STABLE ISOTOPE ANALYSIS OF MULTIPLE TISSUES FROM CHICK CARCASSES OF THREE PYGOSCELID PENGUINS IN ANTARCTICA

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ABSTRACT

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Many types of animal tissues are increasingly being used for stable isotope analysis, with their application dependent on the time frame they reflect and their availability for collection. Here, we investigated the isotopic values (δ^{13}C and δ^{15}N) of four tissues (feather, skin, toenail, and bone) collected from fledgling-period chick carcasses of three species of pygoscelid penguins to compare the variability and accuracy of the data among tissues. Samples were collected at 25 de Mayo/King George Island during the 2017/18 austral summer. Chick carcasses are commonly found at active penguin colonies, and "opportunistic sampling" can easily be performed without disturbing nesting penguins. A total of 25–36 carcasses per species were sampled at active colonies of Adélie *Pygoscelis adeliae*, Gentoo *P. papua*, and Chinstrap *P. antarcticus* penguins. A linear mixed model showed that δ^{13}C values varied significantly between tissues, presumably due to tissue-specific isotopic discrimination. In contrast, the only tissue with significantly different δ^{15}N values was toenail. Stable isotope data revealed dietary differences among species, with Gentoo Penguins having higher average isotopic values in tissues compared to Adélie and Chinstrap penguins. In addition, Chinstrap Penguins showed a consistent, but not statistically significant, trend in having higher δ^{13}C values compared to Adélie Penguins. Gentoo Penguins displayed the highest isotopic variability of any species for all tissues. Isotopic composition was most variable in skin in all three species, making skin the least reliable tissue for isotope analysis, whereas isotopic values were least variable in toenails. Comparison of isotopic values between two bones (tibiotarsus and coracoid) showed no significant differences in isotopic values, indicating that when the same bone is not available for sampling from carcasses, sampling of any major skeletal element is likely to provide a meaningful comparison. These results allow for more informed opportunistic sampling to accurately estimate and compare penguin diet among species and between ancient and active colonies.

Key words: 25 de Mayo/King George Island, δ^{15}N, δ^{13}C, *Pygoscelis* penguins, dietary shifts, opportunistic sampling

Varios tipos de tejidos animales son empleados para el análisis de isótopos estables, considerando el período de tiempo que cada tejido refleja y dependiendo de su disponibilidad para colección. Aquí, hemos investigado los valores isotópicos (δ^{13}C y δ^{15}N) de cuatro tejidos (pluma, piel, uña y hueso) colectados de cadáveres de pichones de las tres especies de pingüinos pigoscélidos con el objetivo de comparar precisión y variabilidad de la información obtenida. Las muestras fueron colectadas en la Isla 25 de Mayo/King George durante el verano austral 2017/18. Los cadáveres de pichones son comúnmente encontrados alrededor de colonias activas y el muestreo oportunista puede fácilmente ser realizado minimizando el disturbio en la colonia. Un total de 25–36 cadáveres por especie fueron muestreados en colonias de Pingüinos Adelia *Pygoscelis adeliae*, Pingüinos Papúa *P. papua,* y Pingüinos Barbijo *P. antarcticus*. Un modelo lineal mixto mostró que los valores de δ^{13}C variaron dependiendo del tejido presumiblemente debido a la discriminación isotópica específica a cada uno de ellos. En cambio, el único tejido significativamente diferente para δ^{15}N fue la uña. La información reveló diferencias dietarias entre especies, exhibiendo el Pingüino Papúa los valores más altos. Asimismo, los Pingüinos Barbijo mostraron valores más altos de δ^{13}C que los Pingüinos Adelia, aunque esta diferencia no fue significativa. Los Papúa mostraron la mayor variabilidad para todos los tejidos. La piel exhibió la mayor variabilidad en las tres especies y fue el tejido menos confiable para análisis de isótopos estables; mientras que las uñas mostraron la menor variabilidad. Los valores obtenidos de dos huesos distintos (tibiatarso y coracoides) no mostraron diferencias significativas, indicando que el muestreo de otros huesos probablemente resultará en valores similares. Nuestros resultados brindan información para poder estimar la composición de la dieta con precisión y comparar diferentes especies y/o colonias abandonadas y activas mediante el muestreo oportunista.

Palabras clave: Isla 25 de Mayo/King George, δ^{15}N, δ^{13}C, pingüinos *Pigoscélidos*, cambios dietarios, muestreo oportunista

INTRODUCTION

Stable isotopes of nitrogen (δ^{15}N) and carbon (δ^{13}C) have been used as tracers to address ecological questions on diet and foraging locations since the 1970s, and their use is still growing today (Fry 2006, Barrett *et al*. 2007). In penguins, isotopic analysis is a more precise, less invasive, and more cost-effective way to establish the trophic level of seabird prey compared to stomach content

analysis, despite the inability of this method to provide taxonomic resolution (Polito *et al*. 2011). Penguin dietary information may reflect variations in prey abundance or reveal changes in foraging areas (Karnovsky *et al*. 2012). Nonetheless, their responses to new and changing conditions are highly site- and species-specific in direction and magnitude, and their responses also depend on local and regional ecosystem changes, the interaction between these changes, and most significantly, on the ability of the species to exploit available resources (Cherel & Hobson 2007, Gorman *et al*. 2014). This last factor varies depending on the specific differences in foraging behavior (e.g., time, area, prey preferences) and life history tactics (e.g., wintering habitat, fasting tolerance, breeding chronology, sexual dimorphism).

The pygoscelid penguins—the Adélie *Pygoscelis adeliae*, Chinstrap *P. antarcticus*, and Gentoo *P. papua* Penguin—are sympatric in the west Antarctic Peninsula, which is experiencing a rapid regional warming trend (Vaughan *et al*. 2003), a decrease in the amount of winter sea ice since the late 20th century (Ducklow *et al*. 2006), and is still recovering from the impact of overfishing and whaling since the 1960–1980s (Ballance *et al*. 2006, Emslie & Patterson 2007, Ainley & Blight 2009, Branch 2011, Barrera-Oro *et al*. 2017). In this environmental scenario, a significantly smaller food supply for pygoscelid penguins is expected. Because their main prey during the breeding season is the Antarctic krill *Euphausia superba*, which is also one of the declining species targeted by commercial fisheries in the Antarctic (Flores *et al*. 2012, but also see Cox *et al*. 2018), the three pygoscelid species are included in the Convention for the Conservation of Antarctic Marine Living Resources (CCAMLR) list of dependent species, and several Antarctic colonies are the subject of monitoring programs (Agnew 1997).

