Validating the use of bulk tissue stable isotope and amino acid $\delta^{15}N$ values measured in molted hair and epidermis of elephant seals to assess temporal foraging niche specialization

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ABSTRACT: The degree of dietary generalization versus specialization exhibited by populations can impact their ability to respond to changing environmental conditions. Naturally shed hair and epidermis are easily collected and may provide a suitable substrate for stable isotope analysis to assess trophic niche specialization. Whether fasting influences the isotopic composition of molted tissues has not been assessed. Here, we gauged the validity of using carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope analysis of molted hair and the outermost epidermis as proxies to study the foraging ecology of southern elephant seals Mirounga leonina at Marion Island in the southern Indian Ocean. Similarity in both bulk tissue ($\delta^{13}C$ and $\delta^{15}N$) and amino acid ($\delta^{15}N$) isotope values of molted tissues and whiskers known to reflect fasting confirmed that physiological changes associated with fasting influenced hair and epidermis isotope values. We recommend caution when using the isotopic composition of molted hair and epidermis to make ecological inferences regarding diet and/or movement. Despite these limitations, molted hair can be used to detect extreme foraging behaviors, which we show using an extensive data set of hair sampled from marked individuals ($n = 331$) that hauled out on Marion Island during the 2012–2013 annual pelage molt. Other factors, such as ontogenetic shifts in diet and potential environmental (baseline) isotopic variation, also influence hair $\delta^{13}C$ and $\delta^{15}N$ values, which complicates the use of isotopic data from molted tissues to assess the temporal consistency of an individuals’ isotopic niche.

KEY WORDS: Epidermis · Marine mammals · Molt · Hair · Seals · Skin · Temporal consistency · Individual trophic niche specialization/conservatism · Trophic ecology

1. INTRODUCTION

Phenotypic and behavioral plasticity influences how organisms respond to changing environmental conditions. It is hypothesized that dietary specialists have a lower ability to adapt than generalist individuals or species that display niche plasticity over time (Bolnick et al. 2003, Clavel et al. 2011). However, it remains logistically challenging to obtain longitudinal data on resource and habitat use for mobile and elusive taxa. Generating a longitudinal isotopic record of sampled metabolically inert keratinous tissues (e.g. whiskers, baleen, nails, hair, and feathers) is increasingly used as an approach to investigate the temporal consistency of an individuals’ isotopic niche (niche conservatism) over periods spanning days to years (Newsome et al. 2007, 2010, Kernaleguen et al. 2012, Grecian et al. 2015, Camprasse et al. 2017, Busquets-Vass et al. 2017, Padayachee et al. 2020). Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic niche space is
often considered to be a quantitative indicator of an individual’s habitat and resource use; traits often used to describe an organism’s trophic niche (Bearhop et al. 2004, Ingram et al. 2018, Sheppard et al. 2018, Petta et al. 2020). Although isotope values measured in keratinous tissues might provide ecologically meaningful data, those values can be altered if synthesized from catabolized endogenous resources (e.g. Habran et al. 2019, Lübcker et al. 2020a). It is important to ascertain the period of biomolecule deposition in sampled tissues before either comparing isotope values measured (1) in different tissue types, (2) along the length of a single tissue (e.g. hair), or (3) in resampled tissue(s) from the same individuals, when aiming to quantify the prevalence of niche conservatism in consumer populations.

Hair growth in the southern elephant seal *Mirounga leonina* (SES) only occurs during the post-breeding foraging trip (~50–80 d) and molting period (~30 d) when they are hauled out on land and fasting (see Fig. 1) (Condy 1979, Jonker & Bester 1998, Kirkman et al. 2003, Ling 2012). Previous work has shown that the stable isotopic composition of elephant seal whiskers grown while breeding or molting on land are likely influenced by fasting when animals are completely relying on endogenous resources (e.g. skeletal muscle and adipose tissue) to fuel metabolism and tissue growth (McHuron et al. 2019, Lübcker et al. 2020a). Specifically, enhanced transamination and deamination of amino acids as substrates for gluconeogenesis enrich bulk tissue δ¹⁵N values of SES whisker segments grown while fasting (Lübcker et al. 2020a). The preferential deamination and excretion of isotopically lighter ¹⁴N amino radicals (~NH₂) during amino acid catabolism leaves the remaining amino acid pool enriched in ¹⁵N. It is, therefore, possible that stable isotope values measured in hair can be affected by fasting if grown while on land from stored endogenous sources.

Hair and skin isotope values obtained from biopsied elephant seals *Mirounga* spp. during the breeding season have been used to investigate the temporal consistency of individual-level trophic niche specialization (Rita et al. 2017). However, it remains to be tested if naturally shed (molted) hair and the outermost epidermis skin layer (see Fig. 1), which are easier to collect, could provide a comparable sample matrix to study isotopic niche specialization. Also, it is yet to be confirmed if these two tissues reflect two distinct time frames that can be used as independent samples to quantify foraging niche specialization with a single sampling event. In addition, it is not known if sequentially sampled hair could provide a time series of ecological data that spans the post-breeding foraging bout. Finally, as described above, the period of biomolecule deposition in molted hair and epidermis remains uncertain and could potentially be affected by fasting.

The goal of this study was to use δ¹³C and δ¹⁵N analysis of naturally molted SES hair and epidermis to evaluate whether longitudinal records of isotopic niche use could be obtained from these tissues. We used a combined bulk tissue and amino acid δ¹⁵N approach to assess when biomolecule deposition occurred in the sampled hair and epidermis and if their isotopic composition is influenced by fasting. Bulk tissue and amino acid isotope data were compared to values previously measured in whiskers sampled from the same individuals (Lübcker et al. 2016, 2020a). A similar approach was also used to assess the period of biomolecule deposition in paired hair and epidermis samples. After assessing the validity of using molt and epidermis isotope values to obtain longitudinal records of isotopic niche space from these tissues, we proceeded to demonstrate what information might be obtainable from molted hair using a representative set of samples collected from the Marion Island SES population during a single season. It remains to be seen if it could be assumed that δ¹³C and δ¹⁵N values measured in molted hair collected from various individuals that are undergoing the same physiological conditions are equally affected by fasting and provide ecologically relevant information. Here, we demonstrate that apart from being useful to identify extreme foraging behaviors, molted tissue isotope values reflect more than just dietary inputs. Both intrinsic (physiological) and extrinsic (diet) factors should be considered when interpreting isotope data from molted hair and epidermis as a proxy for individual trophic niche specialization and an individual’s ability to adapt to environmental change.

