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Using multiple-stable isotopes ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) to assess past and present Adélie penguin foraging grounds in the Ross Sea region, Antarctica

Megan Reaves, Shannon Powers, Steven D. Emslie*

Department of Biology and Marine Biology, University of North Carolina Wilmington, 601 S. College Road, Wilmington, North Carolina 28403, USA

ABSTRACT: We completed multiple-stable isotope analyses ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$) of Adélie penguin *Pygoscelis adeliae* chick-bone collagen to characterize differences in foraging behavior among 15 colony locations across the Ross Sea region. Foraging behavior was represented by $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values and classified into groups using *k*-means cluster analyses. Additionally, we report the first stable isotope values for the Adélie penguin colony on Sabrina Island, Balleny Islands. Cluster analyses revealed distinct isotopic signatures for the northernmost and central colonies; however, owing to spatial and temporal variability, isotopic signatures were not strong enough to distinguish the southernmost colonies. Results also indicated that $\delta^{15}N$ values increased with latitude (66–77° S), corresponding to higher krill consumption at colonies that foraged in sensible heat polynyas or the open ocean and increased fish consumption for those foraging in latent heat polynyas to the south. Generally, $\delta^{34}S$ values are used to distinguish foraging grounds, specifically inshore/offshore foraging or foraging over the continental slope versus the continental shelf, in marine animals. Although the southern and central colonies currently forage along the continental shelf and the northern colonies forage over the shelf, slope, and/or open ocean, we found no significant difference in $\delta^{34}S$ values among colonies. While a positive correlation between $\delta^{15}N$ and $\delta^{34}S$ values was evident, $\delta^{34}S$ signatures did not exhibit distinct patterns specific to individual colonies or regions. The absence of a clear trend reflecting inshore/offshore foraging underscores the need for additional research to bridge this knowledge gap.

KEY WORDS: Adélie penguin · *Pygoscelis adeliae* · Stable isotopes · Polynya · Foraging behavior · Antarctica

1. INTRODUCTION

The Adélie penguin *Pygoscelis adeliae* is an endemic seabird in Antarctica with a circumpolar distribution. It is one of most studied seabirds in the world and approximately 30% of its total population occurs in the Ross Sea (Lynch & LaRue 2014); most colonies (62%) are associated with polynyas (Ainley 2002, Santora et al. 2020). Polynyas are recurring areas of partially or completely ice-free ocean that are surrounded by sea ice and serve as the primary and sometimes only access to open water during the breeding season (Van Woert 1999, Ainley 2002). Polynyas in the Ross Sea tend to expand during late November to early December and are at their largest extent in early January. This timing is critical, as the polynyas align with the Adélie penguin breeding season, October to February, making them essential for their breeding success and survival (Ainley 2002). There are 2 types of polynyas: sensible heat, also known as open-ocean polynyas, and latent heat, also referred to as coastal polynyas (Martin 2001).

*Corresponding author: emslies@uncw.edu

The more northern Adélie penguin colonies in the Ross Sea largely rely on sensible heat polynyas, specifically the Ross Passage or Pennell Bank polynyas, which are formed by upwelling of warmer circumpolar deep water (CDW; Jacobs & Comiso 1989). There is evidence that suggests that these polynyas were present during the Last Glacial Maximum (Thatje et al. 2008), possibly supporting any northern colonies that might have existed at that time.

Most colonies in the southern to central Ross Sea rely on latent heat polynyas, specifically the McMurdo Sound (MSP), Ross Sea (RSP), or Terra Nova Bay (TNBP) polynyas. Latent heat polynyas form as cold katabatic winds force sea ice away from the coast, causing oceanic heat loss and rapid sea ice formation (Martin 2001). The TNBP and possibly the RSP formed when the Ross Ice Shelf retreated to the south of Terra Nova Bay by ~7600 calendar years before present (cal yr BP), followed by the MSP as the ice shelf retreated to its current southerly position (Conway et al. 1999, Emslie et al. 2007). Katabatic winds directed toward Ross Island diverge and form the MSP and RSP. The polynya at which colonies on Ross Island forage can change depending on the sea ice cover over McMurdo Sound, which varies in extent throughout the breeding season (Emison 1968, Kim et al. 2018, Leonard et al. 2021). The Drygalski Ice Tongue, an extension of the David Glacier, and strong persistent katabatic winds together form the TNBP. On average, the Drygalski Ice Tongue extends ~90 km into the Ross Sea, and this length determines the maximum southern extent of the TNBP by preventing pack ice from entering the bay from the south (Indrigo et al. 2021).

Traditionally, stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes are used to evaluate foraging behavior, specifically foraging grounds and diet composition, in wide-ranging species (Fry 2006). These isotopes have helped reconstruct current and past diets, foraging grounds, and prey patterns in Adélie penguins (Emslie & Patterson 2007, Strickland et al. 2008, Tierney et al. 2008, Juáres et al. 2016). On average, parents feed their chicks until 7–8 wk of age, after which the chicks fledge and forage for food on their own (Ainley 2002, Whitehead et al. 2015). Based on this timeline, 1–2 mo of parental foraging behavior is accurately reflected in the tissues of chicks that do not survive past the crèche period (4–7 wk old; Vasil et al. 2012). Sampling of tissues from chick carcasses (e.g. breast feathers, toenails, or bone) is a simple and non-invasive method (Ainley et al. 2003, Ciriani et al. 2021), with large sample sizes possible, as chick mortality ranges around 50% at most colonies (Ainley 2002). Chick tissues can accurately represent parental foraging behavior, as the isotopic composition of tissues remains consistent, regardless of the cause of death, sex, and age (Vasil et al. 2012).

The isotopic composition of Adélie penguin chick tissues provides an overview of parental foraging behavior and surrounding environmental conditions. Northern, central, and southern colonies in the Ross Sea region likely have an individual isotopic signature due to the differences in primary productivity, sea ice conditions, upwelling of carbon sources, and polynya size (Arrigo & Van Dijken 2003, Arrigo et al. 2015, St John Glew et al. 2021). We suspect that these isotopic signatures can be identified by comparing isotope values of chick tissues that record parental foraging behavior associated with each region of the Ross Sea.

Analysis of sulfur stable isotopes ($\delta^{34}S$) in tissues is a recent addition to understanding inshore/offshore foraging or foraging over the continental slope versus the continental shelf, and migratory behavior in avian species (Morkūnė et al. 2016, Steenweg et al. 2017, Szpak & Buckley 2020). The majority of sulfate in the Antarctic originates from marine biogenic sources (Pruett et al. 2004, Nehlich et al. 2013). Sulfate is reduced to sulfide (S^{2-}) by bacteria, which strongly discriminates against ^{34}S, resulting in the enrichment of the remaining sulfate. Due to continual mixing of oceans, seawater sulfate has remained constant during the last million years with a mean isotope ratio of 20.3‰ (Bottrell & Newton 2006, Nehlich 2015, Ishino et al. 2019). The mean isotope ratio of oceans is reflected in tissues of marine-dwelling organisms with $\delta^{34}S$ values of 15–20‰, while $\delta^{34}S$ values $\leqslant$15‰ in tissues reflect freshwater sulfates, indicating foraging in estuarine or terrestrial freshwater environments (Fry 1988, Pruett et al. 2004).

In the Ross Sea, inshore areas on the continental shelf would undergo less oceanic mixing and receive greater freshwater input from terrestrial ice melt. It is expected that the tissues of penguins that forage in polynyas over the continental shelf would reflect these freshwater sulfates with $\delta^{34}S$ values of $\leqslant$15‰. Colonies located along Northern Victoria Land forage farther from the colony, possible due to increased intraspecific competition from a higher density of penguins in that region compared to the south (Lyver et al. 2011). Foraging distances closer to the continental shelf break in sensible heat polynyas, formed by upwelling of CDW, may be reflected in tissues with $\delta^{34}S$ values at or above 15‰.

