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RESEARCH ARTICLE

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The influence of biopsy site and pregnancy on stable isotope ratios in humpback whale skin

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Abstract

Rationale: Stable isotope analysis (SIA) of free-swimming mysticetes using biopsies is often limited in sample size and uses only one sample per individual, failing to capture both intra-individual variability and the influence of demographic and physiological factors on isotope ratios.

Methods: We applied SIA of $\delta^{13}C$ and $\delta^{15}N$ to humpback whale (*Megaptera novaeangliae*) biopsies taken during the foraging season along the western Antarctic Peninsula to quantify intra-individual variation from repeatedly sampled individuals, as well as to determine the effect of biopsy collection site, sex, and pregnancy on isotope ratios.

Results: There was substantial variability in $\delta^{13}C$ from multiple biopsies taken from the same individuals, though $\delta^{15}N$ was much more consistent. Side of the body (left versus right) and biopsy location (dorsal, anterior, ventral, and posterior) did marginally affect the isotopic composition of $\delta^{15}N$ but not $\delta^{13}C$. Pregnancy had a significant effect on both $\delta^{13}C$ and $\delta^{15}N$, where pregnant females were depleted in both when compared to non-pregnant females and males.

Conclusions: These results indicate that isotopic signatures are influenced by multiple endogenous and exogenous factors and emphasize value in accounting for intra-individual variability and pregnancy status within a sampled population. Placed within an ecological context, the endogenous variability in $\delta^{13}C$ observed here may be informative for future isotopic analyses.

1 | INTRODUCTION

The study of free-swimming mysticetes, particularly those in high-latitude oceans, presents researchers with several financial and logistical challenges. Research on these species has benefitted from the development of molecular techniques (e.g., genomics, stable isotope analysis (SIA)[1–3]), recent advances in technology (drones, biologging devices, acoustics[4–7]), and often opportunistic sampling designs.[8] SIA is a cost-efficient and relatively accessible method to gain insight into the foraging ecology of an animal.[9–11] It has repeatedly proven its use for free-swimming marine megafauna.[12] The elements most frequently examined are carbon and nitrogen, often expressed in delta notation as $\delta^{13}C$ and $\delta^{15}N$, respectively, where delta values are presented as the ratio of heavy to light isotopes in a sample to the ratio of heavy to light isotopes in an internationally accepted standard. $\delta^{13}C$ is often used as an indicator of habitat in the marine environment as variability is driven largely by primary production and is affected by growth rates and community

composition of primary producers, nutrient availability, and dissolved inorganic carbon.[13] Additionally, $\delta^{13}C$ is of particular relevance for high-latitude environments, as $\delta^{13}C$-enriched sea-ice-associated algae are linked to the amount of sea ice present.[14] $\delta^{15}N$ is also driven to some extent by primary production and nitrogen processes at the base of the food web; however, this element fractionates heavily with each increasing trophic level, making it a strong indicator of trophic position.[15,16] When paired together, $\delta^{13}C$ and $\delta^{15}N$ taken from tissues can provide insight into an individual's or population's biogeography,[17] foraging ecology and diet,[18] population structure,[19] drivers of stress,[20] trophic position,[16] tissue turnover rates,[21] and other key life history events such as fasting and migration.[22]

While isotope ratios in animal tissues allow researchers to answer a broad range of questions, variability introduced by unknown factors (e.g., pregnancy, fasting, tissue physiology) could confuse or confound study results.[23] Therefore, researchers must understand a focal species' physiology, life history, demographics, and foraging environment and how these factors influence stable isotope ratios measured from tissues to interpret data correctly and rule out alternative explanations. For example, in mysticetes, pregnancy has been hypothesized to result in depleted values of $\delta^{15}N$ due to tissue synthesis and reduced nitrogen excretion and similarly depleted values of $\delta^{13}C$ due to reliance on ^{13}C-depleted lipid stores for energy.[24] In other animals, pregnant and/or lactating individuals have been shown to experience $\delta^{15}N$ depletion in their tissues.[25–27] Consequently, if a study incorporating stable isotope ratios has an uneven distribution of samples by pregnancy status, yet does not consider pregnancy, the study may incorrectly attribute observations of $\delta^{13}C$ and $\delta^{15}N$ to another unrelated variable. Additionally, it is well documented that ratios of $\delta^{13}C$ and $\delta^{15}N$ can vary substantially between tissue types of the same species due to physiological differences (e.g., keratinous structures, muscle, and skin),[28–30] yet little has been explored regarding the homogeneity of $\delta^{13}C$ and $\delta^{15}N$ from the same tissue type taken from different body locations.[31,32] Finally, it is exceedingly rare for studies utilizing SIA of tissues from living, free-swimming cetaceans to include multiple samples from the same tissue type, from the same individual, and from the same period of time.[33] The majority of all isotopic studies have assumed that one sample acts as a static representation of an individual whale's diet, environment, and physiology, yet only in rare cases are these conditions truly static.

