1 Interpretation of southern hemisphere 2 humpback whale diet via stable isotopes; 3 implications of tissue-specific analysis

4 June Eggebo*^a, Jasmin Groß ^a and Susan Bengtson Nash ^a

5
6 ^a Southern Ocean Persistent Organic Pollutants Program, Centre for Planetary Health and
7 Food Security, School of Environment and Science, Griffith University, Nathan, QLD 4111, Australia

8
9 Corresponding author:
10 s.bengtsonnash@griffith.edu.au

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23 Abstract

24 Blubber and skin are commonly used tissues in stable isotope analysis for the purpose of investigating
 25 cetacean diet. Critical comparison of tissue-specific isotopic signals is, however, lacking resulting in
 26 uncertainty surrounding the representativeness and therefore utility of different tissues for accurate
 27 determination of recent foraging. This study used remotely biopsied blubber and skin tissues from
 28 southern hemisphere humpback whales for strategic comparison of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Samples were
 29 collected 2008-2018 as part of long-term monitoring under the Humpback Whale Sentinel Program.
 30 Blubber tissues were lipid-extracted prior to analysis, whilst mathematical lipid-correction was
 31 performed on skin samples. Isotopic values from paired blubber and skin samples from the same
 32 individuals were compared to assess whether tissues could be used interchangeably for isotope analysis
 33 and dietary interpretation. Significant differences were observed for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, flagging
 34 previously undocumented methodological considerations, and the need for method validation and
 35 standardisation in application of these approaches. This study therefore advances methodological aspects
 36 of cetacean dietary analysis. This is of elevated importance in the context of rapidly changing ocean
 37 ecosystems.

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41 1. Introduction

42 Southern hemisphere humpback whales (*Megaptera novaeangliae*; SHHWs) have been implemented as
 43 a sentinel species for the circumpolar surveillance of pollution and climate change in the Southern Ocean
 44 (1,2). As capital breeders, these populations rely on intensive summer feeding on Antarctic krill
 45 (*Euphausia superba*; hereafter ‘krill’; Groß et al., 2021; Waugh et al., 2012), to sustain their annual
 46 winter migrations to lower latitudes for breeding and calving. The narrow foraging niche of SHHWs,
 47 results in a distilled connection between ecosystem productivity and energetic provisioning (both prey
 48 type and foraging success; Castrillon and Bengtson Nash, 2020). Their ecophysiology thus renders these
 49 populations powerful indicators of ecosystem productivity and change.

50 Krill are a sympagic species, with sea-ice providing feeding habitat and refuge for early life stages (6,7).
 51 Polar ecosystems are undergoing rapid change, manifesting in sea-ice melt (8), ocean acidification (9),
 52 and a rise in sea water temperature (10). These physio-chemical characteristics of the krill ecosystem, in
 53 turn, impact krill recruitment and survival (11,12). Any change in the abundance or availability of krill
 54 is expected to carry significant implications for krill consumers (Tulloch et al 2019, Seyboth et al. 2016).
 55 Humpback whale (HW) populations globally show a high degree of plasticity in both their target prey
 56 and foraging behaviour (13,14). As such, SHHWs may be expected to respond to a change in krill
 57 availability through diversified foraging, including changes to both prey and foraging range.
 58 Longitudinal monitoring of SHHW diet has therefore been identified as a core sentinel parameter under
 59 the Humpback Whale Sentinel Program (HWSP), with interannual variation and drift assumed to reflect
 60 a change in krill availability.

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 62 Ecologists use bulk stable isotope analysis (BSIA) to directly identify and trace elemental cycling in the
 63 biosphere (Fry, 2008). Over the last few decades, BSIA has played a significant role in research
 64 involving animal migration (15,16), diet (17,18), reproduction (19,20) and food web connectivity