2. MATERIALS AND METHODS

2.1. Study area and sampling

Tissue collection occurred at Marion Island (46.88° S, 37.87° E) in the south Indian sector of the Southern Ocean. Samples of molted hair and epidermis were collected from 364 actively molting marked SES individuals of various age classes with sampling occurring between 2009 and 2015. Hair grown during the molt in the preceding year is shed together with the epidermis, and pieces were plucked off unrestrained individuals. The sampled age classes in-
cluded adult males (>6 yr old), adult females (≥3 yr old), subadult males (2–6 yr old), subadult females (2 yr old), yearlings (1 yr old) and young-of-year (YoY) (<1 yr old) SES sampled during their first molt. All SES born on Marion Island are tagged with uniquely numbered, color-coded, plastic Dal 008 Jumbotags® (Dalton Supplies) as part of a long-term SES monitoring program (Bester et al. 2011, Pistorius et al. 2011).

2.2. Stable isotope analyses

Surface contaminants and lipids were removed from the collected molted hair and epidermis by washing with deionized (DI) water, followed by 3 rinses in a 2:1 chloroform:methanol solvent solution. Samples were then rinsed thoroughly with DI water to remove solvents and air-dried in an oven at 50°C. Tissue δ13C and δ15N values were measured with Thermo Scientific Flash 1112 Series elemental analyzer/infrared mass spectrometer (EA-IRMS) in the Stable Isotope Laboratory at the University of Pretoria. In-house reference materials (Merck gel) were used to ensure reproducibility and to quantify analytical error. The results are expressed in parts per mil (‰) relative to the international standard atmospheric N2. Analytical error (SD) was ±0.2‰ for both δ13C and δ15N based on the within-run variance (SD) of measured reference materials. We also collected 2 separate hair samples from a subset of individuals (n = 8) during a single molt event to assess the reproducibility of our sampling and analytical approach. The reproducibility (SD) of our sampling approach matched the instrumental analytical error (±0.2‰ for δ13C, ±0.1‰ for δ15N).

A subset of lipid-extracted samples was hydrolyzed and derivatized for amino acid δ15N analysis (Lübcker et al. 2020a). Samples were first hydrolyzed in 1 ml 6 N hydrochloric acid (HCl) for 20 h at 110°C and then derivatized with 2-isopropanol and N-TFAA (Fantle et al. 1999). A Thermo Scientific Trace 1310 gas chromatograph with a SGE BPx5 column BPx5 (60 m) coupled to a Thermo Scientific GC Isolink II and Thermo Scientific Delta V Plus isotope ratio mass spectrometer was used to measure amino acid δ15N values at the University of New Mexico Center for Stable Isotopes. All samples were analyzed in duplicate and were bracketed by an internal stock reference material consisting of pure amino acids that were derivatized in batches alongside unknown samples (Sigma-Aldrich). The δ15N SD for multiple injections of the same sample averaged 0.4‰ (0.2–0.5‰) while the δ15N SD of amino acids in the stock reference material averaged 0.7‰ (0.4–0.9‰). This method provided reliable δ15N measurements of 13 amino acids, which can be divided into 3 general categories: (1) trophic amino acids, which includes alanine (Ala), isoleucine (Iso), leucine (Leu), valine (Val), proline (Pro), glutamic acid (Glx), and aspartic acid (Asx); (2) source amino acids, which includes phenylalanine (Phe), lysine (Lys), and tyrosine (Tyr); and (3) metabolic/physiological amino acids, which includes glycine (Gly), serine (Ser), and threonine (Thr). During derivatization, glutamate and glutamine are converted to Glx while aspartate and asparagine are converted to Asx, and therefore one value (Glx or Asx) is provided for each pair.

2.3. Effects of fasting on hair stable isotope values

A sub-sample of the tip, middle, base, hair follicle, and attached epidermis (Fig. 1) was obtained from ~7–10 mm long molted hair samples collected from 5 adult female SES to determine if fasting influenced the bulk tissue stable isotope values and whether biomolecule deposition in hair and epidermis occurred at different times. Samples of the protruding hair and complete molted hair samples, which included the epidermis and hair follicle, were also analyzed to inform future sampling protocols (Fig. 1). In 2 individuals, the hair was too short to obtain a middle section, so a total of 33 samples were prepared for bulk tissue δ13C and δ15N analysis. To directly compare epidermis and hair isotope values, we applied a nominal tissue-specific correction (δ15Nskin-hair: ±0.7‰ and δ13Cskin-hair: −0.1‰), based on captive phocid seals (Hobson et al. 1996). This correction was applied to the epidermis samples to ensure that our approach was comparable to that of Rita et al. (2017). A Shapiro-Wilk test was used to test normality before using an ANOVA and post hoc Tukey’s HSD to confirm if the mean δ13C and δ15N values between the hair segments differed significantly. Significance was inferred if p < 0.05, and all mathematical and statistical computations were performed using R v.3.4.4 (R Development Core Team 2018) coupled with the RStudio interface v.1.0.153. We calculated the differences between the isotope values measured in each segment of an individual’s hair (e.g. base and tip) to obtain the average difference between the different hair segments. A 1-sample Student’s t-test was then used to assess if these mean differences were significantly different from zero. If p < 0.05, then the differences between the isotope values of the various
segments calculated for each individual could be considered to be significant.

