SAPIENZA
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ISOTOPIC DETERMINATION OF THE DIET OF ADÉLIE AND EMPEROR PENGUINS IN ANTARCTICA THROUGH ANALYSIS OF EGG TISSUES, PLUMAGE, AND FAECES

By

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To the Department of Environmental Biology:

The members of the Committee appointed to examine the dissertation of VAHIDEH JAFARI find it satisfactory and recommend that it be accepted.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 2

CHAPTER 3

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

ISOTOPIC DETERMINATION OF THE DIET OF ADÉLIE AND EMPEROR PENGUINS IN ANTARCTICA THROUGH ANALYSIS OF EGG TISSUES, PLUMAGE, AND FAECES

Abstract

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The Antarctic Sea is the largest protected biodiversity hotspot area in the world and plays an important role in the Southern Ocean ecosystem. The structure and dynamics of the Ross Sea ice coverage have a fundamental role in production processes. During the Antarctic Winter, temperature decreases, and the sea ice coverage increases. The later phenomenon limits the availability of food resources for the entire community. With the onset of the Antarctic Summer, the sea ice extension and thickness decrease, resulting in an increase of trophic resources availability (phytoplankton, diatoms, cryptophytes, and krill), supporting large populations of upper-trophic levels. The Emperor (*Aptenodytes forsteri*) and Adélie (*Pygoscelis adeliae*) Penguins are two meso-predators, which represent key species in the Antarctic food web. The objectives of this thesis are to determine how the spatial and temporal changes of the sea ice coverage in Ross Sea coastline affect the trophic ecology of penguin species during a year and to compare these changes over two consecutive breeding and moulting periods. The results of the Carbon and Nitrogen stable isotopes analysis show that fish are the most important component of the Emperor Penguin both in Winter and Summer diets. Krill, instead, represent the dominant food resource for the Adélie Penguin in Summer. In Summer 2016-17 I found that Adèlie colonies at highest latitude, foraged more on krill than on fish. Emperor penguins fish consumption in the pre-moulting period decrease significantly from 2016-17 to the following year. The 2016 year, with the lowest atmospheric pressure, highest wind speed, and temperature over the past thirty years had the lowest ice expansion. Based on these conditions, adult krill abundance, which had dependence life cycle on the ice expansion, decreased in 2016 Summer. This decrease in krill availability determined a shift from krill-based diet towards fish-based diet during the Adèlie pre-breeding (Summer), and pre-moulting (Winter) seasons.

Keywords: Antarctica, Adèlie and Emperor penguins, Food webs, Stable Isotopes Analysis

CHAPTER 1

INTRODUCTION

1.1. Introduction

The Ross Sea (Antarctica, Figure 1-1), the largest marine protected area in the world, is considered a pristine ecosystem and an important biodiversity hotspot. It is also a critical climate-change reference area, and a climate refugium for ice-dependent species (Ballard et al., 2012; Rossi et al., 2019). The structure and dynamics of the sea ice have a fundamental role in primary production processes. The ice restrains organisms, such as algae, diatoms and bacteria, and its melting for a short period of the year determines the period which a greater abundance of resources temporarily becomes available for the planktonic and benthic compartment (Cattaneo-Vietti et al., 1999; Sedwick and DiTullio, 1997).

Figure 1-1: The Antarctic's map on the earth

The Ross Sea ice increase for hundreds of miles from the shoreline during the Antarctic Winter (Figure 1-2) supporting large populations of upper-trophic levels by its high productivity (Arrigo et al., 2002), such as two meso predators which represent key species in the Antarctic food web: The Emperor (*Aptenodytes forsteri*) and the Adélie penguin (*Pygoscelis adeliae*). The availability of their trophic resources ranging from small krill to fish may be influenced by the cover breaking of the sea ice. It is complicated to study all aspects of krill and fish in an integrated temporal and spatial manner (Huang et al., 2013; Polito et al., 2011a), so I study the diet of these penguins to reflect somehow what happens to their trophic networks.

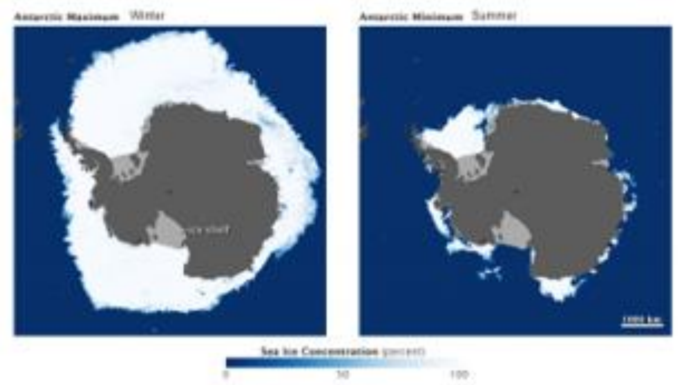


Figure 1-2: The differences of Sea Ice concentration in Antarctic Winter and Summer ("World of Change," 2009)

The hypothesis of this thesis is that the diet of penguins is related to sea ice dynamics. According to our hypothesis, the objectives of this thesis are: determine the effect of temporal and spatial changes in sea ice coverage on Penguin trophic ecology and compare the diet of Adèlie and Emperor penguins with different reproductive and feeding behavior

I used Stable Isotope Analysis (S.I.A.) to investigate intra- and inter-colony seasonal variation in the foraging ecology of Adélie and Emperor penguins. The S.I.A. has become increasingly established as one of the main techniques for analysing the structure of trophic networks, trophic level, and provides a complementary method for determining the trophic position (Jafari et al., 2018). The isotopic analysis provides clear ideas integrated into space and time, on the trophic relationships that are established between the organisms and therefore can easily develop models of trophic structures (Jafari et al., 2018). The isotopic composition of an animal's tissue reflects that of its prey (Bodin et al., 2007). The δ^{13}C values of penguin show the reflection of their foraging area (Cherel, 2008), and can evaluate source primary production in the marine system (Bodin et al., 2007), while their δ^{15}N values increase with trophic level and serve as indicators of a predator trophic position (Cherel, 2008). By sampling tissues produced at different locations or times of the year, it is possible to examine variations in an animal's diet over both time and space. The isotopic ratio of tissue reflects the time period which it was synthesized (Bearhop et al., 2004). By analysing the stable isotope of the egg and the feather of penguins, it can be estimated that what have they eaten before laying and moulting(Polito et al., 2011a, 2009a). Also, by faeces isotope analysing, it is possible to assess what did they eat over the past 24 hours. The variation of the isotopic values is originated from the fractionation processes. The trophic fractionation between resource and consumers is created in the metabolic reactions of assimilation, respiration, and excretion of nutrients within organisms. Consequently, fractionation depends on the elemental composition of the diet, type of tissue analysed (particular tissue versus whole organisms), and the analysis method (acidification versus non-acidification; lipid extraction versus non-extraction) of the samples (McCutchan et al., 2003). The CO_2 produced by cellular respiration processes is depleted compared to the carbon introduced by diet, therefore the animals are enriched about 1‰ in $\delta^{13}C$ compared to their resources (Deniro and Epstein, 1981). Similarly, the excreted nitrogen is typically depleted in $\delta^{15}N$, therefore the animals are enriched about 2.5-5‰ respect to their resources (Deniro and Epstein, 1981).

The poorly known Winter foraging ecology of penguins was investigated by egg samples analysing. The egg-laying time in the emperor's penguin is mid-May (Antarctic Winter) and their moulting time is January (Antarctic Summer). Further, laying and moulting time in Adélie penguin is November (Antarctic Summer) and March (Antarctic Winter), respectively. By adding faeces data can be estimated the food pattern of the year.

The second aim of this thesis is to compare the diet composition of Emperor and Adélie penguins to evaluate annual variations in the foraging ecology of adults over two consecutive breeding and moulting seasons (2016-17 to 2017-18). The knowledge of the Victoria land and the Ross Sea is limited and thus incomplete, so this research could be fundamental to the understanding of the biogeography of this region, and will provide valuable information on the foraging ecology, also, could be some attempts to understand the impacts of climatic change on the Antarctic. Annual variations could also complicate the comprehension of diet changing pattern (Peterson and Howard-Wiliams, 2000). To achieve this aim, I used stable isotope analysis (S.I.A.) again, which is the main technique for analysing the diet composition by researchers. Because animal biomass stable isotope signatures are determined mainly by the animal's food isotopic abundances (Jafari et al., 2018). $\delta^{15}N$ and $\delta^{13}C$ provide an average estimate of an organism's preferred diet. The animal carbon and nitrogen isotopes change with altering animal feeding on prey with different isotopic composition versus the previous intake (Bodin et al., 2007). The feather and egg tissue samples used to provide information on adult diets undergo moult, and on female diets during a brief period before breeding, respectively. This study research provides important information for its conservation management. It is a way to understand how that ecosystem reacts to the variation in the sea ice cover, and it gives the importance and its value.

CHAPTER 2

MATERIALS AND METHODS

2.1. Studied species

The Emperor penguin (*Aptenodytes forsteri*) is the largest of all penguins with 110-130 cm height and 22-45 kg weight which vary by season and sex (Jadwiszczak, 2001; Kooyman et al., 2004). Their dorsal side of the body is black, while the front is white with yellow breast and ear patches. Emperor chicks have a silver-grey body with a black head and a white mask (Figure 2-1). They are one of the two species of penguin that solely residents the Antarctic continent, and their colonies mostly are located on the frozen sea-ice (Colin et al., 1987). Their wings are stiffened and flattened into flipper to be able to dive, swim, and feed in the marine habitat (Ponganis et al., 2001). They merge 150-500 meter depth and their unusually structured hemoglobin which function with low oxygen, declining heart rate, solid bones that reduce barotrauma (Ponganis et al., 2015), and finally their ability to reduce its metabolism or shut down non-essential organ functions are their adaptations that facilitate to remain submerged up to 18 minutes on a single breath. Although they do not have gills, they can tolerate blood pH (Ponganis et al., 2001) (Figure 2-2).

Figure 2-1: Emperor Penguin (photo by @paulnicklen)

Figure 2-2: Emperor penguin's merging (photo by @paulnicklen)

Emperor penguins principally feed on fish, cephalopods, and crustaceans (Cherel, 2008; Kirkwood and Robertson, 1997) and are in turn important prey for larger predators such as a leopard seals and sharks (Kooyman et al., 2000) (Figure 2-3), that's why they are important members of the Antarctic ecosystem.

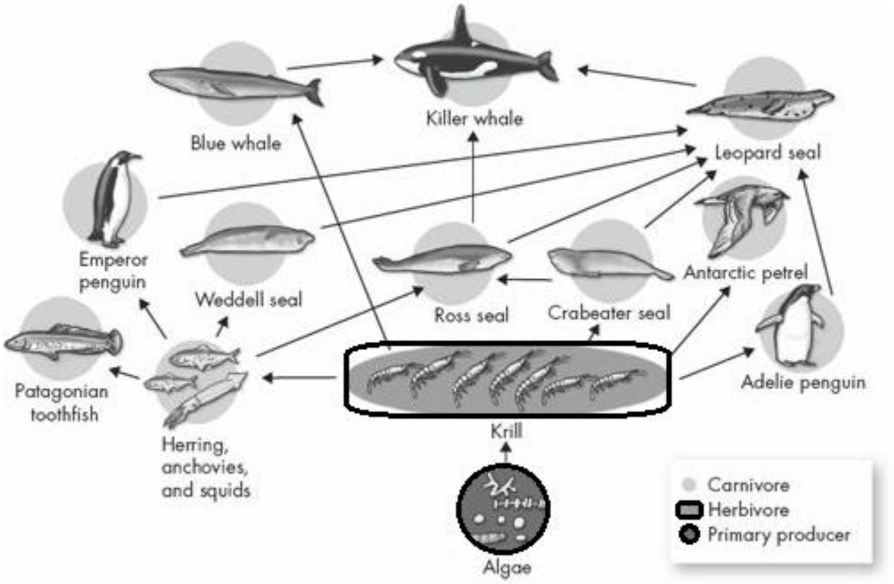


Figure 2-3: The Antarctic's trophic level

The Emperor penguin is the only Antarctic species penguin that does not require ice-free land to breed (Harris et al., 2015). They mate in April-May (Antarctic Winter) on ice (Kooyman et al., 2004) after a 100-160 kilometer march with -40 centigrade of temperature and 144 km/h wind speed, and moving away from the sea (Figure 2-4). The feathers, a layer of subdermal fat up to 3 cm thick, and a minimum of mobility, play a critical role to insulate penguins from cold. Also, muscles allow the feathers to be held direct and by trapping a layer of air, decreasing skin heat loss (Kooyman et al., 2000). They do not have a fixed nest site and use a complex set of calls for partner identification. The female penguins deposit a single egg (Figure 2-5) to the male penguin after laying and goes to the sea to feed. Males incubate egg during the months June-July for 64 days and are fast until the hatching (Kooyman et al., 2004). The male penguin feeds the chick until a female penguin returns from the sea, with its secretion from its mouth. When the mother returns, she will raise the swallowed fish and feed her chick, and this is a good opportunity for the male penguin to go to the sea and feed. This cycle is repeated six or more times, and each cycle lasts almost 20-24 days. In October-November, chicks gather together to warm up, and in December (Kooyman et al., 2004) that's the warmest month in the Antarctic with about -2 centigrade of temperature, they break into the ice and go to the sea to feed while they are five months old (Figure 2-3). They prefer to live in a colony for chicks' security and tolerance extreme cold. Mature Emperor penguins usually moult in January (Antarctic Summer) during approximately 30 days, which is faster than other species (Kooyman et al., 2000, 2004). They must find a large enough, protected, and stable location to moult, which is near and accessible abundant food resources (Kooyman et al., 2000, 2004) (Figure 2-6).

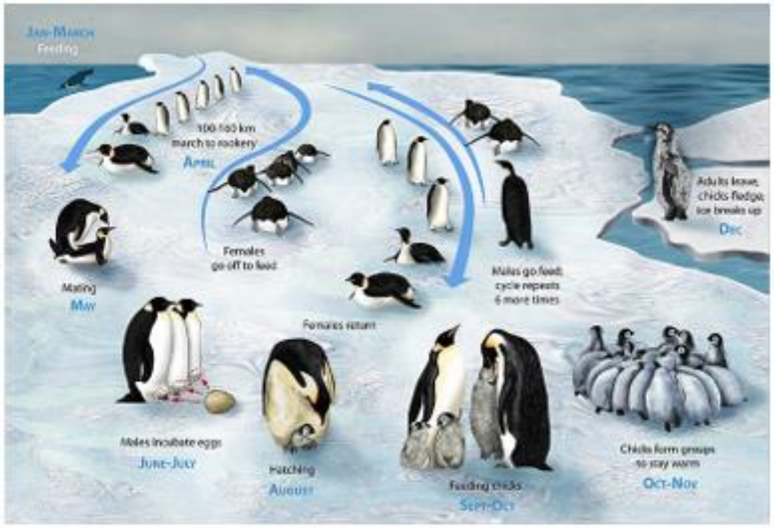


Figure 2-4: Emperor Penguin’s life cycle (Deretsky, 2006)

Figure 2-5: Emperor penguin’s egg

Figure 2-6: Emperor penguin's moulting

The Adélie penguin (*Pygoscelis adeliae*) is one of the most widespread around the coastline and the smallest penguin species that has a key component of the Antarctic ecosystem (Ainley et al., 2003; Micol and Jouventin, 2001) with 46-71 cm in height and 3.6-6 Kg in weight (Salihoglu et al., 2001). Adèlie penguin has a tail which is a little longer than other species and has a white ring surrounding the eye (McClung et al., 2004) (Figure 2-7). They build rough nests of stones on ice-free and breed from October to February (Antarctic Summer) (Figure 2-8). Two eggs are laid and incubated almost for 30 days by the parents taking turns (Tenaza, 1971) (Figure 2-9). Adult penguins feed their chicks by regurgitating the pre-digested krill by removing a portion of the saline ions like sodium and mixing with their cloacal fluids to protect chicks from heavy salt loads (Janes, 1997; Schmidt-Nielsen, 1960). Their nasal salt excretions have a significant role in reducing the load on their kidneys, also are crucial in the maintenance of the Antarctic ecosystem. These concentrations of salt wastes facilitate the material flow from the sea to the land, which makes habitats for those bacteria that live in the soil (Schmidt-Nielsen, 1960). Their juveniles go out to sea after 50-60 days to feed (Salihoglu et al., 2001). Adélie penguins consume mainly Krill (*Euphausia superba* in pelagic waters, and *Euphausia crystallorophias* in neritics waters), less fish (22.5%) (Figure 2-10), and the rest amphipods and others (Ainley et al., 2003; Clarke et al., 1998; Kirkwood and Robertson, 1997; Tierney et al., 2008; Trivelpiece et al., 2011), and are prayed on by leopard seal, skuas, and killer whales. Adélie penguin's diets vary within and between colonies in the year (M. Beaulieu et al., 2010).

Figure 2-7: Adélie Penguin

Figure 2-8: Adélie penguin's life cycle (Michaël Beaulieu et al., 2010; Kooyman et al., 2000)

Figure 2-9: Adélie penguin's egg

The Adélie penguin moult begins in March-April when temperature decline and sea-ice expand. They need a massive and tick pack-ice to moult (Kooyman et al., 2000) (Figure 2-8).

Adélie penguins have a Summer breeding, while Emperor penguins breed in the Winter. When Adélie penguins begin their breeding cycle, Emperor penguins rear their chicks (Cherel, 2008) (Figure 2-4; 2-8). The Antarctic silverfish (*Pleuragramma antarctium*) as dominant pelagic fish in Antarctic shelf areas (Granata et al., 2009), Antarctic krill (*Euphausia superba*), and Crystal krill (*Euphausia crystallorophias*) play the important role of the Antarctic marine ecosystem because of their significant role as a link in food web structure (Granata et al., 2009; Zhang et al., 2017). They have differences in nitrogen isotopic signatures because of their trophic levels, so make them distinguishable from each other (Ainley et al., 2003; Quillfeldt et al., 2005; Strickland et al., 2008) (Figure 2-10).

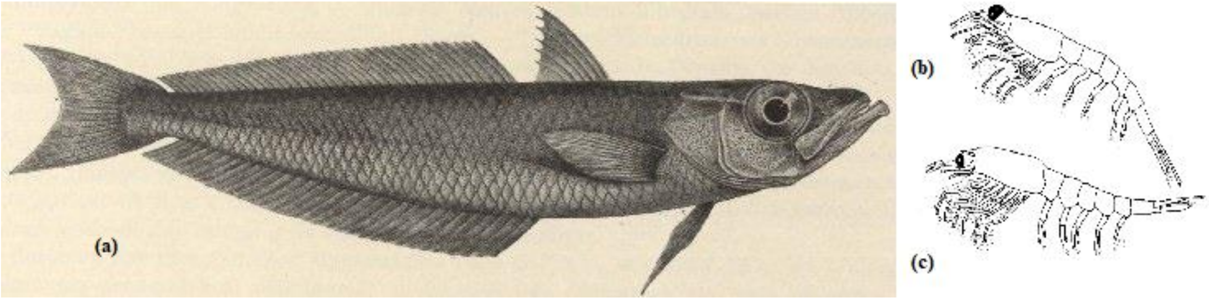


Figure 2-10: Antarctic silverfish; (a) *Pleuragramma antarctium* (Everson, 2009), and Antarctic Krill; (b) *Euphausia crystallorophias* , and (c) *Euphausia superba* (Daly and Zimmerman, 2004; Dana, 1852)

2.2. Study location

The study was performed at Inexpressible Island (163°43’02” E, 74°54’01” S), Adèlie Cove (164°00’35” E, 74°45’51” S), Edmonson Point (165°05’44” E, 74°19’32” S), and Cape Hallett (170°13’31” E, 72°19’13” S) (Harris et al., 2015) for Adèlie colonies, and Cape Washington (164°58’02” E, 74°37’07” S) for Emperor colony, in the coastal Antarctic Ross Sea (Figure 2-11).

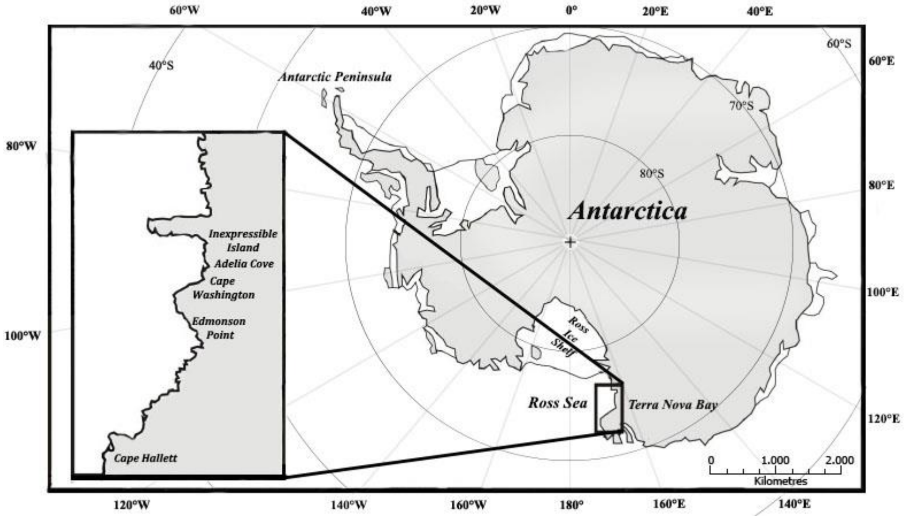


Figure 2-11: Sampling area
*latitude information was used from (Cavalieri and Parkinson, 2008; Harris et al., 2015)

Inexpressible Island (163°43’02” E, 74°54’01” S), is situated in Terra Nova Bay, Ross Sea. The concentration of seabirds, in particular, Adélie penguin colony, are presented in an ice-free ground, approximately 24450 breeding pairs(Harris et al., 2015).

Adèlie Cove (164°00’35” E, 74°45’51” S), is located on the coast of the Northern Foothills, Victoria Land. The concentration of seabirds as Adélie penguin colony has been observed in the area with an average of 11234 breeding pairs (Harris et al., 2015)

Cape Washington (164°58’02” E, 74°37’07” S), is situated on the lower slopes of Mount Melbourne, Victoria Land, in northern Terra Nova Bay, Ross Sea. The Emperor penguin colony is one of the largest populations known in this area with population range from 13000-25000 breeding pairs. The colony typically breeds on sea ice up to 0.5 km west of Cape Washington and move several km from this site throughout the breeding season (Harris et al., 2015).

Edmonson Point (165°05’44” E, 74°19’32” S), is placed in Wood Bay, at the foot of the eastern slopes of Mount Melbourne with the majority nesting close to the Adélie penguin colony (Harris et al., 2015).

Cape Hallett (170°13’31” E, 72°19’13” S), is located at the northern extremity of the Hallett Peninsula, northern Victoria land with an average of 42628 breeding pairs of Adélie penguin (Harris et al., 2015).

The figure 2-12, show the spatial Sea ice persistence. There is a gradient of ice breaking, which start to break from Inexpressible Island as Polynya (ice free) and hand by hand along red arrow ice break delay (Figure 2-12).

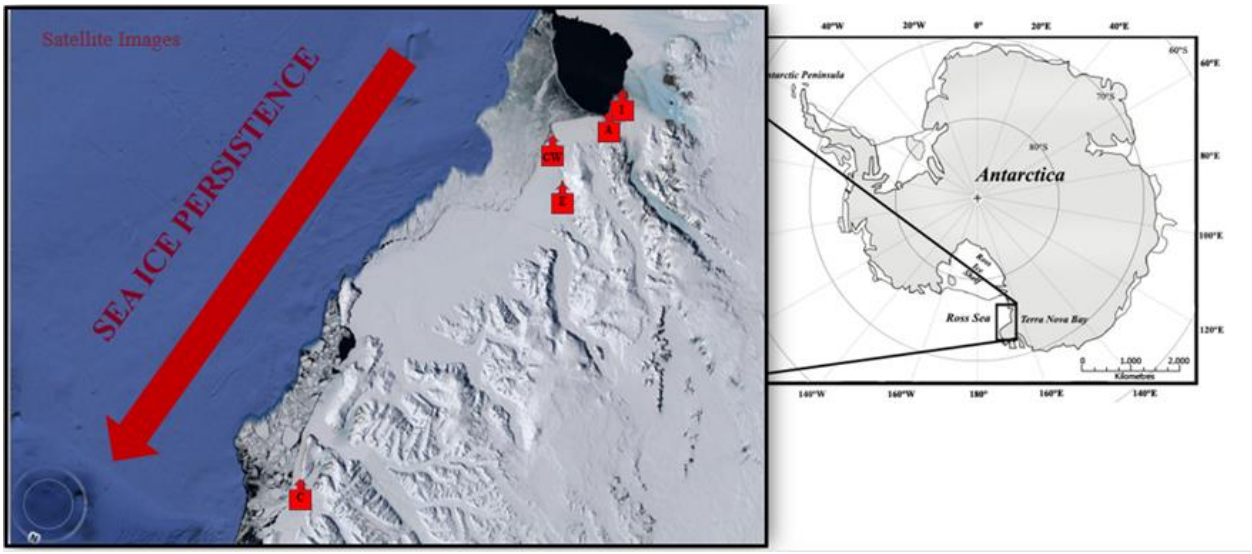


Figure 2-12: The spatial ice breaking time-delay by using satellite images

As show in Table 2-1, the ice of Adèlie Cove break 31 days later than Inexpressible Island (Polynya), and in continuous, the ice of Edmonson Point and Cape Hallett become free 82 and 91 days later than Inexpressible Island. The persistence is measured as the number of days of delay in the breaking of the ice respect to the day of ice-break observed in the area of Polynya in 2016.

Table 2-1: The ice breaking time-delay in different colonies (T0=Polynya opening in 2016)

Colonies	**2016**	**2017**
Inexpressible Island	0 (T0)	+ 49 days
Adèlie Cove	+31 days	+72 days
Edmonson Point	+82 days	+115 days
Cape Hallett	+91 days	+106 days

Comparing 2016 and 2017, the ice of Inexpressible Island of 2017, became free 49 days later than previous year (Table 2-1). After a period of increase sea ice extent since 1980, the sea ice was started to decrease from 2014, which arrive to 2016 with the minimum sea ice coverage (Figure 2-13).