65 (21,22). The stable isotopes of carbon (^{13}C , ^{12}C) and nitrogen (^{15}N , ^{14}N) have, in particular, become
 66 valuable in diet research of marine mammals (23–26). The use of stable isotope analysis to investigate
 67 the structure of food webs is based on two assumptions: namely that the isotopic composition of
 68 consumer tissue reflects the isotopic composition of their diet, and that consumers are slightly enriched
 69 in ^{15}N and to a lesser extent in ^{13}C compared to their food (27,28). The phenomenon is called ‘trophic
 70 discrimination’, also referred to as ‘trophic fractionation’ and averages 0.5-1.0 ‰ for carbon ($\Delta^{13}\text{C}$;
 71 DeNiro and Epstein, 1978; Fry, 2008; Zuev et al., 2019) and 2-4 ‰ for nitrogen ($\Delta^{15}\text{N}$; Minagawa and
 72 Wada, 1984; Fry, 2008; Zuev et al., 2019). Trophic levels (TLs) are a hierarchical way of classifying
 73 organisms according to their theoretical feeding relationships within an ecosystem (32). Nitrogen
 74 isotopes ($\delta^{15}\text{N}$) increase as a function of mean TL (Minagawa and Wada, 1984) due to the relatively
 75 faster metabolic loss of ^{14}N compared to ^{15}N leaving animals at higher trophic levels with higher $\delta^{15}\text{N}$
 76 values (Fry, 2008). Carbon isotopes ($\delta^{13}\text{C}$) in marine environments can be traced from producers such
 77 as particulate organic matter (POM) and phytoplankton, to consumers to determine primary carbon
 78 sources (27,33). These values are often used to distinguish between two geographically distinct food
 79 webs. Altabet and Francois (1994) demonstrated that surface water $\delta^{13}\text{C}$ values of POM lay at
 80 approximately -22 ‰ in temperate latitudes but decrease to -25 ‰, sometimes down to -35 ‰ (35) closer
 81 to Antarctica. Thus, animals feeding in Antarctic food webs demonstrate correspondingly low carbon
 82 isotope values (36–39), compared to those feeding temperate food webs (40,41).

83 The interpretation of bulk stable isotope (BSI) signals is, however, not without uncertainty. In addition
 84 to the prey type and foraging range, the trophic position (TP) is known to be influenced by endogenous
 85 factors such as nutritional stress, metabolic activity of tissues, diet quality, body size, excretory
 86 mechanisms and feeding rate (42–46). Further, the isotopic signals associated with tissues of different
 87 biomolecular composition (i.e. lipids, carbohydrates, proteins) have frequently been recorded (30,46,47).
 88 The extent to which tissue types within an individual differ in their $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values thus carries

89 inherent uncertainty for robust quantification of diet and represents a methodological aspect of cetacean
90 dietary investigation that has not been thoroughly addressed.

91 In cetacean research, blubber and skin tissue are the most commonly used tissue types for dietary
92 investigation as they are metabolically active and can easily be obtained via non-lethal biopsies from
93 healthy, free-swimming individuals (48,49). Marine mammal blubber is principally composed of lipids
94 and contains small amounts of protein (46,50). By contrast, skin mainly contains protein and a limited
95 amount of lipids (46,51,52). In BSIA, lipids confound analyses by decreasing the tissue $^{13}\text{C}/^{12}\text{C}$ and
96 hence lowering measured $\delta^{13}\text{C}$ values (30). As such, the influence of lipid content on whole tissue $\delta^{13}\text{C}$
97 values, and following dietary interpretation must be accounted for (53). Two approaches are commonly
98 used to account for lipids. The first methods is the physical removal of lipid fractions through solvent
99 extraction prior to BSIA. Alternatively, where the relationship between lipid-containing and lipid-
100 depleted tissues of a species is known, mathematical corrections have been developed and applied (Post
101 et al., 2007; Ryan et al., 2012, Groß et al. 2021).

102 In an effort to further strengthen data obtained from long-term monitoring of SHHW diet, the current
103 study sought to compare the BSI measurements obtained from lipid-adjusted blubber and skin tissues
104 respectively. In order to test the hypothesis that $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of blubber and skin taken from the
105 same individual could be used interchangeably, 171 paired samples were investigated, providing new
106 insights into method application, data interpretations, and species physiology.