To further assess if physiological changes associated with fasting during the molt influenced protruding hair bulk tissue isotope values, we compared previously published whisker isotope data for individual adult female (n = 4) and YoY SES (n = 16; Lübcker et al. 2016, 2020a) to the temporally matched molted hair isotope data of the same individuals (paired sampling). Before comparing the isotope values of hair and whiskers, it was important to confirm that corrections for tissue-specific (hair-to-whisker) isotopic discrimination were not required. The SES hair-to-whisker bulk tissue stable isotopic offsets presented herein (see Section 3.1) were comparable in magnitude to instrumental error ($\Delta^{15}N_{\text{hair-whisker}} = -0.2$ to $0.0\%$; $\Delta^{15}C_{\text{hair-whisker}} = -0.4$ to $0.4\%$). Therefore, no corrections were applied when comparing hair and whisker isotope values. Hair growth in YoY occurs during the post-weaning fast, which temporally coincided with that of ~10 mm segment of whisker growths as described in Lübcker et al. (2017). Temporally overlapping whisker and hair growth of adult female SES occurs during the annual molt, and it was assumed that the overlapping hair growth occurred with the distal ~10 mm segment of their whiskers that preceded the onset of the post-molt (winter) foraging bout (Ling 2012, e.g. Fig. 3 in Lübcker et al. 2020a). Whisker isotope data were also compared to that of epidermis sampled from 5 unrelated (unpaired) adult females. Whisker sample treatment and analytical procedures followed the methods described in Lübcker et al. (2016, 2017, 2020a).

Lastly, hair (n = 8) and epidermis (n = 8) amino acid $\delta^{15}N$ values of adult female SES samples were used to assess whether the hair and epidermis isotope values were influenced by fasting. Similar to our approach with the bulk tissue data (see above), amino acid $\delta^{15}N$ data for hair and epidermis were also compared to whisker amino acid $\delta^{15}N$ values from adult female SES (n = 10). This comparison aimed to assess if the amino acid isotopic composition of the epidermis and hair tissues produced similar patterns to whisker segments that were either synthesized during a known period of fasting or when actively foraging (Lübcker et al. 2020a).

A non-parametric Kruskal-Wallis chi-squared test was used to assess the difference in the bulk tissue isotope values as well as the $\delta^{15}N$ values of amino acid pairs measured in the hair, epidermis, and whiskers during particular periods, with significance confirmed using a post hoc Wilcoxon rank-sum test (‘coin’ package in R; Zeileis et al. 2008). Differences between the $\delta^{15}N$ values of the trophic amino acid Glx and source amino acid Phe ($\Delta^{15}N_{\text{Glx-Phe}}$) are widely used to estimate the trophic position of consumers (e.g. Chikaraishi et al. 2009). Phe $\delta^{15}N$ values are assumed to reflect the isotopic composition of the baseline (Chikaraishi et al. 2009) yet can also be affected by fasting as previously observed in SES whiskers (Lübcker et al. 2020a). We reported the $\Delta^{15}N_{\text{Glx-Phe}}$ values measured in hair and epidermis to assess if fasting could influence estimates of trophic position from these tissues.

2.4. Age-related trophic niche partitioning

The molted hair samples collected from 331 tagged individuals (n = 187 females, n = 144 males) during 2012–2013 (Dec–Feb) were used to assess age-related trophic niche partitioning during the post-breeding foraging bout when resources used to synthesize the hair were consumed. Combined, this sample size represents ~82% of the Marion Island tagged SES population that hauled out during the 2012–2013 molt (Table S1 in the Supplement at www.int-res.com/articles/suppl/m673p229_suppl.pdf).
To avoid confounding factors associated with interannual variability in diet composition and/or baseline isotope values, we limited the analysis to samples collected in a single molt season (2012–2013). Samples with isotope values 1.5 times below or above the interquartile range of the associated lower and upper 95% confidence intervals were considered outliers. Bivariate isotopic niche widths (units: ‰²) were estimated with the widely used Stable Isotope Bayesian Ellipses in R library (SIBER, version 2.1.4; Jackson et al. 2011). The credible interval was set to include ~40% of the data of each individual. The small sample size corrected ellipses (SEA_c) were used to visualize the data in bivariate δ¹³C versus δ¹⁵N space (Jackson et al. 2011). The overlap between ellipses was evaluated using the built-in SIBER functions (Jackson et al. 2011). In addition, significant differences between the δ¹³C and δ¹⁵N values of age classes were tested using a Kruskal-Wallis chi-squared test.

2.5. Trophic niche specialization

In addition to the hair samples (n = 331) collected during the 2012–2013 molting season, we also measured δ¹³C and δ¹⁵N values in hair from an additional 25 individuals of various ages that were sampled during the molt in 2009–2010, 2010–2011, and 2014–2015 (see Table S4). These samples were analyzed to assess interannual variation in baseline isotope values. Lastly, of all the individuals sampled (excluding individuals sampled as YoY), 11 were resampled across 2 or more years to assess if inter-annual trophic niche specialization at the individual level could be characterized with hair isotope data.

3. RESULTS

3.1. Effects of fasting on hair stable isotope values and temporal resolution of epidermis

Mean (±SD) corrected epidermis δ¹³C values (~22.0 ± 0.9‰) were 2.1 ± 0.5‰ lower than measured in hair (protruding hair, −19.9 ± 0.7‰; ANOVA: F₅,₉₆ = 3.91, p < 0.01; Fig. 2a) with significance confirmed using a post hoc Tukey’s HSD test (p < 0.01). Epidermis δ¹³C values were 1.9 ± 0.9‰ lower than that of the base of the hair (p < 0.05). Epidermis δ¹⁵N values (12.5 ± 0.2‰) were 2.0 ± 0.5‰ higher than that of the protruding hair (10.5 ± 0.6‰; ANOVA: F₅,₉₆ = 10.25, p < 0.001; Tukey’s HSD test: p < 0.001; Fig. 2b). Follicle δ¹³C values were 0.8 ± 0.4‰ lower compared to the rest of the hair shaft while δ¹⁵N measured in the follicle was 0.9 ± 0.5‰ higher than measured in the protruding hair (Fig. 2a,b).