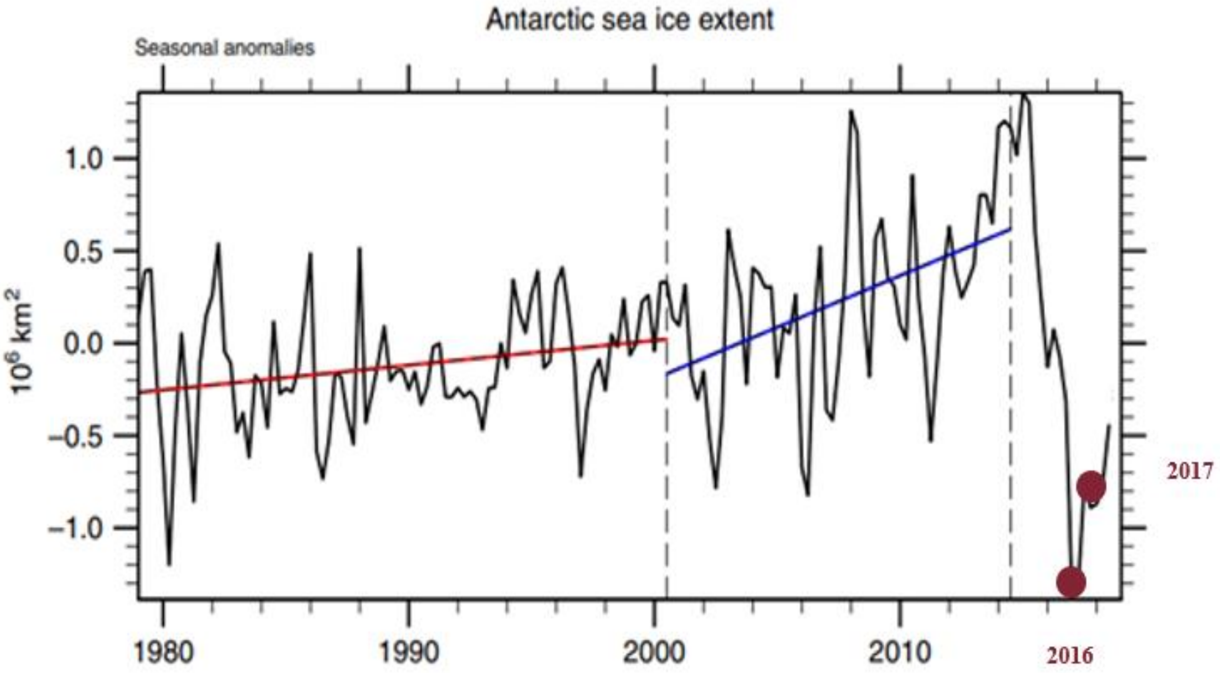


Figure 2-13: The Antarctic Sea Ice extent over 40 years (Meehl et al., 2019)

2.3. Sample preparation

The sampled tissues were eggs (shell membrane), feathers, and faeces. Penguin eggs samples involved Adélie Cove, Edmonson Point, Cape Hallett, and Cape Washington colonies. In the laboratory, egg samples have divided into small pieces and separated eggshell membrane from eggshell using a sharp blade (Figure 2-14), then freeze-dried (Figure 2-15). Afterward, samples were dried at 60 °C overnight. Shell membranes were weighted 0.5-1 mg of powder in a tin capsule (Figure 2-16) using an analytical ball mill (Fritch Mini-Mill Pulverisette 23) (Figure 2-17). Inorganic calcium carbonate from the eggshell was removed through a process of acidification. Approximately 10 mg of cleaned eggshell fragments were dissolved in an Eppendorf tube using the drop-by-drop method employed by Jacob et al. (Jacob et al., 2005). A minimum of four 20 mL aliquots of 6 N HCl was added, one at a time after each aliquot stopped bubbling (Figure 2-18). Acid was then evaporated at room temperature under a fume hood for 24 hr, and then for 48 hr in an oven at 60°C (Strickland et al., 2008). Almost 5 mg of eggshell acidified powder were weighted in a tin capsule (Figure 2-16).

Figure 2-14: Separating eggshell and shell membrane

Figure 2-15: Freeze dryer (photo by author)

Figure 2-16: The laboratory scale

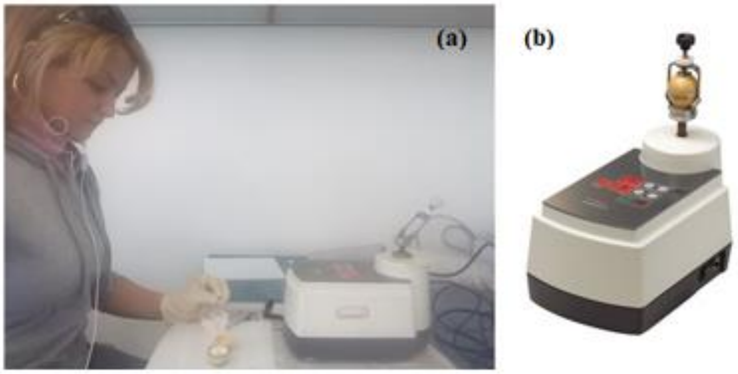


Figure 2-17: The analytical ball mill (b), ground samples into powder (a) (Author photo)

Figure 2-18: Egg acidification using the drop-by-drop method (Author photo)

Penguin feathers samples consisted of feathers of Adélie Cove, Edmonson Point, Cape Hallett, Inexpressible Island, and Cape Washington colonies. In the laboratory, feathers samples were freeze-dried (Figure 2-15) and dried at 60 °C overnight. Afterward, samples were cleaned of surface contaminants using a 2:1 chloroform: methanol solution (Figure 2-19). Feathers were rinsed in distilled water to remove chloroform and methanol solution (Graves et al., 2018), then cut into small fragments using stainless steel scissors. Approximately 0.5-1 mg of feathers fragments were weighted in a tin capsule (Cherel et al., 2005) (Figure 2-16).

Penguin faeces samples included faeces of Inexpressible Island, Adélie Cove, Edmonson Point, Cape Hallett, and Cape Washington colonies. In the laboratory, faeces samples were freeze-dried dried at 60 °C overnight. Next, ground into powder using an analytical ball mill (Fritch Mini-Mill Pulverisette 23) (Figure 2-17, a and b). Approximately 1-1.5 mg of faeces were weighted in a tin capsule (Figure 2-16).

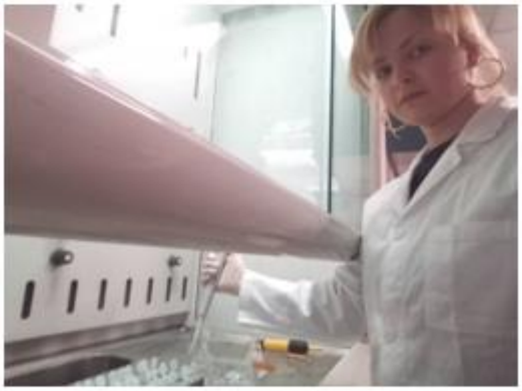


Figure 2-19: Cleaning feathers' samples using chloroform-methanol solution (Author photo)

2.4. Isotopic analysis of samples

The prepared samples were analysed for their carbon and nitrogen isotope content ($\delta^{13}C$ and $\delta^{15}N$) using a CN analyser (Vario Micro-Cube, Elementar Analysensysteme GmbH, Germany) coupled with an isotope ratio mass spectrometer (IsoPrime100, Isoprime Ltd., Cheadle Hulme, UK) (Figure 2-20). A ratio of the heavy to the light isotope was measured, and these ratios were normalized to VPDB (Vienna PeeDee Belemnite) for $\delta^{13}C$ and atmospheric (AIR) N_2 for $\delta^{15}N$. Stable isotope values followed the δ notation and were derived from the following equation (Hobson, 1995):

$$\delta X=[(R_{sample}/R_{standard})-1]*1{,}000 \qquad \text{(Formula 1)}$$

where X is ^{13}C or ^{15}N and R is the ratio of $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

Figure 2-20: The mass spectrometer analyser

2.5. Statistical analysis

All data analysis was conducted in the Statistical Package for the Social Science (SPSS) software, with analysis of variance (ANOVA) and the graphs were done in excel Microsoft office. To test the pairwise comparisons among means was used the Tukey's Honest Significant Difference (Tukey's HSD) test (Abdi and Williams, 2010). The isotopic value fractionations used among feathers, and shell membrane were 0.35‰, and 2.83‰ in $\delta^{13}C$

and 3.62‰, and 4.33‰ in $\delta^{15}N$, respectively (Cherel et al., 2005; Polito et al., 2011b, 2009b; Quillfeldt et al., 2009). To estimate and determine the relative contribution of food items consumed by penguins, used the mixing models in R software with simmr package. Activities were carried out within the trophic ecology laboratory of the Environmental biology department in Sapienza University.

CHAPTER 3

RESULTS

3.1. The spatial and temporal changes of the sea ice coverage effect on Antarctic trophic ecology

In the following are expressed the results of the first objective of this thesis, which is to determine how the spatial and temporal changes of the sea ice coverage in Ross Sea coastline affect the trophic ecology of penguin species during a year. Table 3-1 and 3-2 are presented Isotopic values of differences tissues that reflect the different time of the year in Emperor and Adèlie penguins.

Table 3-1: Stable Isotopes signature, Ratio, and percentage of Carbon and Nitrogen of various tissues at different time of year 2016-17 in Emperor penguins

Site	Date	Tissue	δ^{13}C Mean±SD	δ^{15}N Mean±SD	%C Mean±SD	%N Mean±SD	C/N Mean±SD
Cape Washington	Jan. 2016	Feathers	-24.06±0.34	10.27±0.72	48.52±0.92	15.69±0.36	3.09±0.11
	May 2016	Shell membrane	-29.56±0.90	8.24±1.16	47.50±5.14	11.92±1.93	4.11±1.05
	Nov. 2016	Faeces	-29.17±0.57	9.59±1.36	39.36±7.02	14.79±1.88	2.70±0.62
	Dec. 2016	Faeces	-28.08±0.66	9.57±1.40	43.37±4.81	12.57±4.99	4.06±1.91
	Prior Jan. 2017	Faeces	-27.07±0.78	10.65±0.63	37.38±5.93	9.68±3.64	4.27±1.31
	-	Krill	-25.75±0.07	6.32±1.15	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-

Table 3-2: Stable Isotopes signature, Ratio, and percentage of Carbon and Nitrogen of various tissues at different time of year 2016-17 in Adèlie penguins

Site	Date	Tissue	δ^{13}C Mean±SD	δ^{15}N Mean±SD	%C Mean±SD	%N Mean±SD	C/N Mean±SD
Inexpressible Island	Late Mar.-Prior Apr.2016	Feather	-23.66±1.48	8.56±1.47	48.63±0.86	15.81±0.26	3.08±0.03
	Late Nov. 2016	Faeces	-28.61±0.69	7.26±0.94	42.13±3.43	9.27±2.61	4.94±1.55
	Prior Dec. 2016	Faeces	-27.38±1.30	4.92±2.88	40.22±3.38	7.89±1.37	5.23±0.90
	Late Jan. 2017	Faeces	-26.76±0.63	6.63±1.79	42.33±6.50	8.99±2.56	5.06±1.58
	-	Krill	-28.64±1.17	4.84±0.66	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-
Adélie Cove	Late Mar.-Prior Apr.2016	Feather	-24.92±0.1	6.99±0.1	46.05	14.82	3.10
	Late Oct. 2016	Shell membrane	-27.48±0.32	5.33±0.85	44.37±1.94	14.04±2.24	3.23±0.47
	Late Nov. 2016	Faeces	-28.57±0.74	8.32±1.43	35.54±8.27	9.72±2.90	3.93±1.43
	Prior Dec. 2016	Faeces	-26.72±1.01	8.03±2.46	33.99±5.61	6.69±2.37	5.68±2.45
	Late Jan. 2017	Faeces	-26.78±0.76	7.36±1.92	41.72±5.67	8.72±2.02	5.01±1.21
	-	Krill	-26.79±1.39	5.30±0.27	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-
Edmonson Point	Late Mar.-Prior Apr.2016	Feather	-22.12±0.53	11.23±1.01	47.72±0.95	15.51±0.39	3.08±0.03
	Late Oct. 2016	Shell membrane	-27.47±1.14	5.89±0.85	47.07±2.64	13.18±0.60	3.57±0.16
	Late Nov. 2016	Faeces	-28.48±1.10	7.90±1.54	37.19±7.56	11.46±3.63	3.51±1.25
	Prior Dec. 2016	Faeces	-27.28±0.90	8.10±1.07	37.97±6.45	13.10±4.24	3.40±1.77
	Late Jan. 2017	Faeces	-26.44±0.78	6.48±1.48	37.50±6.24	8.42±2.30	4.75±1.40
	-	Krill	-25.75±0.07	6.32±1.15	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-
Cape Hallett	Late Mar.-Prior Apr.2016	Feather	-24.38±0.44	5.87±0.75	48.54±1.70	15.56±0.10	3.11±0.11
	Late Oct. 2016	Shell membrane	-27.72±0.66	5.24±0.60	45.01±0.84	12.84±0.84	3.51±0.27
	Late Dec. 2016	Faeces	-27.77±0.69	4.88±1.84	39.92±6.74	10.72±3.69	3.97±0.93
	Prior Feb. 2017	Faeces	-28.45±1.03	4.59±1.97	38.45±3.98	9.04±2.62	4.54±1.15
	-	Krill	-28.08±0.95	5.56±0.32	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-

3.1.1. Intra-colony variation in foraging pattern during different seasons of the year for Adélie and Emperor Penguin's colonies

The carbon and nitrogen isotopes of Emperor penguin in the year 2016-17 at Cape Washington site have shown that there was a significant (Table 3-3) δ^{13}C and δ^{15}N range increase from Winter to Summer (Figure 3-1, a and c). As shown in Table 1 (Appendix), the δ^{13}C and δ^{15}N of Winter had a significant difference with Summer months.

Table 3-3: The analysis of variance test results of $\delta^{13}C$, and $\delta^{15}N$ between seasons of each penguin colony

Colony		Variable	df	F	P-value
Emperor Penguins	Cape Washington	$\delta^{13}C$	3	166.61	0.000
		$\delta^{15}N$	3	9.72	0.000
Adèlie Penguins	Inexpressible Island	$\delta^{13}C$	3	49.35	0.000
		$\delta^{15}N$	3	5.93	0.001
	Adélie Cove	$\delta^{13}C$	4	21.90	0.000
		$\delta^{15}N$	4	8.86	0.000
	Edmonson Point	$\delta^{13}C$	4	66.49	0.000
		$\delta^{15}N$	4	31.27	0.000
	Cape Hallett	$\delta^{13}C$	3	27.26	0.000
		$\delta^{15}N$	3	0.80	0.498

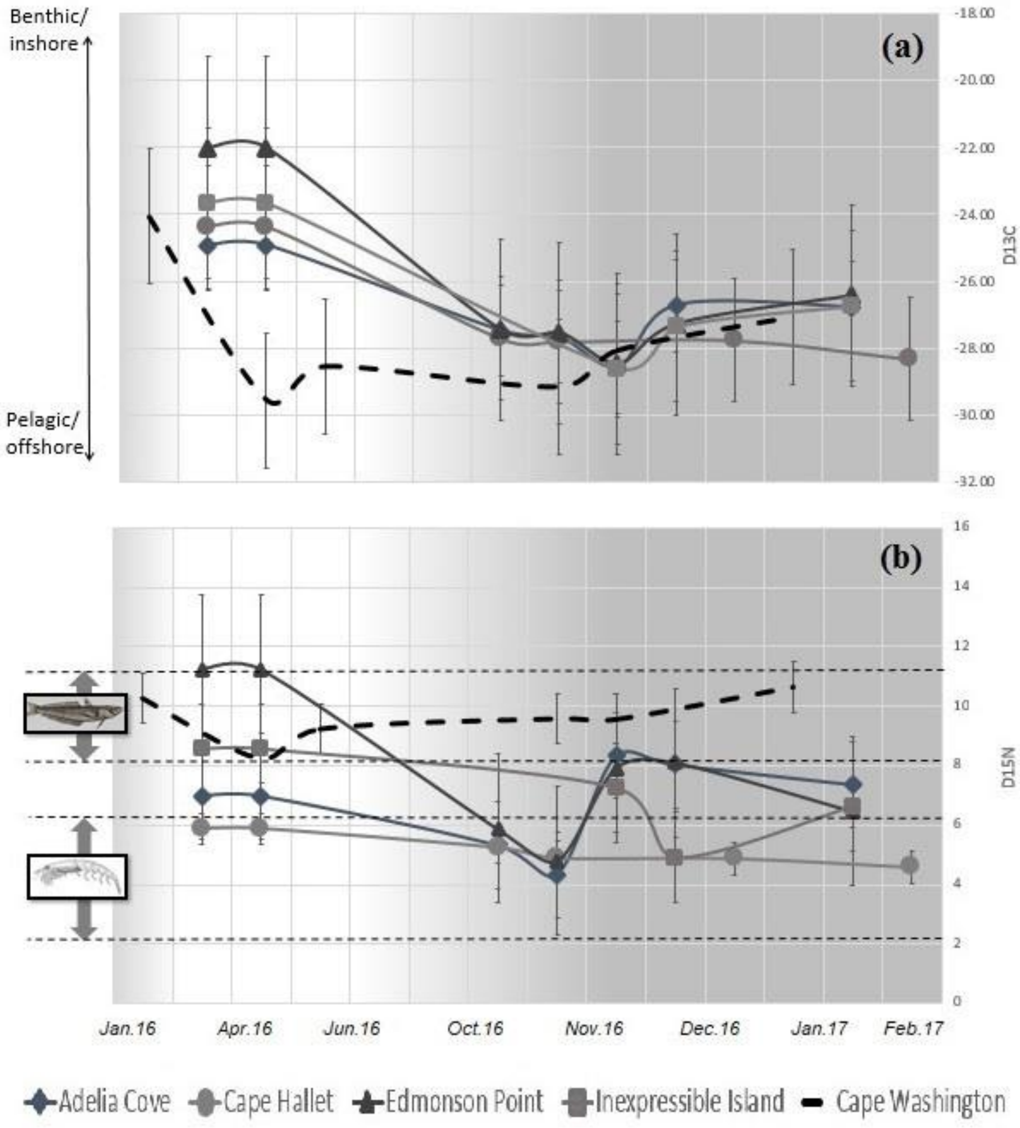


Figure 3-1: Stable Isotopes signature of Carbon (a) and Nitrogen (b) of various colonies in the year 2016-17;
* The white and gray part of the field represent the Winter and the Summer season, respectively.

Based on the mixing model analysis (Table 3-4), when the Emperor penguins are on the ice to breed in Winter consumed 64.2% fish. By increasing sea ice breaking, in the Summer, the penguins go offshore and forage in open waters, where can find mostly fish. The minor nitrogen range variations inside of trophic level whole the year, indicate that the major part of Emperor penguin's diet was fish during the year (Figure 3-1, b; 3-2).

Table 3-4: Diet composition of Emperor and Adélie colonies in different seasons of the year 2016-17

		Winter		Spring		Summer	
2016-17		**Fish %**	**Krill %**	**Fish %**	**Krill %**	**Fish %**	**Krill %**
Emperor Penguins	Cape Washington	64.2	35.8	81.1	18.9	95.1	4.9
Adélie Penguins	Inexpressible Island	81.4	18.6	-	-	43.4	56.6
	Adélie Cove	42.1	57.9	3.0	96.2	26.8	73.2
	Edmonson Point	89.4	10.6	3.1	96.9	13.2	86.8
	Cape Hallett	36.5	63.5	5.0	94.7	3.3	96.7

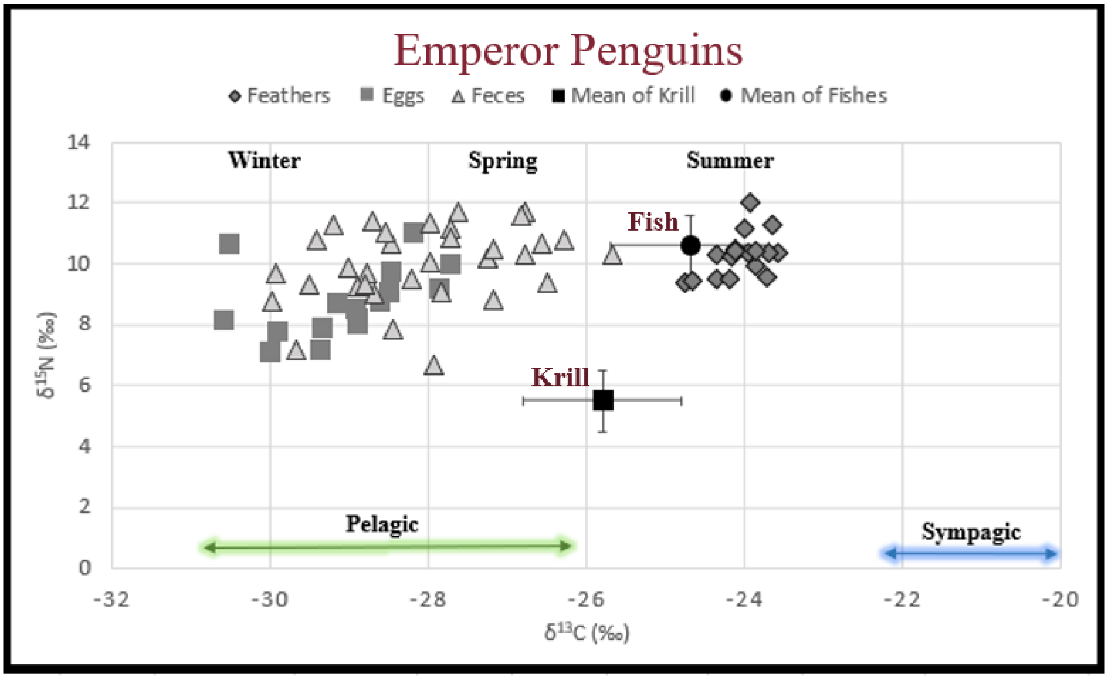


Figure 3-2: Comparing of Carbon and Nitrogen Stable Isotopes signature of different Emperor penguin tissues with the mean of krill and fish in the year 2016-17

*Emperor's feather, egg, and faeces sample presented the Antarctic Spring, Winter, and Summer, respectively.

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton

As shown in Figure 3-1, there was a significant (Table 3-3) reduction carbon and nitrogen isotope signatures in Adélie colonies from Winter to Summer. As shown in Table 1, (Appendix), the δ^{13}C of April as Winter was significantly less than Summer months in all Adélie colonies. There were not significant differences in δ^{15}N of Adélie Cove and Cape Hallett during the year (Table 3-3). Based on the mixing model analysis in Winter, Inexpressible Island, Adélie Cove, Edmonson point, and Cape Hallett colonies consumed 81.4%, 42.1%, 89.4%, and 36.5% fish, and fed 18.9%, 57.9%, 10.6%, and 63.5% on krill, respectively. In the Spring and Summer, the large part of Adélie penguins’ diet was krill (Table 3-4). These results are visible also in Figure 3-1 (b) and Figure 3-3 with samples of feces and eggs.

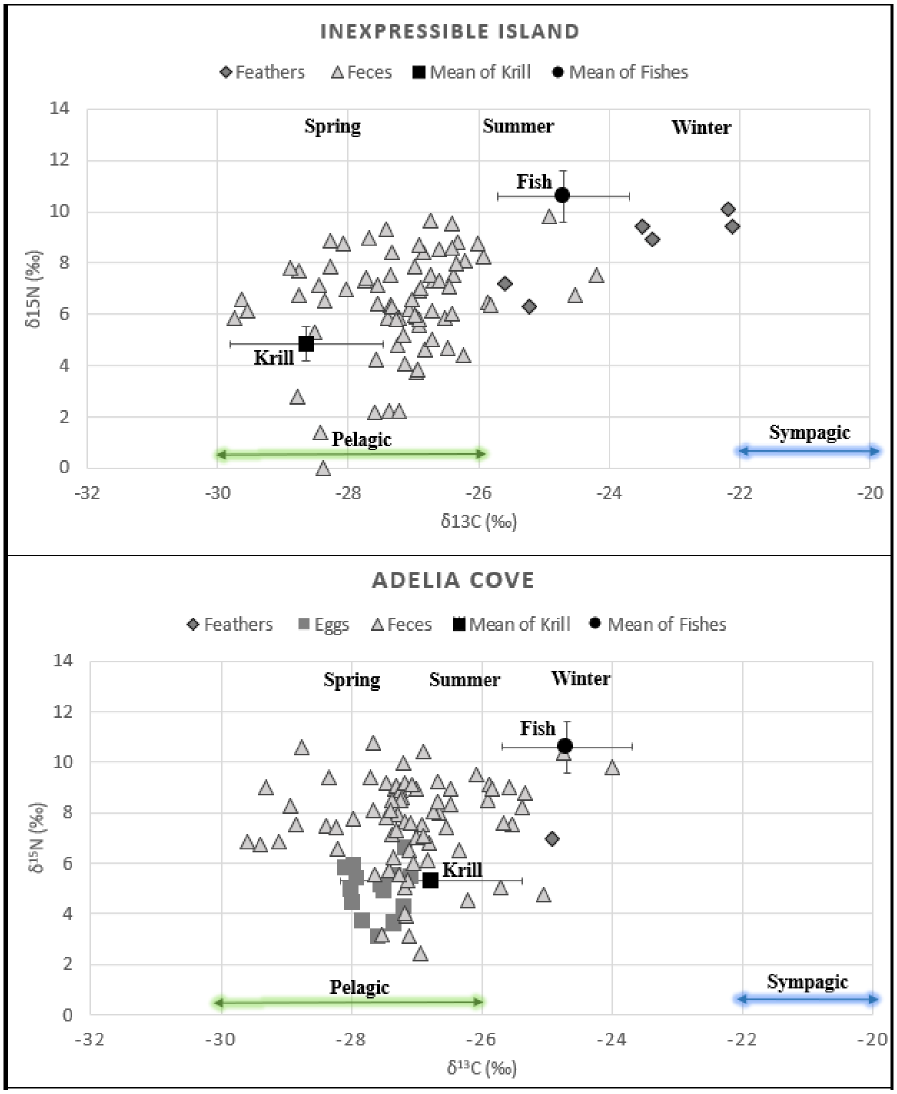
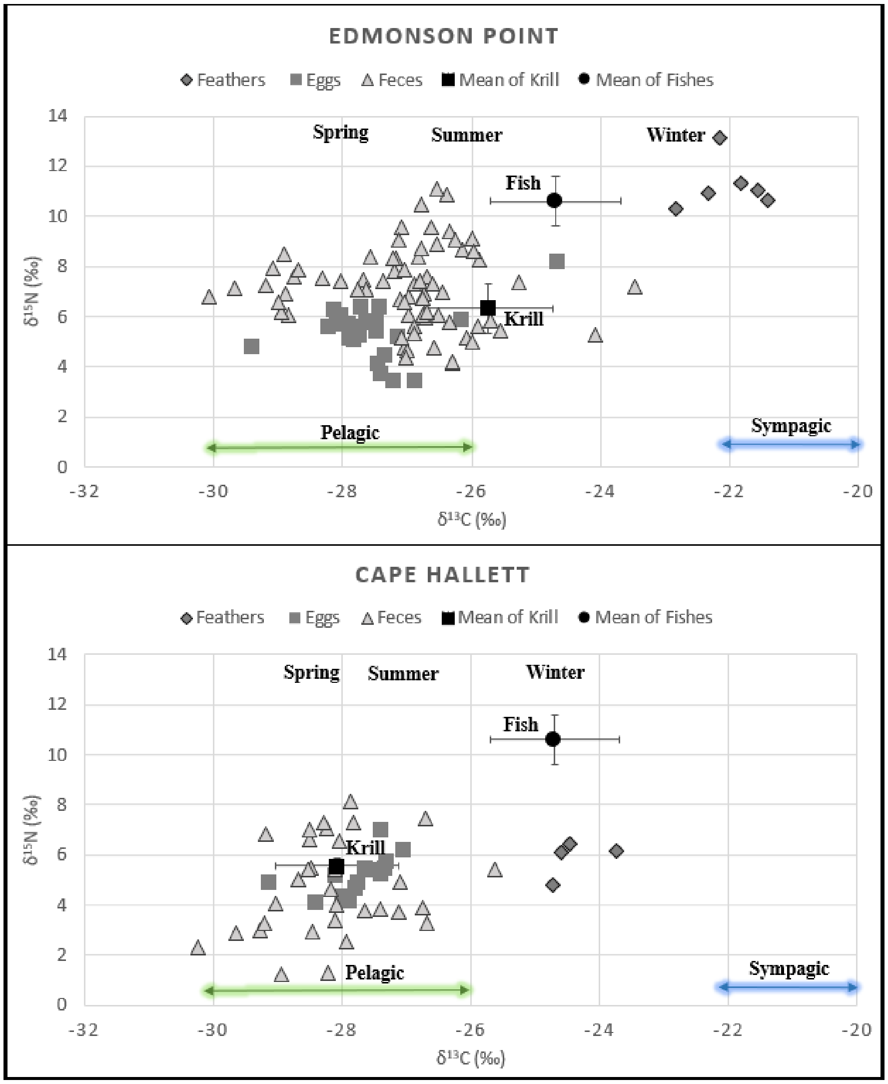


Figure 3-3: Comparing of Carbon and Nitrogen Stable Isotopes signature of various Adélie penguin tissues with the mean of krill and fish in the year 2016-17

*Adélie’s feather, egg, and faeces sample presented the Antarctic Winter, Spring, and Summer, respectively.

