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, Antarctica, supports large populations of Emperor Penguin (Aptenodytes forsteri) and Adélie Penguin (Pygoscelis adeliae), two key meso-predators that occupy high trophic levels. Despite these species are largely studied, little is known about their diet outside the breeding period. In the present study, we investigated the intra-annual diet of Adélie and Emperor Penguins belonging to five colonies in the Ross Sea through the stable isotope analysis of different tissues (feathers and shell membranes), synthetized in different seasons, and guano that indicates recent diet. Penguin samples and prey (krill and fish) were collected during the Antarctic spring–summer. δ13C and δ15N of tissues and guano indicate spatio-temporal variation in the penguin diet. The krill consumption by Adélie Penguins was lowest in winter except in the northernmost colony, where it was always very high. It peaked in spring and remained prevalent in summer. The greatest krill contribution to Emperor Penguin’s diet occurred in summer. The relative krill and fish consumption by both species changed in relation to the prey availability, which is influenced by seasonal sea ice dynamics, and according to the penguin life cycle phases. The results highlight a strong trophic plasticity in the Adélie Penguin, whose dietary variability has been already recognized, and in the Emperor Penguin, which had not previously reported. Our findings can help understand how these species might react to resource variation due to climate change or anthropogenic overexploitation. Furthermore, data provides useful basis for future comparisons in the Ross Sea MPA and for planning conservation actions.

Keywords Penguin ecology · Ross Sea · Sea ice · Krill · Fish

Introduction
The Ross Sea is one of the most biologically productive regions of the Antarctic continent (Arrigo et al. 2002). It is characterized by high biodiversity (Laffoley et al. 2019) and since October 2016 it is the largest marine protected area in the world (Brooks et al., 2020). The area supports large populations of species occupying high trophic levels, including the two key meso-predators, Emperor Penguin (Aptenodytes forsteri) and Adélie Penguin (Pygoscelis adeliae). Emperor Penguin lives and reproduces on the pack ice. It has a diet based mainly on fish and, to a lesser extent, on crustaceans and cephalopods (Kirkwood and Robertson 1997; Cherel and Kooyman 1998; Burns and Kooyman 2001; Cherel 2008). The diet of the smaller Adélie Penguin, a species that spends part of its life cycle on sea ice but reproduces on land, is mainly based on crustaceans belonging to the genus Euphausia, primarily Antarctic krill (Euphausia superba) and ice krill (Euphausia crystallorophias) and, to a lesser extent, on fish (Ainley 2002; Ainley et al. 2003). The Antarctic silverfish (Pleuragramma antarctica) represents the main fish prey of both penguin species (Wienecke and Robertson 1997; Cherel 2008; Tierney et al. 2008). The availability of krill and P. antarctica as prey for penguins is related to sea ice dynamics (Ainley et al. 1998). In fact, increased availability of krill as prey for penguins seem to occur in seasons or areas characterized by a greater pack ice presence while there is an increase in fish availability in areas without sea ice (Ainley et al. 1998, 2003; Ainley 2002; Strickland et al., 2008; Olmastroni et al. 2020).
Although these penguin species are extensively studied due to their considerable ecological and conservation importance, studies related to their trophic ecology are limited to specific periods of the year, such as the chick-rearing period that corresponds to summer for Adélie Penguin and spring for Emperor Penguin (Tierney et al. 2008; Juáres et al. 2016). As a result, for the Adélie Penguin there is a paucity of diet information in spring, and for the Emperor Penguin little is known about its trophic ecology in summer (Cherel 2008). Very few data are available on the winter diet of both species (Wienecke and Robertson 1997; Burns and Kooyman 2001).

The scarcity of data about these predators in specific periods of the year is constrained by their life cycle (Juáres et al. 2016). Indeed, these species perform long migrations in different phases of their life cycle, making it difficult to obtain continuous observations and sample collections (Juáres et al. 2016). Such migrations, coupled with the extreme conditions of the Antarctic environment, in particular during winter, make the reconstruction of the penguins’ diet difficult outside their breeding areas (Cherel 2008; Tierney et al. 2008; Juáres et al. 2016). Stable isotope analysis (SIA) of Carbon ($^{13}$C/$^{12}$C) and Nitrogen ($^{15}$N/$^{14}$N) has been increasingly and successfully applied to penguins (Cherel 2008; Strickland et al. 2008; Tierney et al. 2008; Juáres et al. 2016). SIA is based on the evidence that isotopic values of consumers (Hobson and Clark 1993) are directly related to the assimilated diet, since tissues are synthesized from the nutrients formerly assimilated, reflecting their isotopic composition in a predictable manner (DeNiro and Epstein 1978; Hobson and Clark 1992a, b; Bearhop et al. 2004; Newsome et al. 2007; Calizza et al. 2018; Rossi et al., 2019; Sporta Caputi et al., 2020) Since different tissues have different isotopic turnover rates and are produced at different times of the year (Hobson and Clark 1992a; Cherel et al. 2005a; Juáres et al. 2016), diet can be inferred at the different times according to the tissues or biological matrices analysed (Hobson and Clark 1993).