Here we perform SIA of $\delta^{13}C$ and $\delta^{15}N$ from free-swimming humpback whale (*Megaptera novaeangliae*) skin biopsies collected as part of a long-term monitoring project along the western Antarctic Peninsula (WAP) to quantify the degree of variability in isotope ratios by sex, pregnancy status, and body position. Importantly, our dataset includes resampling events of the same genetically confirmed individuals, in many cases with less than 1 week between resampling events, allowing us to explore isotopic variability at a timescale short enough to eliminate skin turnover rates, behavioral shifts, or changes in environmental conditions as confounding variables. Specifically, our goals were to (1) quantify isotopic variability within genetically confirmed individuals sampled less than 1 week apart, (2) explore the effect of body biopsy site on ratios of $\delta^{13}C$ and $\delta^{15}N$, comparing left versus right side of the body and among four body locations (dorsal, ventral, posterior, and anterior), and (3) determine the effect of sex and pregnancy status on ratios of $\delta^{13}C$ and $\delta^{15}N$. Ultimately, these goals fall under the overarching theme of quantifying internal and inherent sources of variability that may impact the use of isotopes in broader ecological and behavioral investigations.

2 | METHODS

2.1 | Field sampling, DNA profiling, and hormone analysis

Methods regarding whale field sampling and subsequent analysis to determine the identity, sex, and pregnancy status are presented in Pallin et al,[2,34] but a summary is as follows. Skin–blubber biopsy samples of humpback whales were collected from individuals foraging along the WAP during the 2010 and 2013–2018 austral summer and autumn using crossbows and modified darts. The majority of biopsies occurred during the months of January to April, although a few did occur as late as June and as early in the season as mid-November. Biopsies were collected opportunistically as whales were sighted and approached during regularly scheduled surveys. All age and demographic classes were sampled, except calves. The genetic sex of whales and individual identification were determined from the biopsies using DNA profiles containing microsatellite loci and sex-specific markers; this allowed for the confirmation of resampling of certain individuals with sampling intervals ranging in time from hours to years. Finally, progesterone was extracted from biopsies and compared against individuals of known pregnancy status to determine whether a given whale was pregnant.[35] Progesterone levels increase, often by orders of magnitude, in pregnant humpback whales and are a reliable indicator of pregnancy.[24] Pallin et al[2] required their predictive model to be 99.9% certain in order to assign pregnancy status for these samples; all other individuals in their study received a status of unknown. Following these analyses, a subset of remaining tissue from each biopsy sample was analyzed for stable isotopes of carbon and nitrogen.

2.2 | Stable isotope analysis

For SIA, a skin subsample was cut from each biopsy and underwent lipid extraction. Lipid extraction is often recommended when working with fatty tissues such as whale skin, as lipids are considerably more depleted in ^{13}C than other macromolecules and can confound the targeted environmental signal. Each sample was soaked in 2:1 chloroform–methanol solution twice, first for approximately 24 h and then a second time for approximately 1 h. Each sample was then submerged in 0.7% NaCl, centrifuged, and dried in a drying oven. Carbon-to-nitrogen ratios (C:N) of samples were used for quality assurance that lipid extraction was successful. Samples were run for

$\delta^{13}C$ and $\delta^{15}N$ at the University of North Carolina Wilmington's (UNCW) isotope ratio mass spectrometer (IRMS) laboratory using an element analyzer coupled with an IRMS and standards Vienna Peedee Belemnite (VPDB) and atmospheric air (AIR), along with acetate, USGS 40, and USGS 41 for calibration. An additional biopsy dataset was obtained from whales sampled in 2010 and 2013 from the same study location and run for SIA at the Duke University Environmental Stable Isotope Laboratory (DEVIL) in Durham, North Carolina. It is crucial to note that whale skin grows in multiple layers, with newly synthesized tissue forming in the innermost basale layer, then gradually being pushed outward to the middle layer (spinosum) and then to the outer externum layer before being sloughed off into the water.[33,36,37] The skin samples in this study were cut from biopsies without consideration of the three skin layers; it is possible that any sample contains a single layer or combination of layers, dependent on the orientation of the cut.

2.3 | Statistical analysis

All statistical analyses were conducted in R (version 4.2.2)[38] and can be broken down to the following objectives: (1) examine isotopic variability within repeat-sampled individuals, (2) examine the effect of body location on biopsy isotope ratios, and (3) examine the effect of sex and pregnancy on isotope ratios. Each objective included a unique subset of individuals from our overall dataset for which data were available.

First, to examine variability in ratios of $\delta^{13}C$ and $\delta^{15}N$ within individuals between paired sampling events (genetically confirmed), absolute values of differences in $\delta^{13}C$ and $\delta^{15}N$ were calculated, as was the time in days between sampling events (Table 1, Figure 1). In an attempt to remove the effects of skin turnover time and changes in environmental or behavioral states on isotope ratios, only paired sampling events that occurred within 1 week from each other were used, as estimates of cetacean skin turnover rates suggest skin is replaced of the order of multiple weeks to months.[21,36,37,39,40] From this subset of data, only a small fraction of samples had associated body biopsy sites; thus, biopsy site was ignored for this objective. Second, the potential effect of body biopsy site on $\delta^{13}C$ and $\delta^{15}N$ was examined. Sampling events for which body biopsy sampling site was noted were pooled together. When a genetically confirmed individual was sampled, either within or between years, both samples were retained to improve the statistical power in examining differences across the body. Biopsies were recorded as either the left versus right side of the body, and further categorized by location: dorsal, ventral, posterior, anterior, and peduncle, the last of which was removed due to a small sample size ($n = 2$; Figure 2A). It is important to note that examination of biopsy site was not part of the original experimental design and thus we retroactively grouped biopsies as well as possible into these locations based on original observer notes and photographs taken at the time of sampling, when available. Data did not meet the assumptions for a parametric test, thus Wilcoxon tests were used to compare $\delta^{13}C$ and $\delta^{15}N$ on the left versus right side of the body and then between each pairing of dorsal, ventral, posterior, and anterior groups, where side of body was not included. Levene's test was run to determine if variance in isotope ratios was even among the four body biopsy sites.