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton



Continuing of Figure 3-3: Comparing of Carbon and Nitrogen Stable Isotopes signature of various Adélie penguin tissues with the mean of krill and fish in the year 2016-17
*Adélie's feather, egg, and faeces sample presented the Antarctic Winter, Spring, and Summer, respectively.
*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton

3.1.2. Inter-colony variation in foraging pattern during different seasons of the year between Adélie penguin's colonies

As showed in Table 3-5, there was a significant difference between δ^{13}C and δ^{15}N of the Adélie colonies. The test Tukey's HSD showed a significant decline between Summer δ^{13}C of Cape Hallett colony with value -28.14‰ versus other Adélie penguin colonies in two subsets, and as well as the same results for δ^{15}N with value +4.69‰ (Table 2, Appendix). As shown in Table 3-4, with increasing latitudinal degree increased the krill in the Summer (Table 3-4). The Winter δ^{13}C and δ^{15}N of Edmonson Point colony was significantly higher than other colonies, but there was no δ^{13}C differences with Inexpressible Island. Adélie Cove and Cape Hallett colonies consumed major krill in Winter

2016-17, whereas Inexpressible Island and Edmonson Point colonies consumed major fish (Table 3-4).

Table 3-5: The analysis of variance test results of δ^{13}C, and δ^{15}N between Adélie penguin colonies

Source		Variable	df	F	P-value
Corrected Model	Summer	δ^{13}C	3	23.05	$6.26*10^{-13}$
		δ^{15}N	3	16.13	$1.81*10^{-9}$
	Winter	δ^{13}C	3	7.35	0.003
		δ^{15}N	3	20.22	$2.33*10^{-5}$

3.1.3. Inter-colony variation in foraging pattern during different seasons of the year between Adélie and Emperor penguin's colonies

As showed in Table 3-6, there were a significant δ^{13}C and δ^{15}N differences between the Adélie and Emperor colonies both in Summer and Winter. The Tukey's HSD test showed that Cape Washington δ^{13}C in Summer and Winter was significantly lower than Adélie colonies, but there was no significant difference with Cape Hallett in Summer (Table 2, Appendix). Emperor penguin δ^{15}N in Summer was significantly higher than all Adélie penguin colonies. The minimum mean of δ^{13}C and δ^{15}N of Adélie penguin colonies were in the Summer, whereas for the Emperor penguin colony were in the Winter (Figure 3-1, a, b, and c). Also, the Emperor penguin colony consumed more fish in the Summer than the Winter, whereas Adélie penguin colonies consumed more krill in the Summer than the Winter (Table 3-4).

Table 3-6: The analysis variance test results of δ^{13}C, and δ^{15}N between Emperor and Adélie penguin colonies

Source		Variable	df	F	P-value
Corrected Model	Summer	δ^{13}C	4	11.17	0.000
		δ^{15}N	4	39.83	0.000
	Winter	δ^{13}C	4	87.57	0.000
		δ^{15}N	4	11.84	0.000

3.2. Compare the spatial and temporal penguin diet changes over two consecutive breeding and moulting periods

Here is expressed the results of the second aim of this thesis that is to compare the diet composition of penguins to evaluate annual variations in the foraging ecology of adults over two consecutive breeding and moulting seasons (2016-17 to 2017-18). Carbon and Nitrogen stable isotopes, Ratio, and percentage of feather, egg, and faeces in the year 2016-17 and 2017-18 are presented in Table 3 and 4 (Appendix).

3.2.1. Diet composition of Emperor penguins over two consecutives breeding season and moulting period

The Emperor penguin carbon and nitrogen isotopes of pre-moulting with feather samples over two years have shown that the $\delta^{13}C$ and $\delta^{15}N$ decreased significantly from 2016-17 to 2017-18 (Figure 3-4, a and b; Figure 3-5; Table 3-7).

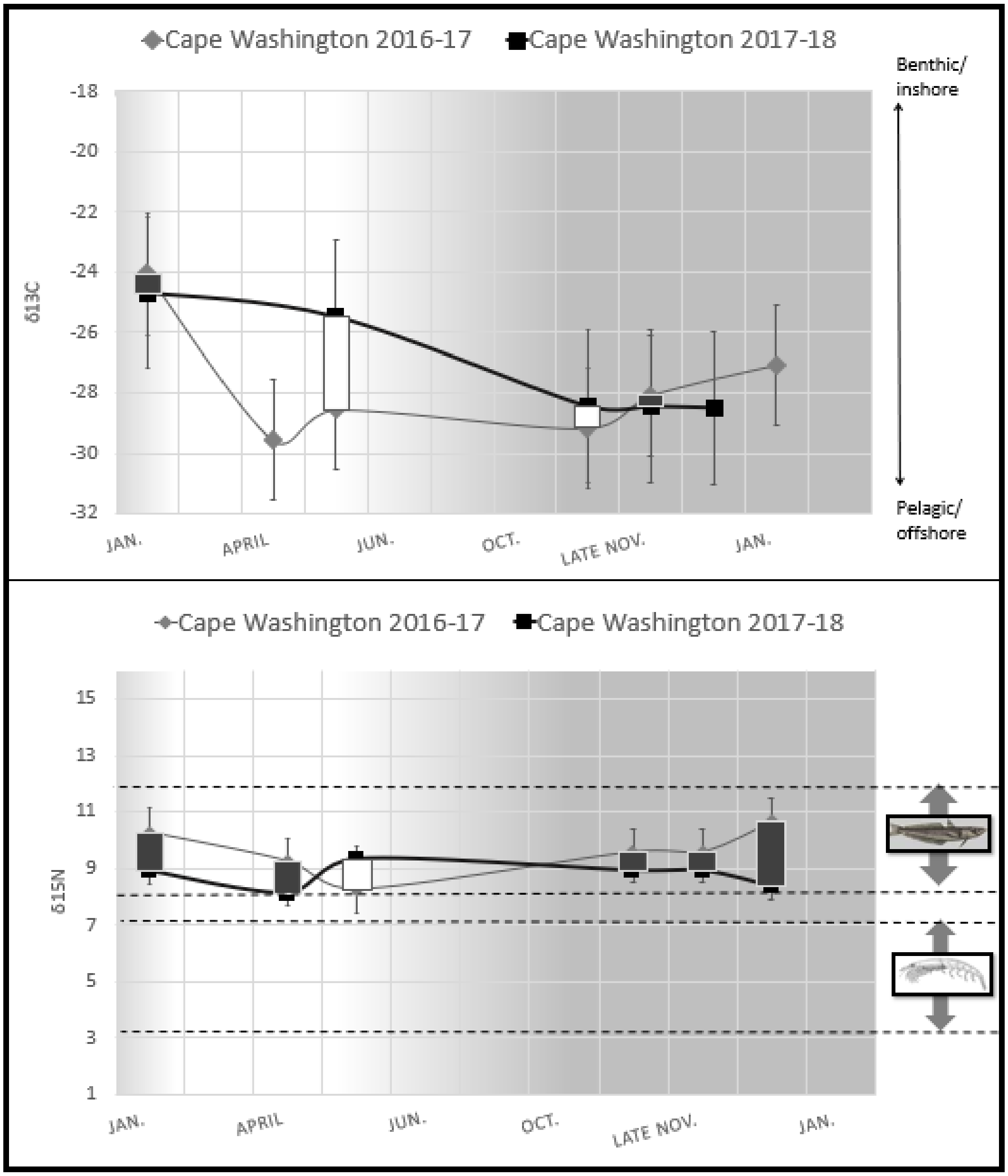


Figure 3-4: Carbon (a) and Nitrogen (b) stable isotopes of Emperor penguin colony over two consecutive breeding and moulting periods

* The white and gray part of the field represent the Winter and the Summer season, respectively.

Table 3-7: The analysis of variance test results of $\delta^{13}C$, and $\delta^{15}N$ of Emperor penguin colony

Colony	Variable	Season	df	F	P-value
Emperor penguins	$\delta^{13}C$	Pre-moulting (Summer)	1	8.006	0.009
		Pre-breeding (Winter)	1	54.01	0.000
	$\delta^{15}N$	Pre-moulting (Summer)	1	13.45	0.001
		Pre-breeding (Winter)	1	4.16	0.064

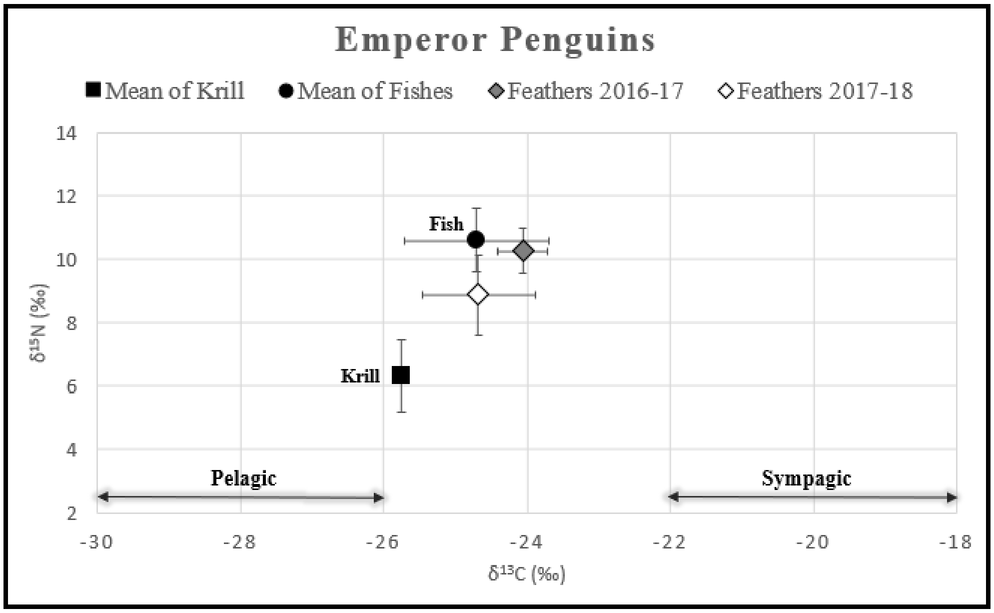


Figure 3-5: Carbon and Nitrogen stable isotopes of Emperor penguin colony over two consecutive pre-moulting periods

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton

As shown in Table 3-8, the Emperor penguin diet was major based on fish in two consequence pre-moulting periods.

Table 3-8: Diet composition of Emperor penguin in pre-moulting and pre-breeding periods between two consecutive years

		Pre-moulting (Summer)		**Pre-breeding (Winter)**	
		Fish %	**Krill %**	**Fish %**	**Krill %**
Cape Washington	**2016-17 low ice**	95.1	4.9	64.2	35.8
	2017-18 high ice	80.8	19.2	66.7	33.3

The mean of the $\delta^{13}C$ of Emperor penguin pre-breeding period over two years have shown an enrichment from year 2016-17 to 2017-18 (Figure 3-4, a), and there was not $\delta^{15}N$ differences (Table 3-7; Figure 3-4, b; Figure 3-6). As shown in Table 3-8, Emperor penguin mainly consumed fish in the pre-breeding period over two years.

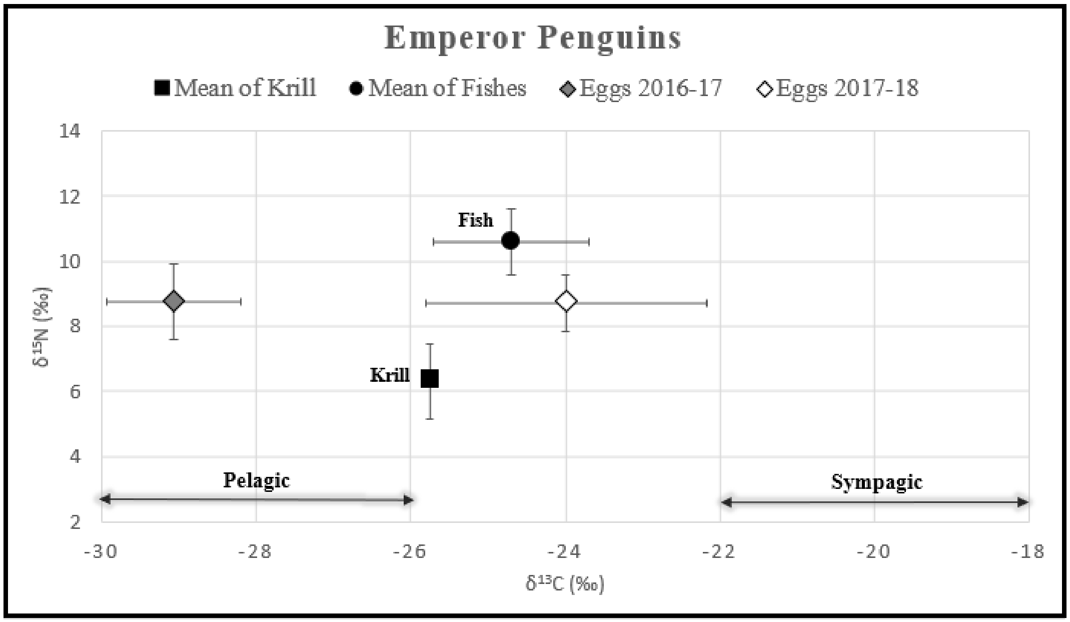


Figure 3-6: Carbon and Nitrogen stable isotopes of Emperor penguin colony over two consecutive pre-breeding periods

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton

3.2.2. Diet composition of Adélie penguins over two consecutives breeding season and moulting period

The $\delta^{13}C$ and $\delta^{15}N$ of Edmonson Point pre-moulting period decreased significantly from the year 2016-17 to 2017-18 (Figure 1, Appendix; a and b; Figure 3-7). Adélie Cove was the only colony, which had increment $\delta^{13}C$ from -24.92‰ in the year 2016-17 to -21.88‰ in the year 2017-18 (Figure 1, Appendix, a; Figure 3-7).

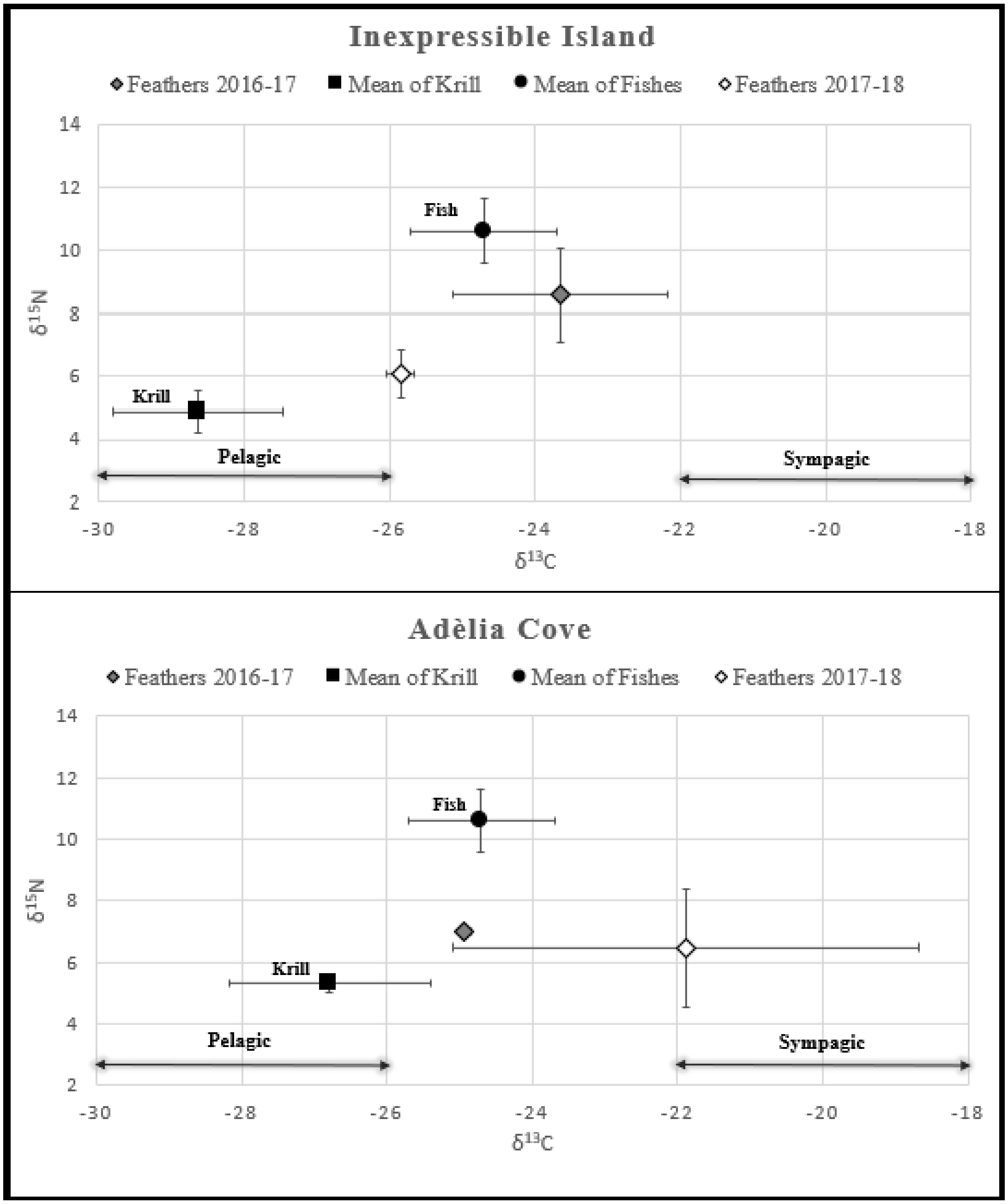
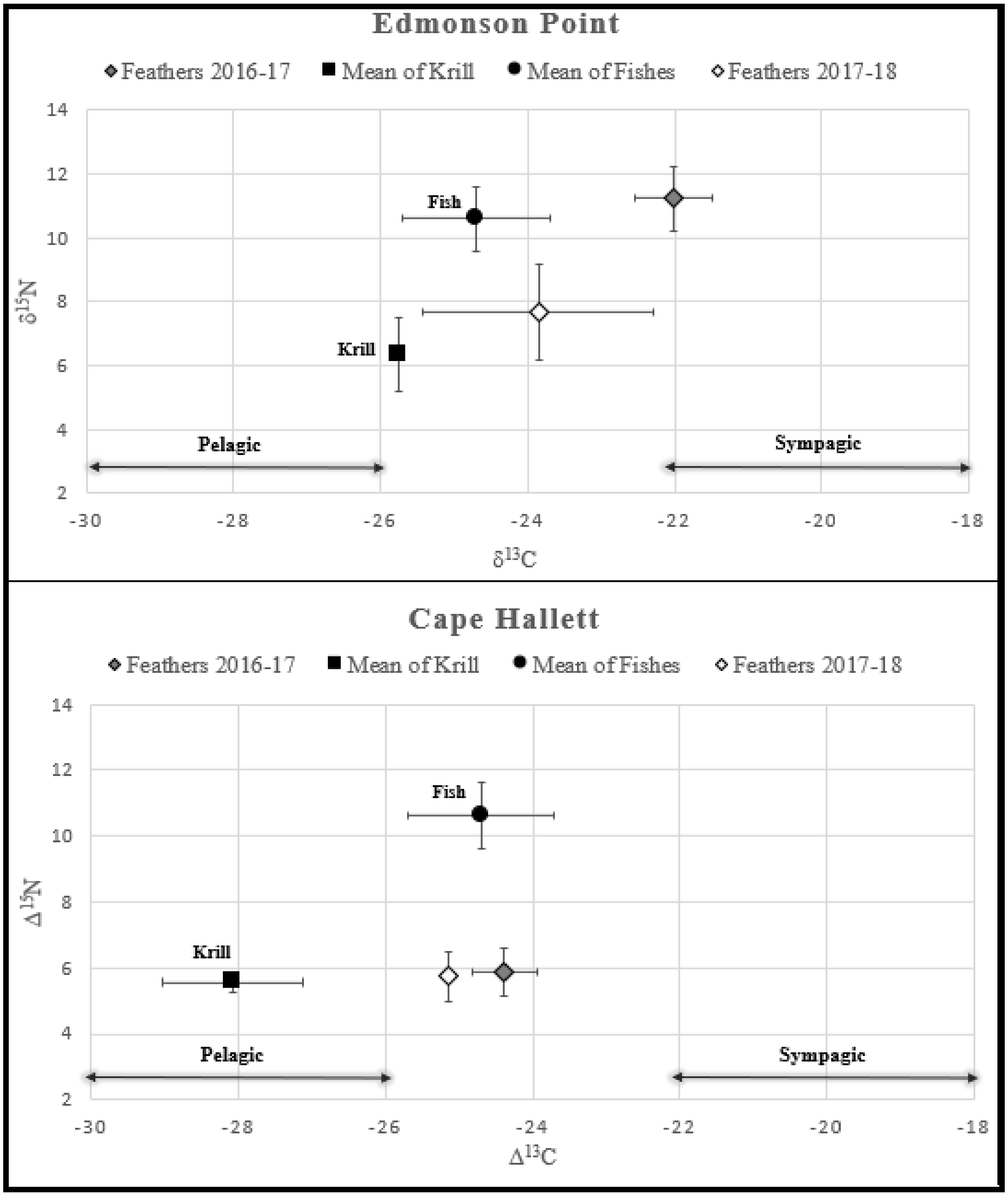


Figure 3-7: Carbon and Nitrogen stable isotopes of Adèlie penguin colonies over two consecutive pre-moulting periods

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton



Continuing Figure 3-7: Carbon and Nitrogen stable isotopes of Adèlie penguin colonies over two consecutive pre-moulting periods

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton

Table 3-9: The analysis of variance test results of $\delta^{13}C$, and $\delta^{15}N$ of each Adélie penguin colony

Colony	Variable	Season	df	F	P-value
Inexpressible Island	$\delta^{13}C$	Pre-moulting (winter)	1	3.92	0.095
		Breeding (Summer)	1	1.41	0.237
	$\delta^{15}N$	Pre-moulting (winter)	1	4.99	0.067
		Breeding (Summer)	1	$8.05*10^3$	0.000
Adélie Cove	$\delta^{13}C$	Pre-moulting (winter)	1	1.78	0.313
		Pre-breeding (Spring)	1	10.70	0.005
		Breeding (Summer)	1	2.55	0.113
	$\delta^{15}N$	Pre-moulting (winter)	1	0.142	0.743
		Pre-breeding (Spring)	1	18.23	0.001
		Breeding (Summer)	1	16.80	0.000
Edmonson Point	$\delta^{13}C$	Pre-moulting (winter)	1	7.47	0.029
		Pre-breeding (Spring)	1	10.75	0.006
		Breeding (Summer)	1	20.99	0.000
	$\delta^{15}N$	Pre-moulting (winter)	1	18.44	0.004
		Pre-breeding (Spring)	1	14.15	0.002
		Breeding (Summer)	1	2.16	0.145
Cape Hallett	$\delta^{13}C$	Pre-moulting (winter)	1	5.14	0.086
		Pre-breeding (Spring)	1	23.72	0.000
		Breeding (Summer)	1	5.87	0.018
	$\delta^{15}N$	Pre-moulting (winter)	1	0.060	0.818
		Pre-breeding (Spring)	1	1.88	0.194
		Breeding (Summer)	1	10.89	0.001

As shown in Table 3-10, in the Inexpressible Island and Edmonson Point colonies there was a significant reduction in fish consumption and increment krill consumption of the pre-moulting period between Antarctic Winter over two years.

As showed in Figure 1 (Appendix; a, b), the carbon and nitrogen isotopes of pre-breeding period over two years differed significantly between two consecutive years (Figure 1, Appendix, a; Figure 3-8; Table 3-9). The nitrogen isotopes of breeding period in all Adèlie colonies (Figure 1, Appendix), decreased significantly from the year 2016-17 to 2017-18 (Table 3-9), except Edmonson Point.