The stable isotope signature in animal tissue provides integrated dietary information and reflects the biochemical and physiological processes that occurred during the synthesis and life of the tissue (Hobson & Clark 1992). Moreover, tissues obtained from closely related taxa are assumed to undergo similar processes during their synthesis and life (Cherel & Hobson 2007). Theoretically, dietary niche over time can be analyzed by examining a combination of tissues from a single individual (Herman *et al*. 2017) due to different time of synthesis and turnover rates across tissues. Here, we chose four tissues from penguin chick carcasses for analysis: bone, feather, skin, and toenail. Our primary goal was to evaluate the potential of these four different tissues to reflect feeding preferences (prey/habitat) among three sympatric pygoscelid penguin species, i.e., whether the tissues that were sampled accurately reflected the dietary segregation of these species during the breeding season, and if certain tissues yielded more variable

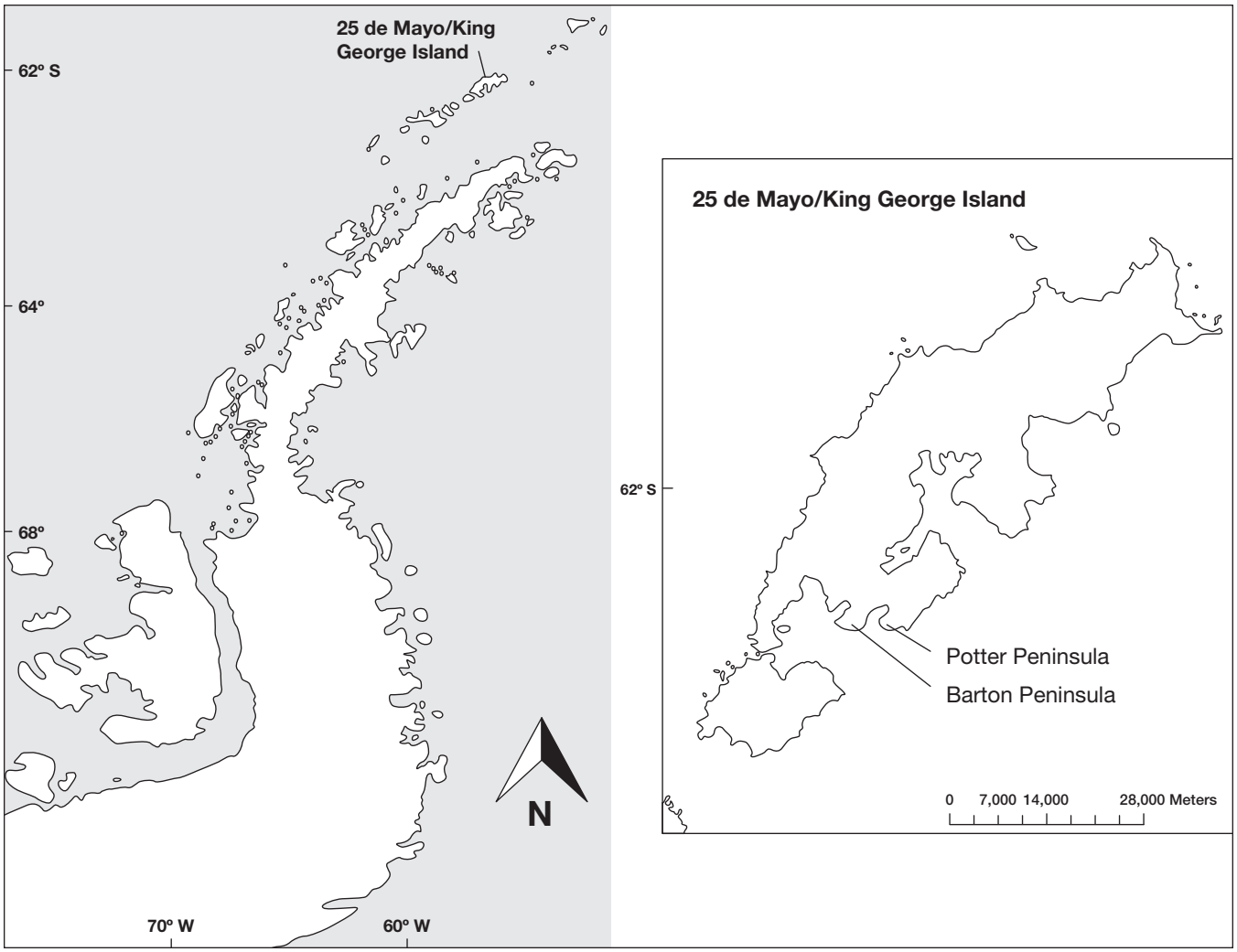

Fig. 1. Location of study sites in the Antarctic Peninsula and on 25 de Mayo/King George Island. Modified after Emslie *et al.* (2020).

and/or less accurate isotopic values than others, making them less useful for detecting dietary patterns.

Bone collagen and integument tend to be the most heavy-isotope enriched tissues among birds and mammals (Kelly 2000), making them target tissues for dietary studies. Bone collagen is turned over much more slowly than other tissues and may reflect lifetime dietary integration in adults, whereas collagen in chicks would reflect diet only during the breeding season (Hobson & Clark 1992). Feathers are one of the most commonly used avian tissues for isotope analysis. This tissue is composed of keratin, which is inert after synthesis, thus "freezing" the isotopic value and diet at the time of feather growth (Cherel *et al*. 2005). Thus, fledgling-aged chick feathers integrate dietary history only during the chick-rearing period (Polito *et al*. 2011). Skin is rarely used in isotopic studies of birds; however, as with feathers, skin in penguin chicks should integrate dietary information only during the chick-rearing period due to the high metabolism of chicks during this period. Toenails are also keratinous, but contrary to feathers, they grow continuously and are worn out during life, reaching equilibrium with diet after several weeks and coinciding with the duration of toenail replacement (Ainley *et al*. 2003, Lourenço *et al*. 2015).

By analyzing bone, feather, skin, and toenail tissues among the three pygoscelid penguin species, we sought (1) to determine whether the dietary segregation that exists among these species can be indicated by $\delta^{13}C$ and $\delta^{15}N$ values in each tissue; (2) to determine if bones (coracoid and tibiotarsus), feathers, skin, and toenails exhibit equal $\delta^{13}C$ and $\delta^{15}N$ values; and (3) to determine variability across tissues. Because the same bone is not always available from carcasses for sampling, we also sampled two bones to test for intraskeletal variation in bone collagen to determine whether different bones can provide the same information. Based on previous research, it was expected that Gentoo Penguins would have higher values of both $\delta^{13}C$ and $\delta^{15}N$ (indicating an inshore foraging habitat and a higher percentage of upper trophic level prey) in all tissues compared to the other two species (Trivelpiece *et al.* 1987, Volkman *et al*. 1980, Kokubun *et al*. 2010, Miller *et al*. 2010, Juáres *et al*. 2013, Polito *et al*. 2015, Dimitrijević *et al*. 2018). Although the tissues that were sampled varied in their rate of growth and in the timing of the life cycle of these species, the isotopic signatures of our samples encompassed, on average, only 4–6 weeks of dietary information, thereby eliminating any variation that could result from dietary shifts or environmental variability over a longer period of life. In other words, the tissues that were sampled mirrored the diet of the chicks during the specific reproductive season and, thus, could be used to assess seasonal changes in diet (Ainley *et al*. 2003). Sampling chicks from the same colonies in the same region also removed any isotopic variability that could have arisen from foraging at different locations or under different marine conditions.