The aim of this study is to reconstruct and compare the year-round diet of the Adélie and Emperor Penguin from different Ross Sea colonies. To achieve this goal, the Carbon and Nitrogen stable isotopes of different biological matrices ( feathers, eggshell membranes, and guano) were analysed, providing information related to different phases of the life cycle of these predators (Hobson and Clark 1992a; Cherel et al. 2005a; Juáres et al. 2016). Shell membrane provides information on diet before the breeding period (Astheimer and Grau 1985; Emslie and Patterson 2007; Strickland et al. 2008; Polito et al. 2009; Juáres et al. 2016) that corresponds to spring for Adélie Penguins. Instead, female Emperor Penguins fast throughout oogenesis (Speake et al. 1999; Connan et al., 2019) and egg components are produced using stored reserves. For this reason, the shell membrane in this species provides information on the pre-reproductive fattening period at sea, which corresponds to the winter months of April and May (Kirkwood and Robertson 1997; Speake et al. 1999; Burns and Kooyman 2001). Feathers, which are metabolically inert after synthesis, reflect the resources incorporated during the discrete time interval in which that tissue was grown, in fact they encapsulate information about a bird’s diet and foraging habitat related to the pre-moult period (Hobson 1999; Cherel et al. 2000). Specifically, stable isotopes in feathers provide information on the summer and winter diet for Emperor and Adélie Penguins, respectively. The guano provides information on the short-term diet, indicating what the animal has consumed in the days preceding the sampling (Bird et al. 2008). This biological material was collected and used to obtain information about the spring and the summer diets for Emperor and Adélie Penguins, respectively.

Few studies on the foraging ecology of penguins during different stages of their annual cycle were conducted using the stable isotope analysis (Polito et al. 2009, 2011b; Hinke et al. 2015; Juáres et al. 2016). Therefore, by sampling these different biological matrices, we aimed at describing the diet of these two species in different colonies in the Ross Sea and thus acquiring new important information during three phases of their life cycle corresponding to different periods of the year. In agreement with previous studies that highlighted a strong trophic plasticity of the Adélie Penguins (Strickland et al., 2008; Olmastroni et al. 2020; Watanabe et al. 2020) we expected season and site-specific variations of their diet due to variations of krill and P. antarctica abundances following the space–time dynamics of sea ice (Ainley et al. 1998, 2003; Ainley 2002; Strickland et al., 2008; Olmastroni et al. 2020). Unlike the Adélie Penguin, which feeds in shallow waters (Cherel and Kooyman 1998; Rodary et al. 2000b; Wienecke et al. 2000), the Emperor Penguin can forage in the whole water column (Cherel and Kooyman 1998; Rodary et al. 2000a; Zimmer et al. 2008), where it can capture a great quantity of deep pelagic fish such as P. antarctica (Cherel 2008; Daneri et al. 2018). Thus, according to literature, we expected Emperor Penguin to maintain a mainly piscivorous diet without significant seasonal dietary shifts (Cherel and Kooyman 1998; Barbraud and Weimerskirch 2001; Cherel 2008).

We also hypothesized seasonal interspecific dietary differences due to the different life cycle patterns of Emperor and Adelie penguins.

Determining the resource use of these two penguin species is critical to better understanding the responses of these krill- and fish-dependent predators to possible resource variations associated with climate change and fisheries (Cherel and Hobson 2007; Juáres et al. 2016) and will help establish effective conservation measures for these two species.
Materials and methods