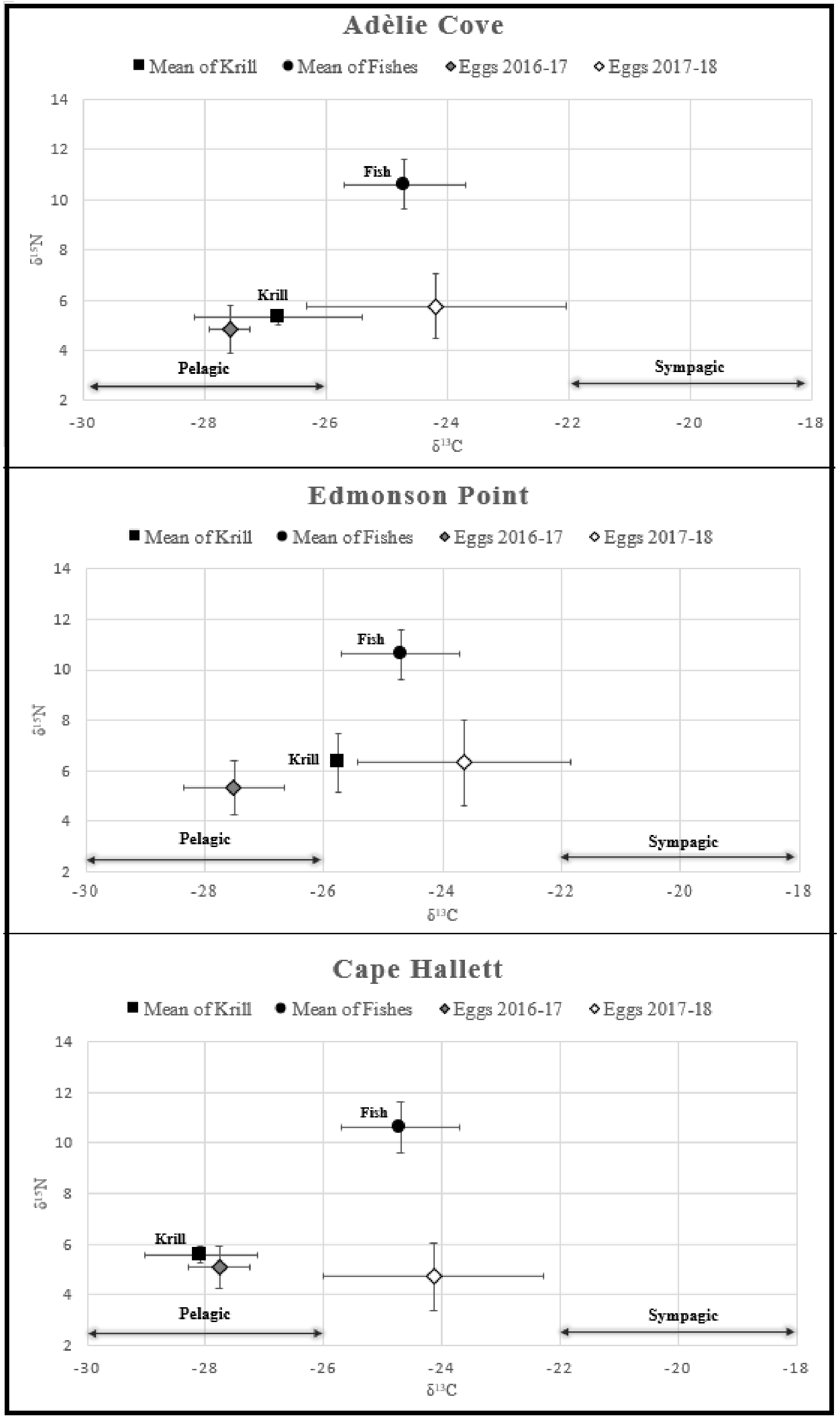


Figure 3-8: Carbon and Nitrogen stable isotopes of Adèlie penguin colonies over two consecutive pre-breeding periods

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton

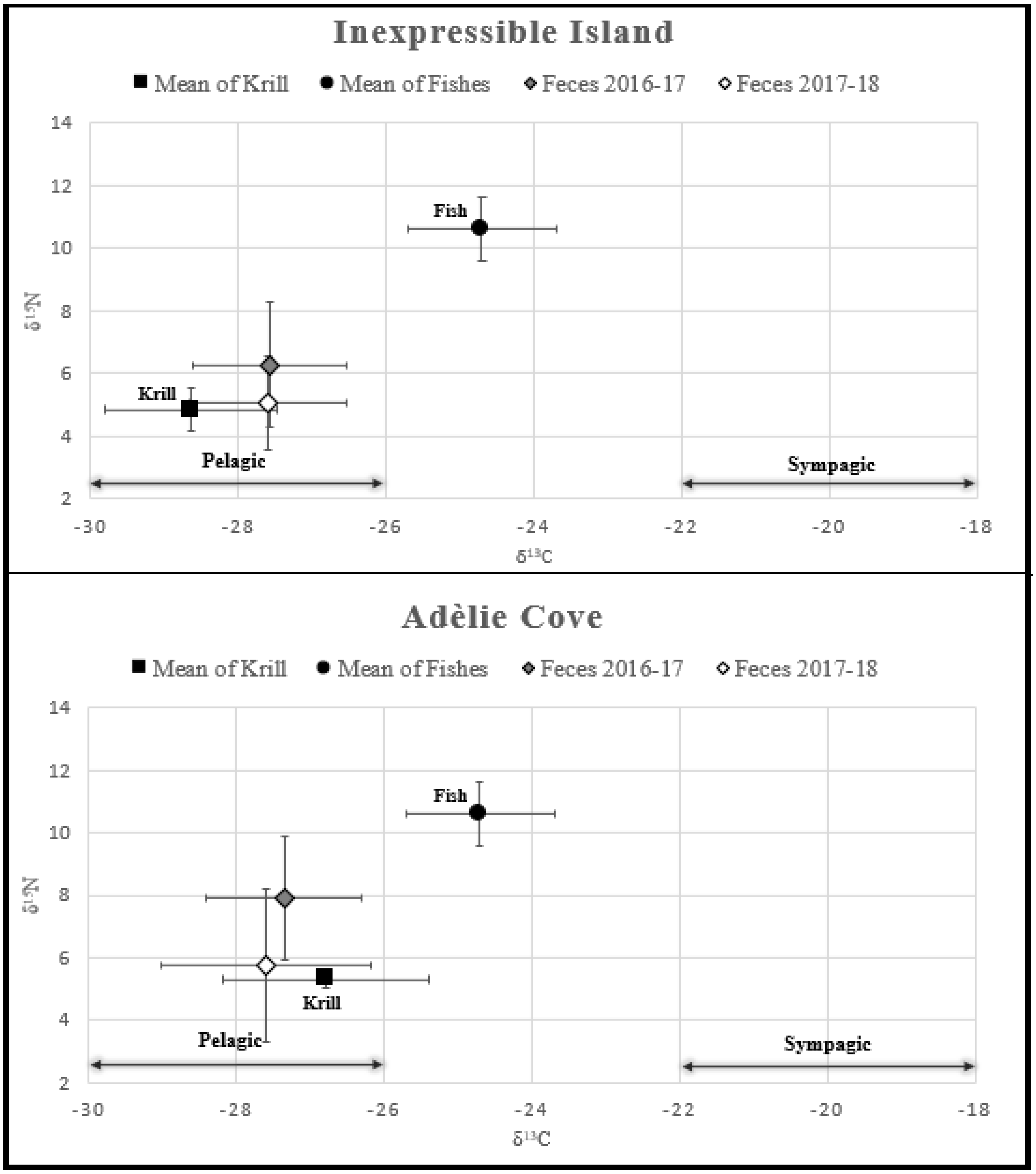
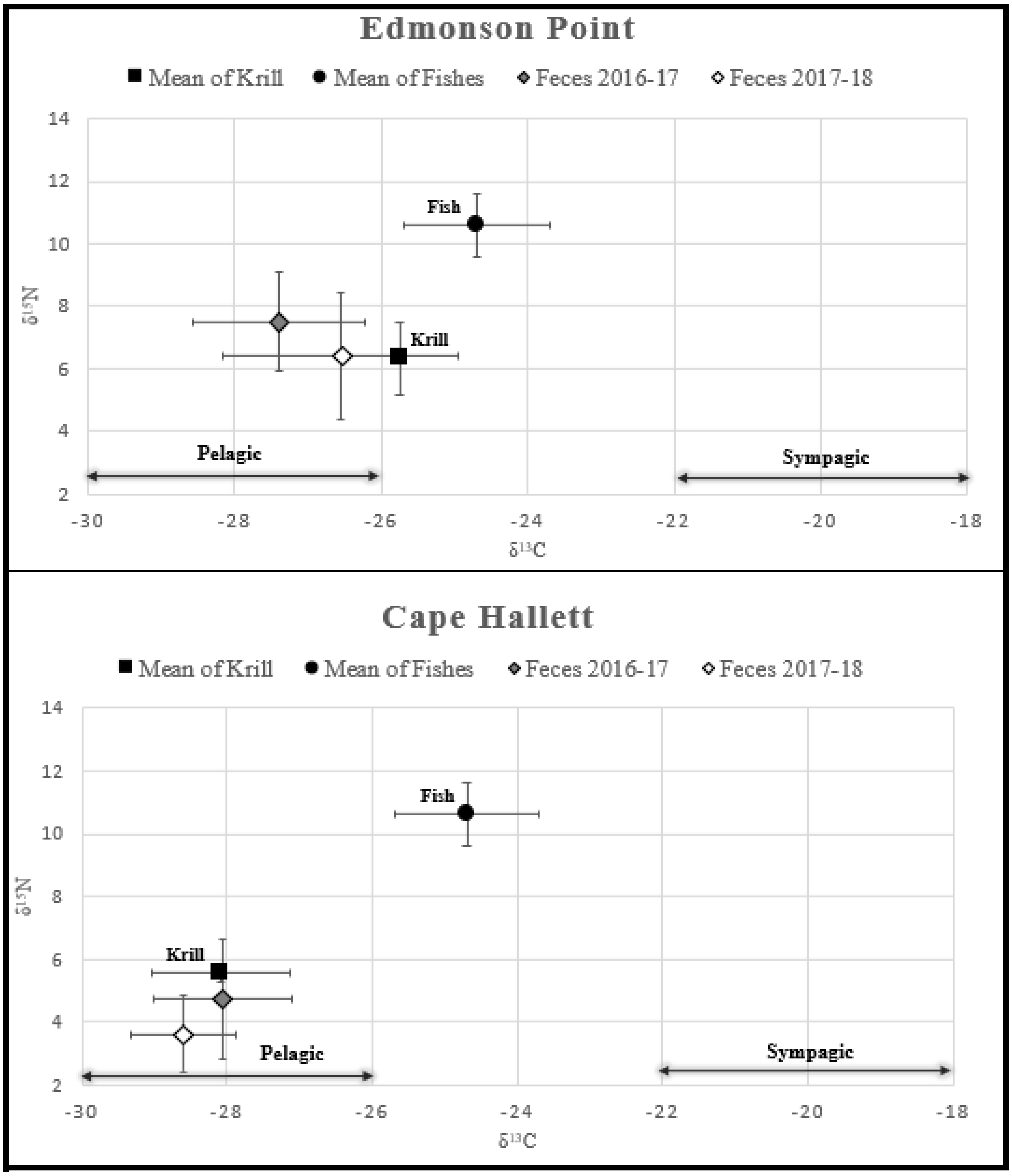


Figure 3-9: Carbon and Nitrogen stable isotopes of Adèlie penguin colonies over two consecutive breeding periods

*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton



Continuing Figure 3-9: Carbon and Nitrogen stable isotopes of Adèlie penguin colonies over two consecutive breeding periods
*Sympagic: Food chain based on sea ice associated alghe; Pelagic: Food chain based on phytoplankton

There was a significant increment in fish consumption and reduction in krill consumption from the pre-breeding period of the year 2016 to the year 2017 (Antarctic Spring), conversely in the breeding season (Antarctic Summer) they consumed minor fish and major krill in the following the year (Table 3-10).

Table 3-10: Diet composition of Adélie penguin colonies in pre-moulting, pre-breeding, and breeding periods between two consecutive years

		Winter (pre-moulting)		**Spring (pre-breeding)**		**Summer (breeding)**	
		Fish %	**Krill %**	**Fish %**	**Krill %**	**Fish %**	**Krill %**
Inexpressible Island	**Low Ice**	81.4	18.6	-	-	43.4	56.6
	High Ice	65.8	34.2	-	-	36.5	63.5
Adélie Cove	**Low Ice**	42.1	57.9	3.8	96.2	26.8	73.2
	High Ice	43.1	56.9	30.7	69.3	4.9	95.1
Edmonson Point	**Low Ice**	89.4	10.6	3.1	96.9	13.2	86.8
	High Ice	43.6	56.4	36.4	63.6	11.7	88.3
Cape Hallett	**Low Ice**	36.5	63.5	5.3	94.7	3.3	96.7
	High Ice	41.0	59.0	32.3	67.7	1.4	98.6

The results showed that the krill consumption increased from low ice year to high ice year in both periods of Winter and Summer. All samples taken in the Adèlie penguin colonies in two years showed there is a linear relationship between the persistence sea ice and krill consumption (Figure 3-10). The persistence is measured as the number of days of delay in the breaking of the ice respect to the day of ice-break observed in the Polynya area in 2016.

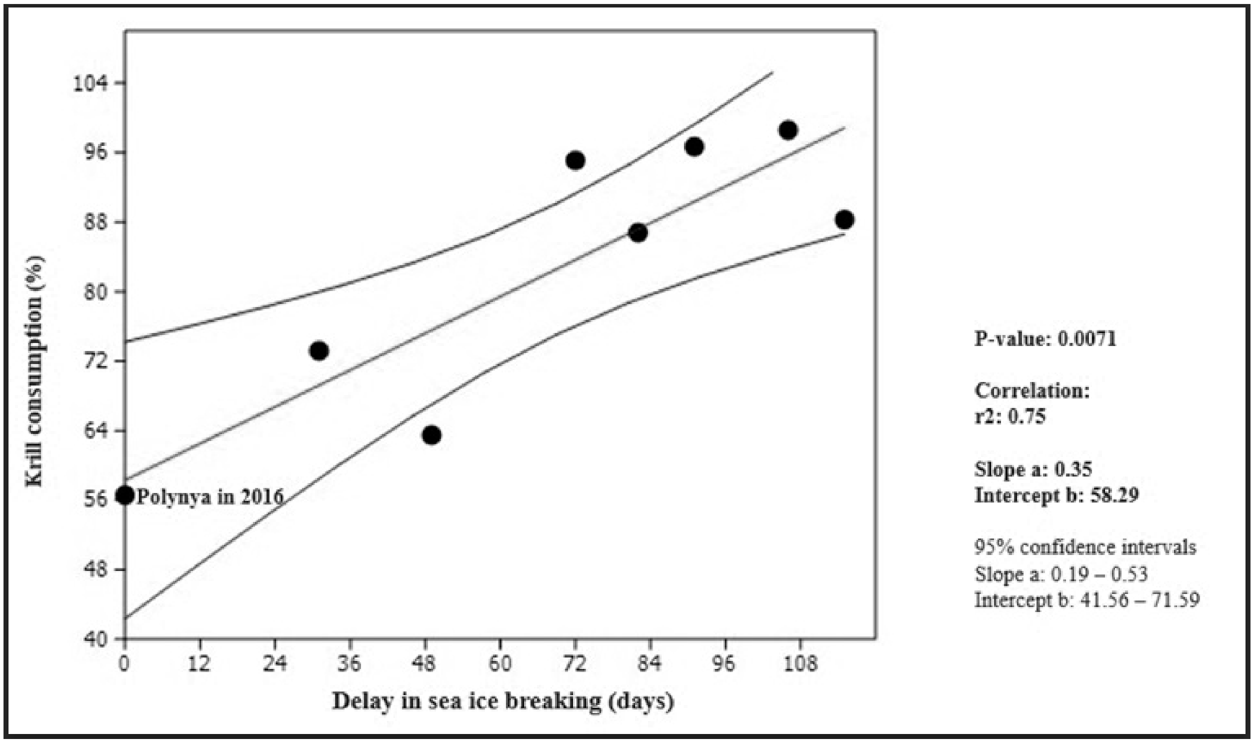


Figure 3-10: The relationship of persistence of sea ice and krill consumption

CHAPTER 4

DISCUSSION

4.1. The spatial and temporal changes of the sea ice coverage effect on Antarctic trophic ecology

4.1.1. Intra-colony variation in foraging pattern during different seasons of the year for Adélie and Emperor Penguin's colonies

According to the results section, Emperor penguins' $\delta^{13}C$ and $\delta^{15}N$ had an increment from Winter to Summer. In Antarctic Winter, by decrement of temperature and expansion of sea ice, food resource availability is limited, and Emperor penguins go offshore to forage. In contrast, from late November and early December (Antarctic Summer) reduce sea ice results in increment of food resources (phytoplankton, diatoms, cryptophytes, and krill) (Figure 4-1). These conditions induce an increment fish availability, in fact the Emperor penguin consumed more fish in Summer, which is in accordance with Cherel, 2008.

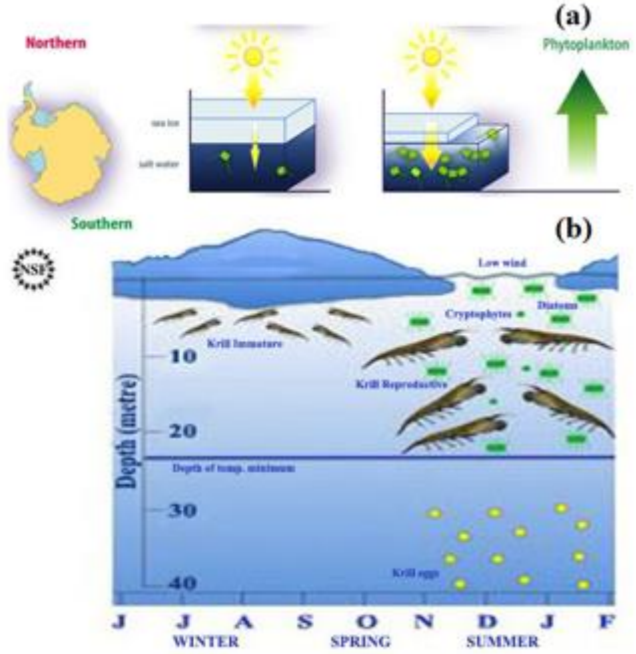


Figure 4-1: The sun effect on sea ice thickness and phytoplankton abundance (a)(Deretsky, 2007) ,
The seasonal sea ice effect on marine resources as diatoms, cryptophytes, and krill abundance (b) (Saba et al., 2014)

The Emperor penguins can dive at different deep and find resources with diverse carbon signatures (Figure 4-2; 4-3) (Davis et al., 2017; Ponganis et al., 2000; Wienecke and Robertson, 1997), corresponding different foraging habitat, in which there are various resources distribution (Fish and krill)(Tierney et al., 2008) (Figure 4-4).

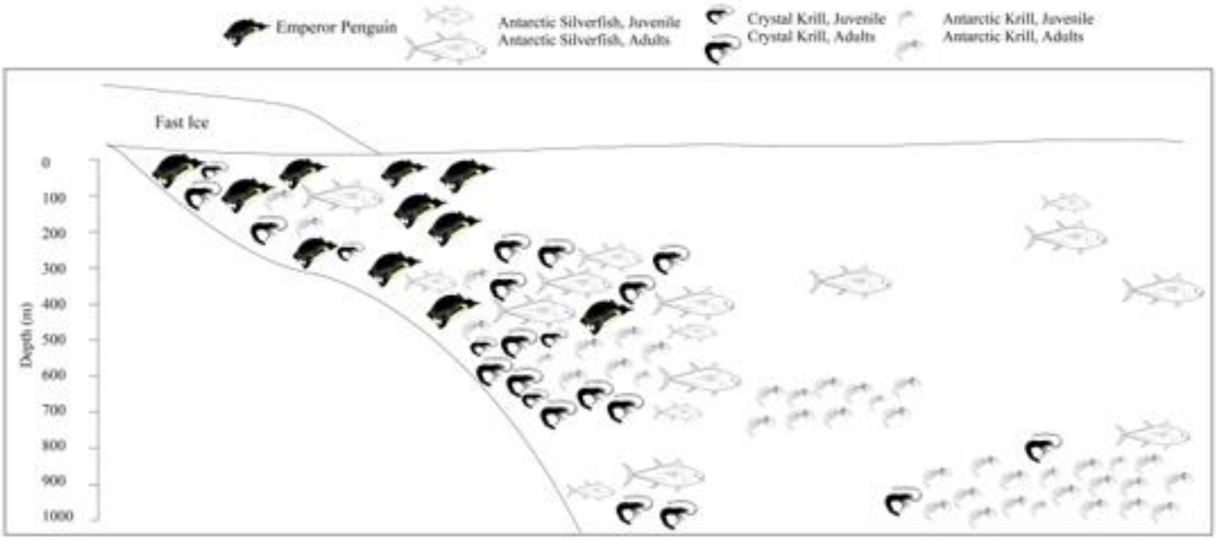


Figure 4-2: The Emperor penguin foraging behavior in the Antarctic Summer (Davis et al., 2017; Ponganis et al., 2000; Wienecke and Robertson, 1997)

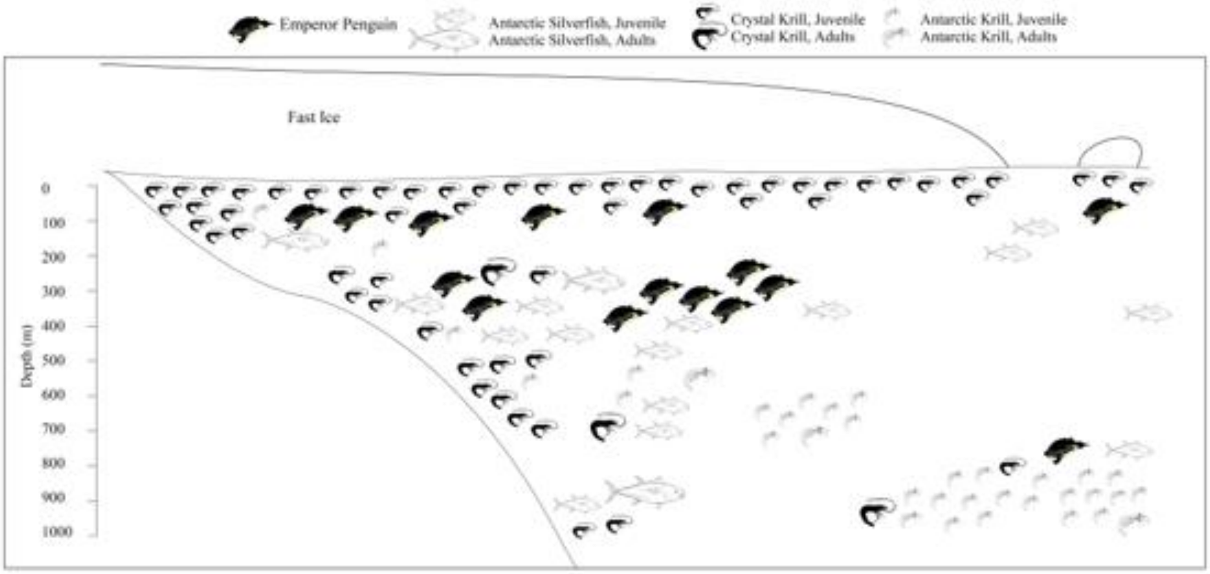
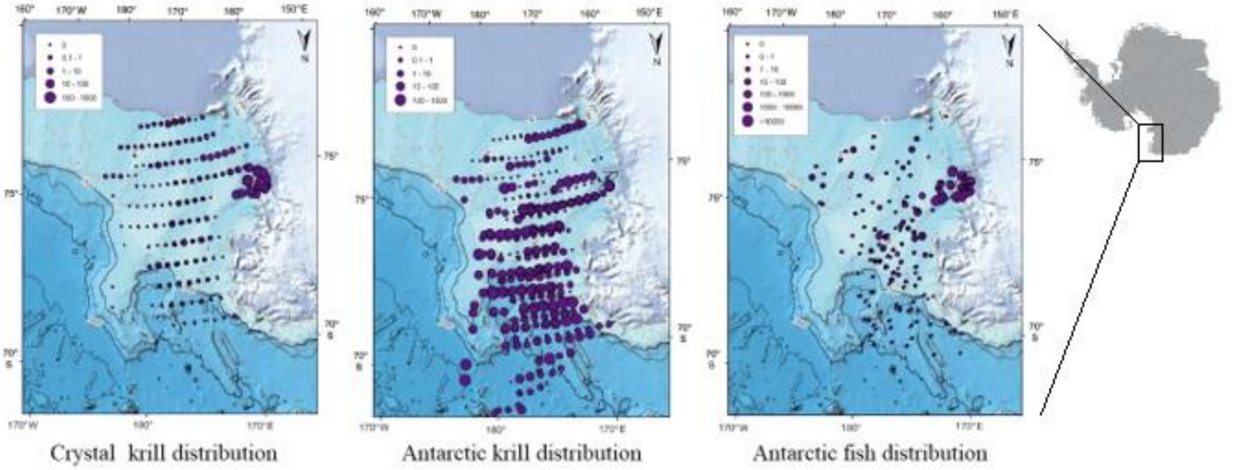


Figure 4-3: The Emperor penguin foraging behavior in the Antarctic Winter (Davis et al., 2017; Ponganis et al., 2000; Wienecke and Robertson, 1997)


Crystal krill distribution

Antarctic krill distribution

Antarctic fish distribution

Figure 4-4: The Antarctic fish, Crystal and Antarctic krill distribution (Davis et al., 2017) in our study locations

There were a decline carbon and nitrogen isotope signature in Adélie colonies from Winter to Summer. Adélie colonies forage over the continental shelf waters all the year (Clarke et al., 1998) (Figure 4-5 and 4-6). In Winter, they can find immature fish and krill (Figure 4-6) versus in Summer; there is an abundance of adult krill to consume (Figure 4-5) (Arrigo et al., 2002; Davis et al., 2017) which is in agreement with Strickland et al., (2008).

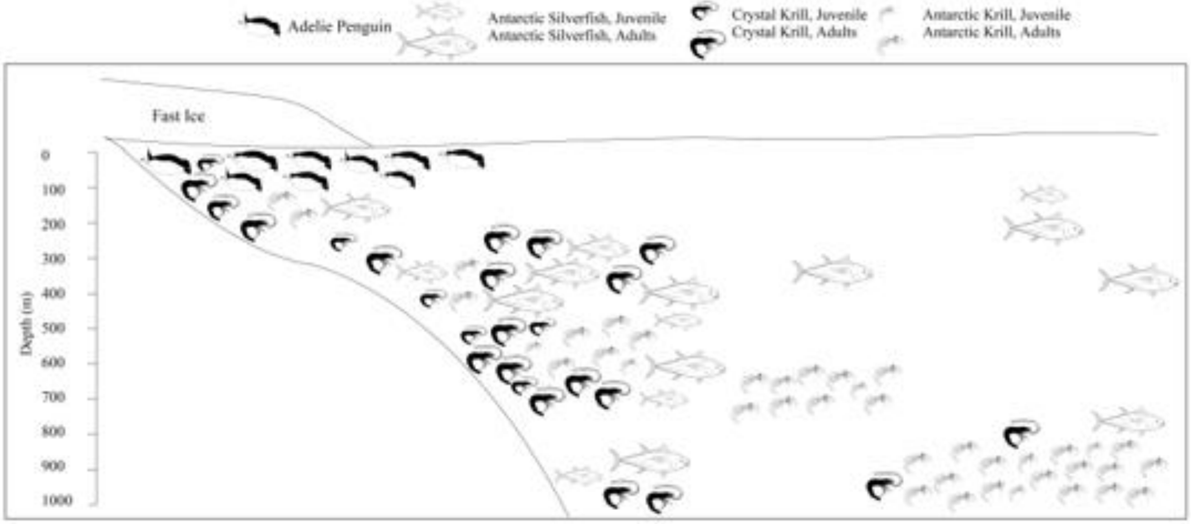


Figure 4-5: The Adélie penguin foraging behavior in the Antarctic Summer (Clarke et al., 1998; Davis et al., 2017)

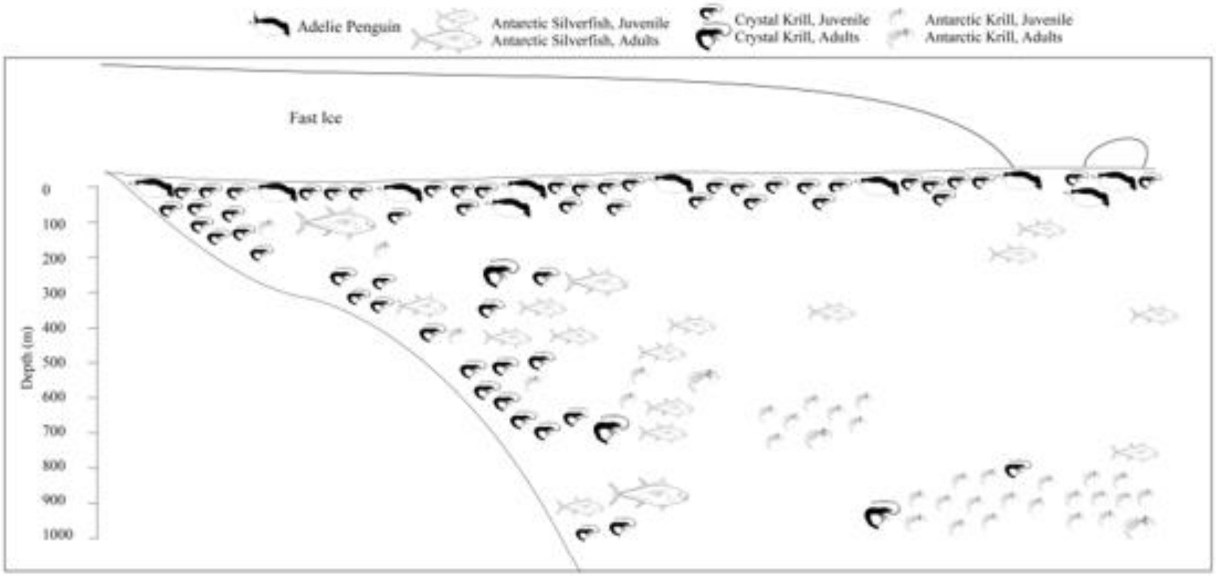


Figure 4-6: The Adélie penguin foraging behavior in the Antarctic Winter (Clarke et al., 1998; Davis et al., 2017)

In the Winter, Adèlie penguins do not have chicks, so march to the edge of sea ice and dive into open sea, where there is the abundance of fish. In the beginning of the Summer, when the sea ice does not melt completely, they dive into crevasse to feed, where is more krill. The δ^{13}C of Cape Hallett and Adélie Cove decrease from Winter to Summer. The reasons could be the passage from the sympagic dives (ice-associated) and benthic dives (deep) to the pelagic one (Cherel, 2008; Riaux-Gobin et al., 2011; Søreide et al., 2006).