METHODS

Study sites

Samples were collected at Antarctic Specially Protected Areas N° 132 "Potter Peninsula" and N° 171 "Narębski Point" (Barton Peninsula), 25 de Mayo/King George Island, South Shetland Islands (Fig. 1). At Narębski Point, a colony of Chinstrap Penguins (~3 000 pairs) coexists with a smaller population of Gentoo Penguins (~2 000 pairs) (Kokubun *et al.* 2010). Since 2012/13, the number of breeding pairs in both species has been steadily declining (CCAMLR 2016).

At the adjacent peninsula, Potter Peninsula, at Stranger Point/Cabo Funes, Gentoo and Adélie Penguins breed on the same beaches as a very small population of Chinstrap Penguins (Juáres 2013). The population trends at Stranger Point are consistent with those exhibited by other colonies in the West Antarctic Peninsula, where a decline in Adélie populations and an increase in the number of reproductive pairs of Gentoo Penguins has been observed (Carlini *et al*. 2009; Juáres *et al*. 2015; Juáres *et al*. 2020). Recently, during austral summer season 2018/19, 5 383 breeding pairs of Gentoo Penguins were counted (Juáres *et al*. 2020); in contrast, the number of Adélie breeding pairs was less than 1 500 during the austral summer of 2014/15 (Juáres *et al*. 2018).

The diets of Adélie and Gentoo Penguins at Stranger Point were examined during the crèche stage for more than 10 years: Antarctic krill appeared in 100% of the samples, accounting for > 99.7% of stomach contents by mass for Adélie Penguins, while fish represented < 0.15% by mass of the total prey, appearing in only 4%–24% of the samples, depending on the year (Juáres *et al*. 2018). Antarctic krill was the main prey of Gentoo Penguins, appearing in 100% of the samples, with a mass > 92% in every season studied (Juáres 2013; M.A. Juáres unpubl. data).

During the period 2003/05, Antarctic krill appeared in 100% of the samples of Chinstrap Penguins' stomach contents in the Barton Peninsula; krill mass accounted for > 97.6% of stomach contents, whereas the frequency of fish in the samples was variable (10.81%–90%); wet mass, however, was never over 2.4% (Rombolá *et al*. 2010).

Field methods

During February 2018, Potter Peninsula was surveyed daily for recently deceased Adélie and Gentoo chicks, while Narębski Point was surveyed once at the end of the season for deceased Chinstrap chicks. All tissues were sampled from recently deceased fledgling-aged chicks to minimize disturbance to breeding colonies, and because carcasses of chicks are commonly found in and around

TABLE 1
Sampling size per tissue for each pygoscelid species

Species/ Tissue type	Number of samples				
	Feather	Toenail	Skin	Coracoid	Tibiotarsus
Adélie	34	21	32	21	21
Chinstrap	19	18	20	20	19
Gentoo	28	30	24	26	30

active penguin colonies. In addition, Vasil *et al*. (2012) found that the cause of death does not affect isotopic values. A total of 36 Adélie, 25 Chinstrap, and 35 Gentoo carcasses were sampled (Table 1). Species were identified visually. The tissues of interest present in each carcass were collected (Table 1) and placed in plastic bags; the remaining carcass was left where it was found. Feathers were collected from the breast when possible, or from the closest area available (back, upper leg, or neck), and the location from which the feathers were taken was recorded. Two to three toenails were collected from each carcass as well. Two types of bone were collected by cutting a portion of the mid-shaft: the tibiotarsus and the coracoid (both bones were collected when it was possible). A small piece of skin was cut from the chest area, or from the closest area available.

Laboratory methods

Samples were rinsed and dried at Carlini Station and transported to the University of North Carolina Wilmington (UNCW) where they were preserved at -20 °C. To remove lipids, two to three feathers were placed in glass vials and soaked with 2:1 chloroform/methanol solution for 24 h, drained, and then rinsed three times with chloroform/methanol solution and dried for 24 h. After drying, the feather vanes were cut into small pieces. Toenails were placed in glass vials and rehydrated to remove attached bone and dried at 60 °C. They were then rinsed with 2:1 chloroform/methanol solution and dried again. The distal third of the claw was removed to avoid tissue formed during the pre-hatching period. The remainder of the tissue was finely ground using a mortar and pestle. Skin samples were cleaned by scrubbing under running distilled water, feathers were removed manually after soaking in boiling water, and samples were dried again. The skin samples were placed in glass vials where they were soaked with 2:1 chloroform/methanol solution for 24 h, drained, and then rinsed three times with chloroform/methanol solution (agitating for 15 min each time; Kristan *et al*. 2019), and dried for 24 h.

Bones were demineralized using the "chunk method" described by Sealy *et al*. (2014), with the addition of a 24-h lipid extraction with 2:1 chloroform/methanol solution (Liden *et al*. 1995). $\delta^{13}C$ values were measured after lipid extraction, whereas $\delta^{15}N$ values were measured before lipid extraction because the effect of lipid extraction on $\delta^{15}N$ values is hard to predict and correct (Sweeting *et al*. 2006). A small piece of bone (0.5 g) was cut and surface-cleaned with sandpaper to remove superficial contamination. For both bones, a piece was cut from the periphery of the mid-shaft of the bone. The surface-cleaned bone fragments were crushed and placed in glass vials for lipid extraction. After drying, 0.2 M HCl was added to the vials at room temperature. The acid was changed every other day for 16 days, or until no bubbles were produced which indicated that decalcification was complete. Subsequently, bones were rinsed three times in distilled water and freeze-dried.

Approximately 0.5 mg of feather, 0.7 mg of toenail, 0.7 mg of skin, and 0.65 mg of demineralized bone were weighed into tin capsules and analyzed with a Costech Elemental Analyzer (EA 4010) paired with a Thermo Electron Delta V Isotope Ratio Mass Spectrometer (EA-IRMS) located at the Center for Marine Science, UNCW. Raw results were normalized on a two-point scale using enriched and depleted glutamic acid reference materials, USGS-40 and USGS-41.