107 **2. Material and methods**

108 *2.1 Sample Collection*

109 Blubber and skin biopsy samples were obtained for long-term monitoring under the HWSP from free-
110 swimming SHHW of the east coast of Australia-migrating stock (E1 as defined by the International
111 Whaling Commission; Acevedo et al., 2013), between 2008 and 2018. The biopsies were collected off
112 North Stradbroke Island, southeast Queensland, Australia (approximately 27°26 S, 153°34 E) during

the annual northward (June/ July) and southward (September/ October) migration according to methods described by Bengtson Nash et al. (2018). The collection of samples was carried out under Scientific Purposes permit, granted by the QLD department of Environment and Heritage Protection and animal ethics permit granted by the Griffith University Animal Ethics Committee. In total, 171 paired blubber and skin biopsy samples were included in this study. Blubber tissue was lipid extracted with solvents prior to analysis while skin tissue was mathematically lipid corrected. Both are referred to as “lipid-adjusted” in subsequent text.

2.2 Lipid Adjustment

2.2.1 Solvent Extraction

Approximately 30 mg of blubber was lipid extracted prior to BSIA. The solvent lipid extraction of blubber tissue was completed using a modified methanol-dichloromethane-water (2:1:0.8 v/v/v MeOH/CH₂Cl₂/H₂O) method pioneered by Bligh and Dyer (1959), as described in detail elsewhere (e.g. Groß et al., 2021).

2.2.2 Mathematical Correction

Previously, Groß et al. (2021) determined the most appropriate isotopic discrimination factor of skin for the study population to be 8.92 ‰. The mass balance approach (MBA) developed by Fry (2002), was considered the best fit for lipid correction of SHHW skin, and was therefore applied in the current study. The correction applied in this study was as follows (Eq. 1):

$$\delta^{13}\text{C}_{\text{LFM}} = \delta^{13}\text{C}_{\text{B}} + D \times \left(1 - \frac{\text{C:N}_{\text{LF}}}{\text{C:N}_{\text{B}}}\right) \quad (1)$$

Where $\delta^{13}\text{C}_{\text{LFM}}$ is the lipid-free (or lipid-corrected) carbon isotope value of skin, $\delta^{13}\text{C}_{\text{B}}$ is the bulk carbon isotope value measured from Balaenopteridae skin, and D is the isotopic discrimination factor. C: N_{LF} is the measured ratio of lipid-free skin tissue, whilst C: N_B is the measured ratio of bulk Balaenoptera skin tissue.

136 2.3 Bulk Stable Isotope Analysis

137 Lipid-adjusted blubber and skin tissue were oven dried overnight at approximately 58°C and
138 pulverized in to 1-2 mg samples which were placed into tin capsules for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis. Stable
139 isotope abundances were calculated in permil using the following equation:

$$140 \delta X = [(R \text{ sample} / R \text{ standard}) - 1] \times 1000 \quad (2)$$

141 Where, X is ^{13}C or ^{15}N , and R is the respective ratio $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. The international reference
142 standards used for carbon and nitrogen are, respectively, Vienna Pee Dee Belemnite and N_2 in air.
143 Laboratory standards, sucrose and $(\text{NH}_4)_2\text{SO}_4$ were calibrated using international standards IAEA- CH_6
144 for carbon and IAEA N1 for nitrogen. The preparation system used is a Europa EA-GSL interfaced to
145 a SERCON Hydra 20–20 isotope ratio mass-spectrometer (IRMS). Based on analysis of replicate
146 standards, the standard deviations for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ averaged 0.1 ‰ and 0.15 ‰, respectively.

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149 2.4 Krill range calculation

150 The krill range i.e., the isotopic range expected for individual whale $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values feeding
151 exclusively on Antarctic krill, was calculated based on isotopic values of krill derived from
152 Eisenmann et al. 2016. Blubber and skin trophic fractionation (TF) estimates were calculated in this
153 study (Table S1, Table S2). The krill range for lipid-extracted blubber was -28.14 to -24.66 and 5.96
154 to 9.34, while for lipid-corrected skin the range was -27.09 to -23.61 and 5.12 to 8.50 respectively for
155 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. This facilitated comparison of blubber and skin foraging results, allowing for an inter-
156 annual evaluation of diet representation within and between tissues throughout sample years.