This study represents the first application of sulfur isotopes to track inshore/offshore foraging in Antarctic penguins in the Ross Sea region. We also provide

new information on the isotopic niche of Adélie penguins on Sabrina Island (Balleny Islands). The main objectives of this study were to (1) analyze $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values in Adélie penguin chick-bone collagen to characterize differences in foraging behavior among 15 colony locations across the Ross Sea region and (2) determine if the northern, central, and southern colonies in the Ross Sea region have distinct $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ isotopic signatures.

2. MATERIALS AND METHODS

2.1. Sample collection and site background

Adélie penguin chick remains were collected from the surface or by excavation of ornithogenic soils between the austral summers of 2000/2001 and 2019/2020 (Table 1). Remains found on the surface of active colonies were categorized as modern or dating within the past ~20 yr from the date the remains were collected. Samples were categorized as ancient if they had radiocarbon ages greater than 1200 BP, which is the youngest limit for providing a 2-sigma calibrated age range in cal yr BP (Table S1 in the Supplement at www.int-res.com/articles/suppl/m756p127_supp.pdf). Samples greater than 20 yr with radiocarbon ages less than 1200 BP were categorized as historic. Ornithogenic soils were excavated in 5 cm levels, and each excavation was assigned a site number or name (see methods in Emslie et al. 2003). Specific site age and mean calibrated radiocarbon dates previously published or reported here for Adélie penguin remains (e.g. eggshells, bones, and feathers) from cor-

Table 1. Number of chick bone samples analyzed for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ isotopes from each Adélie penguin colony grouped by northern, central, and southern regions. Locations are listed from north to south (see Fig. 1); an asterisk (*) indicates samples collected from active sites representing modern foraging behavior. BI: Beaufort Island; SCBE: South colony beach exposure; NC: North colony; GS: Gully site. See Table S1 for more details

Location	Site	n		Coordinates	
		$\delta^{13}C/\delta^{15}N$	$\delta^{34}S$	Latitude (°S)	Longitude (°E)
Northern					
Sabrina Island	Surface*	18	10	66° 54′ 26.00″	163° 22′ 34.3″
Cape Adare	Surface*	32	10	71° 17′ 53.00″	170° 13′ 25.15″
Cape Hallett	Surface*	42	2	72° 19′ 10.57″	170° 12′ 54.68″
Central					
Edmonson Point	Surface*	5		74° 19′ 31.84″	165° 7′ 16.13″
	1	5	3	74° 19′ 53.29″	165° 8′ 30.41″
	2	1	1	74° 19′ 40.30″	165° 8′ 7.80″
Campo Icarus	2	5	2	74° 42′ 45.22″	164° 6′ 53.60″
	3	6	3	74° 42′ 44.60″	164° 6′ 43.42″
North Adelie Cove	1	1	1	74° 44′ 4.88″	164° 6′ 37.01″
	3	4	3	74° 43′ 58.40″	164° 6′ 21.71″
Adelie Cove	Surface	5		74° 46′ 0.90″	163° 59′ 37.32″
	3	3	3	74° 46′ 5.80″	164° 0′ 35.80″
Southern					
Cape Irizar	Surface	6	1	75° 32′ 60″	162° 56′ 60″
	1	3	3	75° 32′ 60″	162° 56′ 60″
	5	1		75° 32′ 60″	162° 56′ 60″
Franklin Island	1	2	1	76° 10′ 00.2″	168° 21′ 23.1″
Cape Ross	2	1	1	76° 43′ 49.56″	163° 0′ 41.79″
	3	1	1	76° 43′ 49.56″	163° 0′ 41.79″
Beaufort Island	1	1	1	76° 55′ 47.50″	166° 53′ 31.70″
	BI 3.1–3.2	11	2	76° 55′ 51.5″	166° 54′ 32.5″
	2	4	2	76° 55′ 52.50″	166° 54′ 15.08″
	Surface	3		76° 57′ 1.18″	166° 57′ 18.49″
	SCBE	7	2	76° 57′ 1.18″	166° 57′ 18.49″
Cape Bird	NC, Surface*	23	5	77° 14′ 31.08″	166° 24′ 1.18″
	GS1	3	3	77° 14′ 31.08″	166° 24′ 1.18″
Marble Point	1	11	4	77° 25′ 56.69″	163° 49′ 17.14″
Cape Crozier	1	2	2	77° 27′ 34.04″	169° 13′ 44.25″
Cape Barne	1	1	1	77° 34′ 50.04″	166° 15′ 14.43″

responding sites and levels to our bone samples can be found in Table S1.

Our analyses included 28 sites within 15 colony locations across the Ross Sea region, including Sabrina Island (Balleny Islands), the most northerly in the Ross Sea region (Fig. 1, Table 1). The Sabrina Island colony forages from the open ocean rather than from any specific polynya. Northern colonies where penguins forage in the Ross Passage and/or Pennell Bank polynyas near the continental shelf break include those at Cape Hallett and Cape Adare (Ainley & Wilson 2023). Central colonies that rely or likely relied on the TNBP for foraging include Edmonson Point, Campo Icarus, North Adelie Cove, and Adelie Cove. Southern colonies presumably relying on the MSP include Cape Ross, Cape Barne, and Marble Point, and those relying on the RSP include Franklin Island and Cape Crozier. Depending on the annual extent of each polynya, abandoned Adélie penguin colonies located on or near Ross Island (Beaufort Island and Cape Bird) would have foraged in the MSP and/or RSP (Fig. 1). Penguins at Cape Irizar, located just south of the Drygalski Ice Tongue, could have foraged in the TNBP or MSP depending on polynya expansion, ice conditions, and presence or absence of the Drygalski Ice Tongue over time.