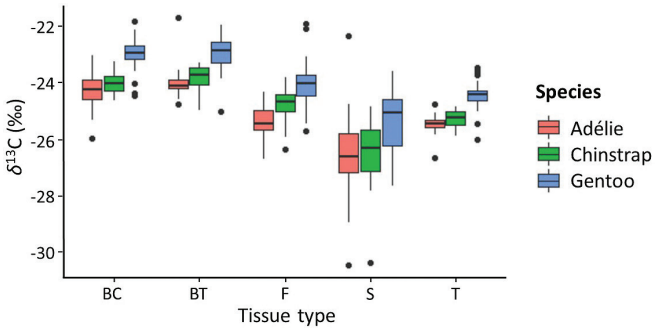

Fig 2. Boxplot showing $\delta^{13}C$ values for each species and tissue type. BC = bone coracoid, BT = bone tibiotarsus, F = feather, S = skin, T = toenail.

TABLE 2
$\delta^{13}C$ (‰) values for each species and tissue type[a]

Species/ Tissue type	$\delta^{13}C$ (‰) Mean ± Standard Deviation				
	Feather	Toenail	Skin	Coracoid	Tibiotarsus
Adélie	–25.5 ± 0.6	–25.5 ± 0.4	–26.6 ± 1.5	–24.3 ± 0.6	–24.1 ± 0.3
Chinstrap	–24.8 ± 0.6	–25.3 ± 0.3	–26.5 ± 1.3	–24.0 ± 0.4	–23.9 ± 0.5
Gentoo	–24.0 ± 0.9	–24.4 ± 0.5	–25.5 ± 1.2	–23.0 ± 0.6	–22.9 ± 0.6

[a] see Table 1 for sample sizes

TABLE 3
$\delta^{13}N$ (‰) values for each species and tissue type[a]

Species/ Tissue type	$\delta^{13}N$ (‰) Mean ± Standard Deviation				
	Feather	Toenail	Skin	Coracoid	Tibiotarsus
Adélie	+8.2 ± 0.3	+6.7 ± 0.3	+8.3 ± 0.5	+8.22 ± 0.3	+8.28 ± 0.3
Chinstrap	+8.2 ± 0.4	+6.5 ± 0.3	+8.2 ± 0.4	+8.10 ± 0.4	+8.17 ± 0.2
Gentoo	+8.5 ± 0.5	+7.0 ± 0.5	+8.6 ± 0.5	+8.64 ± 0.5	+8.58 ± 0.5

[a] see Table 1 for sample sizes

Twenty percent of the samples were replicated and showed a sample precision of 0.3‰ and 0.2‰ for δ^{13}C and δ^{15}N, respectively.

Statistical analysis

Statistical analyses were completed using Microsoft Excel and R (R Core Team 2019). Descriptive statistics were used to characterize δ^{13}C and δ^{15}N values and variances for the different tissues among the three species.

In addition, to identify whether a tissue reflected the known dietary differences between species, we performed a parametric ANOVA and post-hoc Tukey test; when assumptions for ANOVA were not attained, we performed a non-parametric Kruskal-Wallis rank sum and post-hoc multiple comparison test (function kruskalmc in R) on each tissue data set.

Comparison between tissues was completed by fitting a linear mixed model (function lmer in R) to the data with species and tissue type as fixed factors and individual as a random factor; posteriori Tukey contrasts Simultaneous Tests for General Linear Hypotheses were performed to determine if tissues exhibited equal values. The coracoid and tibiotarsus were considered different tissues to determine if they underwent different fractionation.

Significance was assumed for all tests if $P < 0.05$. The following R packages were used: "lme4" (Bates *et al*. 2015), "pgirmess" (Giraudoux 2018), "lmeteset" (Kuznetsova *et al.* 2017), "multicomp" (Hothorn *et al*. 2008), and "ggplot2" (Wickham 2016).

RESULTS

Carbon

Mean δ^{13}C values in the four tissues (including the two bones sampled from each carcass) ranged from –22.9‰ (Gentoo tibiotarsus) to –26.6‰ (Adélie skin; Table 2). Gentoo Penguins had the highest δ^{13}C values in all four tissues, followed by Chinstrap and Adélie Penguins (Table 2).

The δ^{13}C values of feathers were significantly different among species (KW = 38.08, df = 2, $P < 0.001$) and the multiple comparison test after Kruskal-Wallis showed significant differences between all species pairs. The δ^{13}C values of toenails (KW = 39.08, df = 2, $P < 0.001$), skin (KW = 11.16, df = 2, $P < 0.005$), coracoid bone (F = 34.02, df = 2, $P < 0.001$), and tibiotarsus bone (KW = 34.66, df = 2, $P < 0.001$) were significantly different among species. Significant differences were found between Gentoo and Adélie/Chinstrap penguins, but not between these latter two species. All data are provided in Appendix 1 (available on the website).

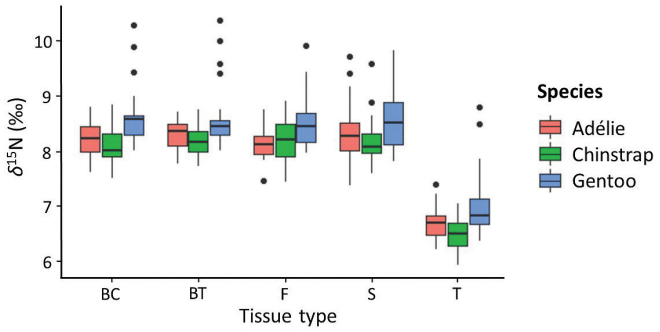

Fig. 3. Boxplot showing δ^{15}N values for each species and tissue type. BC = bone coracoid, BT = bone tibiotarsus, F = feather, S = skin, T = toenail.

The "tissue type" and "species" factors in the linear mixed model had a significant effect on the carbon isotopic signature. Post-hoc analysis showed that δ^{13}C values in all tissue types were significantly different from each other ($P < 0.05$), except for the tibiotarsus–coracoid pair ($P = 0.24$).

Skin had the lowest δ^{13}C values followed by feathers and toenails. Bones had the highest δ^{13}C values (Fig. 2). Regarding variability, skin was the most variable (± > 1‰ standard deviation (SD)), whereas toenail was the least variable. The most variable species was the Gentoo Penguin for all tissues except skin.

Nitrogen

The mean value of δ^{15}N in the four tissues sampled (including the two bone tissues sampled from each carcass) ranged from +6.5‰ (Chinstrap toenail) to +8.6‰ (Gentoo coracoid; Table 3). Gentoo Penguins had the highest values in all tissues followed by Adélie Penguins in all tissues except feathers (Table 3). However, the differences in δ^{15}N values between Chinstrap and Adélie Penguins were not significant in any of the tissues examined.