157 2.5 Trophic position calculation

158 Trophic position for SHHWs was calculated from lipid-adjusted blubber and skin tissues, relative to
159 krill using the following equation:

$$160 \text{ TP} = 2 + (\delta^{15}\text{N}_T - \delta^{15}\text{N}_A) / \Delta^{15}\text{N}_T \quad (3)$$

161 Where, 2 is the TP of the primary consumer, T is tissue type (lipid-extracted blubber or lipid-corrected
162 skin), A is Antarctic krill (prey) and $\Delta^{15}\text{N}$ is the TF value. Mean lipid-corrected skin and lipid-
163 extracted blubber $\delta^{15}\text{N}$ isotope values were derived from BSIA in this study, the mean Antarctic krill
164 $\delta^{15}\text{N}$ value of 3.2 ‰ was derived from literature estimates as shown in Table S1.1, and TF values for
165 lipid-extracted blubber (4.45 ‰) and lipid-corrected skin (3.61 ‰) were calculated as shown in Table
166 S1.2.

167 2.6 Statistics

168 Data analyses were performed in R version 1.3. 1093 (61) and GraphPad Prism version 9.0.2 (62). A
169 Shapiro-Wilk test and a Levene's test were used to test the data for normality and homogeneity of
170 variance, respectively. All statistical results were interpreted using a significance level of $\alpha = 0.05$. The
171 $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic values across sex and migration showed no significant difference ($p=0.1841$
172 and $p=0.1184$ respectively), thus all samples were treated as a homogenous cohort. A Shapiro-wilks
173 test demonstrated non-normality for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotopic values within and between lipid-adjusted
174 blubber and skin, thus non-parametric statistical tests were further applied. Two separate Wilcoxon
175 matched pair signed rank tests were used to test for differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between the
176 two tissue types. The test structure used $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ as test variables for differences in the factor
177 'tissue type' with fixed values for lipid-adjusted blubber and skin tissue. A non-parametric Kruskal-
178 Wallis test with multiple comparisons was applied to investigate trends across sample years for $\delta^{13}\text{C}$
179 and $\delta^{15}\text{N}$ values.

180 3.0 Results and Discussion

181 The present study is the first to investigate tissue specific BSI measurements and implications for
182 interpretation of SHHW diet. Our results showed that there are significant differences in $\delta^{13}\text{C}$ and
183 $\delta^{15}\text{N}$ values obtained from lipid-adjusted blubber and skin from the same individuals. Such

184 differences were more prominent in some individuals, thus occasionally led to different down-
 185 stream interpretation of trophic position. There was greater variability in $\delta^{15}\text{N}$ values of lipid-
 186 extracted blubber compared to lipid-corrected skin. The tissue-specific variation in $\delta^{15}\text{N}$ values
 187 was surprisingly not reflected in tissue-specific TP estimates as lipid-adjusted blubber and skin
 188 tissue demonstrated a similar TP of 3.0. These findings underscore that tissue-specific variation
 189 must be thoroughly investigated before comparing dietary results obtained via BSIA using two
 190 different tissues and caution against interchangeable use of tissues or comparison between them.

191 3.1 Bulk differences

192 For both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of lipid-adjusted tissues, significant differences were observed ($\delta^{13}\text{C}$
 193 $p=0.0001$ and $\delta^{15}\text{N}$ $p=0.0001$; Figure 1). Lipid-extracted blubber values showed greater variability for
 194 both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ compared to lipid-corrected skin (Table 1., Figure 1).

195 Table 1: Table overview of the mean, standard deviation (SD) and range for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values
196 for lipid-extracted blubber and lipid-corrected skin tissue of E1 humpback whales (n=171).

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Lipid-free skin					Lipid-extracted blubber				
Isotope	n	Mean	SD	Range	Isotope	n	Mean	SD	Range
C	171	-25.35	0.60	-27.35 - -23.52	C	171	-26.40	1.83	-31.56 - -20.77
N	171	6.81	0.46	5.21 - 8.22	N	171	7.65	1.14	3.65 - 13.87

Figure 1: Box plot showing the distribution of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for lipid-extracted blubber and lipid-corrected skin tissue (n=171).