Study area and sampling procedures

The study was carried out at five sites 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 Adélie Penguins (Pygoscelis adeliae) and Cape Washington (164°58′02″ E, 74°37′07″ S) for Emperor Penguins (Aptenodytes forsteri) (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 around 24,500 nesting pairs of Adélie Penguins (Harris et al. 2015). Adélie Cove is located on the coast of the Northern foothills of Terra Nova Bay and hosts a colony of Adélie Penguins with approximately 11,200 reproductive pairs (Harris et al. 2015). Edmonson Point, situated in Wood Bay, at the foot of the Eastern slopes of Mount Melbourne, hosts an Adélie Penguin colony of about 1,900 breeding pairs (Harris et al. 2015). Cape Hallett, located at the northern end of the Hallett Peninsula in northern Victoria Land, hosts on average 42,600 breeding pairs of Adélie Penguins (Harris et al. 2015). Cape Washington, in the Northern part of Terra Nova Bay, on the slopes of Mount Melbourne, houses an important colony of Emperor Penguins, with a population of 13,000–25,000 breeding pairs (Harris et al. 2015). The selected colonies lie in a sector of the Ross Sea characterised by marked spatio-temporal variations in sea ice coverage. The strong katabatic wind characterizing the southern portion of the study area and the block of the ice floe by Drygalski Ice Tongue favour the formation a polynya in the area of Inexpressible Island, with an average surface of 1300 km² (Bromwich and Kurtz 1984; Van Woert 1999). As a consequence, sea ice generally breaks up first at Inexpressible Island. Then, due to dominant winds and sea currents, seasonal sea ice break-up proceeds northward in the direction of Cape Hallett (Petrelli et al. 2008), where sea ice generally persists longer during the summer season.

Samples of eggs, white feathers, and guano were opportunistically collected from each colony during the austral spring 2016-summer 2017 to take into account the two penguins’ seasonal diet variation (Fig. 2). At each colony, guano samples were collected in 15 ml-Falcon tubes using a spatula. Feathers and abandoned unhatched eggs were collected near the nests by hand and placed in sterile plastic bags. In the colony of Inexpressible Island no unhatched eggs were found. In order to avoid pseudoreplication of samples, samples of guano, eggs and feathers were collected approximately at a minimum of 50 m from each other and each

Fig. 1 Sampling area in the Ross Sea (Antarctica). In the small box, the place names indicate the location of the four Adélie Penguin (Pygoscelis adeliae) colonies, Inexpressible Island (I.I.), Adélie Cove (A.C.), Edmonson Point (E.P.), and Cape Hallett (C.H.), and the Emperor Penguin (Aptenodytes forsteri) colony, Cape Washington (C.W.). Information on latitude from Cavalieri and Parkinson (2008) and Harris et al (2015)
sample was classified as an independent observation (each sample was assigned to a different individual). To obtain isotopic values of penguins’ prey, spontaneous vomits of Emperor and Adélie Penguins containing undigested krill and whole fish were opportunistically collected on sea ice and on the ground in spring and summer. Since an oceanographic cruise was not possible, this non-invasive sampling approach made it possible to collect the preys belonging to the specific krill swarms and fish schools actually preyed by penguins in the study area. Specifically, samples of krill were found in all colonies while samples of fish were found only at Edmonson Point and Inexpressible Island. All samples were stored in a refrigerated box until the transport to the Italian scientific station Mario Zucchelli (MZS) in Antarctica. All samples were then enumerated and stored at -20 °C until they were transferred and processed in Italy.

**Laboratory procedures and stable isotope analysis**

The shell membranes were separated from the eggshells using a surgical blade, dipped in distilled water and cleaned of surface debris, dried and finally cut into small pieces in accordance with Quillfeldt et al. (2009). Guano samples were cleaned, the residues of uric acid rich in inorganic nitrogen were removed with a scalpel in order to reduce any alteration during the nitrogen isotope analysis (Bird et al. 2008). In order to remove surface oils and impurities, feathers were washed with a sonic bath in a 2:1 chloroform–methanol, rinsed with distilled water, dried at 60 °C in oven for at least 24 h and then cut into small fragments (Graves et al. 2018).

Samples of penguin prey were identified at the lowest possible taxonomic level: fish at the species level (P. antarctica) and krill at the genus level (Euphausia). A sample of dorsal muscle was taken from each individual of Pleuragramma antarctica (Darnaude et al. 2004; Wolters et al. 2018). Krill was not acidified to remove carbonates as each sample was stripped of the exoskeleton to collect only krill muscle. Samples of eggshell membrane, guano and penguin prey were pulverized using a ball mill (Mini-Mill Frits Pulverisette 23: Fritsch Instruments, Idar-Oberstein, Germany). All samples were then weighed and pressed in tin capsules for the Stable Isotopes Analysis. Specifically, aliquots of 1.0–1.5 mg were used for the analyses. Each sample was analyzed in two replicates using an Elementar Vario Micro-Cube elemental analyzer (Elementar Analysen Systeme GmbH, Germany) coupled with an IsoPrime100 continuous flow mass spectrometer (Isoprime Ltd., Cheadle Hulme, UK), and the mean values were used in subsequent analyses. Carbon (C) and Nitrogen (N) isotopic values were expressed in δ units (δ13C; δ15N) as parts per-thousand (‰) deviations from international standards: Vienna Pee Dee Belemnite (PDB) for C and atmospheric N2 for N. Isotopic ratios were computed according to the equation:

$$δX(‰) = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where $X$ is the C or N isotope and $R$ is the heavy-to-light isotope ratio of the respective element ($^{13}\text{C}/^{12}\text{C}; ^{15}\text{N}/^{14}\text{N}$;Coplen 2011). Results were calibrated to International Atomic Energy Agency reference materials (IAEA-CH-3, IAEA-CH3 and USGS24 for δ13C; IAEA-N1, IAEA-N2 and USGS25 for δ15N). Caffeine IAEA-600 (C8H10N4O2) was used as the internal laboratory standard (δ15N = 1.00‰ and ‰ δ13C = − 27.77‰). Measurement errors associated...
with the linearity and stability of the mass spectrometer were typically smaller than 0.05‰, while the standard deviation of repeated measurements of lab standard material (one replicate every 10 analyses) was typically ±0.02‰ for δ¹³C and ±0.07‰ for δ¹⁵N. Finally, δ¹³C values of fish and krill with C:N ratio > 3.32% were corrected for lipid content in accordance with Post et al. (2007) and the corrected values are reported in the text, tables and figures. No lipid correction was applied for guano, feathers and shell membrane samples.

**Data analysis**

Data analysis was conducted with the Statistical Package for the Social Science (SPSS) software. The R package SIMMR (Stable Isotope Mixing Models in R, Parnell and Inger 2019) was used to estimate the relative contribution of prey (i.e., fish and krill) to the penguins’ diet.

The Analysis of Variance (ANOVA) was performed to compare δ¹³C and δ¹⁵N values among Adélie Penguin colonies within the same season (by comparing the same biological matrix), as well as to compare isotopic values of the same colony among seasons.

The t-test was used for comparisons between krill and fish isotopic values and between the Emperor Penguin and Adélie Penguin of the Edmonson Point colony (the closest Adélie Penguin colony to Cape Washington) in winter and summer.

To compare different tissues, which are characterized by different Trophic Enrichment Factor (i.e., TEF, the isotopic differences between the consumer’s tissue and its food sources due to metabolism; McCutchan et al. 2003), the TEF values reported in literature were subtracted from the isotopic values of samples. Since in the literature there are not specific TEFs for the feathers and the shell membranes of Adélie and Emperor Penguin, we have published TEFs of penguin species belonging to the same genera of the two species. Different TEF values were used for the feathers of Emperor Penguin (Δ¹³C = 0.7 ± 0.8‰; Δ¹⁵N = 3.1 ± 0.8‰ of Aptenodytes patagonicus; Cherel et al. 2005b) and Adélie Penguin (Δ¹³C = 1.3 ± 0.5‰; Δ¹⁵N = 3.5 ± 0.4‰ of Pygoscelis papua; Polito et al. 2011a for), while the same TEF values of eggshell membrane were used for both species (Δ¹³C = 2.8 ± 0.5‰; Δ¹⁵N = 4.4 ± 0.5‰; Polito et al. 2009 for Pygoscelis papua), as they were the only values reported in the literature. The fractionation of guano was considered equal to 0 for both isotopes (Bird et al. 2008). Indeed, the guano, and in particular the solid component, 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 ( Mizutani et al. 1992; Bird et al. 2008). These values have been already used in several studies (Davies et al. 2009; Raya Rey et al. 2012; Juárez et al. 2016). Nevertheless, we acknowledge that variations in the TEF values potentially affect mixing model outputs (Parnell et al., 2010). Thus, we performed a sensitivity analysis of the outputs to variations in TEFs in accordance with Costantini et al. (2018). Due to the scarcity of information on specific TEF values referring to the two penguin or congeneric species, the TEF of each tissue was independently increased or decreased by 15% and 30% for δ¹⁵N and δ¹³C values, resulting in nine different TEF combinations to be used in mixing models (Online Resource 1). The results obtained with different TEFs were compared using ANOVA for repeated measures and the associated Tukey’s post hoc comparison.