The δ^{15}N values of feathers and coracoids were significantly different among species (KW = 11.72, df = 2, $P < 0.005$; and KW = 16.64, df = 2, $P < 0.001$, respectively), and the multiple comparison test showed no significant difference between Adélie and Chinstrap Penguins, but Gentoo values were significantly higher than those recorded in its congeners. The δ^{15}N values of toenail, skin, and tibiotarsi were also significantly different among species (KW = 14.98, df = 2, $P < 0.001$; KW = 6.58, df = 2, $P < 0.05$; and KW = 12.00, df = 2, $P < 0.005$; respectively), but the multiple comparison test showed only a significant difference between Chinstrap and Gentoo Penguins. All data are provided in Appendix 1.

The "tissue type" and "species" factors in the linear mixed model had a significant effect on δ^{15}N. Post-hoc analysis showed that the only tissue that had a significantly different δ^{15}N from the others was toenail ($P < 0.05$). Toenail is, on average, 1.6‰ lighter (± 0.4‰ relative to coracoid, ± 0.3‰ relative to tibiotarsus, ± 0.3‰ relative to feathers, and ± 0.5‰ relative to skin) than the other tissues (Fig. 3). Regarding variability, skin was the most variable; however, all tissues showed similar values. The most variable species was the Gentoo Penguin for all tissues except skin.

DISCUSSION

The results of this study provided sufficient data to address our three primary objectives: (1) to determine whether the dietary segregation that exists among the three penguin species can be indicated by δ^{13}Cand δ^{15}N values in each tissue; (2) to determine if all tissues have similar isotopic values; and (3) to determine the variability in isotopic values across tissues.

Our first objective—to determine whether the dietary segregation that exists among the three penguin species can be indicated by δ^{13}C and δ^{15}N values in each tissue—was supported, as expected. The isotopic evidence from all four tissues analyzed in this study is consistent with

known diets and foraging behavior of pygoscelid penguins to a certain degree. For carbon, all tissues showed a significant difference between the Gentoo Penguin and the other two species, with a tendency for the Chinstrap Penguin to have higher $\delta^{13}C$ values than the Adélie Penguin. This could be related to the size class of krill consumed by each penguin species, or local foraging preferences; in some cases, Chinstrap Penguins may forage more inshore than Adélie Penguins, or vice versa, depending on the colony location and distance to preferred prey (Williams 1995). Foraging range in penguins is also positively correlated with colony size. However, there are no data from large colonies of Chinstrap Penguins, so the foraging range of this species is not completely understood (Santora *et al*. 2020). Gentoo Penguins had significantly higher $\delta^{15}N$ values in all tissues compared to Chinstrap Penguins, but only feathers and the coracoid bones showed significant differences between Gentoo and Adélie Penguins. However, a general trend can be seen in all tissues, wherein Adélie Penguins have lower isotope values than Gentoo Penguins but higher isotopic values than Chinstrap Penguins. The lack of significance among these mean values could reflect insufficient sample sizes; alternatively, Chinstrap Penguins may rely more strictly on krill than Adélie Penguins during the breeding season, in accordance with numerous other dietary studies within the South Shetland Islands (Herman *et al*. 2017), which would result in lower $\delta^{15}N$ values for Chinstrap Penguins. In general, the three pygoscelid penguins in this study currently rely mostly on krill, and this is, at least in part, due to the decline in several fish species that has occurred with fishing (see above), as well as the decline in silverfish that has accompanied reduced sea ice in recent decades (La Mesa *et al*. 2015). Therefore, the differences in $\delta^{15}N$ observed in this study reflect only the proportion of fish that complement their krill-based diet.

Our second objective—to determine if all tissues have similar isotopic values—was partially refuted because $\delta^{13}C$ values were significantly different across tissues; in contrast, there were no significant differences in $\delta^{15}N$ values across tissues except toenails. We also confirmed that the tibiotarsus and coracoid bones had similar isotopic values, and therefore, either bone can be used to assess diet. The isotopic differences among tissues can be attributed to fractionation alone because it was assumed that no dietary change occurred during the life of the chicks. Therefore, there were no discrepancies in the time frame of isotope integration into tissues sampled that would have affected measured isotopic values. It has been suggested that feathers and toenails should exhibit similar fractionation (Lourenço *et al*. 2015) because both tissues are keratinous; however, this study showed that toenails yielded different discrimination factors for ^{13}C and ^{15}N compared to the other three tissues, including feathers. Previous research has suggested that there is a biochemical difference in the keratins that comprise feathers and toenails (Barquete *et al*. 2013), which could cause differences in fractionation and may explain the differences in isotope values found in this study. Fractionation in each tissue depends on the physiological pathways of tissue synthesis (Hobson & Clark 1992) and can be empirically described by discrimination factors, some of which are available in the literature and are usually determined in controlled laboratory or zoo experiments. However, it is often not clear how accurate these discrimination factors are when they are applied to the wild (Polito *et al*. 2009). Our descriptive comparison of isotopic signatures during a constrained time frame in the wild can complement available data on fractionation and may assist in the interpretation of data that is currently available from a variety of tissues.

In addressing our third objective—the variability in isotopic values across tissues—we found that skin was highly variable. Thus, we do not recommend sampling skin for isotope analysis. This recommendation applies especially when the timing of death of the organism is unknown because the extent of decomposition likely contributes this high variability. As vertebrate tissues decay, they become progressively heavier in ^{15}N. Thus, when used postportem, they should be used with caution for inferring trophic ecology (Keenan & DeBruyn 2019). We believe this could also be true for ^{13}C. Keenan & DeBruyn (2019) found that neither bones nor hair exhibited significant changes in isotope values as decomposition progressed, suggesting that more recalcitrant components of an animal require longer time frames to show any postmortem change. Penguin bone, toenail, and feather are recalcitrant tissues that, in addition, were exposed to the low temperatures of Antarctica before collection; therefore, decomposition would have little effect on their isotopic signatures. The data presented here includes samples from carcasses that were relatively fresh, but some carcasses had been decaying for some period of time before they were sampled; consequently, the carcasses were in different states of decomposition, generating high variability in the most labile tissue, which in this study was skin. Supporting this idea, the skin samples collected from older carcasses showed the most aberrant values. In addition, the Antarctic Peninsula is the warmest and most humid area in Antarctica, and the speed of decomposition is faster than in more southern areas of the continent. Variability in the skin isotopic signatures of penguin carcasses in much drier conditions, such as in the Ross Sea area, however, may be less variable and more reliable for opportunistic sampling.

Isotope values were most variable in Gentoo Penguins for all tissues sampled. This is consistent with Gentoo Penguins having a wider foraging niche compared to Chinstrap and Adélie Penguins (Polito *et al*. 2015). Gentoo Penguins are considered Type "B" generalists, where individuals within the population specialize and have a narrow range of prey compared to the population as a whole (Camprasse *et al*. 2017).