206 As the tissues were obtained from the same individual whale, the extent of the variability in both
207 isotope signatures was not expected. There is limited research on the comparison of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$
208 values between HW blubber and skin tissue, however, a significant difference between the two
209 tissues either for one or both isotopes has been documented (e.g Groß et al., 2021; Todd et al.,
210 1997). However, the reasons for this variation are not clear, and thus we attempt to evaluate several
211 factors that may have contributed to the significant differences found in this study.

212 3.2 Inter-annual differences

213 Large inter-annual variability in isotopic signatures has previously been evidenced via fatty
214 acid analysis for this population (Groß et al. 2020). When samples were separated by year,
215 limiting analysis to those years where >10 paired samples were available for analysis (2013-

2018), significant inter-annual differences were observed in selected years. Of the six within year cohorts available, three demonstrated a significant difference in the $\delta^{13}\text{C}$ values between the two tissue types (2016; $p=0.0216$, 2017; $p=0.0335$ and 2018; $p=0.0001$; Figure 2A). Similarly, three years, albeit three different years, showed significant differences in $\delta^{15}\text{N}$ values between tissue types (2013; $p=0.0003$, 2014; $p=0.0001$ and 2017; $p=0.0001$; Figure 2B).

Figure 2: Isotopic values of blubber and skin (n=171) across all sample years. (A) illustrates comparison between both tissues for $\delta^{13}\text{C}$ and (B) for $\delta^{15}\text{N}$ values.

As the significant differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between lipid-adjusted blubber and skin did not occur in the same sample years, there may be underlying tissue-specific variations that could be driving the variability in isotope signatures. Figure 2 illustrates an overall low variability in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values for lipid-corrected skin across all sample years, compared to lipid-extracted blubber that has a greater variability with more prominent oscillations in some years like 2014. The differences in isotopic signatures between the tissues may lead to issues for interpretation because we cannot be certain whether the variability present in blubber $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values is caused by variability in prey type or foraging location, or whether the observed variability is introduced by endogenous factors or method artefacts. Hence, we do not know if we lose information about foraging variability when we just interpret results from skin, or if we introduce variability to results when we just interpret results from blubber tissue.

3.3 Trophic position comparison

Trophic position estimates were calculated to investigate whether the observed differences between blubber and skin $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values also leads to differences in the dietary information derived from the two tissue types. Overall, when samples were considered as a single cohort, TP did not vary significantly between the two tissues (Wilcoxon $p=0.988$). The mean TP was 3 ± 0.26 for blubber and 3 ± 0.13 for skin. However, as with single year cohorts for BSI values, significant differences in tissue-specific TP were found in the years 2013 ($p=0.0211$), 2014 ($p=0.0113$) and 2016 ($p=0.0218$; Figure 3).

Figure 3: Trophic position estimates for blubber and skin tissue across all sampling years.

Although there was an overall significant difference in $\delta^{15}\text{N}$ values between lipid-adjusted blubber and skin tissue, this difference was not reflected in the TP values calculated for both tissues (TP = 3.0). The calculated TP values are congruent with the classical feeding paradigm of a high-fidelity krill diet in SHHWs (63–66). However, as some variation was observed in 2013, 2014 and 2016 (Figure 3), there are small, but minor underlying differences that reflect the significant differences in $\delta^{15}\text{N}$ values between the two tissues.

The equation used to estimate TP has limitations, which can lead to errors in interpretation. First, the trophic fractionation factors ($\Delta^{15}\text{N}$) used in the equation, 4.45 ‰ for lipid-extracted blubber and 3.61 ‰ for lipid corrected-skin tissue, are only based on estimates. The true TP for E1 humpback whale tissues are unknown. Additionally, $\Delta^{15}\text{N}$ vary between and within species and tissues, introducing error when estimates are based on other tissues or species. Secondly, an average $\delta^{15}\text{N}$ value for krill was used in the equation, which introduces errors as there are spatial and temporal differences in $\delta^{15}\text{N}$ values of krill (67–70). Some introduced uncertainty could be reduced by

analysing compound specific nitrogen isotope composition of amino acids (68–70), however we were unable to analyse compound specific isotopes results due to cost restrains.