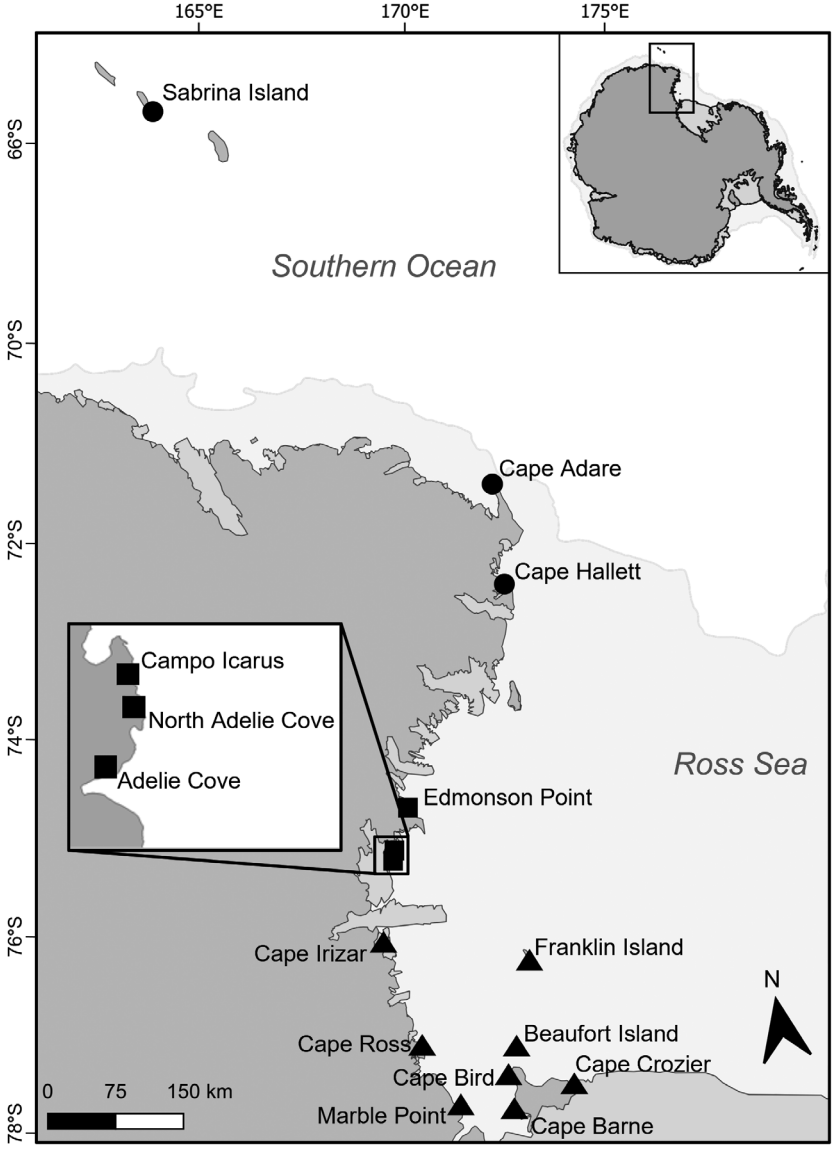

Fig. 1. Northern (circles), central (squares), and southern (triangles) Adélie penguin colony locations where chick bones were collected in the Ross Sea region between austral summers 2000/2001 and 2019/2020

2.2. Sample preparation and quality control

Cleaning, isolating, and purifying bone collagen for stable isotope analysis were completed using the preparation methods outlined by Tuross (2012) and Sealy et al. (2014). The surface of each bone sample was cleaned using a Dremel tool with a sand drum attachment to remove any superficial contamination. Bones were then rinsed with deionized water and placed in a drying oven at 65°C for up to 24 h. Approximately 0.5–1.0 g of the bone was removed using a saw or wire cutters, then crushed into smaller pieces using a mortar and pestle. The bone pieces were then placed in 50 ml Falcon tubes with 40–45 ml of 0.5 M EDTA to isolate bone collagen. The EDTA solution was replaced every 3 d until all bone apatite fully demineralized, isolating the bone collagen. Complete demineralization was determined when the bone pieces were bendable and could easily be cut with forceps or a scalpel. Once demineralization was complete, the resulting bone collagen was rinsed in ultrapure water 15–20 times, then left to soak in ultrapure water overnight. Purification methods involved removing humic acids and lipids, as they can skew $\delta^{13}C$ values (Post et al. 2007, Guiry & Szpak 2021). After overnight soaking, ultrapure water was removed, and the bone collagen was soaked in 0.1 M NaOH for 24 h to remove any re-

maining humic acids. After 24 h, 0.1 M NaOH was removed with a pipette and replaced with ultrapure water. Ultrapure water was replaced every day for 8–10 d or until the pH was neutral. The collagen was then transferred to a −80°C freezer for 24 h and then freeze-dried for 48 h. After drying, it was transferred into a glass vial and soaked in a 2:1 chloroform:methanol solution for 24 h to remove lipids (Liden et al. 1995). Samples were then placed in a fume hood to dry for 24 h.

The atomic carbon to nitrogen (C:N) ratio of bone collagen was used to assess quality. A C:N ratio between 2.9 and 3.6 indicates successful demineralization of bone apatite, lipid extraction, humic acid removal, and well-preserved bones (Ambrose 1993, Tuross 2012). The EDTA and purification methods were repeated for samples that fell outside of this range. There were 5 samples with consistent C:N ratios >3.6 (3.71–3.91) after both EDTA and purification methods were repeated 2 additional times. There were no noticeable differences in C:N isotope values before and after treatments, suggesting no impurities or bone apatite were present. The $\delta^{13}C$ and $\delta^{15}N$ values were consistent with the remainder of samples from corresponding locations and were included in the statistical analyses.

2.3. Stable isotope analyses

A total of 193 bone collagen samples were prepared and analyzed for carbon and nitrogen isotopes. Stable carbon and nitrogen isotope analyses were conducted at the University of North Carolina Wilmington Isotope Ratio Mass Spectrometry facility. Approximately 0.7 mg of sample was weighed in a tin capsule and flash-combusted using a Costech 4010 Elemental Analyzer interfaced with a Thermo Delta V Plus Stable Isotope Ratio Mass Spectrometer. Additional $\delta^{13}C$ and $\delta^{15}N$ values of 14 bone collagen samples from colonies reported by Kristan et al. (2019) were added to our own; thus, 207 samples were analyzed. For sulfur isotope analysis, 10 mg of sample was weighed in a tin capsule and analyzed at the University of California Davis Mass Spectrometry facility using an Elementar vario ISOTOPE cube elemental analyzer connected to an Elementar PrecisION isotope ratio mass spectrometer. Sulfur isotope analysis was completed on 67 of the 193 bone collagen samples.

Isotopic compositions of samples were expressed in standard δ notation in parts per mil (‰) using the following equation:

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

where X is ^{13}C, ^{15}N or ^{34}S, and R is the corresponding ratio of $^{13}C/^{12}C$, $^{15}N/^{14}N$, or $^{34}S/^{32}S$. The $\delta^{13}C$ and $\delta^{15}N$ isotopic compositions were calibrated relative to the international standards of Vienna Pee Dee belemnite and atmospheric N_2 (air). Raw $\delta^{13}C$ and $\delta^{15}N$ values were normalized on a 2-point scale using standard reference materials enriched and depleted in glutamic acid (USGS-40 and USGS-41). Samples were run in duplicate for stable carbon and nitrogen isotope analyses. If differences fell outside of the instrument error, samples were run in triplicate. The $\delta^{34}S$ isotopic composition is reported on the Vienna Canyon Diablo Troilite scale; 5 reference materials (cysteine, hair, mahi-mahi muscle, whale baleen, and taurine) were used for quality control, scale normalization, and linearity correction.

2.4. Statistical analyses

All statistical analyses and plots were performed or created using R version 4.1.2. (R Core Team 2021). A Shapiro-Wilk test was used to assess normality and Levene's test was used to assess equality of variances. Statistical comparisons of isotopic compositions between locations involved a one-way ANOVA followed by a post hoc Tukey's HSD test. Locations with at least 3 samples were included in this comparison (n = 12). All inferential statistics were significant at the <0.05 level. Pearson's correlations were tested between the $\delta^{13}C$ and $\delta^{15}N$ values of all 207 samples and again between the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values of the 67 samples.

We characterized isotopic signatures using *k*-means cluster analyses using the R packages 'cluster' (version 2.1.4) and 'factoextra' (version 1.0.7). The *k*-means cluster analysis assigned samples based on $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values (Cluster Analysis A; n = 67) or $\delta^{13}C$ and $\delta^{15}N$ (Cluster Analysis B; n = 207) to the closest cluster centroids by minimizing Euclidean distance. Cluster Analysis A applied 3 variables ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) and was therefore represented by a principal component analysis (PCA). The PCA allows for visual interpretation of *k*-means clusters using 3 or more variables where principal components (PC1 and PC2), referred to here as dimensions (Dim1 and Dim2), are represented by observable variability (between sum of squares/total sum of squares; %).