In order to quantify the contribution of the different prey in the diet of both penguin species in summer, spring and winter, we used the SIMMR Bayesian stable isotope mixing model in R environment (Parnell and Inger 2019). This model requires the isotopic values of consumers and potential resources (mean and standard deviation), and an appropriate Trophic Enrichment Factor (TEF), as inappropriate TEF values will lead to inaccurate interpretations (Bond and Diamond 2011). We performed a mixing model using two resources, krill and fish, calculated by using the mean δ¹³C and δ¹⁵N values of Euphausia and Pleuragramma antarctica samples, respectively (see “Result” section). The output of the model is a density function distribution of plausible proportion values, whose central tendency (mode, mean, median) and upper and lower limits of credibility intervals (CI: 50%, 75%, 95%) reveal the range of contributions of each resource to penguins’ diet (Parnell et al. 2010).

To investigate the seasonal differences in diet in each colony and between Emperor Penguins and Adélie Penguins, the consumption of krill was compared across groups using the function ‘compare groups’ from the SIMMR package (Parnell and Inger 2019). This function gives the probability, based on Bayesian information criterion (P⁰BIC), that the proportion of any diet source in one treatment is greater than in another treatment, where P⁰BIC > 0.95 indicates a significant difference (Masson 2011; da Silva Santana et al. 2020).

**Results**

Mean δ¹³C and δ¹⁵N values differed significantly among fish and krill (Figs. 3 and 4; t-test, t₆¹₃C = - 4.41 and t₆¹⁵N = - 10.16, p always < 0.0001).

δ¹³C and δ¹⁵N values of Emperor Penguin’s guano were depleted compared to feathers and shell membranes (Table 1). Comparisons of δ¹³C and δ¹⁵N values corrected by TEF indicated significant differences between the three biological matrices, and thus between seasons (Table 2, Figs. 2 and 3). In accordance, the consumption proportions of krill and fish were found to vary seasonally. Specifically,
The krill contribution to Emperor Penguin’s diet was remarkably greater in summer than in spring (70% vs 29%; $P_{BRIC} = 0.79$; Table 3), indicating krill-based diet and fish-based diet in the two seasons, respectively. Krill and fish contributions were more similar in winter, although krill was still the preferred prey (Table 3).

In the three southernmost colonies, feathers of Adélie Penguins had enriched $\delta^{13}C$ and $\delta^{15}N$ values compared to eggshell membranes, while in Cape Hallett, these tissues had similar isotopic values (Table 1). The $\delta^{13}C$ and $\delta^{15}N$ values of feathers, eggshell membranes and guano corrected by the respective TEF differed significantly in every colony, except Cape Hallett for the $\delta^{15}N$ values, thus indicating diet differences among seasons (Table 2). Except for the eggshell membranes, which indicate spring feeding, they differed also among colonies (Table 3). Guano in summer always had intermediate isotopic values (Fig. 4). In accordance with the different isotopic values of the three biological matrices, the Adélie Penguin’s diet varied seasonally in all the colonies. Specifically, krill consumption was highest in spring and lowest in winter, although in Cape Hallett it was notably
high in all the seasons and the seasonal differences observed were very small (Inexpressible Island, $P_{\text{BIC}}=0.93$; Adélie Cove, $P_{\text{BIC}}=0.98$; Edmonson Point, $P_{\text{BIC}}=0.99$; Cape Hallett, $P_{\text{BIC}}=0.43$; Table 3). The consumption of prey did not change significantly with different combinations of TEFs of feathers and eggshell (ANOVA for repeated measures and Tukey’s post hoc comparison, feathers: $F_{8,36}=1.30$, $p=0.28$; eggshell: $F_{8,36}=1.36$, $p=0.26$; for sensitivity analysis, Online Resources 2 and 3).

From comparisons of diets between Emperor and Adélie Penguins in the two neighboring colonies (i.e., Cape Washington and Edmonson Point, respectively), no significant differences were found for either $\delta^{13}C$ or $\delta^{15}N$ values of feathers and guano, corrected by their TEFs, which indicated, respectively, the summer diet of the two species ($t$ test: $t=-0.95$, $p=0.35$ and $t=-0.11$, $p=0.92$, respectively). These values were associated with similar high percentages of krill consumption ($P_{\text{BIC}}=0.53$; Table 3).