Which tissues are best for sampling?

Toenail grows continuously and has a high potential to reveal temporal variation in penguin diet, both within a season as revealed by sampling chicks at various stages of growth (Ainley *et al.* 2003), or during the annual cycle (Lourenço *et al.* 2015). This use of tissue is feasible because inert tissue that has progressive growth retains isotopic values in a chronological order (Dalerum & Angerbjörn 2005). Lourenço *et al.* (2015) showed that the isotopic turnover in avian toenails indeed follows nail growth rate; however, there is inconsistency in the literature because other studies have indicated that bird toenails could be insensitive to short and medium-term dietary shifts (Barquete *et al.* 2013). Although the deposition of keratin is not a linear process, avian toenails show both longitudinal and lateral growth, i.e. conical growth, which causes any part of the toenail to contain a blend of materials formed at different times (Lourenço *et al.* 2015). Thus, the nail tip probably contains a combination of old and new keratin, providing information over a medium temporal scale, from weeks to a few months in the life of the bird (Bearhop *et al.* 2003). Consequently, it is recommended to avoid using toenail for a time-series until pygoscelid penguin toenail growth can be investigated in more detail.

In general, the information in a toenail tip reflects a lag of four to six months (Barquete *et al*. 2013), so the tips sampled from newly arriving penguins at a breeding colony—before wear and tear from terrestrial movements occur—could provide integrated information

about the non-reproductive diets of penguins as they foraged at sea. This type of sampling is particularly important because traditional sampling methods are not easy to perform during winter. Another advantage of this method is that it allows comparison of toenail tissue samples from the same individual over time, allowing longitudinal comparison without having to correct different tissues' signatures with a discrimination factor, which would accumulate error; typically, diet during the pre-breeding period is assessed using one tissue type (e.g., egg membrane), whereas diet during the reproductive season is assessed using another tissue type (e.g., blood, feathers). A remaining question is whether it is feasible to sample toenail sections from live animals. The sample mass required for isotopic analysis is very small, so it may be possible to obtain a sufficient amount of sample by cleaning the nail *in situ* and directly scratching a small amount of sample from any section of the nail. If this is possible, specific individuals could be sampled routinely, allowing non-migratory species (e.g., Gentoo Penguins) to be followed over the whole year.

In contrast to toenail, adult penguins molt their feathers only once per year after the breeding season, and thus, adult feathers only record a short period of the post-breeding diet. Sampling feathers is therefore useful for addressing issues over the post-breeding period, but it is of limited utility for answering ecological questions outside of this time frame. As with bone and toenail, breast feathers from fledgling-aged chicks are valuable for assessing the breeding season diet from the local environment near the colony where adults forage.

Feathers and bones showed similar variability in isotope values, and both $\delta^{13}C$ and $\delta^{15}N$ values reflected the expected dietary patterns of each penguin species. When sampling at different stages of the life cycle, feathers are a more reliable tissue for penguins because they are inert after synthesis. In contrast, bone, due to its slow turnover rate, integrates over a long time frame in the life of the animal and thus reflects an average of seasonal and annual fluctuations in diet (Hobson & Clark 1992). Chick bones, however, are more specific to the breeding season diet, and here it was found that two different bones yielded comparable isotope values. These results confirm that ancient chick bones are a good proxy for studying the paleo diet, even when comparing different bones.

CONCLUSIONS

This study is the first to examine stable isotopic variability among three pygoscelid penguin species sampled from the same region during the same year. This work was encouraged by the increasing use of multiple tissues to assess different time windows of information and the recurrent suggestion to incorporate the opportunistic sampling method of the CCAMLR monitoring program (Dimitrijević *et al*. 2018). This opportunistic method can facilitate regional surveys because samples can be easily collected at remote colonies that are difficult to reach, and dietary information can be gathered during a single visit, even after the breading season is over (Ainley *et al*. 2003). This opportunistic approach can also help to understand the variation and fractionation of isotopes in penguin bone in order to support paleo-dietary reconstructions, because most of the remains recovered from abandoned and ancient penguin colonies are chick bones, in addition to eggshells and membranes (Emslie *et al*. 2014). For dietary reconstruction, it is recommended that at least two tissues should be analyzed, or that a bulk isotope study should be combined with other sampling techniques. Observing the same trend in several tissues provides more confidence in dietary interpretations, as demonstrated in this study. Toenail is considered the best tissue to begin a long-term database for pygoscelid penguins. In addition, sampling breast feathers is easily accomplished at the same time and can complement data from toenails. Results of this study will aid in the establishment of a regional baseline for the four tissues in pygoscelid penguins and will inform future research into how to best interpret diverse tissue values and direct sampling efforts.

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REFERENCES

AGNEW, D. J. 1997. Review: the CCAMLR Ecosystem Monitoring Programme. *Antarctic Science* 9: 235–242. doi:10.1017/S095410209700031X

AINLEY, D.G., BALLARD, G., BARTON, K.J. ET AL. 2003. Spatial and temporal variation of diet within a presumed metapopulation of Adélie Penguins. *The Condor* 105: 95–106. doi:10.1093/condor/105.1.95

AINLEY, D.G. & BLIGHT, L.K. 2009. Ecological repercussions of historical fish extraction from the southern ocean. *Fish and Fisheries* 10: 13–38.

BALLANCE, L., PITMAN, R., HEWITT, R.P., SINIFF, D.B., TRIVELPIECE, W.Z., CLAPHAM, P.J., & BROWNELL, R.L. 2006. The removal of large whales from the Southern Ocean: evidence for long-term ecosystem effects? In: ESTES, J.A., DEMASTER, D.P., DOAK, D.F., WILLIAMS, T. E., BROWNELL, R.L. (Eds.) *Whales, Whaling and Ocean Ecosystems*. Berkeley, USA: University of California Press, pp 215–230.