Temporal differences in both δ¹³C and δ¹⁵N measured in the tip and middle (1-sample Student’s t-test, δ¹³C: t = 0.19, df = 2, p = 0.864; δ¹⁵N: t = 1.08, df = 2, p = 0.393), middle and base (δ¹³C: t = −0.90, df = 2, p = 0.464; δ¹⁵N: t = 0.76, df = 2, p = 0.525), as well as between the tip and base of the molted hair (δ¹³C: t = −0.71, df = 2, p = 0.518; δ¹⁵N: t = −0.54, df = 2, p = 0.620) were not significantly different from zero (Fig. 2c,d). Observed differences were comparable to the reported instrumental precision and shown to be negligible.

The δ¹³C and δ¹⁵N values measured in the hair, corrected epidermis, and whiskers of YoY and adult female SES during known periods of fasting or at-sea foraging differed significantly (δ¹³C: Kruskal-Wallis χ² = 18.22, df = 6, p < 0.01; δ¹⁵N: Kruskal-Wallis χ² = 45.55, df = 6, p < 0.001; Fig. 3, Table 1). Temporally overlapping hair and whisker δ¹³C and δ¹⁵N values of YoY and adult female SES were used to calculate the hair-to-whisker δ¹³C and δ¹⁵N differences (Δ¹³C_hair-whisker, Δ¹⁵N_hair-whisker). YoY and adult female Δ¹³C_hair-whisker values did not differ significantly and were +0.4 ± 0.6 and +0.4 ± 0.2‰, respectively (Wilcoxon rank-sum test: p = 1.000 for both), while the corresponding Δ¹⁵N_hair-whisker values were 0.0 ± 0.5 and −0.2 ± 0.7‰ (Wilcoxon rank-sum test: p = 1.000 for both). Epidermis δ¹³C values of different adult females (n = 5) were 2.8‰ lower than the hair δ¹³C values and 2.4‰ lower than the fasting-related whisker δ¹³C values while being 2.3‰ lower than the foraging-related whisker δ¹³C values, although these differences were not statistically significant (Fig. 3a). Corresponding adult female epidermis δ¹⁵N values were similar to the whisker δ¹⁵N values measured while fasting (p = 1.000) while being 1.9‰ higher than those measured in their whiskers while foraging (p = 0.191; Fig. 3b). Adult female hair δ¹⁵N values were comparable to the δ¹⁵N values measured in the portion of whisker synthesized while fasting (0.2 ± 0.7‰ lower; p = 1.000) but were 1.4 ± 0.2‰ higher than measured in the portion of their whiskers that were synthesized while foraging at sea (Wilcoxon rank-sum test: p = 0.286). Lastly, δ¹³C values measured in the whiskers of YoY SES during their first year at sea (basal segments) did not differ significantly (p = 1.000) from that measured in the whiskers of adult females during their post-molt foraging trip, while the corresponding δ¹⁵N values were 2.0‰ lower (p < 0.001).

Lübcker et al.: Molted hair and epidermis stable isotope values
Fig. 2. Boxplot of (a,c) carbon ($\delta^{13}$C or $\Delta^{13}$C) and (b,d) nitrogen ($\delta^{15}$N or $\Delta^{15}$N) isotope values ($\delta$) or offsets ($\Delta$) measured in different portions of molted hair (tip, middle, base) and epidermis collected from southern elephant seals ($n = 5$). The subsampling regime used is illustrated in Fig. 1. Horizontal line: median; box: 25 and 75% confidence interval (CI); whiskers: 5 and 95% CI; dots: outliers. Asterisks and associated dashed lines denote significant differences which were confirmed with ANOVA, followed by a post hoc Tukey’s HSD (a,b) or Student’s t-test (c,d). Red dashed line indicates zero, and we tested if the difference between tissues were significantly different from zero. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$
Table 1. Bulk tissue mean (±SD) carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) isotope values of molted hair, epidermis (n = 5), and whiskers sampled from young-of-year (n = 16) and adult female (n = 4) southern elephant seals from 2009–2015. The physiological status (fasting or foraging) is also provided for each tissue type and statistical differences were assessed using Kruskal-Wallis chi-squared test followed by a Wilcoxon rank-sum test. Different superscript letters indicate significance levels, a, b: p < 0.001; c: p < 0.01

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3.2. Hair, epidermis, and whisker amino acid $\delta^{15}$N values

Median hair (n = 8) and epidermis (n = 8) amino acid $\delta^{15}$N values were contrasted against the amino acid $\delta^{15}$N values measured along the length of whiskers sampled from adult females (tip, middle, and base of whiskers; Fig. 4). Whisker, hair, and epidermis Ala (Kruskal-Wallis $\chi^2 = 12.13$, df = 4, p < 0.05), Asx (Kruskal-Wallis $\chi^2 = 31.84$, df = 4, p < 0.001), Pro (Kruskal-Wallis $\chi^2 = 36.13$, df = 4, p < 0.001), Glx (Kruskal-Wallis $\chi^2 = 14.90$, df = 4, p < 0.01), Val (Kruskal-Wallis $\chi^2 = 26.13$, df = 4, p < 0.001), Tyr (Kruskal-Wallis $\chi^2 = 25.58$, df = 4, p < 0.001), Lys (Kruskal-Wallis $\chi^2 = 19.66$, df = 4, p < 0.001), Gly (Kruskal-Wallis $\chi^2 = 43.06$, df = 4, p < 0.001), Ser (Kruskal-Wallis $\chi^2 = 37.06$, df = 4, p < 0.001), and Thr (Kruskal-Wallis $\chi^2 = 24.22$, df = 4, p < 0.001) $\delta^{15}$N values were significantly different (Fig. 4). Hair, epidermis, and whisker Leu, Iso, and Phe $\delta^{15}$N values did not differ significantly. Further statistical analyses and discussion of the differences between the whisker segment amino acid data can be found elsewhere (Lübcker et al. 2020a; also see Fig. 4). Overall, the hair and epidermis $\delta^{15}$N values were more comparable to $\delta^{15}$N values of the portion of the whisker that was grown on land during molt (tip of whisker), although differences occurred.

