIX G

FIORDLAND PENGUIN
FEATHER ISOTOPE VALUES

Tawaki Feather δ^{13}C and δ^{15}N Values Among Sites					
Colony	**δ^{13}C (‰)**		**δ^{15}N (‰)**		**Mean C:N Ratio**
	Mean ± SD	**Range**	**Mean ± SD**	**Range**	
Harrison Cove	-18.7 ± 0.5	-20.5 to -16.7	12.7 ± 0.5	11.6 to 14.8	3.5
Gorge River	-18.8 ± 0.5	-19.4 to -18.0	12.7 ± 0.5	11.9 to 13.4	3.8
Jackson Head	-19.0 ± 0.7	-20.6 to -17.5	12.8 ± 0.5	11.7 to 14.4	3.5
Whenua Hou	-19.2 ± 0.6	-20.43 to -18.1	12.7 ± 0.6	11.1 to 14.0	3.7
All Tawaki	**-19.0 ± 0.7**	**-20.6 to -16.7**	**12.8 ± 0.6**	**11.1 to 14.8**	**3.5**

Appendix G. Tawaki feather δ^{13}C and δ^{15}N mean values and ranges from all sites studied.

APPENDIX H
CONNECTING LETTER REPORT FOR FEATHER ISOTOPES BY YEAR

δ^{13}C	
2010	AB
2015	A
2016	B
2017	AB
2018	B
δ^{15}N	
2010	AB
2015	C
2016	BC
2017	A
2018	A

Appendix H. Connecting letter report of pair-wise combinations of δ^{13}C and δ^{15}N values from feathers among years sampled.