3. RESULTS

The $\delta^{13}C$ and $\delta^{15}N$ values for all 207 samples ranged from −26.2 to −18.2‰ (mean ± SD = −22.6 ± 1.4‰)

Table 2. $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ signatures (average ± SD and range; ‰) of chick-bone collagen from each foraging group (in **bold**) and location

Location	n	$\delta^{13}C$	Range $\delta^{13}C$	$\delta^{15}N$	Range $\delta^{15}N$	n	$\delta^{34}S$	Range $\delta^{34}S$
Northern	**92**	**−23.4 ± 1.0**	**−26.2 to −21.3**	**9.1 ± 0.9**	**7.1–12.6**	**22**	**15.8 ± 1.3**	**13.3–19.1**
Sabrina Island	18	−22.4 ± 0.6	−23.4 to −21.3	8.7 ± 0.4	8.0–9.5	10	16.6 ± 1.1	15.0–19.1
Cape Adare	32	−23.9 ± 0.7	−25.6 to −22.4	9.1 ± 0.8	7.6–11.1	10	15.2 ± 1.0	13.3–16.6
Cape Hallett	42	−23.4 ± 0.9	−26.2 to −21.6	9.4 ± 1.1	7.1–12.6	2	14.9 ± 1.5	13.4–16.3
Central	**35**	**−21.9 ± 1.6**	**−25.5 to −19.2**	**13.5 ± 2.1**	**8.9–16.6**	**16**	**16.4 ± 0.7**	**14.5–17.7**
Edmonson Point	11	−21.8 ± 1.0	−23.3 to −19.6	13.1 ± 2.3	8.9–16.1	4	16.9 ± 0.3	16.7–17.4
Campo Icarus	11	−21.1 ± 1.4	−23.3 to −19.2	13.6 ± 1.7	11.0–15.6	5	15.7 ± 0.6	14.5–16.3
North Adelie Cove	5	−21.6 ± 1.6	−23.1 to −19.3	15.9 ± 0.5	15.2–16.6	4	16.4 ± 0.5	15.7–17.1
Adelie Cove	8	−23.3 ± 1.4	−25.5 to −20.5	12.1 ± 1.7	10.3–15.3	3	17.0 ± 0.5	16.6–17.7
Southern	**80**	**−22.1 ± 1.4**	**−24.8 to −18.2**	**13.2 ± 1.6**	**7.2–16.2**	**29**	**16.5 ± 0.8**	**15.5–18.1**
Cape Irizar	10	−20.2 ± 1.3	−23.4 to −18.2	14.5 ± 0.8	12.6–15.4	4	16.7 ± 1.0	15.5–18.1
Franklin Island	2	−22.0 ± 0.7	−22.7 to −21.3	14.7 ± 0.2	14.4–14.9	1	16.1	
Cape Ross	2	−20.8 ± 0.3	−21.1 to −20.5	15.3 ± 0.9	14.3–16.2	2	17.4 ± 0.7	16.6–18.1
Beaufort Island	26	−22.3 ± 0.8	−23.9 to −20.9	12.5 ± 1.6	7.2–14.5	7	16.5 ± 0.8	15.7–17.8
Cape Bird	26	−22.9 ± 1.1	−24.8 to −20.4	12.3 ± 0.9	10.8–14.5	8	16.5 ± 0.6	15.8–17.7
Cape Crozier	2	−23.5 ± 1.0	−24.4 to −22.5	13.0 ± 0.5	12.4–13.5	2	16.3 ± 0.2	16.1–16.5
Marble Point	11	−21.0 ± 1.1	−22.4 to −18.5	14.7 ± 1.0	13.1–15.9	4	15.9 ± 0.2	15.7–16.2
Cape Barne	1	−22.1		13.3		1	17.3	

and 7.1 to 16.6‰ (11.4 ± 2.5‰), respectively (Table 2). The $\delta^{13}C$ and $\delta^{15}N$ values for the 67 samples analyzed for sulfur isotopes ranged from −25.2 to −18.5‰ (−22.2 ± 1.5‰) and 7.6 to 16.6‰ (12.3 ± 2.7‰), respectively. Among the 67 samples, $\delta^{34}S$ values were homogeneous, ranging between 13.3 and 19.1‰ (16.2 ± 0.99‰; Table 2). There were no significant differences in $\delta^{34}S$ values between any of the 11 locations compared (1-way ANOVA: $F_{9,50} = 1.81$, $p = 0.08$). Cape Ross had the highest average $\delta^{34}S$ values (17.4 ± 1.0‰) while Cape Hallett had the lowest average values (14.9 ± 2.0‰). Sabrina Island had the highest $\delta^{34}S$ value of 19.1‰, while Cape Irizar had the highest variation at ±2.6‰.

There were significant differences in $\delta^{13}C$ and $\delta^{15}N$ values among central Ross Sea colonies associated with the TNBP (1-way ANOVA, $\delta^{13}C$: $F_{10,189} = 19.88$, $p < 0.0001$; $\delta^{15}N$: $F_{10,189} = 60.87$, $p < 0.0001$; Tables S2 & S3). For example, the $\delta^{15}N$ values from Adelie Cove were significantly lower than those from North Adelie Cove (Tukey's HSD: $p < 0.001$), while the $\delta^{13}C$ values of Adelie Cove were significantly lower than those of Campo Icarus (Tukey's HSD: $p < 0.0001$). There were significant differences among colony locations associated with the MSP and with those which could be associated with either the MSP or RSP. The $\delta^{13}C$ and $\delta^{15}N$ values from Marble Point were significantly different from Cape Bird and Beaufort Island (Tukey's HSD: $p < 0.05$, $p < 0.001$).

There was a strong significant positive correlation between $\delta^{13}C$ and $\delta^{15}N$ values in all samples ($r(207) = 0.66$, $p < 0.001$; $r(67) = 0.66$, $p < 0.001$; Fig. 2), and between $\delta^{15}N$ and $\delta^{34}S$ for those with sulfur analysis included ($r(67) = 0.34$, $p < 0.01$). The correlation between $\delta^{34}S$ and $\delta^{13}C$ values was weak and close to being statistically significant ($r(67) = 0.23$, $p = 0.057$). There was a significant positive correlation for $\delta^{13}C$ and $\delta^{15}N$ values with increasing latitude (66–77° S) among all samples ($r(207) = 0.27$, $p < 0.001$; $r(207) = 0.69$, $p < 0.001$; Fig. 3).

The optimal number of clusters determined for both *k*-means cluster analyses, Cluster Analyses A and B, was 4. The *k*-means cluster analysis (Cluster Analysis A) between $\delta^{13}C$ and $\delta^{15}N$ values (n = 207) explained 83.6% of variability (Fig. 4). Samples from individual locations were separated into 2 or 3 clusters, except for samples from Sabrina Island, North Adelie Cove, Cape Ross, and Franklin Island, which were assigned to 1 cluster (Table 3). The *k*-means cluster analysis (Cluster Analysis B) using $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values (n = 67) explained 77.6% of variability (Fig. 4). Dim1 explained 61.4% and Dim2 explained 27.1% of the remaining variance. The addition of $\delta^{34}S$ values to Cluster Analysis B created overlap among 3 of the 4 clusters, and samples from individual locations containing more than 1 sample were separated into 2 to 3 clusters.