Adélie Cove significantly differed in both $\delta^{13}C$ and $\delta^{15}N$ values of eggshell membranes and feathers, corrected by TEF, which indicated the winter diet of the two species, respectively ($t=-13.09$ and $t=5.18$, $p<0.0001$ respectively). These values revealed significantly higher percentages of krill consumption by Emperor Penguin than Adélie Penguin.
The percentages of krill consumption differed also in spring as indicated by the significant isotopic difference between the guano of Emperor Penguins and the eggshell membranes of Adélie Penguins ($t = -3.54$, $p = 0.001$ and $t = 8.70$, $p < 0.0001$). However, in this season Adélie Penguin consumed a greater proportion of krill than the other species ($P_{BIC} = 0.99$).

### Discussion

Emperor (Aptenodytes forsteri) and Adélie (Pygoscelis adeliae) Penguins, which inhabit the southernmost latitudes on our planet, are extremely 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 and seasonal migrations as well as a key foraging habitat (Ainley et al. 1998; Bluhm et al. 2010; Lyver et al. 2011).

In order to access open waters throughout the year, penguins may have to migrate thousands of kilometers along the edge of the sea ice (Ainley 2002; Kooyman et al. 2004; Juárez et al. 2016). This makes the study of these species extremely difficult, especially in winter, when darkness, sea ice extent and low temperatures prevent direct observations and sample collections. As a result, there are only few documents describing the feeding behavior of these two species throughout the year (Cherel 2008; Juárez et al. 2016).

Thanks to SIA of guano, eggshell membranes, feathers and the main prey (i.e., krill and P. antarctica) of Emperor and Adélie Penguins, we reconstructed the intra-annual diet of these two predators in five colonies along the coast of the Ross Sea. Since the Euphausiids occupy a lower trophic position than P. antarctica (Quillfeldt et al. 2005; Cherel 2008; Strickland et al. 2008), variations in the consumption of these preys by penguins were reflected in variations in the isotopic values of penguin tissues (Quillfeldt et al. 2005; Cherel 2008; Strickland et al. 2008; Tierney et al. 2008).

Our results clearly indicate spatio-temporal variations in the isotopic values of penguin tissues and guano, reflecting changes in diet across seasons and colonies as well as differences between the two species. The study area was an ideal laboratory for comparing penguin diets under different sea ice conditions as these colonies are located in sites characterized by differences in ice persistence, which in turn influences the abundance of krill and fish, the two main resources of Emperor and Adélie Penguins (Knox 1994; Ainley et al.1998, 2003; 2018; Ainley 2002; Watanuki et al. 2002; Koubbi et al. 2011; Polito et al. 2011b; Meyer et al. 2017). Specifically, a greater availability of krill as prey for penguins occurs in periods or areas characterized by greater persistence and extension of the ice pack, while the fish P. antartica is more frequent in open sea areas without ice cover (Ainley et al.1998, 2003; Ainley 2002; Wienecke et al. 2000; Strickland et al. 2008).

Some studies have indicated that the Emperor Penguin’s diet is mainly based on fish consumption and this food preference is explained by its foraging pattern (Cherel and Kooyman 1998; Barbraud and Weimerskirch 2001; Cherel 2008). Unlike the smaller Adélie Penguins, which feed in shallower pelagic waters, where krill is concentrated (Cherel

### Table 3

| Colony          | Winter Krill | Winter Fish | Spring Krill | Spring Fish | Summer Krill | Summer Fish |
|-----------------|--------------|-------------|--------------|-------------|--------------|-------------|
| Emperor Penguin | 59% (34–89)  | 41% (21–66) | 29% (12–66)  | 71% (35–88) | 70% (59–79)  | 30% (21–40) |
| Adélie Penguin  | 31% (15–67)  | 69% (33–95) | NA           | NA          | 64% (53–75)  | 36% (25–47) |
| Inexpressible Island | 14% (2–46)  | 86% (55–98) | 88% (69–99)  | 12% (2–31)  | 61% (52–70)  | 39% (30–49) |
| Adélie Cove     | 9% (1–26)    | 91% (74–98) | 93% (83–99)  | 7% (1–17)   | 70% (52–87)  | 30% (13–48) |
| Adélie Cove     | 72% (40–97)  | 28% (13–50) | 92% (76–99)  | 8% (1–24)   | 90% (76–98)  | 10% (2–24)  |

The table shows the percentage in the diet (mean 2.5–97.5% C.I.) of fish (Pleuragramma antarctica) and krill (genus Euphausia) for each colony. Shell membranes in Inexpressible Island not available (NA)