3.4 Tissue-specific krill space

The implications of tissue-specific variability in BSI values for the interpretation of diet was further investigated by creating a krill space (isotope range) for each tissue. The shaded areas in Figure 4 illustrate the tissue-specific krill space in which SHHW $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are expected to fall if the individual whales were feeding primarily on krill the austral summer prior to sampling. The figure only shows the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of lipid-adjusted blubber and skin from two sample years, 2013 and 2015, as these years highlight the two different scenarios that we have observed between 2008 and 2020; lipid-corrected skin isotope values fall within the calculated krill space while either the majority of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of lipid-extracted blubber fall outside the krill space or the majority of just $\delta^{13}\text{C}$ values of blubber fall outside the krill space.

Figure 4: Scatterplot illustrating the tissue-specific krill space for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of lipid-adjusted blubber and skin tissue of 2013 (n=24) and 2015 (n=30).

For lipid-extracted blubber, in 2013 only 29.2% and 53.3% of the isotopic data points fell within the krill space in 2013 and 2015, respectively. This was half of what was observed for lipid-corrected skin, where 100% of the data fell within the krill space in 2013, and 96.7% in 2015. This leads to different interpretations about the diet plasticity of SHHW. If we would make inferences based on skin isotope results, we would conclude that SHHW exclusively feed on krill in the Southern Ocean. However, if we would only interpret blubber isotope results, we would conclude that SHHW exhibit a much greater diet plasticity than expected for a high-fidelity krill diet species. Interestingly, the observed variability does not translate into different interpretations of TP.

3.5 Factors influencing variability

3.5.1 Endogenous factors

To properly interpret stable isotope signatures of animal tissues, it is essential to account for temporal dynamics of isotopic integration such as tissue turn-over rate and diet-tissue incorporation.

Isotopic turnover time describes the time it takes for a tissue layer to be replaced entirely by a new layer of tissue (71,72). It is an important consideration when assessing different tissues as isotopic incorporation occurs during tissue growth, resynthesis and breakdown, and can vary among tissue types (47,73). The turnover time for blubber and skin of SHHW is unknown, however SHHW blubber turnover is suggested to be <9 months, because the blubber lipid store is almost entirely depleted over the course of their annual migration, due to prolonged fasting (66). Isotopic turnover time for skin $\delta^{15}\text{N}$ has been estimated to be approximately 180 days for bottlenose dolphins (*Tursiops truncatus*) and 163 days for blue whales (*Balaenoptera musculus*), while $\delta^{13}\text{C}$ has been estimated to be approximately 104 days for bottlenose dolphins (74,75). Based on taxonomy and size, we therefore expect SHHW skin to have an isotopic turnover time that ranges from approximately 104 to 180 days. In the present study, there were roughly 60 days between E1 humpback whales leaving

their Antarctic feeding grounds in March and the time they were sampled in June/ July, and roughly 150 days until they were sampled in September/ October. Although, turnover time for either SHHW tissue is unknown, we can assume that both tissues reflect a similar diet intake timeframe based on available information.

Aside from tissue turnover differences, the variation in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between lipid-adjusted skin and blubber tissues may be linked to differences in tissue-specific metabolic routing, which is expected to produce a consistent offset between the stable isotope values of individual tissues. Metabolic routing of different biomolecules during tissue synthesis and metabolism impacts diet-tissue isotope discrimination. This means that some tissues may primarily reflect individual diet components such as carbohydrates and lipids derived from one dietary source and proteins derived from another (76,77). By way of example, a study by Misra et al. (2019) on bottlenose dolphins found that blubber tissue likely represents metabolic patterns linked to fatty acids and ketogenic amino acids related to fat synthesis and deposition within the tissue, whilst skin showed metabolites involved in gluconeogenic pathways pointing to active anabolic energy-generating metabolism. By extension, it is possible that the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of SHHW blubber tissue may be drawn from a more direct energy pool, where lipids are immediately stored in the blubber, while secondary pathways may be involved in the growth of skin tissue. The complexity of tissue-specific metabolic routing and discrimination can also lead to uncertainty in lipid normalization models due to unknown protein-lipid discrimination values.