4. DISCUSSION

We applied multiple stable isotope ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) analyses to characterize differences in foraging

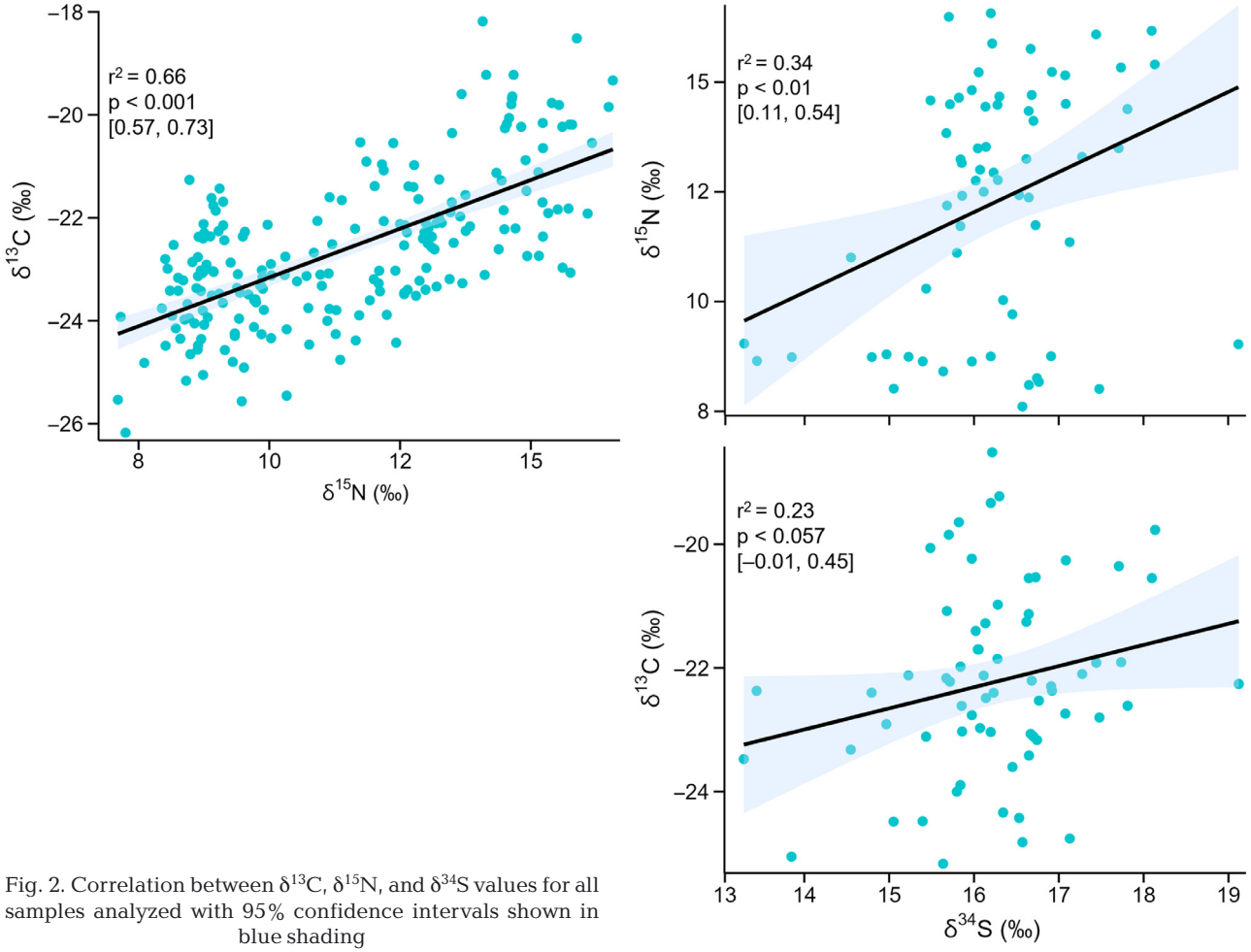

Fig. 2. Correlation between $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values for all samples analyzed with 95% confidence intervals shown in blue shading

Table 3. Number of samples from each foraging group (in **bold**) and location assigned to each cluster

Group	Cluster Analysis A					Cluster Analysis B				
	Cluster 1	Cluster 2	Cluster 3	Cluster 4	n	Cluster 1	Cluster 2	Cluster 3	Cluster 4	n
Northern		**7**	**83**	**2**	**92**	**22**				**22**
Sabrina Island			18		18	10				10
Cape Adare		3	29		32	10				10
Cape Hallett		4	36	2	42	2				2
Central	**18**	**8**	**2**	**7**	**35**	**1**	**4**	**4**	**7**	**16**
Edmonson Point	6		2	3	11			1	3	4
Campo Icarus	6	3		2	11	1	2	2		5
North Adelie Cove	5				5		2		2	4
Adelie Cove	1	5		2	8			1	2	3
Southern	**22**	**15**	**2**	**41**	**80**	**1**	**7**	**14**	**7**	**29**
Franklin Island	2				2				1	1
Cape Crozier		1		1	2			2		2
Cape Ross	2				2		1		1	2
Marble Point	7			4	11		2	2		4
Cape Irizar	9			1	10		3		1	4
Beaufort Island		1	2	23	26			4	3	7
Cape Bird	2	13		11	26	1	1	5	1	8
Cape Barne				1	1			1		1

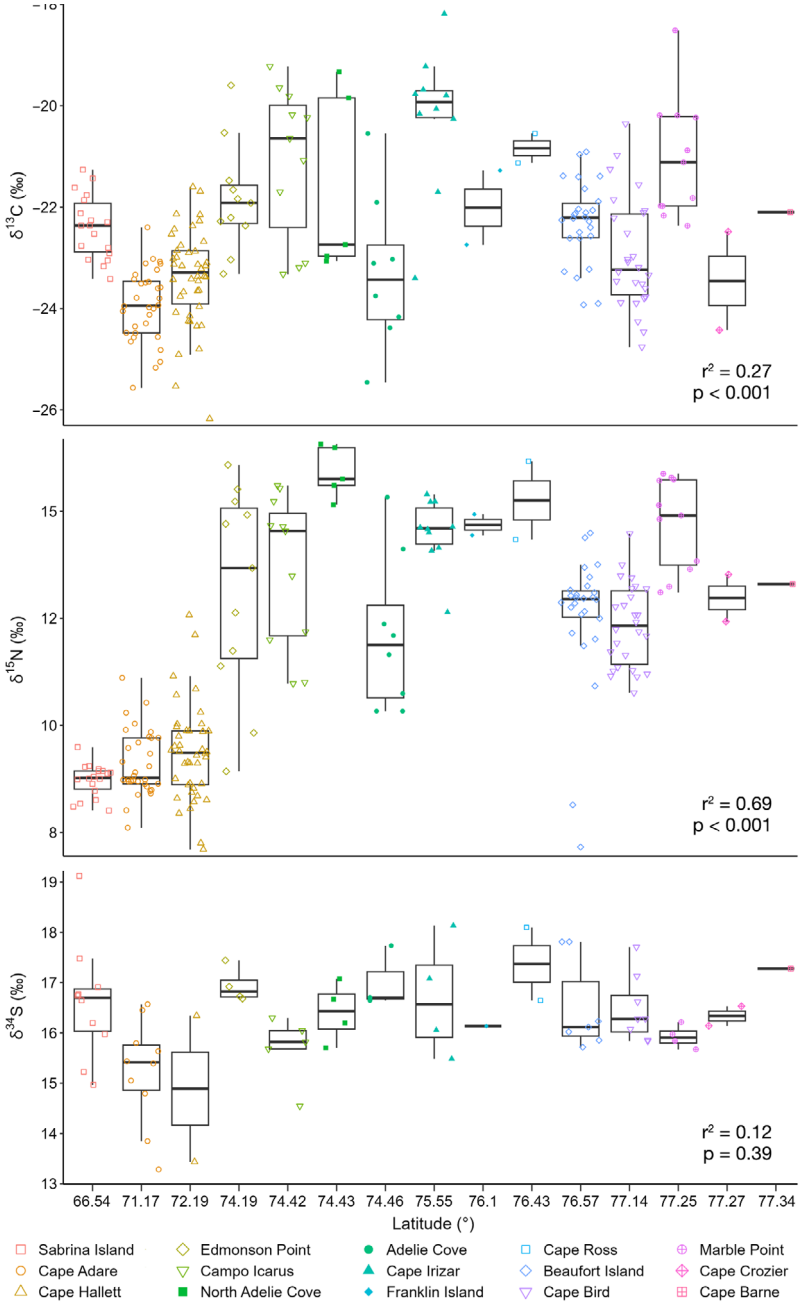

Fig. 3. Relationship between $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values and latitude. The boxes represent the interquartile range, whiskers represent the minimum and maximum isotope values, and thick horizontal lines represent the medians

behavior among 15 Adélie penguin colonies located in the northern, central, and southern Ross Sea region. The addition of sulfur isotope analysis ($\delta^{34}S$) was expected to reveal inshore/offshore foraging, thereby providing a better understanding of Adélie penguin foraging patterns and how changes in the dynamics (e.g. ice cover and size) of polynyas can affect foraging behavior, past and future.