The Cape Hallett and Adélie Cove colonies consumed mainly krill (Figure 4-4) both Winter and Summer. The reason could be the ice break-out time, because in Cape Hallett the ice starts to break the end of December (Gordon, 2003), instead of October (Urbini et al., 2019). So, this late icebreaking has been affected on the penguin's krill availability in Cape Hallett. Adélie Cove is rather deep bay, small, and separated from the open sea, which decrease fish availability and they consumed more krill (Majewska et al., 2016).

4.1.2. Inter-colony variation in foraging pattern during different seasons of the year between Adélie penguin's colonies

As stated before, there was a significant difference between δ^{13}C and δ^{15}N of the Adélie colonies. The δ^{13}C of Cape Hallett colony was significantly less than other Adélie penguin colonies. The reason could be due to latitude difference between these colonies. The Cape Hallett colony with higher latitude have the lower δ^{13}C values which agree with Strickland et al., (2008) and Quillfeldt et al., (2005) (Quillfeldt et al., 2005; Strickland et al., 2008). Cherel and Hobson (2007) suggested that δ^{13}C values and geographical latitude are not inevitably linked in a linear relation, but unexpected changes in carbon values appear in particular fronts where water masses change (Cherel and Hobson, 2007). Krill have a lower trophic level than fish, so by consuming more krill; also, consumer's $\delta^{15}N$ are diminished, which agrees with Strickland et al., (2008). In the Summer, with increasing latitude and ice coverage, the krill consumption increases in Adélie colonies. It may be due to the krill dependence on the ice, which results in their abundance and their availability (Malmquist, 2019).

Moving the Victoria Land Coast, changes of environmental parameters affect all the inland and marine environments and leading to substantial change in community structure and function at any trophic level and over a wide latitudinal range have the effects of potential changes in regional climate and provide a fundamental field of environmental conditions (Lee et al., 2019).

4.1.3. Inter-colony variation in foraging pattern during different seasons of the year between Adélie and Emperor penguin's colonies

There was difference between $\delta^{13}C$ and $\delta^{15}N$ of the Adélie colonies and Emperor colony. The low $\delta^{13}C$ values of Emperor penguins indicate that they foraged in more neritic waters than Adélie penguins. Because Emperor penguins have shallow and deep feeding dives, versus Adèlie penguins have coastal feeding dives (Clarke et al., 1998; Wienecke and Robertson, 1997). These results are agreed with Cherel (2008).

The $\delta^{15}N$ values of Emperor penguins were higher than those of Adélie penguins in the Summer. This difference indicates that Emperor penguins mostly fed on fish, while Adélie penguins preyed primarily upon krill. Our results, which agrees with Cherel (2008), also confirmed that Adélie and Emperor penguins are in a different trophic level ($\delta^{15}N$ of Emperor penguins=10.27±0.72; $\delta^{15}N$ of Adèlie penguins=7.49±1.36).

4.2. Compare the spatial and temporal penguin diet changes over two consecutive breeding and moulting periods

4.2.1. Diet composition of Emperor penguins over two consecutives breeding and moulting periods

As shown in the results section, there was reduction in $\delta^{13}C$ and $\delta^{15}N$ of Emperor penguin pre-moulting (Summer) from low ice to high ice year, which indicate that they consume more krill in the year with high ice, but they consumed mainly fish over two years. According Fretwell and Trathan's (2019), Ludescher et al. (2019), Wang et al. (2019), and Meehl et al. (2019) findings, the year 2016 with the lowest atmospheric pressure, the highest wind speed and temperature over the past thirty years, had the least expansion of ice (Fretwell and Trathan, 2019; Ludescher et al., 2019; Meehl et al., 2019; Wang et al., 2019).

In the pre-breeding season of Emperor penguins, there was an increment in the $\delta^{13}C$ values from the year 2016-17 to 2017-18, which explain that they passed from sympagic and benthic dives in 2016 to pelagic dives in the year 2017 (Cherel, 2008). Emperor penguins in the Antarctic Winter, foraged majorly on fish over two years pre-breeding season (Winter).

4.2.2. Diet composition of Adélie penguins over two consecutives breeding and moulting periods

The δ^{13}C and δ^{15}N of Adèlie colonies pre-moulting decreased from the low ice year to the high ice year. Fretwell and Trathan (2019), Ludescher et al. (2019), and Meehl et al. (2019) showed that in 2016 there were the maximum temperature and winds, and minimum sea ice expanding over thirty years (Fretwell and Trathan, 2019; Ludescher et al., 2019; Meehl et al., 2019). These conditions make the environment suitable for decreasing krill abundance than 2017 (Davis et al., 2017) (Figure 4-7; 4-18).

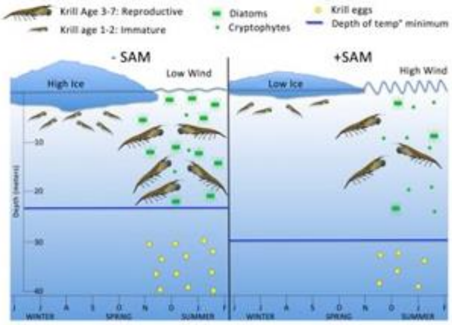


Figure 4-7: The krill abundance differences in two different weather conditions (Malmquist, 2019)

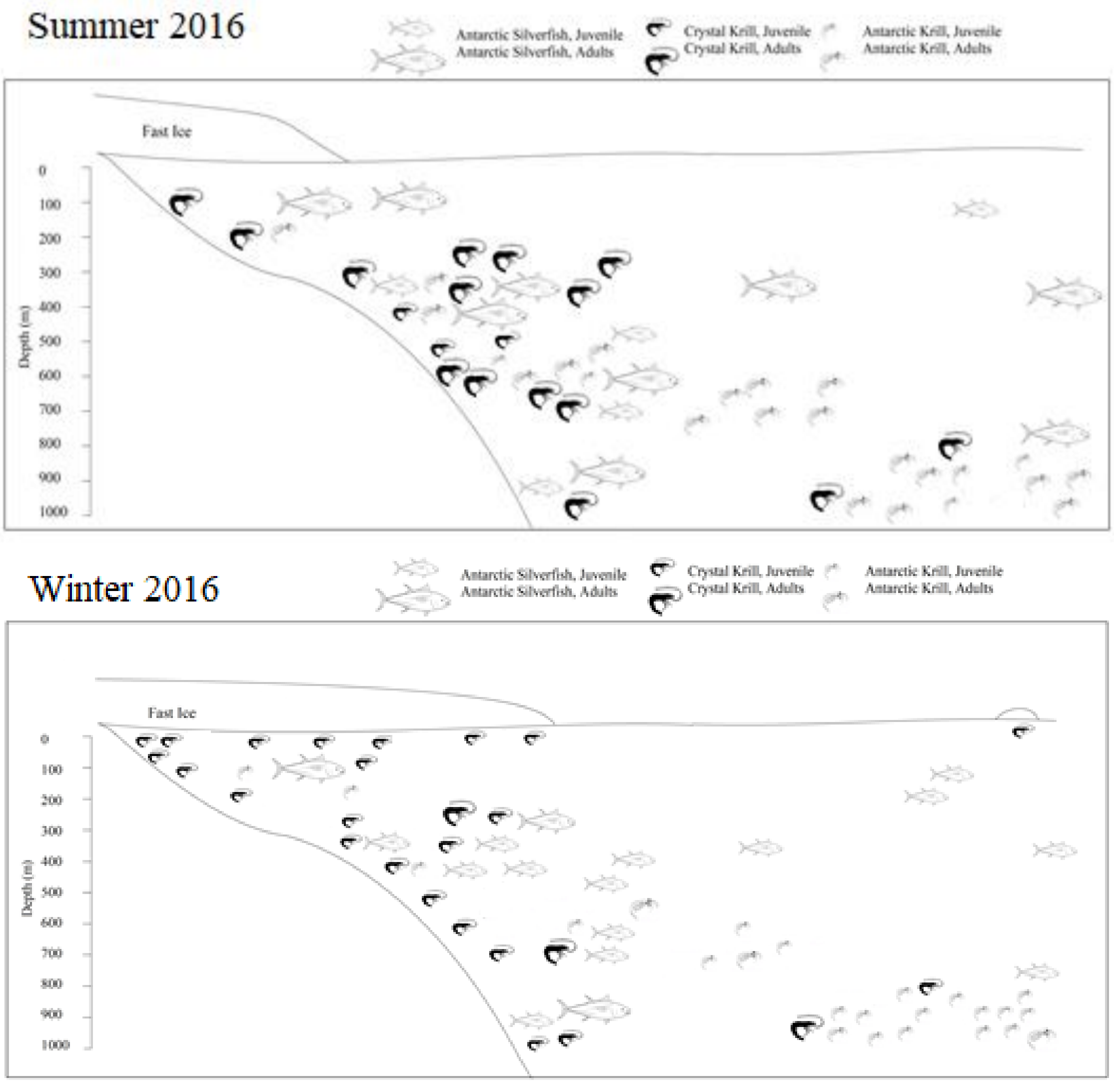
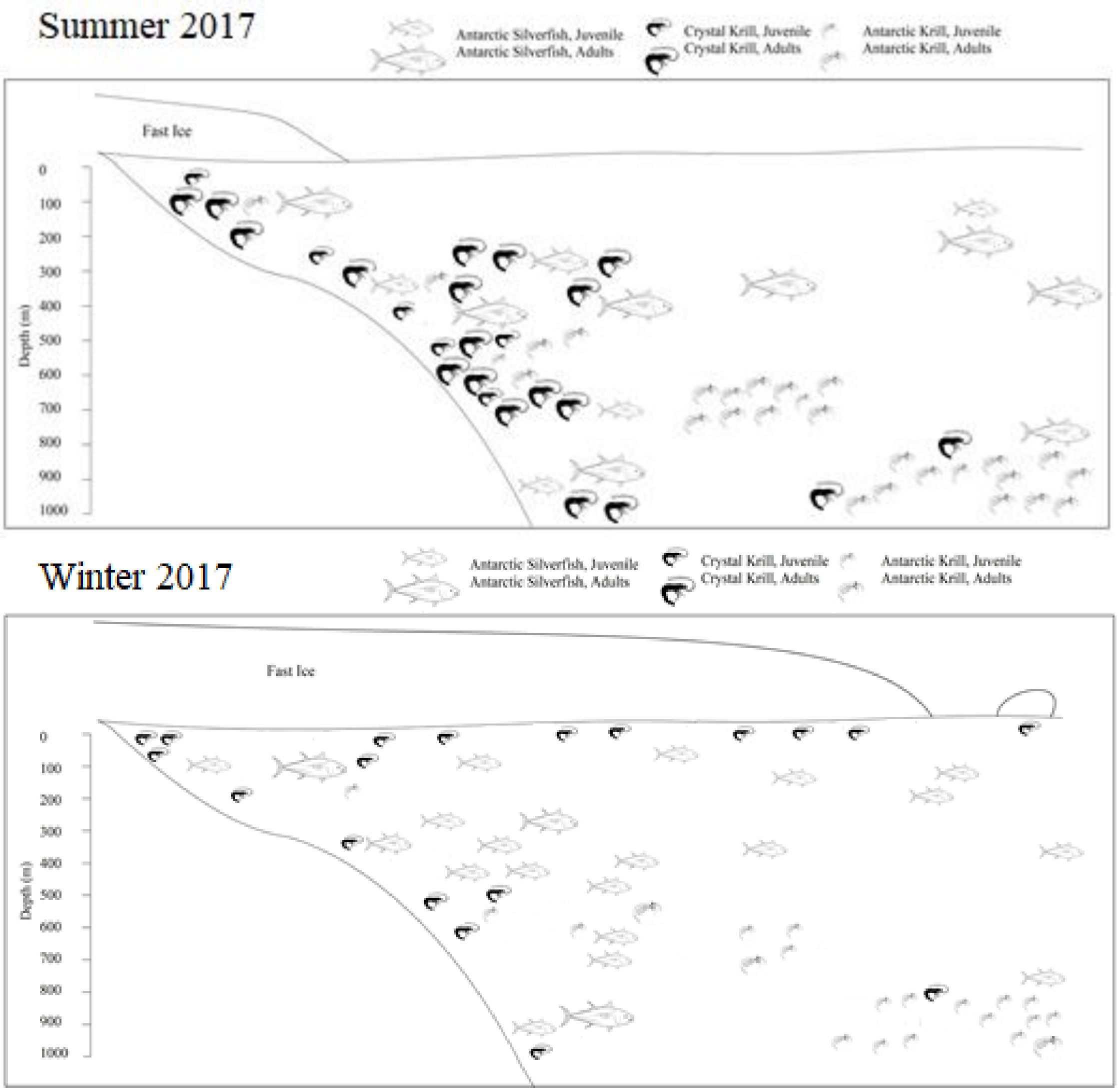


Figure 4-8: Krill and fish distribution in Antarctic Summer and Winter in two consequence year (Davis et al., 2017; DeVries and Eastman, 1981; Malmquist, 2019; White and Piatkowski, 1993)



Continuing Figure 4-8: Krill and fish distribution in Antarctic Summer and Winter in two consequence year (Davis et al., 2017; DeVries and Eastman, 1981; Malmquist, 2019; White and Piatkowski, 1993)

In the breeding season (Antarctic Summer), with increasing latitude and the ice expansion, increased krill consumption in the year 2017, like the year 2016. The principal diet consumption in all colonies were krill over two years. The Inexpressible Island was the first colony, which became ice free and had the minimum of krill consumption in two consecutive low and high ice years. Compare two different years with various ice persistence, in two periods; Winter when Adèlie penguins' diet depends on open water ecosystem and Summer when their diet depends on coastal ecosystem, allow us to determine the relationship between their diet and the ice persistence.

It is the first time that a quantitative relationship is being produced which links the sea ice to krill consumption. The coefficients obtained (intercept and slope) are useful to make predictions on the effect of future variations of sea ice on the trophic ecology of Adèlie penguins and for future monitoring in the protected area of the Ross Sea.

CHAPTER 5

CONCLUSION

5.1. Conclusion

In this study, I found that the sea ice persistence is a significant driver of predator-prey interaction among penguins and krill, two key species of the Antarctic food web. The increase in krill consumption observed over space, seasons, and among two consecutive years can be associated to increased sea ice persistence, which is strongly influenced by global warming.

Adèlie penguins were characterised by a higher trophic plasticity than Emperor penguins, hence representing potential useful sentinels of climate change. In turn, Emperor penguins occupied a higher trophic position, which implies a higher vulnerability to perturbations (i.e. lower resilience due to a longer food chain).

The sea ice represents a fundamental structural component for the Emperor penguins. In fact, this species lay on the fast ice and the pack ice dynamic variations can lead to breeding failure of this fundamental predator.

For the first time, I obtained a quantitative description of the effect of sea ice coverage duration (in days) on the consumption of fish and krill by penguins. Observed coefficients will be useful to model expected changes in penguins feeding choices in response to changing sea ice conditions. The knowledge of the Victoria land and the Ross Sea is limited and thus incomplete, spatial whether temporal, so our results could be fundamental to understand the biogeography of this region, and provided valuable information on the foraging ecology, and also, some attempts to understand the impacts of climatic change on the Antarctic. The extensive isotopic sampling of Adèlie penguin colonies in the Ross Sea marine protected area allowed us to obtain up to date baseline information useful for future monitoring.

Climate change by altering the sea ice persistence, extension, and seasonality could be affected on penguin fitness, modify their trophic ecology and reproductive habitat. This can alter the delicate Antarctic ecosystem balance.

Appendix

Table 1: Tukey's HSD test of δ^{13}C, and δ^{15}N in different times of Emperor and Adélie penguin colonies

Colony					May 16	Nov. 16	Dec. 16	Jan. 16	
Emperor Penguins	Cape Washington	δ^{13}C	Subset	1	-29.06	-28.65			
				2			-27.06		
				3				-24.06	
					May 16	Nov. 16	Jan. 16	Dec. 16	
		δ^{15}N	Subset	1	8.75	9.58			
				2		9.58	10.27		
				3			10.27	10.65	
					Nov. 16	Dec. 16	Jan. 17	Apr. 16	
Adélie Penguins	Inexpressible Island	δ^{13}C	Subset	1	-28.60				
				2		-27.37	-26.76		
				3				-23.66	
					Dec. 16	Jan. 17	Nov. 16	Apr. 16	
		δ^{15}N	Subset	1	4.92	6.63			
				2		6.63	7.26	8.56	
					Nov. 16	Oct. 16	Jan. 17	Dec. 16	Apr. 16
	Adélie Cove	δ^{13}C	Subset	1	-28.57	-27.58			
				2		-27.58	-26.78	-26.72	
				3					-24.92
					Oct. 16	Apr. 16	Jan. 17	Dec. 16	Nov. 16
		δ^{15}N	Subset	1	4.83	6.99	7.36		
				2		6.99	7.36	8.03	8.32
					Nov. 16	Oct. 16	Dec. 16	Jan. 17	Apr. 16
	Edmonson Point	δ^{13}C	Subset	1	-28.48				
				2		-27.50	-27.28		
				3			-27.28	-26.44	
				4					-22.01
					Oct. 16	Jan. 17	Nov. 16	Dec. 16	Apr. 16
		δ^{15}N	Subset	1	5.34	6.48			
				2			7.90	8.10	
				3					11.23
					Feb. 17	Dec. 16	Oct. 16	Apr. 16	
	Cape Hallett	δ^{13}C	Subset	1	-28.33	-27.77	-27.76		
				2				-24.38	
		δ^{15}N		1	4.60	4.88	5.08	5.87	

* The values in the same subset mean there are NOT significant differences.

Table 2: Tukey's HSD test of $\delta^{13}C$, and $\delta^{15}N$ between Emperor and Adélie penguin colonies

Season	Colony			Cape Hallett	Cape Washington	Inexpressible Island	Adélie Cove	Edmonson Point
Summer	δ13C	Subset	1	-28.14	-28.04			
			2			-27.17	-27.07	-27.02
				Cape Hallett	Inexpressible Island	Edmonson Point	Adélie Cove	Cape Washington
	$\delta^{15}N$	Subset	1	4.69				
			2		6.46	7.13		
			3			7.13	7.64	
			4					9.98
				Cape Washington	Adélie Cove	Cape Hallett	Inexpressible Island	Edmonson Point
Winter	$\delta^{13}C$	Subset	1	-29.06				
			2		-24.92	-24.38	-23.65	
			3				-23.65	-22.01
				Cape Hallett	Adélie Cove	Inexpressible Island	Cape Washington	Edmonson Point
	$\delta^{15}N$	Subset	1	5.87	6.99			
			2		6.99	8.55	8.74	
			3					11.23

* The values in the same subset mean there are NOT significant differences.

Table 3: Stable Isotopes signature, Ratio, and percentage of Carbon and Nitrogen of various tissues over two consecutive pre-breeding and pre-moulting periods in Emperor penguins

Site	Date	Tissue	$\delta^{13}C$ Mean±SD	$\delta^{15}N$ Mean±SD	%C Mean±SD	%N Mean±SD	C/N Mean±SD
Cape Washington	Jan. 2016 (Summer)	Feathers	-24.06±0.34	10.27±0.72	48.52±0.92	15.69±0.36	3.09±0.11
	May 2016 (Winter)	Shell membrane	-29.56±0.90	8.24±1.16	47.50±5.14	11.92±1.93	4.11±1.05
	Nov. 2016 (Spring)	Faeces	-29.17±0.57	9.59±1.36	39.36±7.02	14.79±1.88	2.70±0.62
	Dec. 2016 (Summer)	Faeces	-28.08±0.66	9.57±1.40	43.37±4.81	12.57±4.99	4.06±1.91
	Prior Jan. 2017 (Summer)	Faeces	-27.07±0.78	10.65±0.63	37.38±5.93	9.68±3.64	4.27±1.31
	Jan. 2017 (Summer)	Feathers	-24.68±0.79	8.89±1.25	48.41±1.76	15.74±0.55	3.08±0.04
	May 2017 (Winter)	Shell membrane	-25.48±1.18	9.33±0.62	42.73±4.44	12.65±1.60	3.41±0.47
	Nov. 2017 (Spring)	Faeces	-28.47±1.16	8.91±1.20	40.35±7.55	9.82±4.73	4.87±1.93
	Dec. 2017 (Summer)	Faeces	-25.51±1.02	8.35±1.72	41.46±6.87	9.81±2.98	4.65±1.62
	-	Krill	-25.75±0.07	6.32±1.15	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-

Table 4: Stable Isotopes signature, Ratio, and percentage of Carbon and Nitrogen of various tissues over two consecutive pre-breeding and pre-moulting periods in Adèlie penguins

Site	Date	Tissue	δ^{13}C Mean±SD	δ^{15}N Mean±SD	%C Mean±SD	%N Mean±SD	C/N Mean±SD
Adélie Cove	Late Mar.-Prior Apr. 2016 (Winter)	Feather	-24.92±0.1	6.99±0.1	46.05	14.82	3.10
	Late Oct. 2016 (Spring)	Shell membrane	-27.48±0.32	5.33±0.85	44.37±1.94	14.04±2.24	3.23±0.47
	Nov. 2016 (Spring)	Faeces	-28.57±0.74	8.32±1.43	35.54±8.27	9.72±2.90	3.93±1.43
	Dec. 2016 (Summer)	Faeces	-26.72±1.01	8.03±2.46	33.99±5.61	6.69±2.37	5.68±2.45
	Jan. 2017 (Summer)	Faeces	-26.78±0.76	7.36±1.92	41.72±5.67	8.72±2.02	5.01±1.21
	Late Mar.-Prior Apr. 2017 (Winter)	Feather	-21.88±3.21	6.48±1.92	48.07±0.54	15.45±0.01	3.11±0.03
	Late Oct. 2017 (Spring)	Shell membrane	-26.10±1.15	6.90±0.65	44.84±3.95	11.65±1.24	3.89±0.59
	Nov. 2017 (Spring)	Faeces	-28.72±0.95	6.32±1.17	38.86±7.14	8.91±3.05	4.67±1.16
	Dec. 2017 (Summer)	Faeces	-27.46±1.55	6.69±2.70	41.80±5.95	11.07±4.07	4.33±1.75
	Jan. 2018 (Summer)	Faeces	-26.64±0.76	4.28±2.11	37.19±5.72	8.10±3.24	4.95±1.13
	-	Krill	-26.79±1.39	5.30±0.27	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-
Cape Hallett	Late Mar.-Prior Apr. 2016 (Winter)	Feather	-24.38±0.44	5.87±0.75	48.54±1.70	15.56±0.10	3.11±0.11
	Late Oct. 2016 (Spring)	Shell membrane	-27.72±0.66	5.24±0.60	45.01±0.84	12.84±0.84	3.51±0.27
	Dec. 2016 (Summer)	Faeces	-27.77±0.69	4.88±1.84	39.92±6.74	10.72±3.69	3.97±0.93
	Feb. 2017 (Summer)	Faeces	-28.45±1.03	4.59±1.97	38.45±3.98	9.04±2.62	4.54±1.15
	Late Mar.-Prior Apr. 2017 (Winter)	Feather	-25.13±0.001	5.73±0.004	48.29±0.001	15.39±0.00	3.13±0.004
	Late Oct. 2017 (Spring)	Shell membrane	-25.79±0.82	5.78±0.86	43.87±6.44	11.83±2.42	3.77±0.42
	Dec. 2017 (Summer)	Faeces	-28.47±0.99	4.12±1.49	35.86±8.52	9.41±1.92	3.89±0.91
	Jan. 2018 (Summer)	Faeces	-28.73±0.41	3.08±0.77	37.73±2.09	10.04±1.44	3.84±0.63
	-	Krill	-28.08±0.95	5.56±0.32	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-
Edmonson Point	Late Mar.-Prior Apr. 2016 (Winter)	Feather	-22.12±0.53	11.23±1.01	47.72±0.95	15.51±0.39	3.08±0.03
	Late Oct. 2016 (Spring)	Shell membrane	-27.47±1.14	5.89±0.85	47.07±2.64	13.18±0.60	3.57±0.16
	Late Nov. 2016	Faeces	-28.48±1.10	7.90±1.54	37.19±7.56	11.46±3.63	3.51±1.25
	Prior Dec. 2016 (Summer)	Faeces	-27.28±0.90	8.10±1.07	37.97±6.45	13.10±4.24	3.40±1.77
	Late Jan. 2017 (Summer)	Faeces	-26.44±0.78	6.48±1.48	37.50±6.24	8.42±2.30	4.75±1.40
	Late Mar.-Prior Apr. 2017 (Winter)	Feather	-23.85±1.56	7.66±1.51	48.04±1.39	15.66±0.40	3.06±0.01
	Late Oct. 2017 (Spring)	Shell membrane	-25.24±0.05	7.84±0.46	43.91±6.77	12.67±2.51	3.48±0.19
	Nov. 2017 (Spring)	Faeces	-28.31±0.96	6.26±1.48	36.56±5.19	9.96±4.05	5.01±2.45
	Dec. 2017 (Summer)	Faeces	-25.44±1.59	7.52±2.36	32.10±7.05	12.40±6.04	3.23±1.66
	Jan. 2018 (Summer)	Faeces	-25.92±0.31	5.47±1.71	35.63±3.93	8.76±1.87	4.20±0.79
	-	Krill	-25.75±0.07	6.32±1.15	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-
Inexpressible Island	Late Mar.-Prior Apr. 2016 (Winter)	Feather	-23.66±1.48	8.56±1.47	48.63±0.86	15.81±0.26	3.08±0.03
	Late Nov. 2016 (Spring)	Faeces	-28.61±0.69	7.26±0.94	42.13±3.43	9.27±2.61	4.94±1.55
	Prior Dec. 2016 (Summer)	Faeces	-27.38±1.30	4.92±2.88	40.22±3.38	7.89±1.37	5.23±0.90
	Late Jan. 2017 (Summer)	Faeces	-26.76±0.63	6.63±1.79	42.33±6.50	8.99±2.56	5.06±1.58
	Late Mar.-Prior Apr. 2017 (Winter)	Feather	-25.84±0.18	6.04±0.77	49.09±0.001	19.90±5.53	3.06±0.01
	Nov. 2017 (Spring)	Faeces	-28.09±0.61	4.90±1.12	34.13±7.22	6.35±1.58	5.58±1.38
	Jan. 2018 (Summer)	Faeces	-27.11±1.08	5.23±1.63	40.17±3.87	8.68±1.75	4.79±0.96
	-	Krill	-28.64±1.17	4.84±0.66	-	-	-
	-	Fish	-24.7±1.0	10.6±1.0	-	-	-