BARQUETE, V., STRAUSS, V. & RYAN, P.G. 2013. Stable isotope turnover in blood and claws: A case study in captive African Penguins. *Journal of Experimental Marine Biology and Ecology* 448: 121–127. doi:10.1016/j.jembe.2013.06.021

BARRETT, R.T., CAMPHUYSEN, C.J., ANKER-NILSSEN, T. ET AL. 2007. Diet studies of seabirds: a review and recommendations. *ICES Journal of Marine Science* 64: 1675–1691. doi:10.1093/icesjms/fsm152

BARRERA-ORO, E., MARSCHOFF, E. & AINLEY, D. 2017. Changing status of three notothenioid fish at the South Shetland Islands (1983–2016) after impacts of the 1970–80s commercial fishery. *Polar Biology* 40: 2047–2054. doi:10.1007/s00300-017-2125-0

BATES, D., MAECHLER, M., BOLKER, B. & WALKER, S. 2015. Fitting Linear Mixed Effects Models Using lme4. *Journal of Statistical Software* 67:1–48. doi:10.18637/jss.v067.i01

BEARHOP, S., FURNESS, R.W., HILTON, G.M., VOTIER, S.C. & WALDRON, S. 2003. A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. *Functional Ecology* 17: 270–275. doi:10.1046/j.1365-2435.2003.00725.x

BRANCH, T.A. 2011. Humpback whale abundance south of 60°S from three complete circumpolar sets of surveys. *Journal of Cetacean Research and Management (Special Issue)* 3: 53–69.

CAMPRASSE, E.C.M., CHEREL, Y., BUSTAMANTE, P., ARNOULD, J.P. & BOST, C. 2017. Intra- and inter-individual variation in the foraging ecology of a generalist subantarctic seabird, the gentoo penguin. *Marine Ecology Progress Series* 568: 227–242. doi:10.3354/meps12151

CARLINI, A.R., CORIA, N.R., SANTOS, M.M. NEGRETE, J., JUÁRES, M.A. & DANERI, G.A. 2009. Responses of *Pygoscelis adeliae* and *P. papua* populations to environmental changes at Isla 25 de Mayo (King George Island). *Polar Biology* 32: 1427–1433. doi:10.1007/s00300-009-0637-y

CCAMLR. 2016. *Report on the monitoring program of chinstrap and gentoo penguins at Narębski Point (ASPA No. 171), King George Island, since 2006.* WG-EMM-16/62. Tasmania, Australia: Commission for the Conservation of Antarctic Marine Living Resources.

CHEREL Y., HOBSON, K.A., BAILLEUL, F. & GROSCOLAS, R. 2005. Nutrition, physiology, and stable isotopes: New information from fasting and molting penguins. *Ecology* 86: 2881–2888.

CHEREL Y. & HOBSON, K.A. 2007. Geographical variation in carbon stable isotope signatures of marine predators: a tool to investigate their foraging areas in the Southern Ocean. *Marine Ecology Progress Series* 329: 281–287. doi:10.3354/meps329281

COX, M.J., CANDY, S., DE LA MARE, W.K., NICOL, S., KAWAGUCHI, S. & GALES, N. 2018. No evidence for a decline in the density of Antarctic krill *Euphausia superba* Dana, 1850, in the Southwest Atlantic sector between 1976 and 2016. *Journal of Crustacean Biology* 38: 656–661. doi:10.1093/jcbiol/ruy072.

DALERUM, F. & ANGERBJÖRN, A. 2005. Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia* 144: 647–658. doi:10.1007/s00442-005-0118-0

DIMITRIJEVIÇ, D., PAIVA, V.H., RAMOS, J.A., ET AL. 2018. Isotopic niches of sympatric Gentoo and Chinstrap Penguins: evidence of competition for Antarctic krill? *Polar Biology* 41: 1655–1669. doi:10.1007/s00300-018-2306-5

DUCKLOW, H.W., BAKER, K., MARTINSON, D.G. ET AL. 2006. Marine pelagic ecosystems: the West Antarctic Peninsula. *Philosophical Transactions of the Royal Society B: Biological Sciences* 362: 67–94. doi:10.1098/rstb.2006.1955

EMSLIE, S.D. & PATTERSON, W. 2007. Abrupt recent shift in δ^{13}C and δ^{15}N values in Adélie penguin eggshell in Antarctica. *Proceedings of the National Academy of Sciences* 104: 11666–11669.

EMSLIE, S.D., POLITO, M.J., BRASSO, R., PATTERSON, W.P. & SUN, L. 2014 Ornithogenic soils and the paleoecology of pygoscelid penguins in Antarctica. *Quaternary International* 352: 4–15. doi:10.1016/j.quaint.2014.07.031

EMSLIE, S.D., ROMERO, M., JUÁRES, M.A. & ARGOTA, M.R. 2020. Holocene occupation history of pygoscelid penguins at Stranger Point, King George (25 de Mayo) Island, northern Antarctic Peninsula. *The Holocene*. 30: 190–196. doi:10.1177/0959683619875814

FLORES, H., ATKINSON, A., KAWAGUCHI S. ET AL. 2012. Impact of climate change on Antarctic krill. *Marine Ecology Progress Series* 458: 1–19.

FRY, B. 2006. *Stable Isotope Ecology*. New York, USA: Springer.

GIRAUDOUX, P. 2018. *pgirmess: Spatial Analysis and Data Mining for Field Ecologists.* R package version 1.6.9. R Foundation for Statistical Computing, Vienna, Austria. https://CRAN.R-project.org/package=pgirmess

GORMAN, K.B., WILLIAMS, T.D. & FRASER, W.R. 2014. Ecological sexual dimorphism and environmental variability within a community of Antarctic penguins (genus *Pygoscelis*). *PLoS One* 9: e90081. doi:10.1371/journal.pone.0090081

HERMAN, R.W., VALLS, F.C.L., HART, T., PETRY, M.V., TRIVELPIECE, W.Z. & POLITO, M.J. 2017. Seasonal consistency and individual variation in foraging strategies differ among and within *Pygoscelis* penguin species in the Antarctic Peninsula region. *Marine Biology* 164: 115.

HOBSON K.A. & CLARK, R.G. 1992. Assessing avian diets using stable isotopes I: Turnover of ^{13}C in tissues. *The Condor* 94: 181–188. doi:10.2307/1368807

HOTHORN, T., BRETZ, F. & WESTFALL, P. 2008. Simultaneous inference in general parametric models. *Biometrical Journal* 50: 346–363. doi:10.1002/bimj.200810425

JUÁRES, M.A. 2013. Biología reproductiva y ecología trófica de dos especies simpátricas del género *Pygoscelis* en las islas Shetland del Sur, Antártida. Phd thesis. La Plata, Argentina: Universidad Nacional de La Plata.