4.1. Tracking foraging behavior

Although most chick-bone collagen samples were found to have a consistent range of $\delta^{13}C$ and $\delta^{15}N$ values, there were large variations among samples from the same sites and same excavated level within a single location (Fig. 5). These large variations in $\delta^{13}C$ and $\delta^{15}N$ values among individuals in one colony may be indicative of a broader and more diverse diet and foraging area (Guiry 2019, Massaro et al. 2020). As summer progresses, the decrease in pack ice and increasing competition during the crèche phase promote farther foraging trips (Lyver et al. 2011, Ainley et al. 2018, Santora et al. 2020). These longer foraging trips could cause differences in the isotopic compositions of bone collagen from chicks of different ages at time of death at the breeding season. Variations in $\delta^{13}C$ and $\delta^{15}N$ values in samples from a similar time frame could be equally interpreted as changes to primary productivity and sea ice cover within one season or year (Michel et al. 2019). These variations suggest that time is highly influential on foraging behavior; however, due to the lack of radiocarbon dates, we were unable to observe any linear trends between time and stable isotope values.

Although $\delta^{13}C$ and $\delta^{15}N$ values were highly variable within some individual locations and/or sites, this variability was not strong enough to remove the positive correlation between latitude and $\delta^{13}C$ and $\delta^{15}N$ values. Sabrina Island, Cape Hallett, and Cape Adare are located at lower latitudes, and penguins at these colonies largely forage in less productive waters for krill (Ainley 2002, Emslie et al. 2018). This was reflected in the tissues with lower $\delta^{13}C$ and $\delta^{15}N$ values averaging at −23.38 and 9.14‰, respectively. The $\delta^{13}C$ and $\delta^{15}N$ values of penguins foraging from polynyas in the central and southern Ross Sea were more enriched, averaging at −22.01 and 13.25‰, respectively, suggesting higher primary production and higher fish consumption for those foraging in latent heat polynyas (Ainley et al. 1998, Ainley 2002).

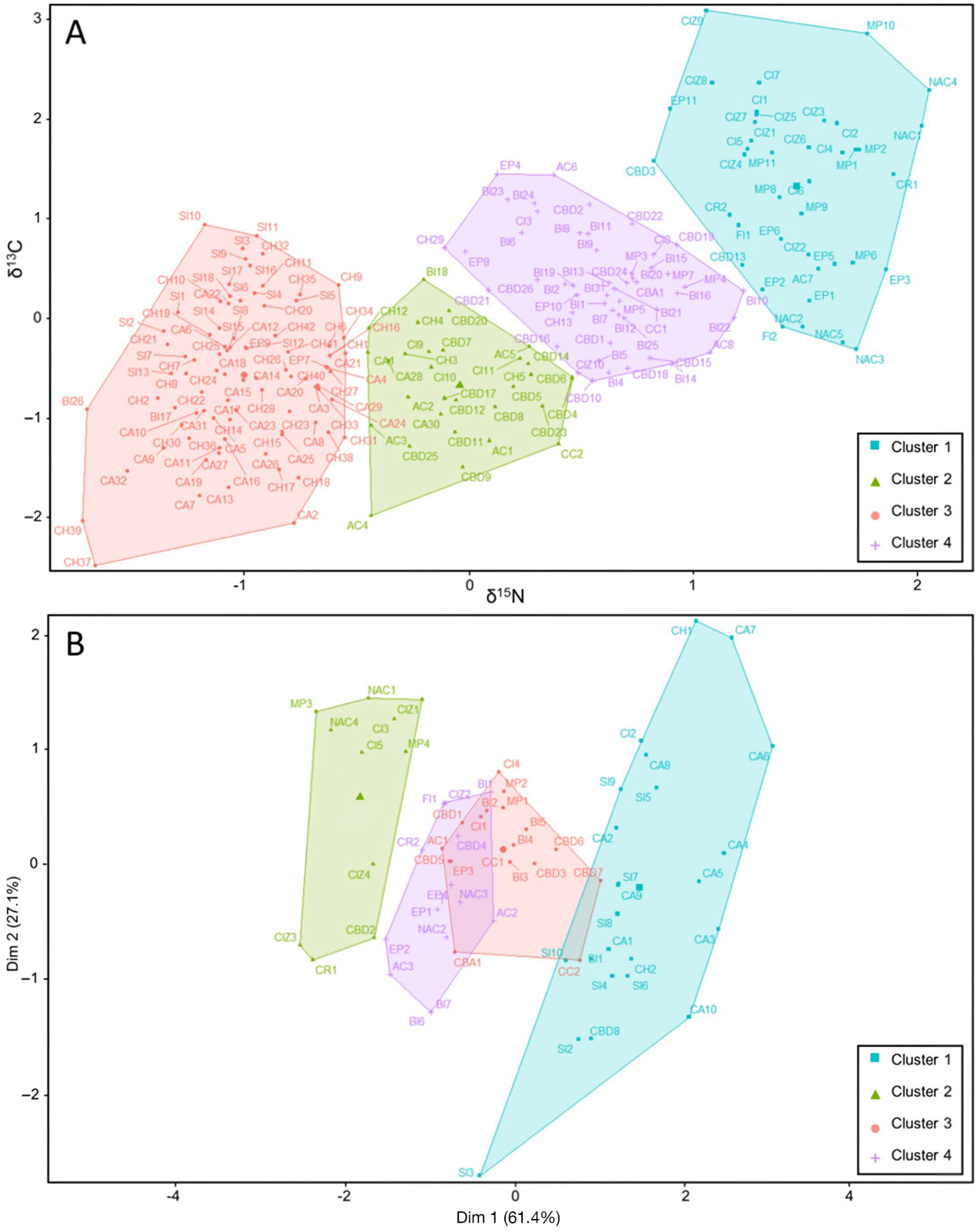

Fig. 4. *k*-means cluster analysis grouping Adélie penguin chick-bone collagen samples into 4 groups (clusters) based on (A) $\delta^{13}C$ and $\delta^{15}N$ values (n = 207) and (B) $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values (n = 67). Cluster Analysis A explained 83.6% variability and Cluster Analysis B explained 77.6% variability. Location names are abbreviated as follows: Sabrina Island (SI), Cape Adare (CA), Cape Hallett (CH), Edmonson Point (EP), Campo Icarus (CI), North Adelie Cove (NAC), Adelie Cove (AC), Cape Irizar (CIZ), Franklin Island (FI), Beaufort Island (BI), Cape Bird (CBD), Marble Point (MP), Cape Ross (CR), Cape Crozier (CC), and Cape Barne (CBA)