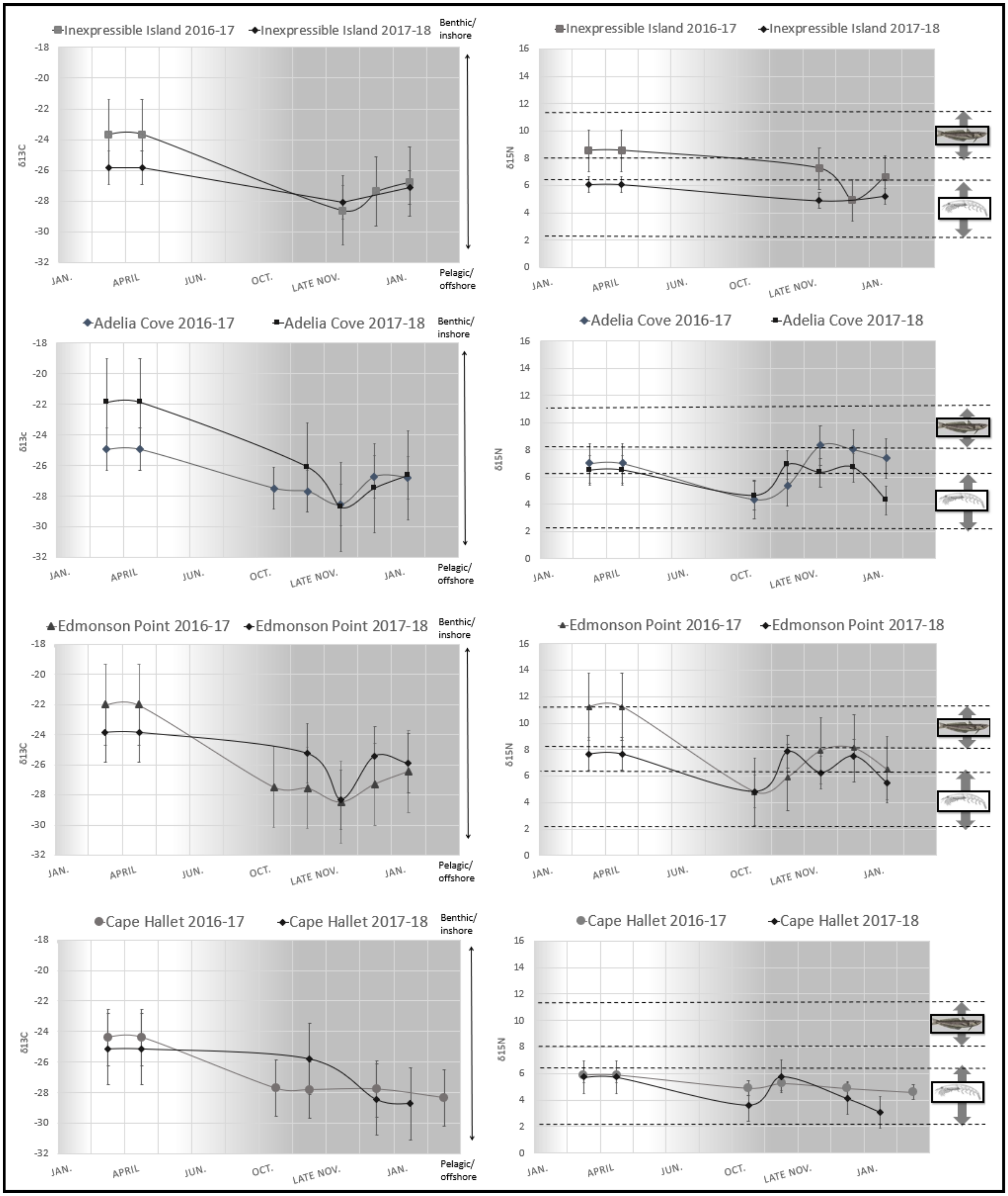


Figure 1: Carbon (a) and Nitrogen (b) stable isotopes of different Adélie penguin colonies over two consecutive breeding and moulting periods

* The white and gray part of the field represent the Winter and the Summer season, respectively.

Additional Information

SPATIAL AND TEMPORAL DIET VARIABILITY OF ADÉLIE (*Pygoscelis adeliae*) AND EMPEROR (*Aptenodytes forsteri*) PENGUIN: A MULTI TISSUE STABLE ISOTOPE ANALYSIS

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Abstract

The Ross Sea is one of the most biologically productive regions of the Antarctic continent and supports large populations of upper-trophic levels such as two key meso-predators: The Emperor (*Aptenodytes forsteri*) and the Adélie penguin (*Pygoscelis adeliae*). Despite the several studies about these species, little is known about their foraging ecology and habitat use during the entire annual cycle. Research into foraging ecology and diets of Antarctic penguins can provide vital information about their vulnerability to ecological pressures and help explain declines in certain species or populations. We investigate spatial and temporal variation in the diet of Adélie and Emperor penguins of five colonies in the Ross Sea. Carbon $\delta^{13}C$ and Nitrogen $\delta^{15}N$ Stable Isotope Analysis (SIA) analysis of feathers, eggshell membrane and faeces was used to obtain diet information during different penguin life phases. First, we observe that, the trophic position of Emperor penguins was higher than Adélie during the entire year. In fact, the estimated contribution of fish to Emperor diet range from approximately 70% in winter to approximately 90% during the Austral summer. Conversely Adélie penguin tends to feed more krill than fish, in particular during the summer. Moreover, for Adelia penguin, we observe an inter-colony diet variation. In particular, the estimated contribution of krill increased latitudinally, moving from south to north. Our results emphasize the high trophic plasticity of Adélie penguin. On the contrary, Emperor penguin appear to be fish specialist consumer. By comparing summer and winter diet we highlight aspects of the feeding habits of Antarctic predators that were not previously studied. These findings have several important implications to understand how these species can react to resources variation due to climate change or anthropic overexploitation. Moreover, knowledge of spatial and temporal variability in their diet can contribute to plan conservation and management actions in the Southern Ocean.

Introduction

The Ross Sea is one of the most biologically productive regions of the Antarctic continent (Arrigo et al., 2002), characterized by high biodiversity (Laffoley et al., 2019) and for these features has recently been designed as the largest protected biodiversity hotspot area in the world (Ballard et al., 2012). Among different species, this region supports large populations of upper-trophic levels such as two key meso-predators: The Emperor penguin (*Aptenodytes forsteri*) and the Adélie penguin (*Pygoscelis adeliae*). The Emperor penguin lives and reproduces on the pack ice, has a diet based mainly on fish, to which are added smaller proportions of crustaceans and, in specific areas, also cephalopods (Kirkwood and Robertson, 1997; Cherel, 2008). The trophic resources of the smaller Adelia penguin, a species that spends part of its life cycle on ice but reproduces on the mainland, are mainly based on crustaceans belonging to the genus *Euphausia* and, to a lesser extent, on fish (Ainley, 2002; Ainley et al. 2003), in particular the Antarctic silverfish (*Pleurogramma antarctica*). In the Ross Sea the climate variations are modifying the dynamics of sea ice. These changes are leading to a change in the availability of the main trophic resources used by the Antarctic predators that are krill and fish (Atkinson et al., 2004; Reiss et al. 2008). Therefore, knowledge of the trophic ecology at different seasons of Adelie and Emperor penguins is crucial to better understand the responses of these krill and fish-dependent predators to marine resource variability (Juáres et al., 2016). Moreover, since these species feed offshore during winter, where human fishing activities are present, quantifying their resources use provides important information for establishing effective conservation measures (Cherel and Hobson 2007).

Although these species are deeply investigated, due to their notable ecological and conservation importance, studies related to their trophic ecology are limited to specific periods of the year (Cherel, 2008). In fact, for the Adelia penguin there is a paucity of diet information in spring, and for the Emperor penguin little is known about its food ecology in summer (Cherel 2008). The availability of data about these predators trophic ecology in specific periods of the year is constrained by their life cycle (Juáres et al., 2016), in fact these species make long migrations in different phase of their life cycle. On a one hand, the Adelia penguins in spring, after having completed the moult, return to the reproductive areas. On the other hand, in summer the Emperor penguins move to the edge of the pack and feed before the mating period. These migrations, coupled with the extreme conditions of the Antarctic environment, in particular, during the Antarctic winter, make complicated the reconstruction of these species diet outside the breeding (for the Adelia penguins) and the pre-mating (for the Emperor penguins) period and breeding areas (Cherel, 2008).

The aim of this study is to reconstruct and compare the diet of the Adelia and Emperor penguin in the Ross sea colonies along the entire annual scale. To achieve this goal, the carbon and nitrogen isotopic signatures of different biological matrices (feathers, eggs, and guano) were used, providing information relating to different phases of the life cycle of these predators. Stable isotopes are widely used in dietary studies because the isotopic ratio of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) in consumer tissues reflect in a reliable and predictable manner those of its dietary components (DeNiro & Epstein 1978, 1981; Hobson & Clark 1992a, b; Calizza et al., 2018). The predators' diet can be inferred at different times according to the sampled matrices (Hobson & Clark, 1993) because different matrices have different isotopic turnover rates (Hobson & Clark 1992a, Cherel et al. 2005a). Therefore, by sampling biological matrices at different locations, or periods of the year, it is possible to examine diet variations over both time and space (Quillfeldt et al., 2005; Polito et al., 2009; Jafari et al., 2018), acquiring crucial information to design effective management plans for the conservation of the two penguin species in the Southern Ocean.

Material and Methods

Study area and sampling procedures.

The study was carried out at various site in the coastal area of the Ross Sea, Antarctica: Inexpressible island (163◦43’02” E, 74◦54’01” S), Adélie Cove (164◦00’35” E, 74◦45’51” S), Edmonson Point (165◦05’44” E, 74◦19’32” S), and Cape Hallett (170◦13’31” E, 72◦19’13” S) for Adèlie, and Cape Washington (164◦58’02” E, 74◦37’07” S) for Emperor penguin (Fig. 1).

Inexpressible island is in Terra Nova Bay and is bordered to the West by the Nansen ice Sheet and to the East by Hells Gate and Evans Cove. This area is home to various species of seabirds, including around 24,450 nesting pairs of Adélie penguins (Harris et al., 2015). Adélie Cove, located on the coast of the Northern foothills of Terra Nova Bay, hosts a colony of Adélie penguins with approximately 11,234 reproductive pairs (Harris et al., 2015). Edmonson Point, situated in Wood Bay, at the foot of the Eastern slopes of Mount Melbourne, has an Adélie penguin colony of about 1890 breeding pairs in the period 1981 – 2012 (Harris et al., 2015). Cape Hallett, located at the northern end of the Hallett Peninsula in northern Victoria Land, host on average 42,628 reproductive pairs of Adélie penguins (Harris et al., 2015). Cape Washington, in the Northern part of Terra Nova Bay, on the slopes of Mount Melbourne house an important colony of Emperor penguins, with a population of between 13,000 and 25,000 nesting pairs.

Samples of eggs, feathers, and guano have been collected from each colony during the austral spring and summer (2016/2017). Egg tissues provide information on female diet before the breeding period (Astheimer & Grau, 1985; Emslie & Patterson, 2007; Strickland et al., 2008) that corresponds to spring for Adelia penguin and to winter for the Emperor penguin. Feathers, as they are metabolically inert after synthesis, encapsulate information about a bird's diet and foraging habitat related to the pre-moult period (Hobson 1999; Cherel et al., 2000; Figure 2). Since the two penguin species moult at different times of the year, the feathers provide information relating, respectively, to the Emperor penguin summer diet and to the Adelia penguin winter diet (Figure 2). The guano provides information on the short-term diet, indicating what the animal consumed in the days preceding the sampling (Bird et al., 2008). This biological material was used to infer information about the Adelia penguin's summer diet and for the Emperor penguin's spring diet (Figure 2).Guano samples were collected using a spatula and 15 ml Falcon tubes. Feather and unhatched eggs were collected by hand and placed in plastic bags. Moreover, regurgitation samples and fish found near the colony were collected opportunistically. All samples were stored in a refrigerate box until the transport to the Italian scientific station Mario Zucchelli (MZS). At MZS all samples were enumerated and stored at -20 °C and shipped to the laboratory of trophic ecology in Italy.

Laboratory procedures and stable isotope analysis.

Egg samples were divided into fragments and the shell membrane were separated from the eggshell using a surgical blade. Eggshell membrane and guano samples were pulverized using a ball mill (Mini-Mill Frits Pulverisette 23: Fritsh Instruments, Idar-Oberstein, Germany).

In order to remove contaminants and oils, feathers were cleaned using a 2:1 chloroform: methanol solution, rinsed with distilled water, air-dried and then cut into small fragments water (Graves et al., 2018).

Aliquots of 0.50-1 mg for the feathers and shell membrane and 1-1.5 mg for guano were pressed into tin capsules for the Stable Isotopes Analysis. Samples were analyzed in two replicates using an Elementar Vario Micro-Cube elemental analyzer (Elementar Analysensysteme GmbH, Germany) coupled with an IsoPrime100 continuous flow mass spectrometer (Isoprime Ltd., Cheadle Hulme, UK), at the Trophic Ecology laboratory (Dept. of Environmental Biology, Sapienza University of Rome). Carbon (C) and Nitrogen (N) isotopic signatures were expressed in δ units ($\delta^{13}C$; $\delta^{15}N$) as parts per-thousand (‰) deviations from international standards: Vienna Pee Dee Belemnite (PDB) for C and atmospheric N_2 for N. Isotopic ratios were computed according to the equation:

δX (‰) = [(Rsample − Rstandard)/Rstandard] × 10^3, where X is the Carbon or Nitrogen isotope and R is the heavy-to-light isotope ratio of the respective element ($^{13}C/^{12}C$; $^{15}N/^{14}N$). Finally, $\delta^{13}C$ values were corrected for lipid content based on the C/N ratio of each sample in accordance with Post et al. (2007). The internal laboratory standard used was IAEA600 Caffeine $\delta^{13}C$ and $\delta^{15}N$ measurement errors were typically smaller than ±0.05‰.

Data Analysis

All data analysis was conducted in the Statistical Package for the Social Science (SPSS) software with the analysis of variance (ANOVA). The R package SIMMR (Stable Isotope Mixing Models in R, Parnel, 2019) was used to estimate the relative proportion of trophic resource (mixing model) to the penguin diet.

Since the different types of biological material analysed present differences in the Trophic Enrichment Factor (i.e. TEF, McCutchan et al., 2003) the following TEFs were considered for the Adelia penguin: δ^{15}N-TEF 3,50‰ (feathers) and 4,33‰ (shell membrane) and δ^{13}C -TEF 3,5‰ (feathers), and 2,83‰ (shell membrane) while for the Emperor penguin: δ^{13}C -TEF 0,14‰ (feathers) and 2,83‰ (shell membrane) and δ^{15}N-TEF 3,5‰ (feathers) and 4,33‰ (shell membrane) (Cherel et al., 2005; Polito et al., 2009, 2011).

For the guano the fractionation was considered equal to 0 for both isotopes (Bird et al.,2008). The guano consists largely of organic material not metabolized by the organism, therefore the phenomenon of fractionation for this type of biological matrix is reduced or absent because there are no differences in isotopic signatures between the ingested and excreted organic substances (Mizutani et al., 1992; Bird et al., 2008). To reconstruct the diet of these two species using the mixing models, the isotopic values of the krill belonging to the genus *Euphausia* and for the fish the values of the Antarctic silverfish (*Pleuragramma antarctica*). For each colony, different krill isotopic values were used, obtained from the analysis of the bolus from each colony, while for the Antarctic silverfish (*Pleuragramma antarctica*), only four samples were found at Edmonson Point, and the mean value was used for all the colonies (Table 1). This can be justified when considering that isotopic signatures in *Pleuragramma antarctica* have been shown to not vary significantly among different areas of the Antarctic Ocean (Hodum & Hobson, 2000; Cherel, 2008; Giraldo et al., 2011; Polito et al., 2011; Lorenzini et al., 2010).

Results

Both δ^{13}C and δ^{15}N values of Emperor penguins were lower in winter and higher and similar in spring and summer (Tables 1 and 2, Fig. 3). Fish was consumed more than krill in all seasons, and the contribution of fish to the diet of Emperor penguins increased from 64.2% in winter to 95.1% in summer (Table 3).

For Adelie penguins, δ^{13}C values varied among seasons in all colonies, being generally higher in winter (Tables 1 and 2, Figure 4). δ^{15}N values varied among seasons in all colonies except Cape Hallet (Table 1). However, observed seasonal variations differed between colonies (Table 2 and Fig. 3). Fish consumption was higher in winter and extremely low in spring (Table 3). In summer, fish consumption decreased with latitude, varying from 47% at Inexpressible Island (in the polynya area) to 3.3% at Cape Hallett, the northernmost colony (Table 3). Both in summer and in winter, differences of δ^{13}C and δ^{15}N among Adelia colonies were observed (Table 4).

When comparing the two species, Emperor penguins had higher δ^{15}N values compared to all the Adélie penguin colonies both in summer and winter (Table 1, one-way ANOVA, F_{summer} 39.83, $F_{winter} = 11.84$, $p < 0.0001$ for both seasons). δ^{13}C values were higher in Emperor than in Adelie penguins in summer, while the opposite was observed in winter (one-way ANOVA $F_{summer} = 11.17$, $F_{winter}= 87.57$, $p< 0.0001$ for both seasons).

Discussion

Emperor and Adelie penguins represent the two bird species that inhabit the southernmost latitudes on our planet and adapted to spend their whole life in the harsh Antarctic environment (Cherel 2008). Here, their life cycle is tightly related to the seasonality of sea ice, which represents a key substrate for breeding, seasonal migrations and a key foraging habitat (Ainley et al., 1998; Lyver et al., 2009; Bluhm et al., 2010). To access open waters during the whole year, penguins may have to migrate for thousands of kilometers while following the sea ice margin (Kooyman et al., 2004; Ainley 2002; Juáres et al., 2016). This makes the study of these species extremely difficult, especially in the winter period, when darkness, sea ice extent, and low temperature imped direct observations and sample collections. As a consequence, there is a paucity of papers describing the ecology of these two species during the whole year (Cherel, 2008; Juáres et al., 2016).

In this study, thanks to the isotopic analysis of diverse biological samples, such as guano, shell membrane of eggs and feathers, we were able to describe the isotopic niches of the two penguin species and obtain information on the consumption of fish and krill (their main prey) throughout the year, including in winter. We focused on five colonies breeding along the Ross Sea coasts. This area is characterised by marked spatio-temporal variations in sea ice coverage, and it represented an exceptional laboratory to compare colonies located in areas differently affected by the persistence of sea ice during spring and summer. In addition, this area represents a biodiversity hotspot and the largest marine protected area (MPA) on our planet (Ballard et al., 2012; Laffoley et al., 2019), and the study of the trophic ecology of high level predators may provide useful information on changes occurring at lower trophic levels in the food web as a consequence of environmental changes (Juáres et al., 2016). Here, the isotopic comparison between and within species allowed us to track changes in the feeding preferences of penguins, providing up-to-date isotopic and diet baseline data useful for future comparisons within the Ross Sea MPA.

Previous studies demonstrated that isotopic variations in penguins effectively reflect changes between a fish- to a krill-based diet (Cherel, 2008, Tienrey et al., 2008; Negrete et al., 2016). For this study, we were able to obtain colony-specific samples of krill, which allowed to take into account potential isotopic variations observed across our study area (Cherel et al., 2007 a). In contrast, while samples of the Antarctic silverfish *Pleuragramma antarctica*, the main fish prey species of both Emperor and Adelie penguins, were found only in two colonies, previous studies have shown that the isotopic signatures in this species are conserved among areas and seasons (Lorenzini et al., 2010; Giraldo et al., 2011; Polito et al., 2011). Accordingly, the measured values closely matched those previously reported in literature for the study area (Hodum & Hobson, 2000; Cherel, 2008).

Our results clearly indicated spatio-temporal variations in the isotopic composition of penguin samples, plausibly reflecting changes in the relative consumption of krill and fish among species, seasons and colonies. Although the diet of the Emperor penguin was based mainly on fish (Cherel, 2008), krill consumption was also observed. Specifically, its consumption was higher in winter, and was associated to lower $\delta^{13}C$ and $\delta^{15}N$ values, indicative of a fully pelagic-based food chain and a lower trophic level occupied by penguins respectively (Cherel 2008; Tienrey et al., 2008). Such winter increase in krill consumption may be due to the greater availability of this resource during the austral winter, caused by the maximum extent of sea ice observed in this season (Nicol, 2006). Indeed, previous studies indicate higher abundances of krill in years or seasons with a higher persistence and extension of the pack ice (Nicol, 2006). In summer, the melting of the ice induces a reduction of krill and an increase of fish, whose availability as prey for penguins is linked to the presence of open sea areas (Ainley et al., 1998). The increase of fish availability, which constitutes a prey characterized by a higher energy value (Hodum & Hobson 2000; Forero et al., 2002; Ainley et al., 2003; Tierney et al., 2008), led to an increase in its consumption by the Emperor penguins. Unlike the Emperor penguin, the Adélie penguin showed the least consumption of fish during spring, while the maximum consumption was observed in winter. During the austral winter, the Adélie penguins migrate from the breeding sites to the moult areas, moving towards the edge of the pack (Juáres et al., 2016). During this phase of their life cycle, Adelia penguins that have access to open sea areas can feed on a greater quantity of fish. This increase in fish consumption, confirmed by the results of the mixing models, leads to an increase in the values of $\delta^{13}C$ and $\delta^{15}N$ in all colonies analysed. During spring this species withdraws to the continent for mating (Tierney et al., 2008). Here, the high extent of sea ice characterizing coastal areas in the Ross Sea prevents this species from reaching the open sea and penguins dive into clefts to search for food under the sea ice. In this phase, their diet is composed almost exclusively of krill, because under the ice there is a great availability of this resource that feeds on the ice algae (Spindler, 1994; Nicol 2006). In summer, melting ice allows couples, engaged in parental cares, to reach the open sea and integrate the fish into their krill-based diet (Tierney et al., 2008). Notably, the differences among the four Adélie colonies in the krill and fish consumption during the summer can be related to their geographic position. Indeed, this colonies were arranged at different latitudes and they were characterized by a different persistence of sea ice. The two southernmost colonies, being in a polynya area, had access to the open sea for the majority of the breeding season (i.e. spring and summer), with a consequent greater availability of fish. In contrast, Edmonson Point and Cape Hallett, the Northernmost colonies were affected by a longer persistence of sea ice and were characterized by a lower consumption of fish.

When comparing the two species, the higher $\delta^{15}N$ value of the Emperor penguin highlighted a more piscivorous diet compared to the Adelia, independently by the season (Volkman et al., 1980; Trivelpiece et al., 2011; Coria et al., 1995; Lynnes et al., 2004; Hinke et al., 2007; Juáres et al., 2016). Such difference was also observed between Emperor penguins and Adelie penguins from Edmonson Point, even if the two colonies were nearly 30 Km apart only, and the foraging areas of the two penguin populations plausibly overlapped.

Isotopic analyses of multiple samples suggested that Emperor and Adélie penguin had a different feeding ecology throughout the year, as expected from the different foraging behavior of the two species. Indeed, the Emperor penguins feed in depth and perform both pelagic and benthic dives (Rodary et al. 2000a; Zimmer et al., 2008) unlike the smaller Adélie penguin which forages in shallow pelagic waters (Rodary et al., 2000b; Wienecke et al., 2000). Reaching greater depths, the Emperor penguin has access to fish throughout the year. This more energetic resource, compared to krill, is therefore an effective prey for this species (Watanuki et al., 2002; Ainley et al., 1998)

Conclusion

In this study, thanks to the analysis of stable isotopes, it was possible to reconstruct the annual diet of the Adelia penguin and the Emperor in colonies that have never been analyzed isotopically. The use of different types of biological matrices has proven to be an effective tool for filling the gaps associated with the trophism of these species in periods such as winter and spring. These biological matrices, providing information relating to different periods (Jafari et al., 2018), have made it possible to obtain a complete picture of the fodder habitat of these two endemic species, characterized by a strong interest in conservation. In this study, a large trophic plasticity of Adelia penguins emerged both spatially and temporally. The strong variability of the diet of this species is linked to the dynamics of sea ice that, determining a different availability of the fundamental resources of the Antarctic Ocean, constitutes a fundamental driver of the predator-predator interaction for this species. On the contrary, the Emperor penguin, while integrating krill into its diet, is a specialized consumer of fish (Cherel, 2008), which represents the main prey throughout the year. From this emerges a minor influence of the dynamics of ice on the trophism of this species, which however constitutes a key factor in its survival, representing a fundamental substrate where to live and breed offspring. The deepening of the knowledge of the trophic ecology of these consumers represents a fundamental step to understand the mechanisms underlying the structural and functional integrity of the Antarctic ecosystem and to predict how these species will react to any changes in the availability of resources due to climate change or over-exploitation, to plan conservation and management actions in the Antarctic Ocean.

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Tables

Table 1. δ^{13}C and δ^{15}N, Ratio and percentage of Carbon and Nitrogen of different biological matrices of Emperor and Adèlie penguins at different sites.