Finally, isotope ratios were compared between pregnant females, non-pregnant females, and males. Any individuals for which sex or pregnancy status was not known were excluded from analysis, and for within-year resample events of the same individual only data from the first sample were included to avoid repeat sampling bias since pregnancy status was unlikely to change while on the feeding grounds within 1 year. As before, data did not meet the assumptions for a parametric test, so a set of Wilcoxon tests were run among the three groups for both $\delta^{13}C$ and $\delta^{15}N$.

TABLE 1 Breakdown of resampling events of the same genetically confirmed individuals. DayDiff is the number of days between resampling events, and the differences in $\delta^{13}C$ and $\delta^{15}N$ between resampling events are reported as absolute values

Individual	Sex	DayDiff	Δ $\delta^{13}C$ (‰)	Δ $\delta^{15}N$ (‰)
gMno13AP016	Male	0	0.48	0.04
gMno13AP024	Male	0	1.43	0.33
gMno13AP025	Female	0	0.50	0.00
gMno13AP061	Female	1	0.38	0.21
gMno13AP069	Male	0	0.62	0.43
gMno14AP013	Male	0	0.19	0.05
gMno14AP025	Male	0	0.12	0.35
gMno14AP051	Male	2	0.29	0.27
gMno14AP102	Male	0	0.34	0.49
gMno14AP114	Male	0	0.02	0.01
gMno14AP134	Female	0	0.13	0.23
gMno16AP011	Female	5	1.22	0.15
gMno17AP045	Male	1	0.23	0.16
gMno17AP068	Male	0	0.27	0.03
gMno17AP136	Male	1	0.05	0.01
gMno17AP168	Male	0	0.18	0.07
gMno18AP010	Female	3	0.12	0.16
gMno18AP015	Female	0	0.40	0.03
gMno18AP086	Male	1	2.86	0.71
gMno18AP157	Male	0	0.83	0.18

3 | RESULTS

Instrument error, measured as standard deviation of internal laboratory standards run at regular intervals, from UNCW's IRMS laboratory was 0.18‰ for $\delta^{13}C$ and 0.07‰ for $\delta^{15}N$ from 20 standards of USGS40 and 0.10‰ for $\delta^{13}C$ and 0.10‰ for $\delta^{15}N$ from 25 standards of USGS41a. For DEVIL, $\delta^{13}C$ and $\delta^{15}N$ was 0.29‰ and 0.23‰, respectively, based on 13 standards of acetanilide.

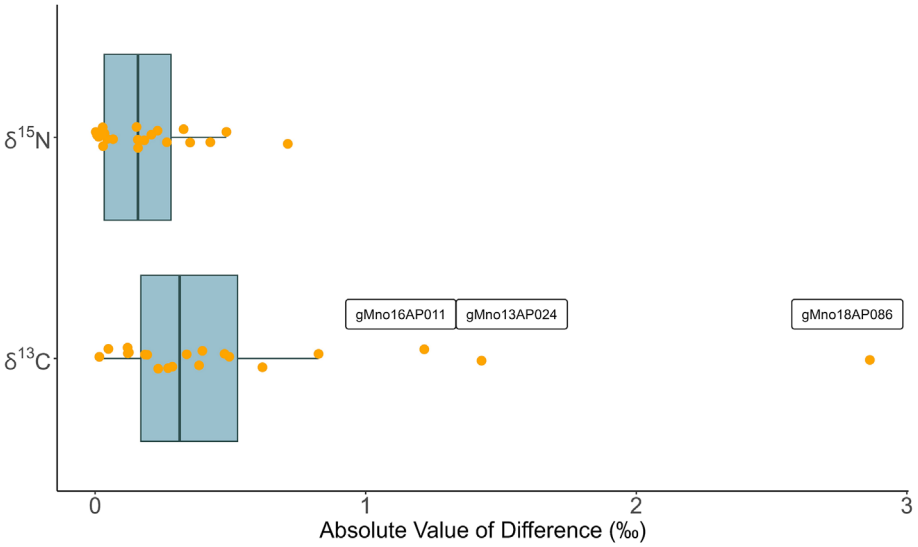

FIGURE 1 Differences in $\delta^{13}C$ and $\delta^{15}N$ between resampling events of the same individuals less than 1 week apart, for objective 1 (individual dots reported as absolute values). The three outlier individuals with surprisingly high $\Delta\delta^{13}C$ are labeled according to their original catalog identifier. Box-and-whisker plots represent first and third quartiles, medians, and maximum and minimum values no greater than 1.5 times the interquartile range [Color figure can be viewed at wileyonlinelibrary.com]