Differences between the amino acid $\delta^{15}$N values measured in the epidermis and whisker segments were as high as 8.5‰ between Gly $\delta^{15}$N values of the epidermis and the base of whiskers. Epidermis $\delta^{15}$N values for Asx, Val, Pro, Ser, and Gly were significantly enriched relative to other tissues (Fig. 4). Epidermis Ala $\delta^{15}$N values were similarly comparable to the whisker tip Ala $\delta^{15}$N values, while epidermis Glx $\delta^{15}$N values were 1.1‰ lower than measured in the tip of whiskers. Significant differences in the amino acid $\delta^{15}$N values of Val (Wilcoxon rank-sum test: p < 0.05), Tyr (p < 0.001), and Thr (p < 0.01) were observed between the whisker tip and hair. Ala (p < 0.01), Asx (p < 0.01), Val (p < 0.05), Pro (p < 0.01), Gly (p < 0.05), Lys (p < 0.01), Tyr (p < 0.05), and Thr (p < 0.01) $\delta^{15}$N values measured in the hair differed significantly from those obtained at the base of whiskers. The hair and whisker tip Ala and Ser $\delta^{15}$N values were comparable but were 1.7‰ lower (p < 0.05) and 1.5‰ higher, respectively, than measured in the base of whiskers. The hair Gly $\delta^{15}$N values were 2.5‰ higher than measured in the base of whiskers (p < 0.05) but were 6.0‰ lower than measured in the epidermis. The Glx values measured in the epidermis were 1.6‰ lower than measured in hair (p < 0.05).

No statistically significant differences were observed between the epidermis, hair, and whisker Phe $\delta^{15}$N values ($\chi^2 = 3.02$, df = 4, p = 0.554). The largest difference in the Phe $\delta^{15}$N values was due to the 1.0‰ enrichment of the whisker tip values compared to that of the base of the whiskers. Differences between the median (±95% confidence interval) Glx and Phe $\delta^{15}$N values ($\Delta^{15}$N$_{Glx-Phe}$) were 14.9 (11.7; 17.1)% for the epidermis, 16.7 (13.2; 18.6)% for hair, 15.1 (14.0; 16.9)% tip of whisker, 15.9 (13.1; 17.8)%
3.3. Age-related trophic niche structuring

Of the 331 individuals sampled during 2012–2013, 6 individuals were considered outliers based on their isotopic bulk tissue $\delta^{13}$C values, which ranged from $-12.8$ to $-15.0\%o$, and were excluded from this analysis and considered separately (see Section 4.3). These 6 outliers consisted of 5 adult females and one 2 yr old subadult male, and their $\delta^{15}$N values (10.9–11.7‰) were within range of the included samples (Fig. 5). $\delta^{13}$C and $\delta^{15}$N values measured in the hair from the remaining 325 individuals (Table S1) differed significantly between age classes ($\delta^{13}$C: Kruskal-Wallis $\chi^2 = 63.04$, df = 7, $p < 0.001$; $\delta^{15}$N: Kruskal-Wallis $\chi^2 = 158.35$, df = 7, $p < 0.001$; Table S2), but SEA$_C$ overlap occurred (Table S3). $\delta^{13}$C values of YoY hair were depleted by 0.5–1.4‰ (Wilcoxon rank-sum test: $p < 0.01$), while $\delta^{15}$N were enriched by 0.6–1.1‰ ($p < 0.001$) compared to the other age class SES (except adult males), and their SEA$_C$ did not overlap with any age class (Fig. 5). $\delta^{15}$N values of male and female yearlings were compara-
ble (49.8% overlap), and δ¹⁵N values of yearling females were 0.5‰ higher than that of adult females (p < 0.01) and their SEAC overlapped by 41.3%. Subadult male and adult female δ¹³C and δ¹⁵N values overlapped (63.5%; Fig. 5), but δ¹⁵N values of subadult males were marginally enriched (0.3‰) compared to adult females (p < 0.05). Adult male and adult female δ¹⁵N values differed significantly (p < 0.05); the male δ¹⁵N values were 1.5‰ higher than those of the adult females, while δ¹³C values differed by 0.7‰ (p = 0.651). Lastly, adult male δ¹⁵N values were enriched by 1.1−1.5‰ compared to all other age class SES (p < 0.05), resulting in <5% SEAC overlap with other age classes.

### 3.4. Temporal consistency in trophic niche specialization

Interannual variation occurred in the δ¹⁵N values measured in molted hair, but not for δ¹³C (Kruskal-Wallis χ² = 30.34, df = 3, p < 0.001; δ¹³C χ² = 1.57, df = 3, p = 0.665). The hair δ¹⁵N values representing the 2014–2015 molt were 1.1‰ higher than measured during the 2009–2010 molt (p < 0.001), and the SEAC overlapped only 10.1% (Table S4). The SEAC of the samples representing the 2011–2012 molt overlapped (49.5%) with the 2009–2010 samples but did not overlap with the 2014–2015 molt (Fig. 6). The δ¹³C and δ¹⁵N values of n = 11 individuals resampled during different years showed interannual, intra-individual variability, as well as ontogenetic responses (increase/decrease) associated with a change in age class (Fig. 7). For example, the hair δ¹³C and δ¹⁵N values of an individual (e.g. RR(2)2400; Fig. 7) sampled as a subadult male during 2009 and resampled twice as an adult male during 2010 and 2011 were affected by both the change in age class between the sampling periods and sampling year (2009–2010).