3.5.2 Artefacts

In addition to endogenous factors, methodological artefacts should also be considered as a source of variation. The observed differences between both tissues may be related to the

lipid-adjustment approaches applied to the respective tissue type. The mass-balance mathematical lipid correction model proposed by Fry (2002) relies on precision, accuracy and reliability in predicting the lipid-free $\delta^{13}\text{C}$ values. The model is based on C:N ratios and thus lipid content, which was estimated to have a mean standard error of ~ 0.05 in predicting lipid-free $\delta^{13}\text{C}$ values for skin tissue of E1 humpback whales (3). A study by Groß et al. (2021) specifically calculated the discrimination value 'D' for skin tissue of individual E1 humpback whales to be applied in the mass balance correction model, which gave a 'D' value of 8.92‰ and a C:N_{LM} of 3.1. The authors recommended the use of these values in conjunction with the mass balance model for E1 humpback whale skin tissue, if the skin tissue has a low lipid content, leading to small lipid corrections that limit errors in interpretation. However, the use of these exact coefficient values for 'D' and C:N_{LM} increases uncertainty if the correction is applied to species and populations where empirical values are unknown. Thus, although the 'D' value has been determined for E1 humpback whales, the accuracy of the value is unknown, given the large interannual variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values (e.g. Bengtson Nash et al., 2017; McConnaughey and McRoy, 1979; Tieszen et al., 1983).

As with mathematical lipid correction, solvent-extraction may be similarly susceptible to the introduction of methodological artefacts. The dichloromethane/methanol solvent combination used in this study has been reported to have little influence on $\delta^{15}\text{N}$ values (80). However, previous studies using various solvent combinations for lipid extraction have detected fluctuations in $\delta^{15}\text{N}$ values as a result of solvents interfering with structural components of the tissue (54,81,82). This could be linked to the high $\delta^{15}\text{N}$ values found in this study where 23% of all lipid-extracted blubber $\delta^{15}\text{N}$ data falls outside the 95% confidence interval. In addition, all figures indicate a high variability and range in lipid-extracted blubber tissue. An increase in $\delta^{15}\text{N}$ resulting from solvent lipid extractions has been linked to the loss of nitrogenous components such as amino acids (AA), which may be extracted unintentionally

from the tissue as the solvents can remove polar and non-polar compounds in the process (83). The hypothesis is that methanol, which removes mostly polar structural fat components that are attached to proteins, also removes amino acids at the same time as structural fats, resulting in enrichment of ^{15}N (Sotiropoulos et al., 2004 and references therein). Although this study did not seek to address this method component, altered $\delta^{15}\text{N}$ values post extraction have previously been observed in fish tissues; muscle and whole body samples (84), and liver tissues (80). A study by Ryan et al. (2012) found significant increases in $\delta^{15}\text{N}$ values post lipid extraction for blubber of fin whales and skin of minke whales (*Balaenoptera acutorostrata*), where the overall changes were more prominent in blubber than skin tissue, which is logical given the respective lipid proportions. Thus, we hypothesize that E1 humpback whale blubber, being an adipose tissue with high lipid content is susceptible to solvent extraction related removal of amino acids resulting in the possibility of distorting the signal of $\delta^{15}\text{N}$ values in BSIA.

4. Conclusion

This study showed that the overall comparison of lipid adjusted blubber and skin $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of SHHW were similar, but not to the extent that we can confidently recommend the interchangeable use of both tissues in this field of research. Although the mean trophic position of each year cohort was similar, the greater variability observed in blubber, which may be interpreted as higher trophic level feeding, is not present in skin values. This variability has been related to variation in lipid content, solvent interference, isotopic discrimination, and metabolic pathways between blubber and skin tissue. All are key factors that can impact the interpretation of stable isotope results. We recommend that future studies incorporate a standard for SHHW blubber and skin tissue, with the application of multiple lipid standardization approaches. Additionally, we suggest the inclusion of multiple solvent lipid extraction trials for blubber tissue to determine the potential impact on isotopic

signatures. This will allow for optimization of dietary investigation and standardization of methodologies, which will improve long-term monitoring of SHHWs to provide new insights into energy utilisation by these populations.

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