Site	Date	Tissue	Period	δ^{13}C Mean±SD	δ^{15}N Mean±SD	%C Mean±SD	%N Mean±SD	C/N Mean±SD
Emperor Penguin								
Cape Washington	Jan. 2016	Feathers	Summer	-24.06±0.34	10.27±0.72	48.52±0.92	15.69±0.36	3.09±0.11
	May 2016	Shell membrane	Winter	-29.56±0.90	8.24±1.16	47.50±5.14	11.92±1.93	4.11±1.05
	Nov. 2016	Faeces	Spring	-29.17±0.57	9.59±1.36	39.36±7.02	14.79±1.88	2.70±0.62
	-	Krill		-25.75±0.07	6.32±1.15	-	-	-
	-	Fish		-24.70±1.0	10.60±1.0	-	-	-
Adèlie Penguin								
Inexpressible Island	Mar.-Apr. 2016	Feather	Winter	-23.66±1.48	8.56±1.47	48.63±0.86	15.81±0.26	3.08±0.03
	Dec. 2016	Faeces	Summer	-27.38±1.30	4.92±2.88	40.22±3.38	7.89±1.37	5.23±0.90
	Jan. 2017	Faeces	Summer	-26.76±0.63	6.63±1.79	42.33±6.50	8.99±2.56	5.06±1.58
	-	Krill		-28.64±1.17	4.84±0.66	-	-	-
	-	Fish		-24.70±1.0	10.60±1.0	-	-	-
Adélie Cove	Mar.-Apr. 2016	Feather	Winter	-24.92±0.1	6.99±0.1	46.05	14.82	3.10
	Oct. 2016	Shell membrane	Spring	-27.48±0.32	5.33±0.85	44.37±1.94	14.04±2.24	3.23±0.47
	Dec. 2016	Faeces	Summer	-26.72±1.01	8.03±2.46	33.99±5.61	6.69±2.37	5.68±2.45
	Jan. 2017	Faeces	Summer	-26.78±0.76	7.36±1.92	41.72±5.67	8.72±2.02	5.01±1.21
	-	Krill		-26.79±1.39	5.30±0.27	-	-	-
	-	Fish		-24.70±1.0	10.60±1.0	-	-	-
Edmonson Point	Mar.-Apr. 2016	Feather	Winter	-22.12±0.53	11.23±1.01	47.72±0.95	15.51±0.39	3.08±0.03
	Oct. 2016	Shell membrane	Spring	-27.47±1.14	5.89±0.85	47.07±2.64	13.18±0.60	3.57±0.16
	Dec. 2016	Faeces	Summer	-27.28±0.90	8.10±1.07	37.97±6.45	13.10±4.24	3.40±1.77
	Jan. 2017	Faeces	Summer	-26.44±0.78	6.48±1.48	37.50±6.24	8.42±2.30	4.75±1.40
	-	Krill		-25.75±0.07	6.32±1.15	-	-	-
	-	Fish		-24.70±1.0	10.60±1.0	-	-	-
Cape Hallett	Mar.-Apr. 2016	Feather	Winter	-24.38±0.44	5.87±0.75	48.54±1.70	15.56±0.10	3.11±0.11
	Oct. 2016	Shell membrane	Spring	-27.72±0.66	5.24±0.60	45.01±0.84	12.84±0.84	3.51±0.27
	Dec. 2016	Faeces	Summer	-27.77±0.69	4.88±1.84	39.92±6.74	10.72±3.69	3.97±0.93
	Feb. 2017	Faeces	Summer	-28.45±1.03	4.59±1.97	38.45±3.98	9.04±2.62	4.54±1.15
	-	Krill		-28.08±0.95	5.56±0.32	-	-	-
	-	Fish		-24.70±1.0	10.60±1.0	-	-	-

Table 2. Analysis of variance (ANOVA) of $\delta^{13}C$ and $\delta^{15}N$ between seasons of each penguin colony.

			$\delta^{13}C$ (‰)		**$\delta^{15}N$ (‰)**	
Species	Colony	Variable	F	p value	F	p value
Emperor Penguin	Cape Washington	Season	166.61	<0.001	9.72	<0.001
Adelia Penguin	Inexpressible Island	Season	49.35	<0.001	5.93	0.001
	Adelie Cove	Season	21.9	<0.001	8.86	<0.001
	Edmonson Point	Season	66.49	<0.001	31.27	<0.001
	Cape Hallett	Season	27.26	<0.001	0.8	0.498

Table 3. Diet composition of Emperor and Adélie colonies in different seasons of the year 2016-17

		Winter		**Spring**		**Summer**	
		Fish %	**Krill %**	**Fish %**	**Krill %**	**Fish %**	**Krill %**
Emperor Penguins	Cape Washington	64.2	35.8	81.1	18.9	95.1	4.9
Adélie Penguins	Inexpressible Island	81.4	18.6	-	-	43.4	56.6
	Adélie Cove	42.1	57.9	3	96.2	26.8	73.2
	Edmonson Point	89.4	10.6	3.1	96.9	13.2	86.8
	Cape Hallett	36.5	63.5	5	94.7	3.3	96.7

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Table 4. Analysis of variance (ANOVA) of $\delta^{13}C$, and $\delta^{15}N$ between Adélie and Emperor penguin in summer and winter.

		$\delta^{13}C$ (‰)		**$\delta^{15}N$ (‰)**	
Period	Variable	F	p value	F	p value
Summer	Site	23.05	$6.26*10^{-13}$	16.13	$1.81*10^{-9}$
Winter	Site	7.35	$3*10^{-3}$	20.22	$2.33*10^{-5}$

Figure 1. Sampling area. Latitude information from Cavalieri and Parkinson (2008) and Harris et al. (2015)

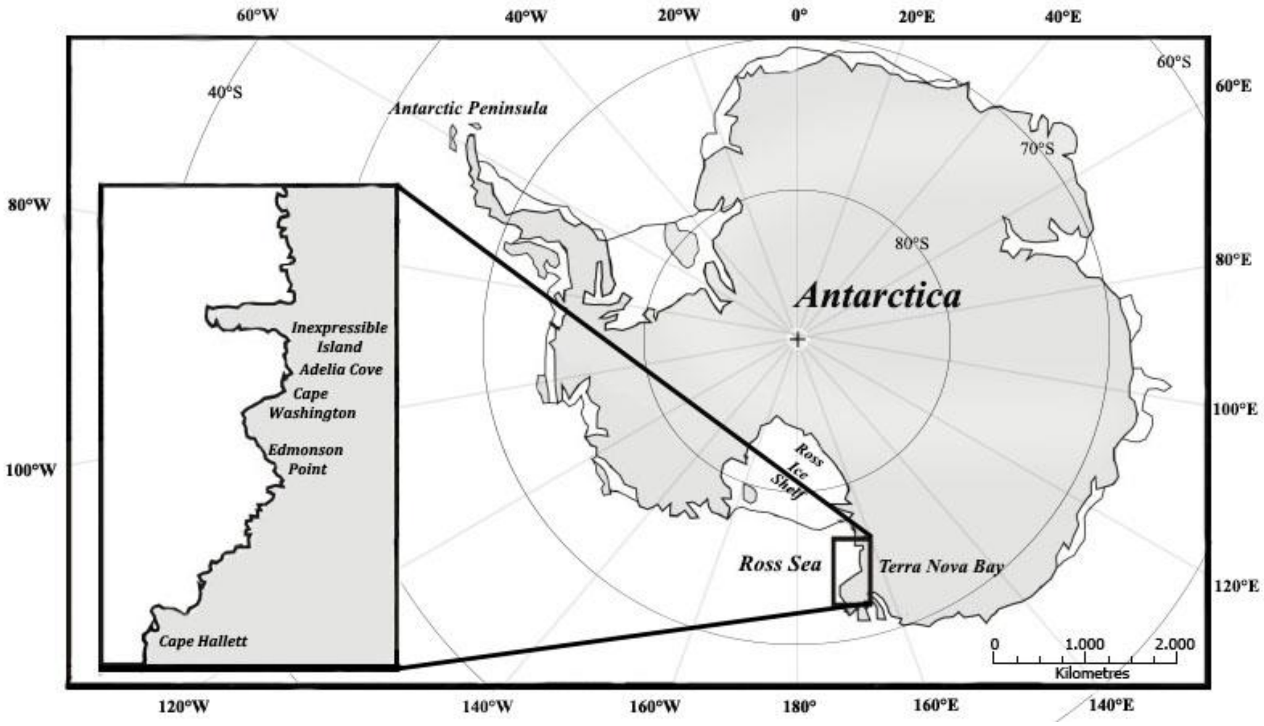


Figure 2. Principal life cycle periods of Adèlie and Emperor penguin and corresponding biological matrices samples.

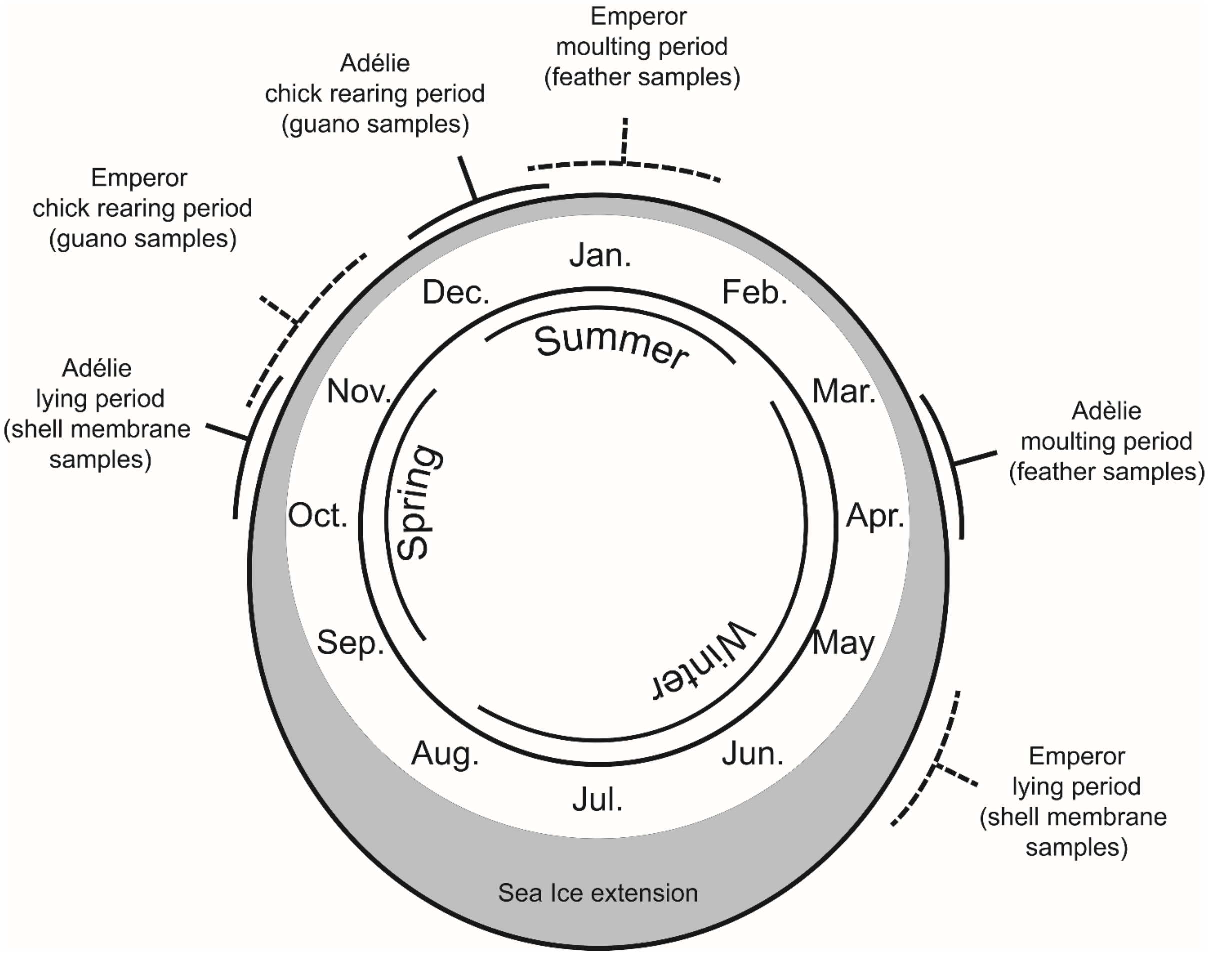


Figure 3. Isotopic biplot of Emperor penguin stable isotopes during winter (shell membrane, dark grey squares) and during spring (feces, light gray triangles) and their principal trophic resources (fish, black circle and krill, black square).

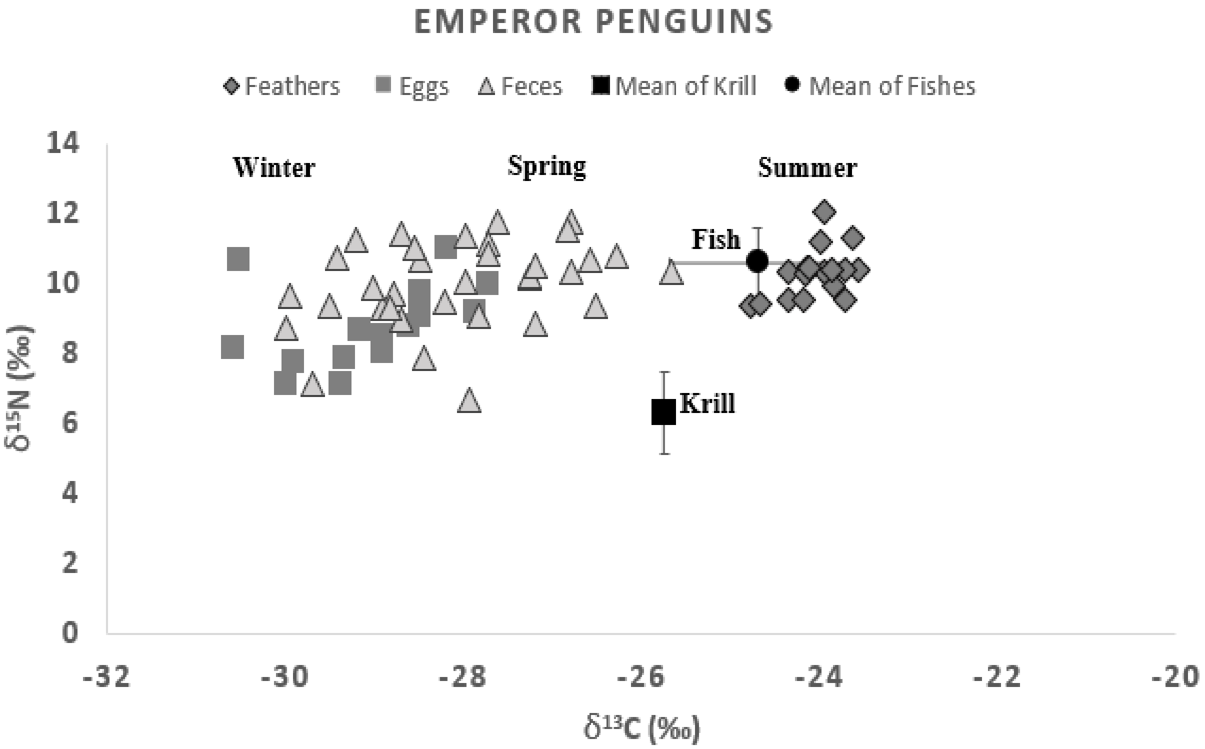
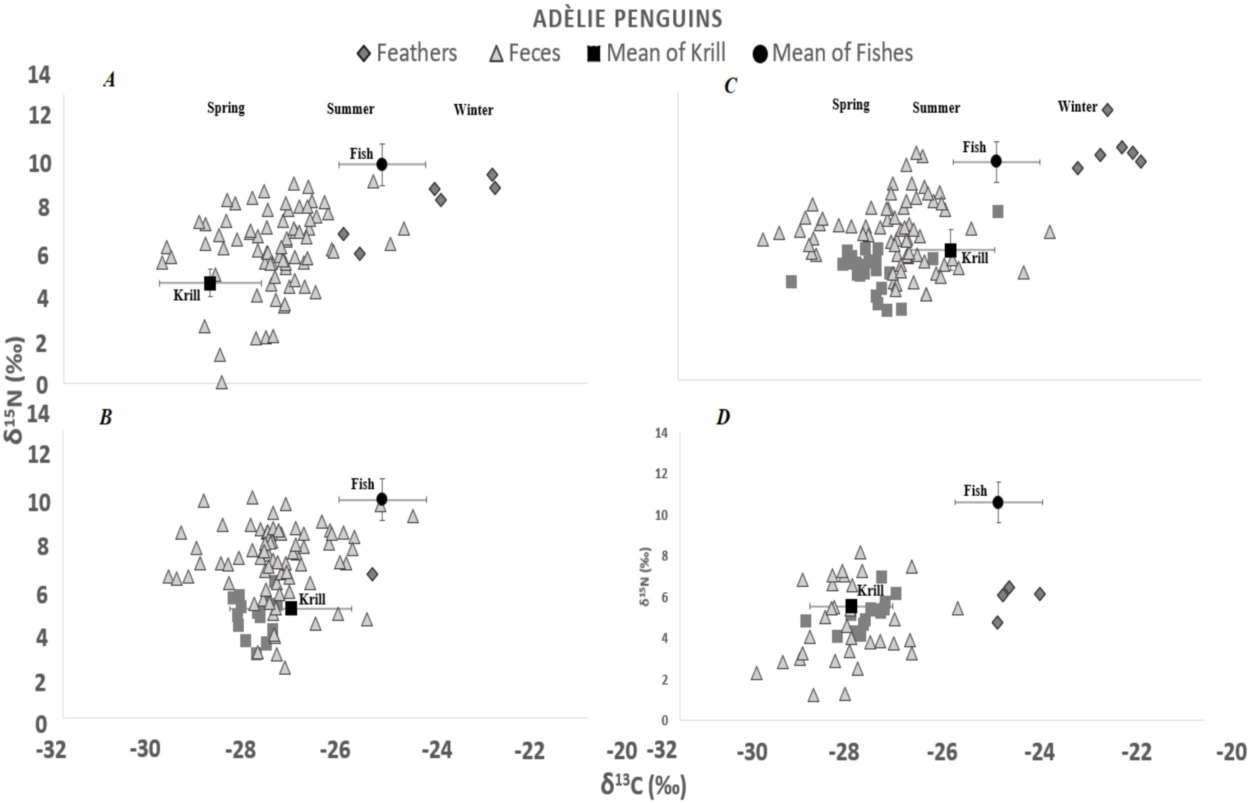
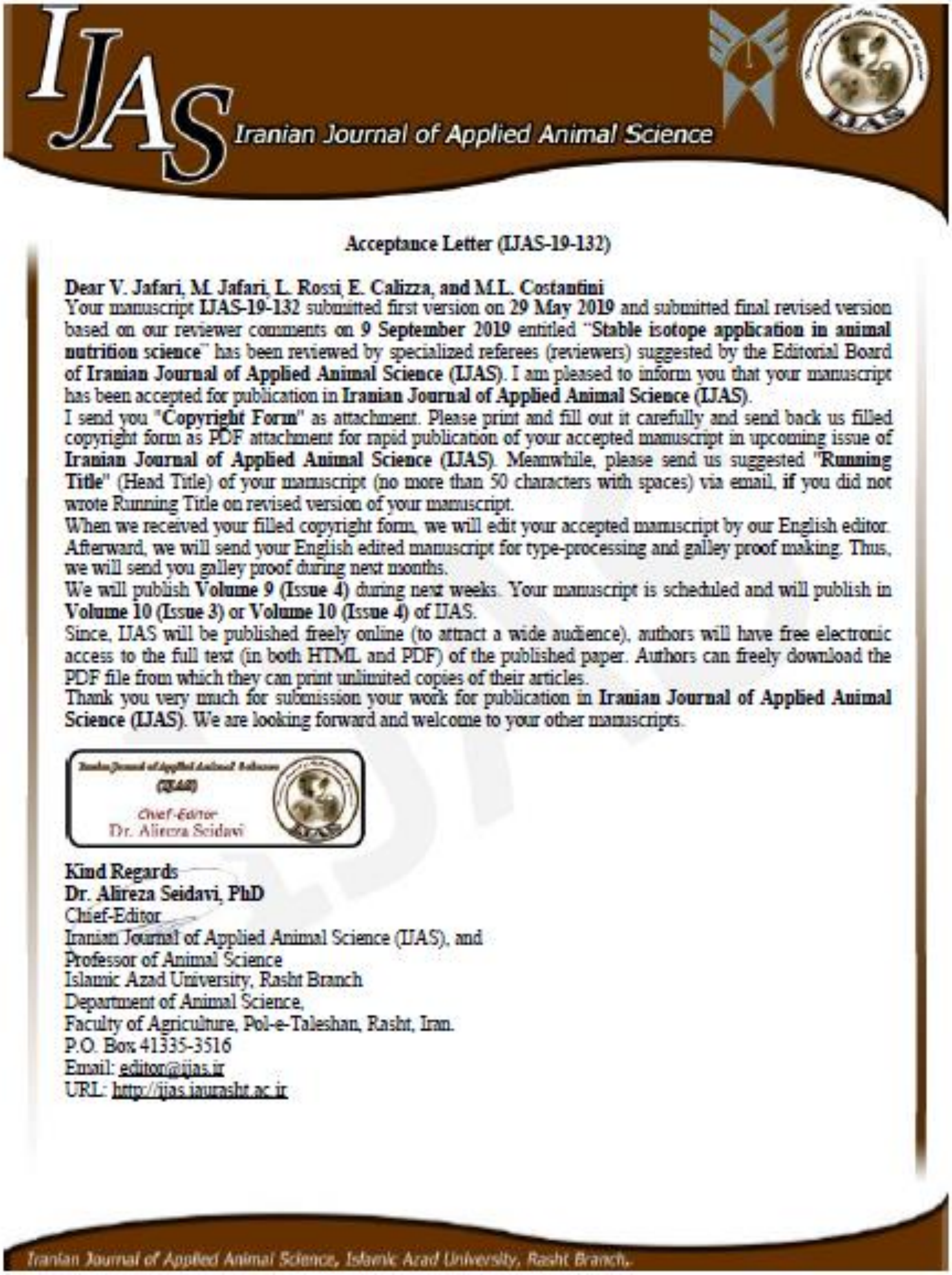


Figure 4. Isotopic biplot of Adelie penguin stable isotopes during winter (shell membrane, dark grey squares) and during summer (feces, light gray triangles) and their principal trophic resources (fish: black circle and krill: black square) in the four colonies (A: Inexpressible Island; B: Adèlie Cove; C: Edmonson Point; and D: Cape Hallett)





Iranian Journal of Applied Animal Science

Acceptance Letter (IJAS-19-132)

Dear V. Jafari, M. Jafari, L. Rossi, E. Calizza, and M.L. Costantini

Your manuscript **IJAS-19-132** submitted first version on **29 May 2019** and submitted final revised version based on our reviewer comments on **9 September 2019** entitled "**Stable isotope application in animal nutrition science**" has been reviewed by specialized referees (reviewers) suggested by the Editorial Board of **Iranian Journal of Applied Animal Science (IJAS)**. I am pleased to inform you that your manuscript has been accepted for publication in **Iranian Journal of Applied Animal Science (IJAS)**.

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Stable isotope application in animal nutrition science

''We are what we eat''

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ABSTRACT

The application of Stable Isotope Analysis (SIA) has become a standard scientific approach in Agricultural and Ecological researches and, more in general, in several disciplines such as Biology, Botany, Zoology, Organic chemistry, Climatology, and Nutrition. The main objectives of this paper are (1) to provide a simple definition of stable isotopes and (2) to illustrate analytical measurement methods and general applications in animal nutrition. The stable isotopes of Carbon ($\delta^{13}C$) and Nitrogen ($\delta^{15}N$) represent powerful tools to evaluate the trophic preferences of organisms and their trophic position. In association with Bayesian Mixing Models, stable isotopes also enable the description of trophic links between species and, thus, of complex food webs. Stable isotope data should be complemented with additional dietary data on feeding behavior to provide information regarding the transfer of energy or nutrients. Nowadays, stable isotope analysis is employed to address animal and human diets around the world.

Keywords: animal nutrition; environment; food web; stable isotopes analysis; trophic position

1. INTRODUCTION

Stable Isotope Analysis (SIA) is considered a helpful tool in a range of researches such as the study of climatic condition (Barnet et al., 2019), agricultural products, biological processes and biogeochemical cycles (Chaffee et al., 2007). In ecological studies, the measurement of stable isotopes in plants and animals is applied to the determination of animal feeding behavior, movement, and trophic position along food chains (Ben-David and Flaherty, 2012; Bouillon et al., 2011). Stable isotopes are safe (non-radioactive) and can be operated by humans. Even infants and pregnant women can be safely examined in medicine and nutrition studies. Naturally occurring stable isotopes are transferred from the physical environment to primary producers, as well as from a resource to its consumer, and emerge in hair, urine, feces, breath, and blood (Rossi et al., 2018; Arndt et al., 2016; Hagen, 1963). Thus, they can be used to trace nutrient uptake in producers and consumers in both terrestrial and marine ecosystems (Madeira et al., 2019; Signa et al., 2019; Calizza et al., 2018). Many measurement techniques depend on natural differences in the way 'light' and 'heavy' isotopes react during metabolic processes through biological and chemical alterations. Other stable isotope techniques depend on adding trace amounts of compounds artificially enriched in the rare (heavy) isotope of the element of interest. These are called isotope tracer methods/techniques. About a century ago, Fredrick Soddy was the first to identify signs of the existence of isotopes (Wilkinson, 2018). Isotopes are classified into 'Stable' and 'Unstable' groups. The unstable isotopes, which are not the subject of this research, are radioactive. Here the question is 'What are the stable isotopes?'. To answer this question, we should start by focusing on the atomic nucleus. Indeed, a different number of neutrons within the nucleus of the heavy and light stable isotopes leads to different atomic masses (Ben-David and Flaherty, 2012). Both light and heavy stable isotopes play a similar role in biological and chemical reactions, but with different response rates. The attractive forces and chemical bonds of the light stable isotope are weaker than the heavier isotope of an element. Thus, the lighter isotope reacts more quickly than the heavier one in both biological and chemical reactions.

Even though Oxygen (O), sulfur (S), and Deuterium (D) are applied in some studies, Nitrogen (N) and Carbon (C) are the two main elements considered in the study of animal diet and food webs.

1.1. Nitrogen (^{14}N, ^{15}N)

Natural nitrogen includes two stable isotopes (^{14}N, ^{15}N). ^{14}N is the most common isotope, while ^{15}N is the rarest. Different nitrogen isotopes (^{14}N and ^{15}N) can be distinguished through thermal diffusion or chemical exchanges. Other isotopes of nitrogen can be found in nature, such as ^{12}N, ^{13}N, ^{16}N, and ^{17}N. However, these isotopes are radioactive. Living organisms through the 'nitrogen cycle' usually transform nitrogen. Microbes convert different nitrogen compounds (like ammonia, NH_3^+) to nitrates for green plants and algae (Finlay and Kendall, 2008). Animals get their required nitrogen by consuming other living organisms (Post, 2002). The measurement of the isotopic signature of nitrogen ($\delta^{15}N$) plays an important role in biochemical, industrial and ecological applications such as food preservation, quantification of ecological processes and feeding interactions among organisms, medical and biomedical research (Signa et al., 2019; Calizza et al., 2018; Schellekens et al., 2011), and climate studies (Dotsika and Diamantopoulos, 2019).

1.2. Carbon (^{12}C, ^{13}C, ^{14}C)

One of the essential elements on earth is carbon, which forms the chemical basis of life. There are three natural isotopes of carbon, with atomic masses of 12, 13, and 14. ^{12}C and ^{13}C are stable and are used as tracers to understand nutrient cycling (Wang et al., 2019), food webs (Telsnig et al., 2019), and air-sea swapping of CO_2 (Lynch-Stieglitz et al., 1995). Plants and phytoplankton have a preferential use of ^{12}C to convert sunlight and carbon dioxide into biomass. The ocean surface is separated from the deeper water. However, when plankton dies, it sinks and removes ^{12}C from the surface (Flannery, 2006). The ^{14}C, or radiocarbon, is unstable. It is produced in the atmosphere and absorbed by living organisms (Marra, 2019). Carbon signatures can be used in agricultural and climate studies, authentication of foodstuff, description of nutrient fluxes in ecosystems, and in the determination of the age of archaeological specimens (Signa et al., 2019; Aitken, 2013; Zeuner, 1958).