### 4. DISCUSSION

#### 4.1. Fasting physiology influences δ¹⁵N values of molted hair

Naturally shed (molted) hair and epidermis samples can be collected from phocids in a minimally invasive fashion; however, it is important to determine whether isotope values measured in these tissues provide ecologically meaningful results. Adult female and YoY hair δ¹⁵N values were indistinguishable from those measured in whisker segments.
new hair is primarily grown from catabolized endogenous stores and does not provide a direct record of changes in whisker composition. That reflect active foraging. Unlike the temporal consistency of an individual’s stable carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) isotope values measured in the molted hair of repeatedly sampled southern elephant seal (SES, n = 11) individuals during different years and from different age classes. Dashed colored lines connect data for samples that were collected from the same individual during 2 or 3 different years. The age class during which the sampled hair was synthesized is labeled for each datapoint for each year sampled. YM: yearling male; YF: yearling female; SAM: subadult male; SAF: subadult female; AF: adult female; AM: adult male. Label colors represent different sampling periods with the direction of the arrows in chronological order from the oldest to most recent sample. Samples collected in the same year are enclosed in a total convex hull area (TA) to show interannual variation in isotope values (2009–2010; 2011–2012; 2014–2015). The 2010–2011 sample size (<10) did not allow the construction of a reliable TA. The year of sampling and age class change between the sampling periods (e.g. RR(2)400) affect the isotope values; yet these patterns are highly variable and confound the practicality of using repeatedly sampled hair samples to assess interannual foraging niche conservatism.

grown during fasting (Fig. 3b,d) but were significantly enriched in comparison to whisker segments that reflect active foraging. Unlike the temporal changes in whisker $\delta^{15}$N values associated with a shift from the use of exogenous (dietary) to endogenous (protein and lipid) resources during the molt (Lübcker et al. 2020a), negligible $\delta^{13}$C and $\delta^{15}$N variation were observed along the length of hair (Fig. 2a,b). Together, these patterns indicate that new hair is primarily grown from catabolized endogenous stores and does not provide a direct record of an individuals’ foraging ecology during the post-breeding foraging bout. While hair growth may commence while SES are still at sea (Ling 2012), most hair growth likely occurs on land near the end of the molt (Hückstädt et al. 2012), a phenomenon also observed in other phocid seals including captive harbor Phoca vitulina and spotted P. largha seals (Ashwell-Erickson et al. 1986). Remobilization of endogenous amino acids stored in structural tissues (e.g. skeletal muscle) likely alter the isotope values of most keratinous tissues that are synthesized while fasting (Cherel et al. 1994, Lübcker et al. 2020a). Rapid hair growth in molting and fasting phocids is associated with variations in steroid levels that promote the mobilization of stored nitrogenous sources and increased blood flow to hair follicles (Ashwell-Erickson et al. 1986, Boily 1996). Our previous work on amino acid $\delta^{15}$N values of SES whiskers described the likely biochemical pathways responsible for synthesizing $^{15}$N-enriched pathways during fasting (Lübcker et al. 2020a). Briefly, the glucose-Ala (Cahill) and glucose-lactate (Cori) cycle likely work in conjunction to cycle glucose in fasting elephant seals (Crocketer et al. 2017). We found that tissue Ala $\delta^{15}$N values are lower during fasting due to enhanced glucose-Ala cycling that uses this non-essential amino acid to transfer nitrogen from the muscle to the liver (Felig et al. 1969, Lübcker et al. 2020a,b). Furthermore, the $\delta^{15}$N values of some non-essential amino acids (e.g. Gly, Ser, Pro, Val) increase during fasting because of their role as substrates for gluconeogenesis and possible de novo synthesis from recycled $^{15}$N-enriched nitrogen pools (Lübcker et al. 2020a). Preferential deamination of amino acids containing $^{14}$N for gluconeogenesis leads to isotopic enrichment of the remaining pool of these amino acids used for tissue synthesis during fasting. Lastly, $\delta^{15}$N values of Glx and branch-chained amino acids (e.g. Leu, Iso, Val) appear to be insensitive to fasting. Branch-chained amino acids are catabolized via a special set of branch-chained amino acid aminotransferase enzymes that are energetically costly to produce and as such are often spared from catabolism in mammals undergoing nutritional stress (Tom & Nair 2006). In contrast, glutamate is the most abundant amino acid in the body and is the hub of nitrogen cycling and subjected to a high degree of transamination and deamination (O’Connell 2017), which may homogenize its $\delta^{15}$N composition. Hair amino acid $\delta^{15}$N values are similar to those in the tip of whisker that was grown on land during the molt. The diagnostically depleted Ala $\delta^{15}$N values, but enrichment of Gly, Ser, and Pro $\delta^{15}$N values (Lübcker et al. 2020a), confirmed that the hair and associated epidermis were grown from catabolized resources.

Offsets in $\delta^{15}$N values between Glx and Phe (i.e. $\Delta^{15}$N$_{Glx-Phe}$), the canonical trophic and source amino acids respectively, can be used to assess trophic position of the consumer without requiring isotope data for potential prey (Chikaraishi et al. 2009). Tissues
grown while in a negative nitrogen balance are expected to produce a decrease in $\Delta^{15}\text{N}_{\text{Glx-Phe}}$, which is related to the enrichment of the remaining Phe $\delta^{15}\text{N}$ values but no change in Glu $\delta^{15}\text{N}$, as described elsewhere for SES (also see Lübcker et al. 2020a). Epidermis collected from adult female SES had the lowest $\Delta^{15}\text{N}_{\text{Glx-Phe}}$ offset (14.9‰) measured in any tissue, yet hair had a $\Delta^{15}\text{N}_{\text{Glx-Phe}}$ of 16.7‰. The differences in $\Delta^{15}\text{N}_{\text{Glx-Phe}}$ offsets were, however, not significant between different segments or tissues and might still be a good trophic level estimator.