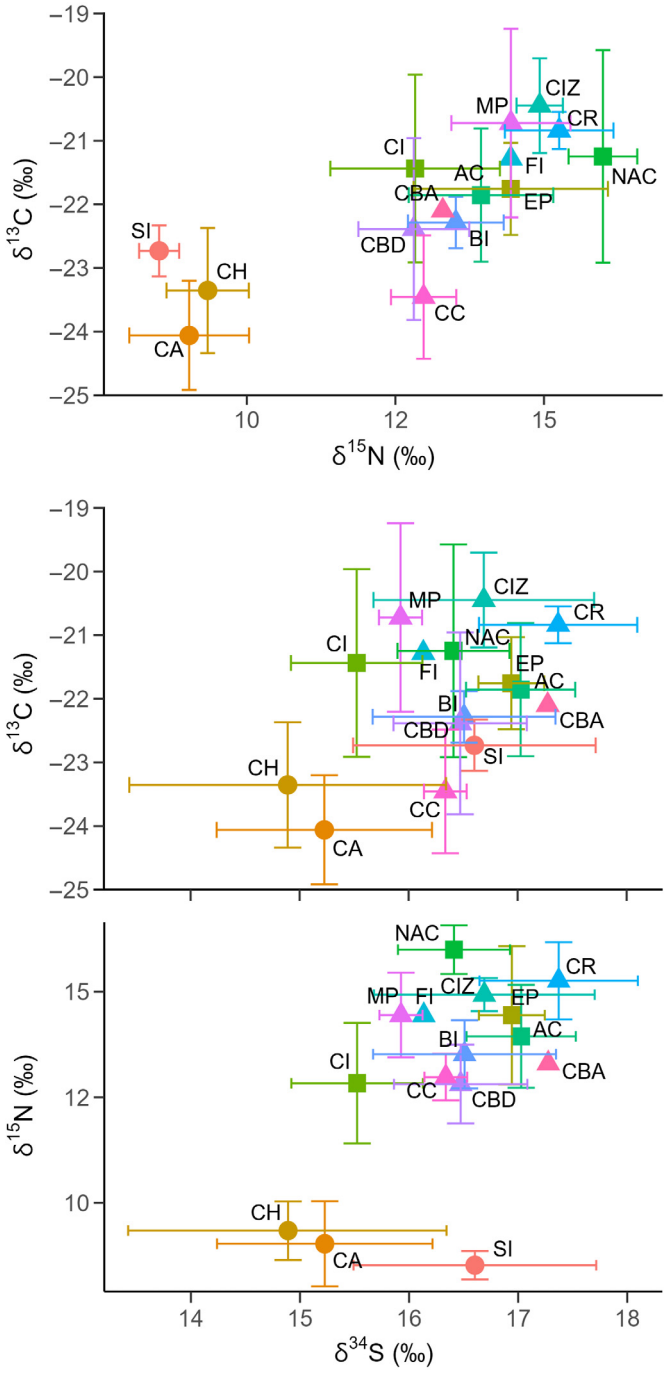

Fig. 5. Carbon, nitrogen, and sulfur isotopic signatures of chick-bone collagen samples from 15 colony locations (color) grouped by area in the northern (circles), central (squares), and southern (triangles) Ross Sea. Values are average ± SD. Location names as in Fig. 4

4.1.1. Sulfur isotopes as tracers

It is expected that higher $\delta^{34}S$ values in bone collagen (~16–18‰) reflect marine sulfates (~21‰), indicating that penguins were foraging farther offshore (Thode 1991). In contrast, lower $\delta^{34}S$ values in bone collagen (⩽15‰) reflect terrestrial freshwater sulfates, indicating inshore foraging (0–10‰; Paytan et al. 2004). Most of the samples had $\delta^{34}S$ values consistent with marine-dwelling organisms (15–20‰). Six of the 67 samples, namely 1 from Sabrina Island, 3 from Cape Adare, 1 from Cape Hallett, and 1 from Campo Icarus, fell below 15‰ (13.3–14.9‰). Low $\delta^{34}S$ values (~14‰) have been observed in Arctic marine mammals and were suspected to be a result of freshwater input from nearby rivers into inshore marine waters (Szpak & Buckley 2020). At minimum, we would expect the Sabrina Island colony, the only colony foraging strictly beyond the continental shelf in the open ocean, to have higher $\delta^{34}S$ values; yet, there were no significant differences in $\delta^{34}S$ values among all 11 colonies tested (1-way ANOVA: $F_{9,50} = 1.81$, $p = 0.08$).

To our knowledge, the only $\delta^{34}S$ values reported for non-captive Antarctic penguin species include Adélie, chinstrap *Pygoscelis antarcticus*, and gentoo penguin *P. papua* feathers collected from Admiralty Bay (King George Island) in the Antarctic peninsula (Padilha et al. 2022, 2023). The previously reported $\delta^{34}S$ feather values were similar in range to those of bone collagen reported in our study (13.3–19.1‰), ranging from 13.5–17.6‰, 12.8–15.2‰, and 14.2–16.3‰ in each species, respectively. Variations greater than 5‰ are to be expected when comparing several species whose migratory and foraging behaviors differ but not from a single species foraging within a small geographic range (Yohannes et al. 2023). While there were no significant differences among colonies, there was a positive correlation between $\delta^{34}S$ and $\delta^{15}N$ values ($r(67) = 0.34$, $p < 0.005$). This correlation may represent a connection between higher fish consumption in polynyas along the continental shelf, but there are too many factors and too much variability to come to a definitive conclusion. Although $\delta^{34}S$ values have been used to distinguish foraging grounds for wide-ranging Arctic marine animals, it may not be suitable for Antarctic species, at least during range-restricted parts of their lifecycle. The extent of isotopic discrimination among penguin tissues (e.g. bone collagen, eggshell, and feather) and how penguin breeding status (non-breeding, molting, pre-egg laying) influences $\delta^{34}S$ values in non-captive penguin species remains largely unknown. While captive gentoo penguins have $\delta^{34}S$ discrimination factors ranging from −0.4 to −1.7‰ (Rosciano et al. 2023), it is possible that even greater differences exist with bone collagen.

Previous research has demonstrated that lipid-extracted eggs of captive gentoo penguins had slightly higher $\delta^{34}S$ values than non-lipid extracted eggs (0.8 ± 0.2‰; Rosciano et al. 2023). Further studies are needed to identify the extent to which different bone collagen extraction methods, including lipid and humic acid extraction, influence $\delta^{34}S$ values.

4.1.2. Sabrina Island

This study was the first to use stable isotope analyses to investigate Adélie penguin foraging behavior on Sabrina Island (Balleny Islands). The Balleny Islands are composed of 3 main islands (Young, Buckle, and Sturge) and several smaller islets. Sabrina Island is a smaller islet composed of sheer cliffs and ridges approximately 3 km south of Buckle Island.

The penguin population is dominated by Adélie penguins, with smaller chinstrap penguin colonies (<200) and occasional sightings of king *Aptenodytes patagonicus* and macaroni penguins *Eudyptes chrysolophus* (Hatherton et al. 1965, Tidemann et al. 2015). Little is known about the population status of Adélie penguins on Sabrina Island due to extreme weather conditions which make it rarely accessible by shore parties.

We found that the Sabrina Island colony had similar diet composition to those at Cape Adare and Cape Hallett. There were no significant differences in $\delta^{15}N$ among these 3 colonies. The average $\delta^{15}N$ values were slightly lower at Sabrina Island (8.71‰) compared to Cape Adare and Cape Hallett, whose averages were 9.10 and 9.35‰. Based on $\delta^{15}N$ values, the Sabrina Island colony consumes more krill compared to any of the other colonies in the Ross Sea region, which may be in part due to small colony size, increased food availability, and lack of intraspecific competition around the Balleny Islands. The $\delta^{13}C$ values of Sabrina Island were slightly but significantly higher than those from Cape Hallett and Cape Adare (Tukey's HSD; $p < 0.05$ and $p < 0.001$, respectively). This significant difference is likely a result of higher primary productivity in the open ocean surface waters where Sabrina penguins forage in comparison to penguins at Cape Hallett and Cape Adare that forage in sensible heat polynyas composed of Ross Sea surface waters mixed with CDW. The widest range of $\delta^{34}S$ values were recorded at Sabrina Island (14.97–19.12‰), which may be a result of changes in the input of CDW by the Antarctic Circumpolar Current (Cincinelli et al. 2008).