3.1 | Individual-level variability

After filtering for paired sampling events of the same individual no more than 1 week apart, the dataset for objective 1 included 20 individuals (14 males and 6 females; Figure 3). Absolute values of the differences in $\delta^{13}C$ ($\Delta\delta^{13}C$) between paired sampling events of the same individuals showed considerably greater variability than for $\Delta\delta^{15}N$ (Figure 1). $\Delta\delta^{13}C$ ranged from 0‰ to 2.9‰ (mean ± SD: 0.5 ± 0.7‰) and $\Delta\delta^{15}N$ ranged from 0‰ to 0.7‰ (mean ± SD: 0.2 ± 0.2‰). Three paired sampling events appeared abnormally high for carbon ($\Delta\delta^{13}C$ > 1‰); these came from individuals gMno13AP024, gMno16AP011, and gMno18AP086. The first individual, gMno13AP024, was a male and sampled twice on the same day in mid-January 2013. Both biopsies were taken from the left side of the whale, with one specifically being marked as ventral and the other as unknown. The next individual, gMno16AP011, was a pregnant female sampled twice 5 days apart in mid-January 2016. No body biopsy site information was recorded for this individual. Last is gMno18AP086, a male with the greatest $\Delta\delta^{13}C$ (2.9‰) reported. This individual was repeat sampled 1 day apart at the very end of February 2018; the first biopsy was taken from somewhere on the right side of the body and the second was taken from the top of the dorsal fin. When these three individuals were excluded from the dataset, $\Delta\delta^{13}C$ ranged from 0‰ to 0.8‰ (mean ± SD: 0.3 ± 0.2‰). The removal of these outliers had minimal effect on $\Delta\delta^{15}N$ (range: 0–0.5‰; mean ± SD: 0.2 ± 0.2‰).

3.2 | Body biopsy site variability

Our dataset for this objective included 58 biopsy samples from 43 individuals (males: $n = 33$; females: $n = 25$). The side of the body from which biopsies were collected (left versus right) did not affect $\delta^{13}C$ and $\delta^{15}N$ ($\delta^{13}C$: $p = 0.9282$; $\delta^{15}N$: $p = 0.1784$), and Levene's test indicated homogeneity of variance among the body positions ($\delta^{13}C$: $p = 0.6892$; $\delta^{15}N$: $p = 0.8156$). $\delta^{15}N$ was significantly different between the dorsal and anterior surfaces ($p = 0.0489$, mean difference = 0.6‰), where anterior samples were depleted (Figure 2). There was also perhaps a marginally significant difference between ventral and anterior surfaces ($p = 0.0673$), where anterior samples were similarly depleted. There were no statistically significant differences in $\delta^{15}N$ between the remaining body biopsy sampling site combinations (p ranged from 0.2265 to 0.749). $\delta^{13}C$ did not differ significantly by body biopsy sampling sites (p ranged from 0.1827 to 0.9366). The range of overall $\delta^{13}C$ values was greater than that of $\delta^{15}N$ (8.0‰ for $\delta^{13}C$ versus 3.8‰ for $\delta^{15}N$; although excluding one enriched outlier reduced the range of $\delta^{13}C$ to 5.4‰) and the absolute difference in dorsal and anterior averages was larger for $\delta^{13}C$ than $\delta^{15}N$ (1.0‰ for $\delta^{13}C$ versus 0.6 for $\delta^{15}N$).

3.3 | Pregnancy variability

After removing all second samples from within-year resampled individuals, sex and, for females, pregnancy status were confirmed for 153 biopsy samples taken from 145 individuals (pregnant females: $n = 36$; non-pregnant females: $n = 29$; males: $n = 88$). Pregnancy did have a significant effect on $\delta^{13}C$ and $\delta^{15}N$ found in humpback skin (Figure 3). Pregnant females were depleted in both elements when compared to non-pregnant females ($\delta^{13}C$: $p = 0.0389$, mean difference = 0.9‰; $\delta^{15}N$: $p = 0.0500$, mean difference = 0.4‰) and males ($\delta^{13}C$: $p = 0.0289$, mean difference = 0.7‰; $\delta^{15}N$: $p = 0.0025$, mean difference = 0.5‰). There was no significant difference between non-pregnant females and males ($\delta^{13}C$: $p = 0.6517$; $\delta^{15}N$: $p = 0.9874$). It is worth noting that, although pregnant females were depleted in both elements when compared to non-pregnant females and males, there was still considerable overlap among individuals in these categories (Figure 3).