Importantly, hair bulk tissue $\delta^{13}\text{C}$ values of adult female SES were comparable to the whisker $\delta^{13}\text{C}$ values, while both fasting and foraging. The $\delta^{13}\text{C}$ values measured in whiskers of adult females reflect variation in foraging latitude (e.g. Lubcker et al. 2017, Walters et al. 2020). The $\delta^{13}\text{C}$ values of adult females are transferred to nursing offspring, which is reflected in molted hair of YoY (Fig. 3a). Lipids have lower $\delta^{13}\text{C}$ values than associated proteins and could lower the whisker and hair $\delta^{13}\text{C}$ values if lipid carbon-skeletons are used to synthesize non-essential amino acids (Newsome et al. 2014). Yet the small number (n = 5) of paired whisker and hair samples, coupled with large variation in their $\delta^{13}\text{C}$ values (Fig. 3a), prevented detailed analyses of the effects of fasting on tissue $\delta^{13}\text{C}$ values.

### 4.2. Shed epidermis stable isotope values

Despite our multi-tissue approach, the period of biomolecule deposition in SES epidermis remains uncertain. After application of a tissue-specific discrimination factor to account for differences in isotopic composition resulting from variation in amino acid concentrations, epidermis bulk $\delta^{13}\text{C}$ values were more than 2.0‰ lower than measured in protruding hair. One possible explanation for this difference is that the hair and epidermis were grown during different periods (Fig. 3a). Alternatively, the difference between epidermis and hair $\delta^{13}\text{C}$ values could also reflect differences in the relative contribution of endogenous source (e.g. skeletal muscle versus blubber) used to synthesize tissues while fasting. However, from the small number of individuals (n = 5) for which we had paired hair and epidermis $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, it was evident that epidermis had the lowest $\delta^{13}\text{C}$ values of any tissue sampled and higher $\delta^{15}\text{N}$ values than any other portion of sub-sampled hair (Fig. 2b). Given that the epidermis isotope values differed from whiskers grown during the post-molt foraging trip (Lubcker et al. 2020b), the epidermis values can only reflect the post-breeding foraging trip if synthesized from exogenous resources while at sea. However, adult female SES are constrained to foraging close (~1000 km) to Marion Island (Jonker & Bester 1998) which is not consistent with the depleted epidermis $\delta^{13}\text{C}$ values (e.g. Lübcker et al. 2017). Thus, it is unlikely that epidermis isotope values solely reflect foraging (exogenous resources) if the applied tissue-specific discrimination factors for phocid seals are accurate (Hobson et al. 1996).

When compared to (unpaired) whiskers, epidermis $\delta^{15}\text{N}$ values were more comparable to the segments of whisker grown while fasting than any other portions of the whisker (Fig. 3b). Moreover, similarity in amino acid $\delta^{15}\text{N}$ values in hair, whisker segments grown while fasting, and epidermis suggested that the latter tissue was synthesized from stored endogenous sources (Fig. 4). The epidermis had depleted Ala $\delta^{15}\text{N}$ values but enriched Gly and Ser $\delta^{15}\text{N}$ values relative to whiskers grown while foraging (Fig. 4b). This represents an amino acid $\delta^{15}\text{N}$ pattern that is typical of a catabolic state in SES (Lübcker et al. 2020a). The only way for an individual to have epidermis Gly and Ser $\delta^{15}\text{N}$ values that are 8.5 and 6.6‰ higher, respectively, than any portion of the whisker is if these 2 non-essential amino acids were synthesized de novo from catabolized endogenous resources (e.g. Lübcker et al. 2020a). The notion that the epidermal layer might have been synthesized while in a catabolic state also supports the findings of Ling (2012), who postulated that the shed cornified outermost epidermis layer in SES consists of several continuous layers of dead keratin (corneocytes) that are formed when the hair growth enters the telogen (static) phase. Combined, these 2 non-essential amino acids represent a large proportion (~40%) of the amino acids in skin and keratin-based tissues (Tabachnick & LaBadie 1970, Wolf et al. 2015). Since any enrichment (or depletion) in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values of Ser and Gly would be reflected in the bulk tissue isotope values, we conclude that epidermis isotope values are influenced by a catabolic state. Exactly when and how the biomolecule deposition occurs, however, remains uncertain. We argue that it is best practice not to interpret isotopic patterns of the epidermis as solely reflective of foraging. In the following section, we further demonstrate why intrinsic and extrinsic factors should be accounted for when using molted hair to make inferences regarding an individual’s isotopic niche specialization.
4.3. Potential ecological relevance of molted hair stable isotope values

Despite evidence that molted hair $\delta^{15}$N values are influenced by fasting and do not directly reflect exogenous dietary resources, it is of interest to determine if age-related trophic niche partitioning can be assessed from molted hair sampled during a single season. The motivation for these analyses was based on the assumption that even if the SES hair isotope values are affected by fasting, all individuals should have the same physiological status, and their isotope values might still provide ecologically relevant information about resource partitioning and niche conservatism. Hair isotope values that reflected the 2011–2012 post-breeding foraging bout, however, showed limited age-related trophic niche partitioning (Fig. 5) but considerable inter-individual isotopic variation, similar to what has been documented in previous studies of SES elsewhere (Hückstädt et al. 2012). SES adult females, subadults, and yearlings showed isotopic niche overlap (Table S3), while adult males had significantly higher $\delta^{15}$N values than other age and sex classes. The higher $\delta^{13}$C and $\delta^{15}$N values in hair collected from YoY relative to adult females (Fig. 5) likely reflect a combination of nursing and the post-weaning fast (Lübcker et al. 2020b) and as such, should be excluded from studies focused solely on assessing foraging ecology. The whisker $\delta^{15}$N values of YoY SES that reflect the post-weaning foraging trip were lower (2.0‰) than the whisker $\delta^{15}$N values of adult females (Fig. 3b), suggesting that YoY used lower trophic level prey (Lübcker et al. 2017). This difference in trophic niche use between YoY and adult female SES, however, was not reflected by the hair $\delta^{15}$N values, which could be due to the influence of fasting on $\delta^{15}$N values.