4.2. Isotopic signatures

We applied $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values of Adélie penguin chick-bone collagen from 15 geographical locations to *k*-means cluster analysis to group colonies into 3 distinct clusters based on where they foraged, that is, the northern (Sabrina Island, Cape Hallett, Cape Adare), central (Edmonson Point, Campo Icarus, North Adelie Cove, Adelie Cove), and southern (Cape Crozier, Cape Ross, Franklin Island, Marble Point) Ross Sea region. Instead, the optimal number of clusters determined for both *k*-means cluster analyses was 4. While the ranges and means of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values among the north, central, and southern colonies varied, values were not distinct enough to separate locations into distinct clusters. Excluding Cape Barne, which was represented by 1 sample, Sabrina Island was the only location whose samples remained in 1 cluster for both Cluster Analyses A and B (Table 3). Overall, cluster analyses were more successful at discriminating clusters when applying $\delta^{13}C$, $\delta^{15}N$ values from all samples and removing $\delta^{34}S$ values from the data set (Fig. 4). The addition of $\delta^{34}S$ values explained less variation and created additional overlap and separation of samples from the same locations. In contrast, when we excluded isotope values from colony locations with smaller sample sizes, it resulted in reduced accuracy. Cluster analysis of $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ was unsuccessful in forming distinct isotopic signatures representing the northern, central, and southern colonies for several reasons.

Multivariate statistical analyses such as clustering, discriminant analyses, and PCA have been successful in previous studies when comparing multiple stable isotope values from a single time period for one species from a distinct geographical region, for several species with distinct foraging behaviors, and isotope signatures among tissue types (Norris et al. 2005, Ortea & Gallardo 2015, Steenweg et al. 2017). In our study, assignment was based on isotope values from one tissue type for one species with similar foraging behaviors at sea, and from several timeframes. The formation of clusters representing isotopic signatures of specific regions relied on factors such as sea ice cover, competition, and food availability, all of which vary through the breeding season.

Additionally, there were large variations among penguin colonies foraging from the same polynya; therefore, isotope values did not reflect just polynyas, but rather a combination of isotope signatures from different time periods, seasons, and locations (Massaro et al. 2020). These factors resulted in significant

differences among central colonies associated with the TNBP, yet no significant differences for southern colonies foraging in the MSP and/or RSP. For example, Adélie penguins from Adelie Cove, North Adelie Cove, and Campo Icarus forage in the TNBP and those from Cape Bird and Beaufort Island could have foraged in the MSP and/or RSP. The $\delta^{13}C$ values of Adelie Cove were significantly different from Campo Icarus (Tukey's HSD: $p < 0.001$), but not so for those from Cape Bird (Tukey's HSD: $p = 0.999$) or Beaufort Island (Tukey's HSD: $p = 0.363$). The $\delta^{15}N$ values of Adelie Cove were significantly different from North Adelie Cove (Tukey's HSD: $p < 0.001$), but not Cape Bird (Tukey's HSD: $p = 0.999$) or Beaufort Island (Tukey's HSD: $p = 0.999$).

This is not to say that northern, central, and southern colonies do not have distinct isotope signatures, but that they were not consistent nor distinct enough to detect in this study. The northern colony group was composed of modern samples while the remaining central and southern colonies were each represented by a combination of modern, historic, and ancient samples. Cluster Analysis A successfully assigned 83 of the 92 samples representing northern colonies into 1 cluster (Cluster 3). Cluster Analysis B successfully assigned all 22 samples representing the northern colonies into 1 cluster (Cluster 1). Distinct isotopic signatures representing each region would likely be present if additional samples were collected from several locations representing each polynya from a smaller time frame.

4.3. Cape Irizar

During ~4000–2000 cal yr BP, there was a warming period followed by an influx of penguins into the southern Ross Sea known as the 'penguin optimum' (Baroni & Orombelli 1994, Emslie et al. 2007, Lorenzini et al. 2010). During the optimum there was little to no fast ice blocking beach access along the Scott Coast which allowed Adélie penguins to occupy Marble Point, Cape Ross, and Cape Irizar. We included 10 samples from Cape Irizar ranging from 2210–795 cal yr BP in this study (Table S1). Stable isotope values from Cape Irizar were by far the most challenging of the locations to interpret because polynya access was dependent on both the growth or calving of the Drygalski Ice Tongue and the expansion of the MSP and TNBP (Emslie 2021). The Drygalski Ice Tongue, an extension of the David glacier, together with strong persistence katabatic winds, forms the TNBP. On average, the ice tongue extends ~90 km into the Ross Sea, and this length determines the maximum extent of the TNBP by preventing pack ice from entering the bay from the south (Indrigo et al. 2021). The calving or partial breakage of the ice tongue has been fully documented 3 times: in the early 1900s, 1956–1957, and 2005–2006 (Frezzotti & Mabin 1994, MacAyeal et al. 2008). These calving events were the result of major storms or iceberg collisions causing the formation of fractures within the ice tongue. During these calving events, the collapse of the ice tongue restricted the full expansion of the TNBP due to high pack ice cover from the south drifting into the TNBP (Frezzotti & Mabin 1994). The collapse of the ice tongue during the penguin optimum would have allowed the TNBP to expand farther south (Emslie 2021), allowing penguins to access Cape Irizar where they would have foraged in an expanded TNBP. Over time, the ice tongue would gradually reform, resulting in heavy ice cover reforming around Cape Irizar and eventual colony abandonment. It is also possible that the reformed ice tongue, which would again form the southern boundary of the TNBP, caused the penguins at Cape Irizar to forage farther south in the MSP. The MSP would have to extend 80 km north of the McMurdo Ice Shelf to allow foraging within 40–70 km from the colony. There were significant differences between $\delta^{13}C$ and $\delta^{15}N$ values from Cape Irizar and some but not all of the central colonies foraging from the TNBP (Tables S2 & S3 in the Supplement).

Overall, there were not enough data to explain the significance of these variations, or what other factors might be driving them, and therefore no definitive conclusion can be made as to where penguins at Cape Irizar once foraged. However, $\delta^{13}C$ and $\delta^{15}N$ stable isotope values were most similar to those representing Marble Point and Cape Ross in comparison to central colonies surrounding the TNBP and those on or near Ross Island (Fig. S1 in the Supplement). Samples from Cape Irizar had the second-widest range of $\delta^{34}S$ values, which may suggest a wider foraging range during its occupation. It is possible that the foraging range of the colony was not consistent for all samples included in our study. In other words, the MSP may have expanded, or the Drygalski Ice Tongue may have collapsed asynchronously for a short amount of time, thereby affecting only a portion of the individuals included in this study. Changes in intraspecific competition with changes in colony size may also have contributed to individual differences in foraging range. Additionally, rapid changes in climate may have influenced seasonal and annual sulfur cycles (Pruett et al. 2004).

5. CONCLUSIONS

With fluctuations in climate, sea ice conditions, access to breeding locations, and prey availability, penguin occupation in the Ross Sea region continues to be a dynamic process. This study was able to track Adélie penguin foraging behavior using stable isotope analyses over the span of thousands of years. Successful studies focusing on the application of sulfur isotopes to track marine animal foraging range require both an in-depth understanding of the surrounding environment and its sulfur cycle, and baseline studies on sulfur isotope analysis of tissues in nonmigratory lower-trophic level organisms. Even with an exceptional understanding of these factors, many studies find inconsistent $\delta^{34}S$ values and remain uncertain of their cause (Hoekstra et al. 2002, Craig et al. 2006, Barros et al. 2010, MacAvoy et al. 2015, Valenzuela et al. 2018). Understanding the heterogeneity of seawater sulfates across waters in the Ross Sea is essential for the interpretation of findings in this and future studies. Overall, sulfur isotopes can be sensitive tracers, and a better understanding in this new field of research is needed to successfully track Adélie penguin foraging grounds.

Data availability. Data associated with this paper is stored at the USAP-DC (doi:10.15784/601913).

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