### Table 4

| Season   | df | F  | p  | F  | p  |
|----------|----|----|----|----|----|
| Summer   | 3  | 8.12 | <0.0001 | 4.02 | 0.011 |
| Winter   | 3  | 7.35 | 0.03 | 20.22 | <0.0001 |
| Spring   | 2* | 0.28 | 0.75 | 2.08 | 0.15 |

*Eggshell membranes in Inexpressible Island not available

($P_{BIC} = 1$; Table 4). The percentages of krill consumption differed also in spring as indicated by the significant isotopic difference between the guano of Emperor Penguins and the eggshell membranes of Adélie Penguins ($t$ test: $t = -3.54$, $p = 0.001$ and $t = 8.70$, $p < 0.0001$). However, in this season Adélie Penguin consumed a greater proportion of krill than the other species ($P_{BIC} = 0.99$).
and Kooyman 1998; Rodary et al. 2000b; Wienecke et al. 2000), the Emperor Penguins make dives that can reach 500 m depth at which it is possible to prey on fish (Cherel and Kooyman 1998; Rodary et al. 2000a; Cherel 2008; Zimmerman et al. 2008). Although our results show that fish is part of the Emperor Penguin’s diet throughout the year, the significant differences in isotopic values between seasons and the results of Bayesian mixing models indicate remarkable krill consumption, in particular in summer and winter. During the austral winter, this species performs long migration towards the breeding sites, far from the open sea areas due to the great sea ice extension (Kirkwood and Robertson, 1997; Wienecke and Robertson 1997; Burns and Kooyman 2001). Since female emperor penguins fast during this phase in order to store energy reserves (Speake et al. 1999; Connan et al., 2019), they spend the pre-breeding fattening period foraging the most available prey in the nearest polynya, if present or, alternatively, in the shelf waters (Kirkwood and Robertson 1997; Speake et al. 1999; Burn and Kooyman 2001; Michelot et al., 2020). In agreement with Kirkwood and Robertson (1997) our results showed that the winter diet of this species consists mainly of krill, which feeds on sympagic algae growing under the surface of the ice pack and, thus, is extremely abundant in this season (Ainley et al. 1998, 2003; Ainley 2002; Wienecke et al. 2000; Strickland et al. 2008). The enriched C and N isotopic values observed in spring indicate that in this season the Emperor Penguin diet was mainly based on fish. For this species, spring represents the chick-rearing period and, since fish is a more energetic resource than krill (Watanuki et al. 2002; Ainley et al. 2003; Tierney et al. 2008), when possible adults feed on this high-quality prey for their chicks and for themselves to recover the energy spent on the care of the offspring (Cherel 2008; Tierney et al. 2008). Summer represents the moulting period for the Emperor Penguins and during this phase the individuals tend to feed heavily to rapidly build up body reserves to cope with the fasting of the following migration period (Kooymans et al., 2004; Wienecke et al., 2004). Results of Bayesian mixing models indicate that Emperor Penguins had a krill-based diet at Cape Washington in summer. Although the presence and abundance of Euphausids is linked to sea ice (Ainley et al. 1998, 2003; Ainley 2002; Wienecke et al. 2000; Strickland et al. 2008), phytoplankton blooms, favoured by increased solar radiation in the water following the summer melting of the sea ice, may have increased the krill local abundance (Saenz et al. 2020; Watanabe et al. 2020) and therefore its consumption by penguins.

The prevalent consumption of krill by Adélie Penguins occurred in spring and summer while in winter we observed a shift to a fish-based diet in all colonies except Cape Hallett. Indeed, in the austral winter the Adélie Penguins, migrating from the breeding sites to the moulting areas, move towards the edge of the pack ice (Juárez et al. 2016), where they have access to open sea areas and then to fish. In Cape Hallett, the northernmost area, the greater persistence of sea ice plausibly led to a greater availability of krill than in the other areas (Ainley et al. 1998; Strickland et al. 2008; Lyver et al. 2011). Unlike the other Adélie Penguin colonies, in Cape Hallett, there were no significant seasonal variations in δ15N values, as krill is the most consumed resource in this site throughout the year. In spring, depleted isotopic values in the three southernmost Adélie Penguin colonies indicated increased krill consumption. Female Adélie Penguins adopted a pre-breeding period foraging strategy similar to that of Emperor Penguins observed in winter. They reach the breeding sites, which are far from the open sea areas due to the great extension of the pack ice (Kirkwood and Robertson 1997). Since Adélie Penguins never travel farther than 100 km from the colony and rarely travel more than 30–50 km (Olmastroni et al. 2020), females forage mainly under sea ice as well as in polynya areas when possible (Kirkwood and Robertson, 1997; Michelot et al. 2020; Watanabe et al. 2020), where there is a large availability of krill (Spindler and Dieckmann 1994; Nicoll 2006).