Whether or not the molted hair isotope values reflect ecologically meaningful age-related niche partition at Marion Island during the post-breeding foraging trip remains uncertain. This uncertainty is due to the lack of other comparable isotope data for SES from this locality during the study period, as well as the unknown inter-individual variation in the influence of fasting on hair isotope values. Age-related variation in the duration of time spent on land during the molt (Kirkman et al. 2003) and individual body mass differences between conspecifics at the start of the molt (Postma et al. 2013) can cause inter-individual variation of $\delta^{15}$N values when fasting, depending on the amount of endogenous nitrogen remobilized for hair synthesis. These factors may dampen any age-related trophic niche partitioning and require further investigation.

Furthermore, our data showed that systemic inter-annual variation in the isotope values of SES tissues can occur (Fig. 6). For example, mean $\delta^{15}$N values of hair representing the 2014–2015 molt were significantly higher (by 1.1‰) than those representing the 2009–2010 molt (Fig. 6), indicative of temporal shifts in either baseline $\delta^{15}$N values, foraging habitat used, and/or prey utilized. Prey isotope data and individual movement data would be needed to constrain the driver(s) for the observed interannual variability, which is also true if the aim was to assess temporal trophic niche conservatism at the individual level between different periods with bulk tissue isotope analysis. Lastly, we suggest that it would be challenging to account for these physiological and environmental factors when using a repeated or multi-tissue sampling scheme to assess individual-level trophic niche specialization at an interannual resolution. Such challenges are further illustrated by hair isotope data from individuals (n = 11) that were resampled in different years (Fig. 7). For example, hair $\delta^{15}$N values of an individual (RR(2)400) sampled as a subadult male during 2009 and resampled twice as an adult male in 2010 and 2011 (Fig. 7) were likely influenced by ontogenetic (subadult to adult) shifts in resource and habitat use as well as by potential interannual differences in baseline $\delta^{15}$N values. In summary, the combined influence of potential interannual variation in the isotopic baseline (Pakhomov et al. 2004, Magozzi et al. 2017), age-related isotopic variation, and potential variations of the fasting effect on the stable isotope values of an individual likely preclude the utility of repeatedly sampled hair to make inferences regarding the temporal consistency of trophic niche specialization at the individual level. While remobilized endogenous stores used to synthesize hair are ultimately sourced from diet, it is unlikely that hair $\delta^{13}$C and $\delta^{15}$N isotope values of SES provide a reliable temporal record of an individual's foraging niche. It is important to consider intrinsic and extrinsic factors that could affect an individual’s isotopic composition, especially when isotope analysis of multiple tissues is used to make inferences regarding niche conservatism and the potential of SES to adapt to climate change. We contend that predictions regarding the potential of SES to adapt to climate change based on comparison of hair and skin isotope values require careful consideration.

Despite these complications, isotope analysis of molted hair may allow for the identification of extreme foraging behaviors at the individual level. Of the 331 SES sampled during the 2012–2013 molt on Marion Island, 6 had hair $\delta^{13}$C values that were con-
considered outliers ranging from $-12.8$ to $-15.0\%$. These relatively high $\delta^{13}C$ values likely indicate use of foraging areas along the southern African coast (Connan et al. 2014), which is not a common foraging area for this SES population based on telemetry data (Jonker & Bester 1998), although SES have occasionally been observed (Oosthuizen et al. 1988, 2011). Specifically, the similarity in $\delta^{13}C$ values between these 6 SES individuals and keratinous tissues collected from Cape fur seals 

Arctocephalus pusillus pusillus that forage near the South African coast (range: $-12.4$ to $-14.5\%$; Connan et al. 2014) confirm that SES from Marion Island venture this far north to forage. Five of the 6 outlying individuals were females, and 3 of these were not recorded on Marion Island on occasion of ‘skipping’ a breeding season (de Bruyn et al. 2011). These females remained at sea throughout the year and based on their $\delta^{13}C$ values did not forage close to Marion Island, the primary foraging ground of this population during the post-breeding foraging period (Jonker & Bester 1998). Individuals from this population are thus foraging in areas outside of their primary foraging habitat, and such individual behavioral plasticity could benefit the adaptive capacity of the population as a whole in response to environmental change (Siniff et al. 2008, van den Hoff et al. 2014).

### 4.4. Analytical and sampling considerations

We recommend that future studies carefully separate hair from epidermis because observed differences in the isotopic composition of these tissues are driven by a variety of factors discussed above. In addition, both $\delta^{13}C$ and $\delta^{15}N$ values of hair follicles differed from other sections of the hair, perhaps due to the presence of compounds (e.g. blood) other than keratin (Lübcker et al. 2016) and are best excluded from analysis. Lastly, our findings extend beyond isotope-based studies of foraging and movement ecology. For example, serum pollutant concentrations increase when stored lipophilic pollutants are remobilized from adipose tissue during prolonged periods of fasting (Peterson et al. 2016, 2018). Consequently, hair that was synthesized while fasting might amplify pollutant concentrations in comparison to tissues grown while animals are actively foraging. Thus, the period in which hair synthesis takes place, and whether it occurs during periods of fasting or foraging, should also be considered when interpreting pollutant loads within or among marine mammal species.

### 4.5. Conclusions

We evaluated the approach of using naturally shed hair and epidermis isotope values to investigate the temporal (interannual) consistency of individual isotopic niche specialization during the post-breeding foraging period prior to the molt. Similar amino acid $\delta^{15}N$ patterns between these tissues and whisker segments grown while fasting confirmed that hair and epidermis are influenced by remobilization of endogenous stores and do not solely reflect (exogenous) dietary resources. However, the period of biomolecule deposition in shed epidermis could not be ascertained. For these reasons, the notion that the isotopic composition of these tissues is solely influenced by diet and can be used to investigate the temporal consistency of isotopic niche specialization needs to be reconsidered. We demonstrated that hair and epidermis isotope values are useful to identify extreme foraging events; however, these values reflect more than just diet, and both intrinsic and extrinsic factors contribute to the unfeasibility of using the isotopic composition of these tissues to examine niche conservatism.

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