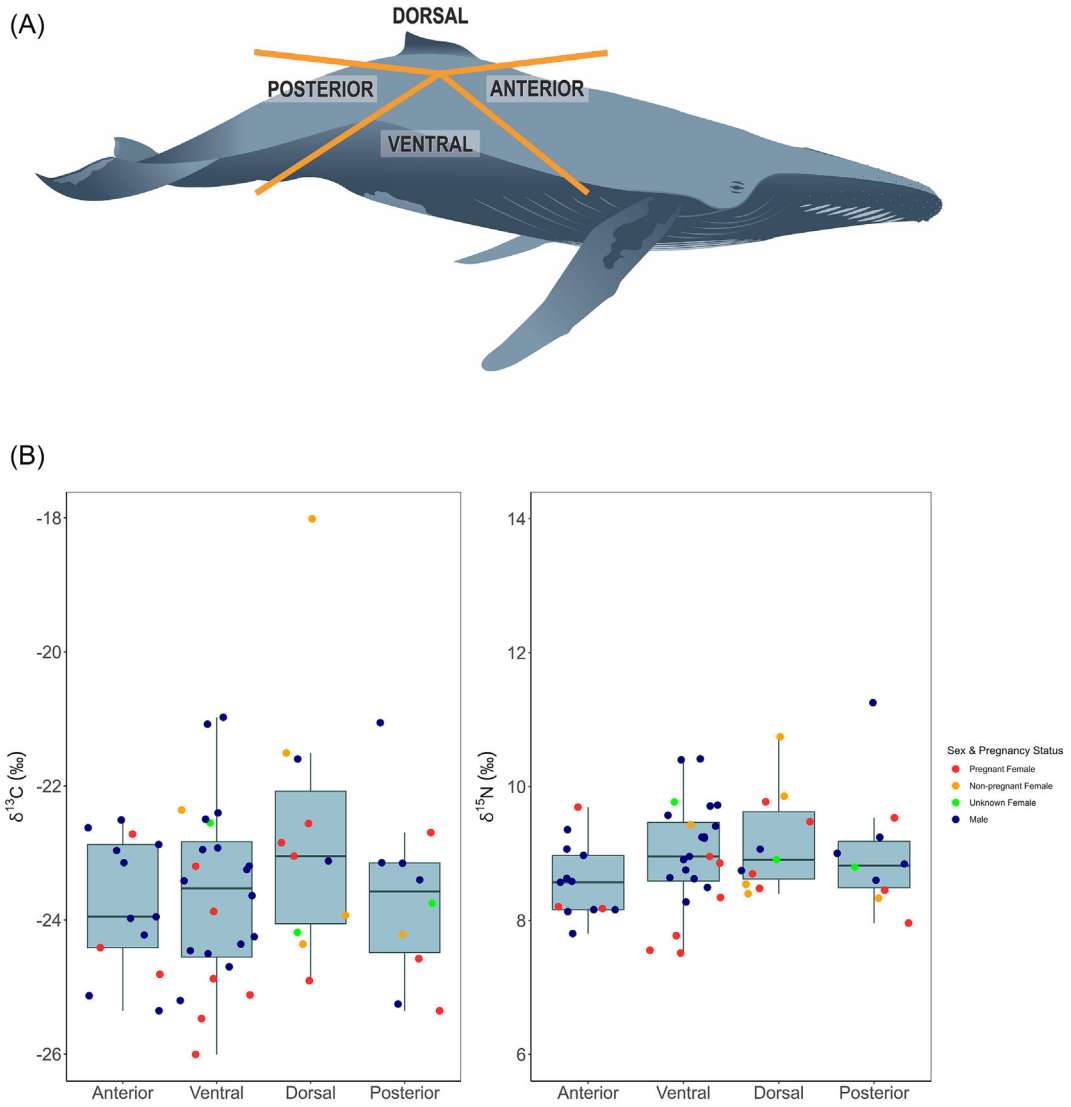

FIGURE 2 Variability in $\delta^{13}C$ and $\delta^{15}N$ from biopsies taken from the four sites, for objective 2. (A) Conceptual diagram showing the four biopsy sites included in analyses. (B) There was no significant effect of body position on $\delta^{13}C$, nor was there any significant difference between samples taken from the left versus right side of the body for either element. $\delta^{15}N$ was significantly depleted in anterior biopsies when compared to dorsal, but not for any other combination of body biopsy locations. Dots represent individual biopsies, while box-and-whisker plots represent first and third quartiles, medians, and maximum and minimum values no greater than 1.5 times the interquartile range. Dot color corresponds to sex and pregnancy status [Color figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Accurate interpretation of isotope ratios for any animal species requires knowledge of sources and scales of variability that may influence isotope ratios. Due to the logistical constraints of studying free-swimming mysticetes, little is known regarding the unexplained variability of ratios of $\delta^{13}C$ and $\delta^{15}N$ in the skin and how the isotopic compositions of these two elements are affected by biopsy site, sex, and pregnancy. The population of humpback whales foraging along the WAP is one of the better-studied Southern Ocean populations and is thought to have a diet consisting almost entirely of Antarctic krill (*Euphausia superba*), due to this species' extremely high abundance in the region and capability of sustaining the energetic demands of large baleen whales.[41–43] The isotopic composition of Antarctic krill will vary to a small extent over space and time, as well as by life stages of krill, yet variability along the WAP is generally low.[44] This presumed stability in prey composition offers the chance to reduce some of the ecological and geographic drivers of isotope change, allowing for investigation of lesser known or quantified drivers. Multiple years of fieldwork in the region have produced one of the largest collections of biopsy samples of any southern hemisphere baleen whale. From this dataset, we were able to successfully investigate new scales and sources of internally driven variability in $\delta^{13}C$ and $\delta^{15}N$.

The degree of variability between skin isotope ratios of individuals sampled repeatedly over a short period of time was