The comparison between the neighboring colonies of Emperor Penguins and Adélie Penguins shows that δ13C and δ15N values of these two species differed in spring and winter. These seasonal differences can be related to the different life cycle patterns of these two species, which lead them to forage in different geographic areas during the high sea ice coverage seasons (Cherel 2008). In contrast, since the two colonies are only 30 km apart, an overlap of the summer foraging areas between the two species is plausible. Indeed, during this season no differences were observed in the δ13C and δ15N values of these two species whose diet was based mainly on krill (Strickland et al. 2008; Watanabe et al. 2020).

In summary, contrary to our predictions, the results show that not only the Adélie Penguin but also the Emperor Penguin exhibits a variation in diet over the course of the year. This temporal dietary variation is due to the phases of the life cycle of the two species and the seasonal dynamics of the sea ice that influence the availability of their prey. Furthermore, the variable sea ice persistence along the coasts of the Ross Sea, influencing the spatial availability of prey, determines dietary variability between colonies in Adélie penguins.

The comparison of the isotopic values of different biological matrices has proved to be a valuable tool for reconstructing the intra-annual diets of Adélie and Emperor Penguins. The advantage offered by this ‘multi tissue approach’ compared to classic methods such as stomach contents analysis or direct observations, is the possibility of collecting information on the trophic ecology of seabirds also when these species are inaccessible for observation or sampling due to their migratory activity or extreme environmental conditions.
Although powerful, this approach has some limitations. The main one concerns the use of tissue-specific turnover and isotope fractionation values, as the use of incorrect values can lead to an incorrect interpretation of the results (Dalerum and Angerbjörn 2005; Bond and Diamond 2011). The large inter-specific variation (Vanderklift and Ponsard 2003) makes it necessary to use species-specific fractionation values or, when not available in the literature, fractionation values obtained from phylogenetically related species (Dalerum and Angerbjörn 2005). The knowledge of species-specific tissue turnover allows definition of the time window in which the tissue reflects the dietary record (Dalerum and Angerbjörn 2005). On the other hand, the use of metabolically inert matrices that integrate dietary information in a discrete period has made it possible to establish with certainty the diet of the two species in different periods of the year, making us confident of the goodness of the results, which were also robust to relevant variations (i.e., up to 30%) in the values of TEF used in mixing models.

Concluding Remarks

In this study, the stable isotope analysis of different tissues made it possible to reconstruct the intra-annual diet of the Adélie Penguin (Pygoscelis adeliae) and Emperor Penguin (Aptenodytes forsteri) in colonies located in the core of the Ross Sea MPA and never studied before through SIA. Feathers, eggshell membranes and guano provided information from different periods (Jafari et al. 2020), filling gaps associated with the trophic behavior of these endemic species of conservation importance in winter and spring.

Knowledge of the trophic ecology of high trophic level predators can provide useful information on changes occurring at lower trophic levels in the food chain as a consequence of environmental changes (Juáres et al. 2016). Here, comparison of C and N stable isotopes between and within species allowed us to detect changes in penguin feeding preferences, providing updated dietary and isotopic reference data useful for future comparisons within the Ross Sea MPA, which represents a biodiversity hotspot and the largest marine protected area on our planet (Ballard et al. 2012; Laffoley et al. 2019). Deepening of the knowledge of the trophic ecology of Adélie and Emperor Penguins represents an important step to understand the mechanisms underlying the structural and functional integrity of the Antarctic ecosystem and to predict how these species will respond to changes in resource availability due to the climate change or overexploitation in the Southern Ocean.

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Author contributions LR, MLC and EC conceived and designed research. GC and EC conducted the sampling activity. VJ and SSC processed the samples. VJ, DM and GC analysed data. VJ, DM and MLC wrote the manuscript. All authors read, reviewed, approved the manuscript.

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Declarations

Conflict of interest The Authors declare no conflict and/or competing interests.

Ethical approval The present study is part of the PNRA2015/AZ1.01 and PNRA16_00291 projects. No living organisms were involved. The sampling activity was agreed with the PNRA (Italian National Antarctic Research Program), which issued permits to collect samples in the study area on behalf of the Italian Ministry of Foreign Affairs. Permits were issued in compliance with the “Protocol on Environmental Protection to the Antarctic Treaty”, Annex II, art.3)

Data availability All the relevant data and information are within the paper.

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