JUÁRES, M.A., SANTOS, M.M., NEGRETE, J. ET AL. 2013. Better late than never? Interannual and seasonal variability in breeding chronology of gentoo penguins at Stranger Point, Antarctica. *Polar Research* 32: 18448. doi:10.3402/polar.v32i0.18448

JUÁRES, M.A., SANTOS, M.M., NEGRETE, J. ET AL. 2015. Adélie penguin population changes at Stranger Point: 19 years of monitoring. *Antarctic Science* 27: 455–461. doi:10.1017/S0954102015000152

JUÁRES, M.A, CASAUX, R., CORBALAN, A. ET AL. 2018. Diet of Adélie penguins (*Pygoscelis adeliae*) at Stranger Point (25 de Mayo/King George Island, Antarctica) over a 13-year period (2003–2015). *Polar Biology* 41: 303–311. doi:10.1007/s00300-017-2191-3

JUÁRES, M.A., CASAUX, R., NEGRETE, J. ET AL. 2020. Update of the population size and breeding performance of gentoo penguins (*Pygoscelis papua*) at Stranger Point/Cabo Funes, South Shetland Islands. *Polar Biology* 43: 123–129. doi:10.1007/s00300-019-02614-0

KARNOVSKY, N.J., HOBSON, K.A. & IVERSON, S.J. 2012. From lavage to lipids: estimating diets of seabirds. *Marine Ecology Progress Series* 451: 263–284. doi:10.3354/meps09713

KEENAN, S.W. & DEBRUYN, J.M. 2019. Changes to vertebrate tissue stable isotope (δ^{15}N) composition during decomposition. *Scientific reports* 9: 9929. doi:10.1038/s41598-019-46368-5

KELLY, J.F. 2000. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. *Canadian Journal of Zoology* 78: 1–27. doi:10.1139/z99-165

KOKUBUN, N., TAKAHASHI, A., MORI, Y., WATANABE, S. & SHIN, H. 2010. Comparison of diving behavior and foraging habitat use between chinstrap and gentoo penguins breeding in the South Shetland Islands, Antarctica. *Marine Biology* 157: 811–825. doi:10.1007/s00227-009-1364-1

KRISTAN, A.K., EMSLIE, S.D. & PATTERSON, W.P. 2019. Stable isotope analyses of ancient and modern Adélie Penguin (*Pygoscelis adeliae*) mummies from the Ross Sea Region, Antarctica. *Polar Biology* 42: 1183–1192. doi:10.1007/s00300-019-02513-4

KUZNETSOVA, A., BROCKHOFF, P.B. & CHRISTENSEN, R.H.B. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software* 82: 1–26. doi:10.18637/jss.v082.i13

LA MESA, M., RIGINELLA, E., MAZZOLDI, C. & ASHFORD, J. 2015. Reproductive resilience of ice-dependent Antarctic silverfish in a rapidly changing system along the Western Antarctic Peninsula. *Marine Ecology* 36: 235–245.

LIDEN, K., TAKAHASHI, C. & NELSON, D.E. 1995. The Effects of Lipids in Stable Carbon Isotope Analysis and the Effects of NaOH Treatment on the Composition of Extracted Bone Collagen. *Journal of Archaeological* Science 22: 321–326. doi:10.1006/jasc.1995.0034

LOURENÇO, P.M., GRANADEIRO, J.P., GUILHERME, J.L. & CATRY, T. 2015. Turnover rates of stable isotopes in avian blood and toenails: implications for dietary and migration studies. *Journal of Experimental Marine Biology and* Ecology 472: 89–96. doi:10.1016/j.jembe.2015.07.006

MILLER, A.K., KAPPES, M.A., TRIVELPIECE, S.G. & TRIVELPIECE, W.Z. 2010. Foraging-niche separation of breeding gentoo and chinstrap penguins, South Shetland Islands, Antarctica. *The Condor* 112: 683–695. doi:10.1525/cond.2010.090221

POLITO, M.J., FISHER, S., TOBIAS, C.R. & EMSLIE, S.D. 2009. Tissue-specific isotopic discrimination factors in gentoo penguin (*Pygoscelis papua*) egg components: Implications for dietary reconstruction using stable isotopes. *Journal of Experimental Marine Biology and Ecology* 372: 106–112. doi:10.1016/j.jembe.2009.02.014

POLITO M.J., TRIVELPIECE, W.Z., KARNOSVSKY, N.J., NG, E., PATTERSON, W.P. & EMSLIE S.D. 2011. Integrating stomach content and stable isotope analyses to quantify the diets of pygoscelid penguins. *PLoS One* 6: e26642. doi:10.1371/journal.pone.0026642

POLITO, M.J., TRIVELPIECE, W.Z., PATTERSON, W.P., KARNOVSKY, N.J., REISS, C.S. & EMSLIE, S.D. 2015. Contrasting specialist and generalist patterns facilitate foraging niche partitioning in sympatric populations of *Pygoscelis* penguins. *Marine Ecology Progress Series* 519: 221–237. doi:10.3354/meps11095

R DEVELOPMENT CORE TEAM (2019). *R: A language and environment for statistical computing*. Vienna, Austria: The R Foundation for Statistical Computing.

ROMBOLÁ, E.F., MARSCHOFF, E. & CORIA, N. 2010. Inter-annual variability in Chinstrap penguin diet at South Shetland and South Orkneys Islands. *Polar Biology* 33: 799–806. doi:10.1007/s00300-009-0757-4

SANTORA, J.A., LARUE, M.A. & AINLEY, D.G. 2020. Geographic structuring of Antarctic penguin populations. *Global Ecology and Biogeography* 29: 1716–1728. doi:10.1111/geb.13144

SEALY, J., JOHNSON, M., RICHARDS, M. & NEHLICH, O. 2014. Comparison of two methods of extracting bone collagen for stable carbon and nitrogen isotope analysis: comparing whole bone demineralization with gelatinization and ultrafiltration. *Journal of Archaeological Science* 47: 64–69. doi:10.1016/j.jas.2014.04.011

SWEETING, C.J., POLUNIN, N.V.C. & JENNINGS, S. 2006. Effects of chemical lipid extraction and arithmetic lipid correction on stable isotope ratios of fish tissues. *Rapid Communications in Mass Spectrometry* 20: 595–601. doi:10.1002/rcm.2347

TRIVELPIECE W.Z., TRIVELPIECE, S.G. & VOLKMAN, N.J. 1987. Ecological segregation of Adélie, gentoo, and chinstrap penguins at King George Island, Antarctica. *Ecology* 68: 351–361. doi:10.2307/1939266

VASIL, C.A., POLITO, M.J., PATTERSON, W.P. & Emslie, S.D. 2012. Wanted: dead or alive? Isotopic analysis (δ^{13}C and δ^{15}N) of *Pygoscelis* penguin chick tissues supports opportunistic sampling. *Rapid Communication in Mass Spectrometry* 26: 487–493.

VAUGHAN, D.G., MARSHALL, G.J., CONNOLLEY, W.M. ET AL. 2003. Recent rapid regional climate warming on the Antarctic Peninsula. *Climatic Change* 60: 243–274. doi:10.1023/A:1026021217991

WICKHAM, H. 2016. *ggplot2: Elegant Graphics for Data Analysis.* New York, USA: Springer-Verlag.

WILLIAMS, T.D. 1995. *The Penguins*. Oxford, UK: Oxford University Press.