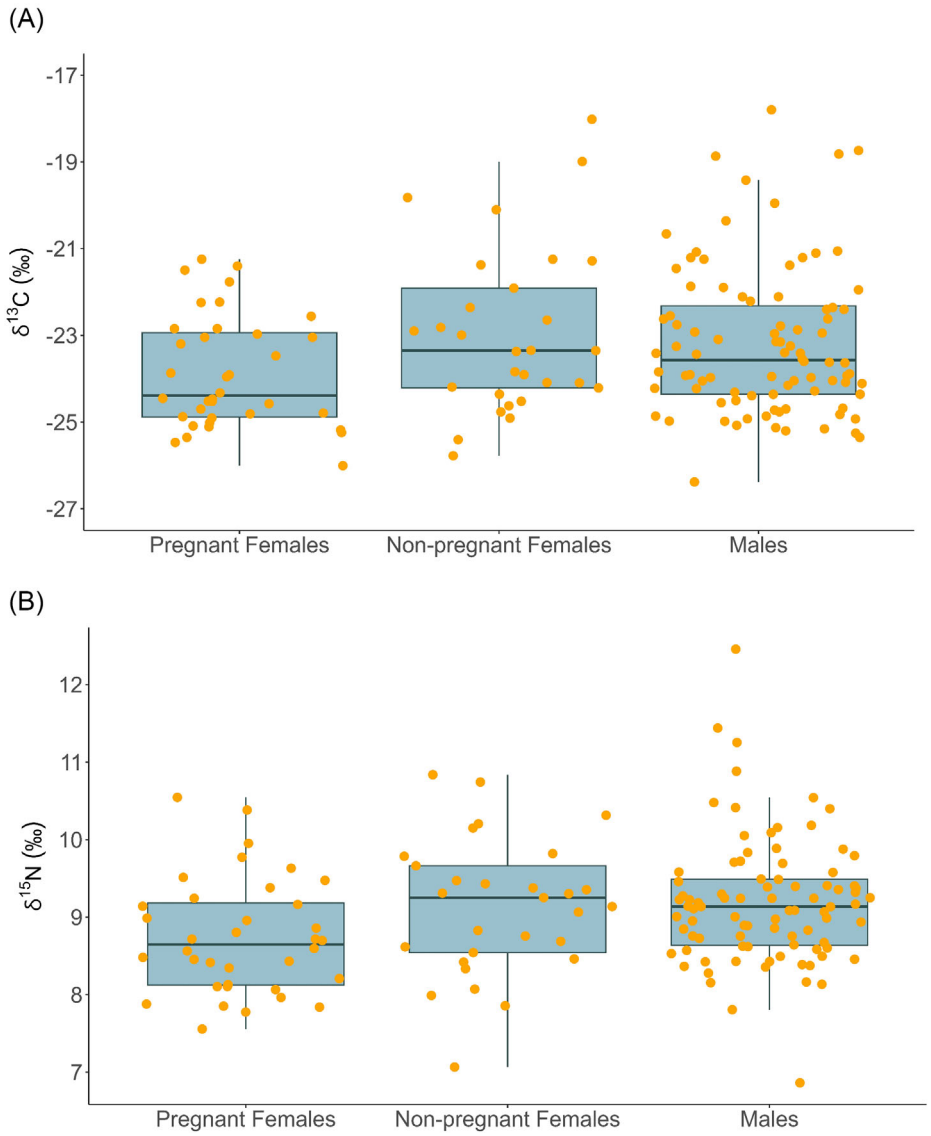

FIGURE 3 $\delta^{13}C$ and $\delta^{15}N$ by sex and pregnancy status, for objective 3. Pregnant females were significantly depleted in both elements when compared to non-pregnant females and males. There was no significant difference in $\delta^{13}C$ or $\delta^{15}N$ between non-pregnant females and males. Dots represent individual biopsies, while box-and-whisker plots represent first and third quartiles, medians, and maximum and minimum values no greater than 1.5 times the interquartile range [Color figure can be viewed at wileyonlinelibrary.com]

considerable for $\delta^{13}C$. The absolute values of the difference between $\delta^{13}C$ for three individuals sampled were greater than 1‰, despite having only a maximum of 5 days between resampling events. Turnover rates of skin for cetaceans have been estimated for bottlenose dolphins (*Tursiops truncatus*)[36,39,40] and belugas (*Delphinapterus leucas*)[37] in captivity and for free-swimming blue whales (*Balaenoptera musculus*) during migration across the northeast Pacific Ocean.[21] Estimates of isotopic turnover times from these studies range from a few weeks to several months, though rates for blue whales would likely be a more accurate comparison to humpbacks than the other species. Busquets-Vass et al[21] reported a mean turnover time of 163 (±91) days for blue whales. Over these timescales, well-documented drivers of changes in isotopic signatures include changes in environment and/or foraging behavior.[45,46] A less than one-week sampling interval was expected to result in consistent stable isotope values. Thus, our results point to two important considerations for designing and interpreting future isotope studies. First, as mentioned in the methods above, it is important to control for skin layer. Cetacean skin contains three layers, the innermost "basale" layer, the middle "spinosum" layer, and the outermost "externum" layer; new skin is synthesized in the basale layer before gradually being pushed outward and sloughed off in the ocean.[33,36,37] A vertical core of our biopsies of all three layers could include different isotopic information from low-latitude breeding grounds as well as from the WAP, depending on how much time had elapsed between an individual arriving to the WAP and being sampled. As this study did not specifically control for skin layer, the two matched samples may have had slightly different layer compositions. Second, if the layer composition was largely similar between matched samples, this suggests there are other sources of variability that we have not identified here that could be skewing interpretations in other studies. Future studies relying on the isotopic composition of whale skin should, if possible, aim to identify additional potential sources of variability. It is also worth noting the possibility that differences in $\delta^{13}C$ could at least partly be attributed to the lipid extraction process; any variability during this step between samples (e.g., the

completeness of extraction) could have influenced our results. Ratios of $\delta^{13}C$ in animal tissues are assumed to increase by approximately 1‰ to 2‰ per trophic position, although there may be substantial variation in the degree of fractionation depending on multiple factors.[14,25] Therefore, differences in $\delta^{13}C$ of 1‰ or higher as seen in our data warrant caution for trophic interpretations. On the other hand, it is generally assumed that $\delta^{15}N$ will increase by approximately 3‰ per trophic position; in this sense the observed differences in $\delta^{15}N$ between resampling events of less than 1‰ are not particularly biologically meaningful.[25]