1.3. Hydrogen (^{1}H, ^{2}H, ^{3}H, ^{4}H, ^{5}H, ^{6}H, ^{7}H)

Hydrogen has two naturally stable isotopes, ^{1}H and ^{2}H. The ^{2}H isotope is called deuterium (D), while ^{3}H is known as tritium (T), which is radioactive. Four other hydrogen isotopes, ^{4}H, ^{5}H, ^{6}H, and ^{7}H, are highly unstable and have been synthesized in the laboratory by bombarding tritium and by fast-moving deuterium or tritium nuclei (Golovkov et al., 2003). Some applications of the hydrogen isotopes could be highlighted in the authentication of foodstuff, agricultural, ecological, geochemical studies, and medical applications (Boschetti et al., 2019; Finlay and Kendall, 2008).

1.4. Oxygen (^{16}O, ^{17}O, ^{18}O)

Oxygen isotopes include three stable forms. The most abundant is ^{16}O, while ^{17}O and ^{18}O are categorized as secondary stable isotopes. The ^{16}O is mostly produced by massive stars composed only of hydrogen. ^{17}O and ^{18}O nucleosynthesis needs seed nuclei. The ^{17}O is produced by hydrogen burning into helium in CNO (Carbon-Nitrogen-Oxygen) cycle, and ^{18}O is made when the ^{14}N catches the ^{4}He nucleus (Emsley, 2011; Meyer, 2005). Oxygen isotopes can be used in the authentication of foodstuff, agricultural, ecological, geochemical, climate, and medical studies (Boschetti et al., 2019; Duffy et al., 2019; Finlay and Kendall, 2008).

1.5. Sulfur (^{32}S, ^{33}S, ^{34}S, ^{36}S)

Sulfur has twenty-four isotopes. Among these, ^{32}S, ^{33}S, ^{34}S, and ^{36}S are stable. Understanding acidic deposition in the forest ecosystems is the major application of sulfur isotopes (Campbell et al., 2006). The ^{34}S values increase with pollution sources and gas emission, which makes sulfur a powerful detector (Mayer et al., 1993). The sulfur input to marine systems mainly arises from seawater sulfate ($\delta^{34}S$= 21‰), whereas terrestrial inputs mainly depend on precipitations ($\delta^{34}S$=2-8‰) (Michener and Kaufman, 2008).

2. MEASUREMENT METHODS

2.1. Instrument

The abundance of stable isotopes in mineral and biological samples is measured as the heavy-to-light isotope ratio (R). For a given element (X), the isotopic signature of a sample (δ) is expressed as the per mill deviation (‰) from an international standard (Philp, 2015; Muccio and Jackson, 2009), according to the following equation:

$$\delta X = (R_{Sample}/R_{Standard} - 1)*1000 \qquad (1)$$

The International Atomic Energy Agency (IAEA, 2004) and the National Institute of Standards and Technology (Muccio and Jackson, 2009) provided an accurate evaluation of the reference standard elements (Brand et al., 2014; Flannery, 2006; Werner and Brand, 2001; Lynch-Stieglitz et al., 1995). The ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, and $^{2}H/^{1}H$ have been used widely to measure stable isotopes in carbon, nitrogen, oxygen, and hydrogen (Muccio and Jackson, 2009). The analytical determination of δ values implies the use of few light gases such as CO_2, CO, N_2, O_2, and SO_2. Accordingly, this standard technology has been called Isotope Ratio Mass Spectrometry (IRMS) (Brand, 2004). This analytical method identifies the chemical substance by ionizing it, focusing the resulting ions into a beam, and by separating the light and heavy atoms according to their net electric charge (Finlay and Kendall, 2008). The classical method of analysis includes two gases that are stored in containers connected via capillaries to a switching unit, the changeover valve. An isotope ratio mass spectrometer uses one gas as its ion source, while other available gas flow to the waste vacuum line (Werner and Brand, 2001). Both gases are used and compared a few times and measured separately through the ion currents. The relative difference in the ratio of light and heavy ions is calculated according to an international relative isotope ratio scale (Paulet al., 2007). The instrument has six basic components (Figure 1) (Edmond and Stroobant, 2013), which include: 1) a vacuum system; 2) an ion source; 3) a mechanism to concentrate ions into a narrower beam; 4) the speeding up of the beam; 5) a mass analyzer; 6) a detector. The material is initially present in the vacuum system, which produces the required low pressure to produce electrons and ions in the gas phase. Then, samples are transformed and concentrated into a narrower beam. (Paul et al., 2007; Meier-Augenstein, 1999; Brenna et al., 1997). Commonly, two connectors are used to introduce samples into IRMS: Elemental Analysers (EAIRMS) and Gas Chromatographers (GCIRMS). For many years, techniques such as gas chromatography (GC) and gas chromatography-mass spectrometer (GCMS) have been used to identify contamination sources (Philp, 2015). Similarly, the Combined Gas Chromatography Isotope Ratio Mass Spectrometry (GCIRMS) technique can be used to determine individual compounds and soil contamination sources.

2.2. Sampling procedures

Before sampling, it is necessary to define what kind of information is needed. This information usually depends on research objectives and the type of samples to be collected. Otherwise, the risk is to waste time and resources in collecting either wrong or not enough data. The sampling design is a tool that is utilized to infer how many data to collect, where, when, and how often they should be collected.

2.2.1. Plant sampling

Samples of vascular plants should be collected in the field and separately kept cold or frozen until processing. Samples of non-vascular plants are divided into two sections: lichens and marine algae. Lichens samples need to be collected directly into paper bags and dried once in the laboratory (Eldridge et al., 2003). If no oven is available, they can be spread out in a warm and well-ventilated place in packets and stored upright in a box. The samples of marine algae may be partially dried in the sun, but the small ones should be placed between sheets of paper, and the large ones should be placed in a box for further drying. Afterward, specimens should be pressed and stored in a dry and warm place (Steinitz and Kurle, 2014) .

2.2.2. Animal sampling

As regards terrestrial animals, it is possible to collect a sample of muscle, skin, feathers, eggshell, egg albumen, fur hairs, bones, *etc*. The samples may reflect diets ingested months before sampling, e.g. during the moulting or laying phase, and they can be collected from live or dead animals. Bone growth rings and whole bone can reflect an annual diet trend, during the whole animal’s lifetime. If bone samples are collected, soft tissues should be removed, and bones should be rinsed to remove impurities. When dry, bone samples can be placed in a paper bag. Feather samples should be cleaned to remove residual dirt and oil using a chloroform-methanol solution (Paritte and Kelly, 2009). Also, inorganic calcium carbonate from eggshell samples should be removed through a process of acidification (Finlay and Kendall, 2008).

As regards aquatic animals, small invertebrates can be collected by using kick nets, grabs, and litterbags, while large predators such as crabs, sea snails, and stomatopods, can be gathered by using traps baited with fresh fish flesh (Careddu et al., 2015). For bigger organisms, the muscle tissue often provides enough biomass to perform stable isotope analyses (Abrantes et al., 2013), while for small invertebrates such as amphipods and polychaetes, the whole body can be used (Ng et al., 2007). In order to study a consumer's diet, sampling should also include any dietary item that has likely been accessed by the consumer. All animal samples should be kept frozen at -20 °C and then lyophilized or dried at 60 °C overnight and kept in dry conditions until analysis (Finlay and Kendall, 2008).

Before isotopic analysis, all samples should be reduced to a fine homogeneous powder with a ball mill (Rossi et al., 2018). Then, powder of animal tissues (0.20±0.05 dry-mg) and vegetal tissues (3.0±0.05 dry-mg) should be weighed into tin capsules and analysed with an isotope ratio mass spectrometer. Thus, based on $\delta^{13}C$ and $\delta^{15}N$ values of consumers and their potential food sources, the animal diet can be determined through the R software (R Core Team, 2013), and Bayesian stable isotope mixing model (Rossi et al., 2018). Specifically, Bayesian mixing models allow the estimation of the proportion of each resource in the consumer's diet. The model requires three inputs: the isotopic signatures of the target consumer, the isotopic signatures of potential food sources, and the trophic enrichment factor, which represents the expected isotopic increase from a resource to its consumer due to metabolic processes (Careddu et al., 2015; McCutchan et al., 2003).

3. STABLE ISOTOPE APPLICATIONS IN ANIMAL NUTRITION

As stated before, Stable Isotope Analysis (SIA) is considered a helpful tool to be used in many disciplines. Among these, stable isotope-based environmental studies have recently flourished. Stable isotope signatures can be used to measure environmental stressors by monitoring plant uptake of carbon dioxide (Zheng et al., 2019) and greenhouse gas emissions (Popa et al., 2014; IAEA, 2004) as well as by tracing the source of water in catchments (Barbieri, 2019; Fiorentino et al., 2017; Philp, 2015) and organic and mineral compounds during biogeochemical processes (Finlay and Kendall, 2008) and cycles (Lichtfouse, 2000). The study of past climatic conditions is essential (Barnet et al., 2019; Jafari and Jafari, 2019) because it enables to modelling climate variability and make predictions of future conditions (Noorollahi et al., 2011). When dealing with animal nutrition, climate variability, ecological transitions, temporal and spatial scales, and individual choices can all affect variation and adaptation in the diet of organisms across trophic levels (Jafari and Jafari, 2019; Calizza et al., 2018; Bentivoglio et al., 2016; Careddu et al., 2015). Therefore, the study of temporal and spatial patterns of animal foraging through stable isotope analysis can provide useful information to predict future variations in feeding preferences according to climate change scenarios (Rossi et al., 2019; Calizza et al., 2018; Finlay and Kendall, 2008). In this perspective, oxygen isotope values can be used to indicate hotter and drier climate (^{18}O enrichment) versus colder and wetter conditions (^{18}O depletion). As an example, results published by Noorollahi et al. (2011) showed that increasing temperature has a positive correlation with rising $\delta^{18}O$ values (Noorollahi et al., 2011). Also, enrichment in ^{15}N has been reported as an indication of arid conditions (Pate and Anson, 2007). The $\delta^{18}O$ and $\delta^{2}H$ values have been shown to vary across geographic regions (Bowen and Revenaugh, 2003) or along environmental gradients (Lee et al., 2019), being thus useful to infer the geographic origin of samples.

SIA has proven to be a beneficial tool for: (1) studying nutrient uptake by humans, nutrient body reserve and nutrient metabolism paths (Schoeller, 2002), (2) describing contaminant flows (Signa et al., 2019), trophic relationships and food web structures (Signa et al., 2019; Calizza et al., 2018; Careddu et al., 2015; Rossi et al., 2015), as well as nutrient status (Calizza et al., 2016), (3) examining animal movement and migration (Cicala et al., 2019; Madeira et al., 2019; di Lascio et al., 2016), (4) clarifying patterns of resource allocation (di Lascio et al., 2013; Stachowicz et al., 2007), (5) identifying primary and secondary food sources (Komorita et al., 2014), and (6) detecting nutrient and mineral uptake by plants (Clewlow et al., 2019). The principal aspects of animal nutrition generally investigated through SI are diet patterns and trophic position of organisms along food chains (Bentivoglio et al., 2016; Boecklen et al., 2011).

As mentioned, stable isotope analysis enables the evaluation of the trophic position of organisms and populations in food webs. However, the isotopic values in the consumer alone do not trace its trophic position (Bentivoglio et al., 2016). Indeed, there is a stepwise increase between the corresponding isotope signature of food consumed and the consumers' tissue. Such expected isotopic increase between consumers and resources is referred to as isotopic discrimination or trophic enrichment (McCutchan et al., 2003). As an example, when an animal, such as a cow or a sheep, eats a specific plant, it will express the plant isotopic value in its muscles, bones or teeth, but the heavy isotope will be relatively more retained in the consumer's tissues than the light one (Reid and Koch, 2017; Cassano et al., 2016; Careddu et al., 2015). However, the plant energy and nutrition values also vary through growth stages (Jafari and Torbatinejad, 2015). Thus, it is essential to consider potential differences in environmental conditions and diet components when studying animal diet (Jafari and Torbatinejad, 2015).

Isotopic fractionation is particularly marked for nitrogen, while the carbon and sulfur isotopic composition of consumers closely reflects that in their diet. Studies with cows, fish, and zooplankton show that animal's feces are enriched in ^{15}N versus the diet, but urinary nitrogen (both NH_3^+ and urea) is depleted in ^{15}N. For example, cow urine can be 1‰ to 4‰ depleted in ^{15}N versus diet, while feces (2‰), and milk and blood (4‰ both) are enriched in ^{15}N. The ratio of sulfur isotopes ($\delta^{34}S$) varies substantially among salt marsh and marine primary producers from -9.6‰ to +12.9‰ (Currin et al., 1995). Thus, the $\delta^{34}S$ can be used to identify resource pools in these ecosystems. Similarly, the $\delta^{34}S$ values have been measured in transitional water ecosystems and marine ecotones to distinguish between marine and freshwater inputs (Finlay and Kendall, 2008; Martinetto et al., 2006; Currin et al., 1995; Peterson and Howarth, 1987).

$\delta^{13}C$ values in phytoplankton can vary markedly with latitude and longitude. Indeed, $\delta^{13}C$ values may vary with temperature, location, and growth rates that can affect the carbon uptake rate by phytoplankton (Zheng et al., 2019). Significant differences in carbon isotopes between animals indicate that consumers rely on different food sources or that their respective food webs are based on primary producers characterised by different isotopic signatures (Michener and Kaufman, 2008). Differences in the processing of carbon, nitrogen, and sulfur isotopes by animals stand out even more clearly, when the whole food web is examined. In many food webs, nitrogen isotope values increase by 10‰ to 15‰ from basal resources to top predators due to 3‰ to 5‰ stepwise increase among subsequent trophic levels. The opposite effect – no change with increasing trophic level – is observed for sulfur (Saggar et al., 1981).

The isotopic differences in consumer $\delta^{13}C$ may arise also by the consumption of C_3 or C_4 plants. The C_3 plants are linked with a wetter and colder climate, while C_4 plants are related to more arid and warmer conditions. As a consequence of metabolic adaptation by plants to such different climatic conditions, C_4 plants generally show markedly higher $\delta^{13}C$ values (from -10‰ to -18‰) than C_3 plants (from -22‰ to -30‰) (Philp, 2015) (Figure 4). In addition, C_3 plants may also show lower $\delta^{15}N$ values than C_4 plants (Figure 4).

DeNiro and Epstein (1981) mentioned that $\delta^{13}C$ values in C_3 plants averaged around -25.5‰, while values around -9.0‰ were reported for C_4 plants (Figure 2). The $\delta^{13}C$ value of meat was around -18.0‰, suggesting that the animal's diet was composed by a mix of C_3 and C_4 plants (Deniro and Epstein, 1981). Differences in $\delta^{13}C$ values have also been reported between terrestrial plants and aquatic algae (Rossi et al., 2010). The latter generally show higher $\delta^{15}N$ values than the former (Figure 3) (Schulting, 1998). This mainly depends on differences between the sources of carbon used for primary production in the two systems (Schulting, 1998). As shown in Figure 4, the $\delta^{13}C$ of terrestrial C_3 primary producers is generally lower than that of marine producers (Vinagre et al., 2011). In addition, $\delta^{13}C$ signatures in plants can be affected by plant development and water management (Barbieri, 2019; Schulting, 1998). Given such expected differences among marine and terrestrial ecosystems, isotopic differences among aquatic consumers can inform on the benthic or terrigenous origin of nutrient inputs at the base of coastal or littoral food webs. Furthermore, dissolved inorganic carbon in estuaries commonly derives from different sources, either CO_2 from the atmosphere or the dissolution of carbonate with approximately zero per mill value of $\delta^{13}C$ (Bouillon et al., 2011; Finlay and Kendall, 2008). Given the predominance of C_3 metabolism in coastal and aquatic vegetation, the $\delta^{13}C$ in aquatic consumers usually display values around -28‰. Chanton and Lewis showed that the $\delta^{13}C$ values in estuaries are closely related to the soluble inorganic carbon and water salinity (Chanton and Lewis, 1999).

At the community level, the range of $\delta^{13}C$ values (Carbon Range) can provide a useful indication of the diversity of basal resources consumed by animals (Wilkinson, 2018) and both $\delta^{13}C$ and $\delta^{15}N$ describe the niche space occupied by all the organisms. To move from the isotopic description of organisms to the quantification of trophic interactions within the food web, Philips et al. (2010) proposed the use of mixing model equations. By explicitly taking into account uncertainties in consumer and resource isotopic signatures, the development of Bayesian approaches has enabled a more robust description of trophic links between species (Rossi et al., 2019; Careddu et al., 2015).

Some tissues, such as the dentine of teeth, hairs, and feathers are metabolically inert. Therefore, the study of these tissues can inform on the isotopic signature of a consumer's diet at the time of tissue deposition. If the rate of tissue deposition is known, these tissues can provide a timeline of the consumer's dietary history (Layman et al., 2012). For example, Hobson and Sease (1998) recorded ontogenetic isotopic shifts in Steller sea lions from tooth annuli (Hobson and Sease, 1998). Newsome et al. (2009) documented temporal changes in resource use by the California sea otter *Enhydra lutris nereis* by using regular sections of whiskers (Newsome et al., 2009). In these cases, information on the inert tissue deposition processes is necessary. Indeed, the process can be continuous over time (e.g., for whiskers of some mammal species), or discontinuous (e.g., for feathers) (Layman et al., 2012). In addition, it must be considered that different tissues are characterised by different turnover rates, thus providing dietary information over different time scales. Therefore, turnover rate data in the distinct tissues are required to conclude the degree of dietary proficiency (Layman et al., 2012). For instance, in some vertebrates, blood plasma integrates the diet over days to weeks, whereas turnover in muscle tissue is on the scale of months (Phillips and Eldridge, 2006; Dalerum and Angerbjörn, 2005).

Nowadays, stable isotope analysis is utilized to address questions about human diets around the world, and it has been said that 'we are what we eat' (Deniro and Epstein, 1981). SIA provides quantitative data that complete floral, faunal, and other information about dietary habits of individuals. This passage through human metabolism is specifically valuable to the quantitative study of human nutrition (Cooper et al., 2019). Humans express different isotope signatures according to the consumption of C_3 and C_4 plants (Figure 2), terrestrial animal proteins like cow, sheep, and goat meat, or aquatic animal resources such as fish and shellfish (Figure 3) (Schulting, 1998). Interestingly, there are diverse plant groups in human nutrition that can be differentiated through $\delta^{13}C$ values in human tissues (such as hair), including C_3 plants such as wheat, barley, soy, potatoes, fruits, vegetables versus C_4 plants such as corn, sorghum, millet, sugar cane. This difference is also reflected in animal-derived food products such as milk carbon signatures ranging from -14‰ (diet-based C_4 plants) to -27‰ (diet-based C_3 plants) (Petzke et al., 2005).

4. CONCLUSION

The present paper has highlighted the stable isotope concept, applications, measurement method, and its relationships with animal nutrition. The many examples cited allow us to conclude that the analysis of stable isotopes of nitrogen and carbon is a powerful tool for evaluating animal feeding choices and trophic position in food webs, as well as the trophic sources supporting aquatic and terrestrial consumers. In addition, coupled with isotopic Bayesian mixing models, stable isotopes are a valuable tool that can provide insights into the structure and the complexity of food webs, as well as into the pathways of nutrient and energy transfer among ecosystem compartments and trophic levels. Nevertheless, it must be noted that many of the ecological questions addressed through the analysis of stable isotopes are reliant on the assumption that source pools have distinguished isotope values. When sources cannot be distinguished, stable isotopes may have little performance in answering questions about trophic relationships. In this case, stable isotope analysis should be complemented with additional information, such as stomach content and/or feces analysis, as well as other data on feeding behaviour including direct observation of feeding preferences in the field. In any case, both source and consumer pools must be sampled on suitable spatial and temporal scales to provide reliable information on diet composition. Isotope signature differences in samples depend on the climate, the isotopic baseline of the food web the consumer is part of, organisms' dietary habits, and body conditions. Therefore, all these aspects should be considered in isotopic studies in order to achieve accurate results. Besides its broad application in environmental and ecological studies, SIA is increasingly used in the study of human diet, and it has the potential to resolve many ambiguities in nutritional and medical studies.

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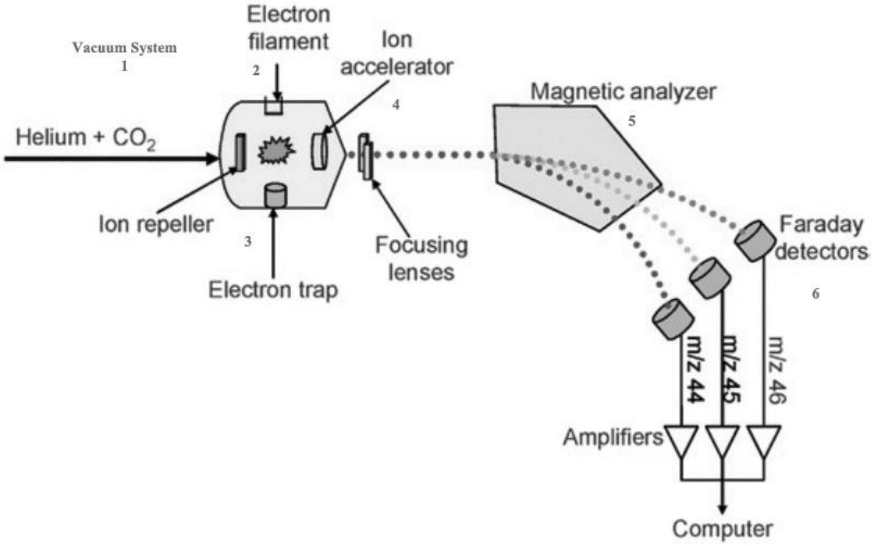

Figure 1. Schematic fundamental components of Mass Spectrometry (Edmond and Stroobant, 2013; Muccio and Jackson, 2009)

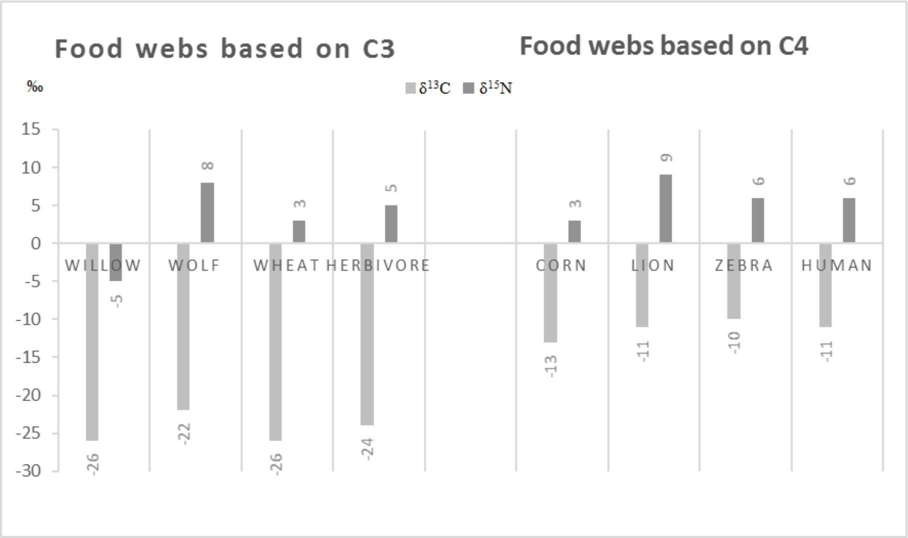

Figure 2. Differences in δ13C (‰) and δ15N (‰) between food webs based on C3 and C4 plants (Schulting, 1998)

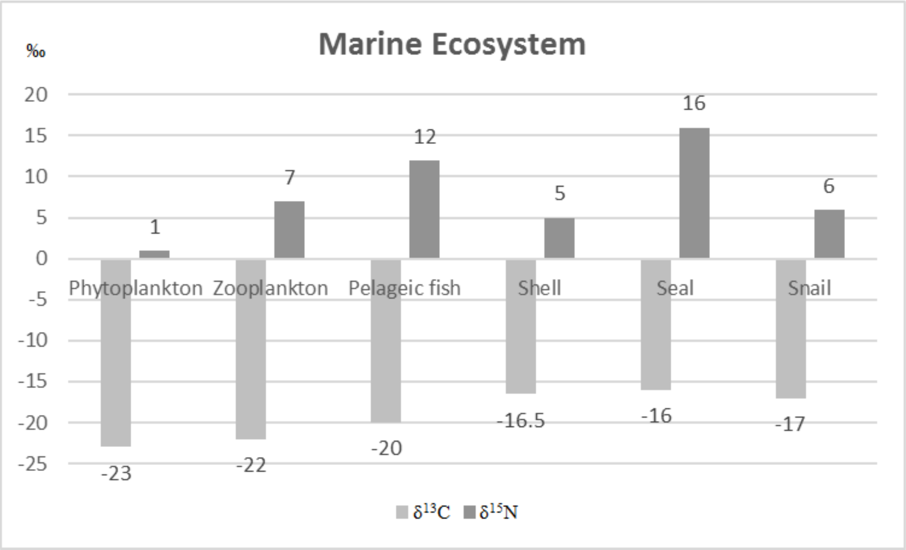

Figure 3. Differences in $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) values in Marine Ecosystem (Schulting, 1998)

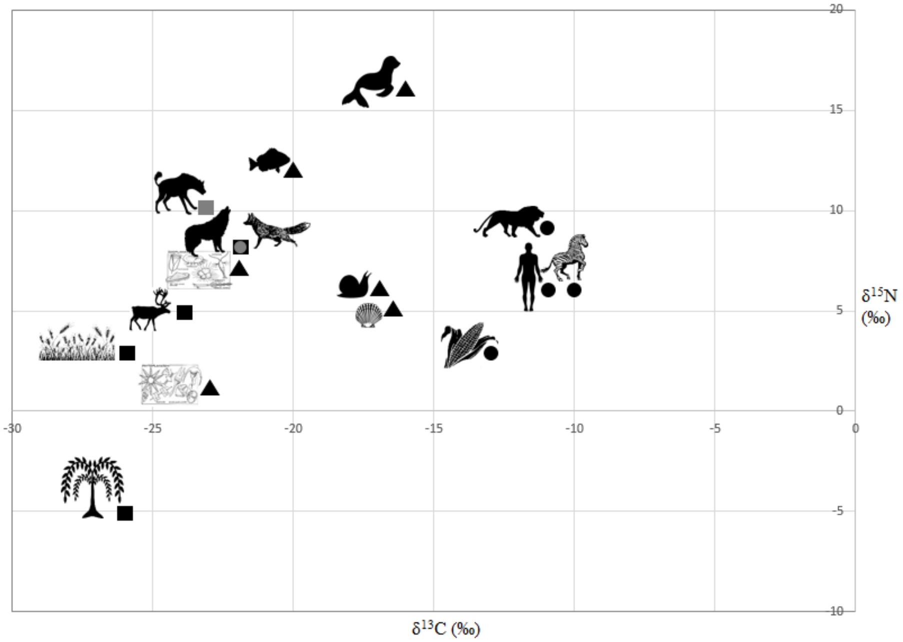

Figure 4. Trophic enrichment in stable carbon and nitrogen values from primary producers, to terrestrial herbivores and predators (circles and squares) and marine ecosystems (black triangles). Differences in δ13C between food webs based on C3 (black square symbols) and C4 plants (black circles symbols), omnivore/carnivore (gray circles), carnivore (gray squares) are also shown (Reid and Koch, 2017; Schulting, 1998)

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