Busquets-Vass et al[21] found differences in $\delta^{15}N$ between the innermost basale layer and the outermost externum layer as well as between the basale layer and sloughed skin in blue whales in the California Current System. Yet the same study did not detect a difference in $\delta^{15}N$ between the same layers for blue whales in the Gulf of California, nor was there any significant difference in $\delta^{13}C$ among skin layers from whales sampled in either location.[21] Similarly, Wild et al[33] tested for differences in $\delta^{13}C$ and $\delta^{15}N$ between three skin layers (basale, spinosum, and externum) for a single stranded humpback whale, a stranded fin whale (*Balaenoptera physalus*), and over two dozen free-swimming sperm whales (*Physeter macrocephalus*). The two stranded whales and one sperm whale with a skin sample large enough for testing layers were included in a first experiment which had mixed results; $\delta^{13}C$ but not $\delta^{15}N$ varied by skin layer in the sperm whale sample, $\delta^{15}N$ but not $\delta^{13}C$ varied by skin layer in the humpback sample, and skin layer was not a significant predictor for either element in the fin whale sample. A second experiment using 28 free-swimming sperm whale biopsies from the Gulf of Alaska found differences in $\delta^{13}C$ and $\delta^{15}N$ by skin layer, with maximum differences between skin layers from the same sample being 1.0‰ for $\delta^{13}C$ and 1.1‰ for $\delta^{15}N$.[33] Three repeat-sampled humpbacks included in our study had greater $\Delta\delta^{13}C$ than any between-layer differences for sperm whales in Wild et al,[33] yet our comparisons are also different. Instead of looking at $\Delta\delta^{13}C$ between layers of one biopsy, we report $\Delta\delta^{13}C$ between two separate biopsies without accounting for skin layer.

It is possible that, in addition to the potential inadvertent sampling of heterogeneous skin layers, biopsy sampling from different body sites might contribute to observed differences in $\delta^{13}C$ between paired samples, although our results suggest that when grouped together body biopsy site does not significantly affect the isotopic composition of $\delta^{13}C$. Our three "outlier" repeat-sampled individuals all had uncertainties regarding sampling location on the body from at least one sampling event, and the individual with by far the greatest $\Delta\delta^{13}C$ was sampled from two separate body sites (an unknown site somewhere on the right side of the body and at the top of the dorsal fin). The dorsal fin is considerably different from other biopsied regions on the flank of the whale in terms of fibrous connective tissue and vasculature; it is entirely possible that this may have contributed to the high variability observed between paired samples from this individual.[47,48]

There have been few studies to date looking at whether ratios of $\delta^{13}C$ and $\delta^{15}N$ in cetacean skin differ based on body biopsy site, and none have thus far detected a significant effect of site on isotopic ratios of either element.[31,32,49] Our study, however, is the first to ask this question for humpback whales and includes a much larger sample size than any previously published for cetaceans ($n = 58$ samples from $n = 43$ individuals). While most body sites were comparable, we did detect a significant difference in $\delta^{15}N$ between anterior and dorsal surfaces only, where anterior biopsies were on average 0.6‰ lower. Borrell et al[31] compared dorsal to ventral skin $\delta^{13}C$ and $\delta^{15}N$ from hunted fin whales ($n = 28$) and found no differences. A similar study was carried out in common (*Delphinus delphis*; $n = 10$) and striped (*Stenella coeruleoalba*; $n = 9$) dolphins, where $\delta^{13}C$ and $\delta^{15}N$ were compared among 11 body sampling locations from stranded or captured individuals and neither element was found to differ among locations.[32] Finally, Williams et al[49] found no differences in $\delta^{13}C$ or $\delta^{15}N$ between body positions in bottlenose dolphins, yet it should be noted that only two individuals were included in their analysis. Our results mostly agree with previous research that body position does not seem to affect $\delta^{13}C$ and $\delta^{15}N$ in cetacean skin, with the notable exception of $\delta^{15}N$ being depleted in anterior biopsies when compared to those taken from the dorsal surface.

Despite our statistically significant results, differences in $\delta^{15}N$ between anterior and dorsal surface biopsies were relatively minor (Figure 2). A mean difference of 0.6‰ is only a small fraction of the approximately 3‰ change that would be expected due to a shift in trophic position. Alternatively, compared to $\delta^{15}N$, $\delta^{13}C$ showed considerably greater variability within and between body biopsy sites with the greatest difference also found between anterior and dorsal samples of 7.3‰. In isotope ecology, a 1‰ change in $\delta^{13}C$ is often interpreted as reflective of a substantial change in trophic position or foraging habitat. Putting this into practice, future studies should consider the magnitude of variability in $\delta^{13}C$ observed here and investigate drivers of this large variability in $\delta^{13}C$.

Although the effect of pregnancy on $\delta^{13}C$ and $\delta^{15}N$ in cetacean tissues is not well understood, it has been hypothesized that during pregnancy $\delta^{15}N$ will decrease due to increased tissue synthesis and a reduction in nitrogen excretion. Further, it is assumed, at least for capital breeders such as mysticetes, that $\delta^{13}C$ will also decrease with pregnancy and fasting due to the reliance on ^{13}C-depleted lipid stores for energy.[24,25] Clark et al[24] tested for differences among pregnant and non-pregnant humpback whales in the California Current across 2 years and suggested both $\delta^{13}C$ and $\delta^{15}N$ were depleted in pregnant females sampled one year but not the other. Yet when samples were combined for both years and constrained for similar time periods throughout the foraging season there was no longer a difference between groups. Borrell et al[50] provided further evidence for a possible depletion in $\delta^{15}N$ with pregnancy in fin whales, but their results were not significant. Moreover, they examined the effect of gestation and lactation on females and their offspring, rather than comparing pregnant females to non-pregnant females and/or males. Placing our data into broader context, a depletion in $\delta^{13}C$ of 0.9‰ and 0.7‰ when comparing pregnant females to non-pregnant

females and males, respectively, is comparable to the ~1‰ difference expected between trophic levels or large oceanographic regions of the Southern Ocean.[14,25,51] These results can have serious implications for stable isotope studies and highlights the importance of accounting for pregnancy, if possible. Pregnant females were also depleted in $\delta^{15}N$ by approximately 0.4‰ and 0.5‰ when compared to non-pregnant females and males, respectively; in terms of estimated trophic position these results are likely less severe, as each of these only represents a fraction of an expected shift in trophic position (approximately 3‰).[25] It is also possible that differences in body biopsy sampling locations could result in us slightly underestimating the depletion of $\delta^{15}N$ in pregnant females. When comparing biopsy sampling locations for objective 2, the only significant difference we detected was between anterior and dorsal surfaces, where the anterior surface had lower values of $\delta^{15}N$ (Figure 2). Interestingly, most of these anterior samples were from males ($n = 10$ males and $n = 3$ females), despite pregnant females being significantly depleted in $\delta^{15}N$ when compared to males. Yet, placing our results in ecological context for isotope applications, the magnitudes of the depletion of $\delta^{15}N$ between pregnant females and non-pregnant females and males (0.4‰ and 0.5‰, respectively) and between biopsies taken from the anterior versus dorsal surfaces (0.6‰) are comparable and represent considerably less than the estimated 3‰ expected of a shift in trophic position. Finally, future studies might also consider utilizing compound-specific isotope analysis (CSIA) to help interpret results. CSIA involves the analysis of specific amino acids, some of which remain reflective of foraging source (e.g., "source" or "essential" amino acids) and some which fractionate heavily and are strong indicators of trophic position (e.g., "trophic" amino acids).[12,52] By making this distinction, CSIA allows for the direct comparison of isotopic data among samples in reference to an environmental baseline and thus helps to isolate the degree of fractionation occurring due to physiological processes. This approach could be particularly useful for determining the effect of sex and pregnancy on ratios of $\delta^{13}C$ and $\delta^{15}N$. Additionally, the application of CSIA could help explain some of the within-individual variability we observe at such short timescales by attributing variability to either foraging habitat or physiological processes affecting fractionation.[53,54]

Here we show that pregnant female humpbacks from this population are depleted in $\delta^{13}C$ and $\delta^{15}N$ as compared to non-pregnant females and males. These results have important implications for studies incorporating stable isotope ratios from cetacean tissues and indicate that pregnancy status should be included in analyses to better interpret results. Our findings also provide insight into the degree of isotopic variability among individuals which may be considered meaningful when interpreting results from skin, and urge caution when it comes to interpreting data from a single sample as a representation of an individual's biology. Consequently, there may be a limit to claims which can be justifiably made from one sample. Finally, while most body surfaces were not significantly different from each other, we did detect a depletion in $\delta^{15}N$ of biopsies taken from the anterior surface of whales compared to those from the dorsal surface. No differences were detected for $\delta^{13}C$, though variability in $\delta^{13}C$ within and between biopsy body positions was greater than it was for $\delta^{15}N$. Future studies should consider subsampling biopsies into the three distinct skin layers when using skin samples as a proxy for a whale's physiological state, foraging behavior, or environment. Stable isotopes offer a powerful tool to better understand the lives of marine mammals in ways that can support greater conservation and management of species and their ecosystems. However, the knowledge that variability in these ratios is subject to a number of endogenous and exogenous factors urges greater specificity in how stable isotopes are both analyzed and interpreted.

AUTHOR CONTRIBUTIONS

Devin C Fraleigh: Conceptualization; investigation; writing - original draft; methodology; validation; visualization; software; formal analysis; project administration; data curation. **Logan J Pallin:** Conceptualization; investigation; methodology; data curation; resources; formal analysis. **Ari S Friedlaender:** Conceptualization; investigation; funding acquisition; methodology; formal analysis; project administration; resources; data curation. **Jay Barlow:** Methodology; validation; formal analysis. **Annette E Henry:** Investigation; resources; project administration. **Danielle M Waples:** Investigation; data curation; methodology; resources. **Teris Oglesby:** Methodology; data curation. **Alyson H Fleming:** Conceptualization; investigation; funding acquisition; methodology; validation; visualization; project administration; supervision; resources; data curation; formal analysis.

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PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/rcm.9746.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS

Permission to carry out the research and procedures for ensuring animal welfare during biopsy collection were approved as part of the scientific research permits (permit no. 14809) issued by the National Marine Fisheries Service (NMFS) under the authority of the Marine Mammal Protection Act of 1972. The National Science Foundation Antarctic Conservation Act permit (2016-024 M#1) was obtained by Dr Ari Friedlaender to conduct tagging and biopsy sampling of baleen whales in the Antarctic Peninsula region. Oregon State University's Institutional Animal Care and Use Committee (IACUC) approved a protocol for the collection of biopsy samples (permits 4513 and 4943). The samples originating from outside the US jurisdiction were imported under the Convention on International Trade in Endangered Species (CITES) import permit numbers 16US50849B/9.

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