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Nutrient allocation in capital breeding baleen whales: a novel tool to infer protein balance and reproductive status

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Author-supplied statements

Relevant information will appear here if provided.

Ethics

Does your article include research that required ethical approval or permits?:
This article does not present research with ethical considerations.

Statement (if applicable):
All whale tissues used in this study were collected and processed under special permits. Biopsy samples were collected during the 1995â€“1998 field seasons under permit from the Department of Conservation (DOC) of New Zealand to C. S. Baker and N. Gales and University of Auckland Animal Ethics Committee approved protocol to C. S. Baker. Biopsy were collected during 2003â€“2005 by the DOC. Biopsy samples were collected during the 2006â€“2009 field seasons under DOC Marine Mammal Research permit and University of Auckland Animal Ethics Committee approved protocol to C. S. Baker. Biopsy samples were collected during the 2020â€“2022 field seasons under DOC New Zealand Marine Mammal Protection Act Permit 84845-MAR and Marine Reserve Act Permit 87513-MAR and University of Auckland Animal Ethics approved protocol 002072 to E. Carroll. All tissues were collected using non-lethal sampling techniques.

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1 **TITLE: Nutrient allocation in capital breeding baleen whales: a novel tool to infer protein**
2 **balance and reproductive status**

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Abstract (200 words)

Assessing reproductive status and the nutrient allocation strategies animals use to reproduce is integral for evaluating the vulnerability of species to environmental change. We measured carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values of bulk skin tissue and constituent amino acids (AAs) collected from southern right whales (*Eubalaena australis*) to assess reproductive status and the impacts of reproduction on protein balance. Most AAs in cows had higher $\delta^{13}C$ but lower $\delta^{15}N$ values in comparison to other adults, suggesting they route fat stores for milk production and use skeletal muscle reserves to maintain tissues. Lower $\delta^{15}N$ is likely associated with protein sparing and/or modifications to the urea cycle to retain or recycle nitrogen during reproduction. Nursing calves had distinctive AAs patterns compared to cows and adults that are likely driven by high metabolic demands associated with rapid growth. Adult males and non-lactating adult females had nearly identical $\delta^{13}C$ and $\delta^{15}N$ patterns, suggesting they use similar nutrient allocation strategies while fasting. Patterns in AAs $\delta^{13}C$ and $\delta^{15}N$ allowed correct classification of demographic groups with 96% accuracy and enabled the identification of lactating cows with 100% accuracy. This approach holds promise for identifying the reproductive status of capital breeding mammals.

Key Words: southern right whales, *Eubalaena australis*, stable isotopes, amino acids, lactation, fasting.

1. Introduction

Animals have evolved a variety of nutrient allocation strategies to grow and reproduce. The terms capital and income breeding are used to represent the extremes along a spectrum of strategies that animals use to allocate nutrients for reproduction [1,2]. Income breeders require a consistent supply of nutrients, while capital breeders utilize stored nutrients for reproduction [1,2]. The latter strategy is frequently observed in a wide variety of migratory animals that take advantage of seasonal pulses in resource availability to fuel reproduction [1,2]. Many capital breeders also fast for several weeks or months during the reproductive period, thus relying on endogenous nutrients to produce or feed offspring and maintain homeostasis [1,2]. Our understanding of the physiological adaptations in nutrient allocation strategies used to overcome these challenging periods remains limited in both terrestrial and marine organisms due to the complexities of tracking the importance of various metabolic pathways involved in these complex life history events. Nevertheless, such information is crucial for assessing the vulnerability of capital breeding species to environmental stochasticity in resource availability and quality.

Migratory baleen whales are often categorized as capital breeders [3], although some species can combine income and capital strategies and opportunistically forage during migration and/or on their breeding grounds [4,5]. Species that use a strict capital breeding strategy may be extremely vulnerable to shifts in resource availability, phenology, or quality, which can result in mass mortalities [6,7]. The southern right whale (SRW, *Eubalaena australis*) is one of the most extreme examples of capital breeding in cetaceans. This species was decimated by historical commercial whaling [8], but is slowly recovering in several genetically distinct wintering grounds [9] that have been monitored over the past five decades [10]. The species summer-fall (Dec-May) foraging grounds are located at temperate to polar latitudes across the Southern Ocean, while the winter-spring (Jun-Nov) calving grounds are

typically located at temperate latitudes in sheltered coastal regions in the south Atlantic, Pacific, and Indian Oceans [10]. During the wintering (breeding) season, adult and subadult whales fast for several months, while calves nurse to fuel rapid growth and development [11]. The demographic group with the highest energetic demands is cows (lactating females), which can lose up to 25% of their body volume during the winter fasting months [12]. Accordingly, cows are potentially the most vulnerable demographic group during periods of low resource availability, with decreased survival linked to climatic events and by extension lower population reproductive output and growth rates [13–15]. Cows that become nutritionally stressed would need to either decrease milk production and endanger their calves or compromise body condition and future reproductive fitness [11]. Reproductive success is therefore strongly dependent on the cows' body condition and resource availability prior to the onset of nursing.

Although body condition has been monitored in this species using morphometric data [12,16], the nutrient allocation strategies used by SRW and other baleen whales to maintain protein balance and support lactation while fasting remain poorly understood. From a physiological perspective, understanding the underlying mechanisms of nutrient allocation in cryptic capital breeding species could provide tools to assess the protein balance, as well as ontogenetic changes in resource use of this potentially vulnerable group. Currently, hormone and stable isotope analysis of bulk tissues have been used to assess pregnancy [17,18], the quality of lipid-content in whale milk [11], and nutritional stress [19]. However, these methods are not optimal to identify or characterize the physiological impacts of gestation and lactation, thus the development of new methods are needed to assess how endogenous nutrients are remobilized and routed during reproduction.

Over the past decade, stable isotope analysis of individual compounds has emerged as a powerful technique to study the foraging ecology and physiology of organisms [20].

Specifically, isotope analysis of amino acids can provide insights into how fasting, and reproduction influence the nitrogen balance in marine mammals [21], elasmobranchs [22], seabirds [23,24], and migratory geese [25]. Examples for marine mammals include changes in the nitrogen isotope ($\delta^{15}N$) composition of AAs in whiskers of southern elephant seals (*Mirounga leonina*) grown in a catabolic (fasting) versus anabolic (foraging) state [21]. Specifically, $\delta^{15}N$ values of several glucogenic AAs (e.g., glycine, serine, proline, phenylalanine) increase during fasting, reflecting the use of ^{15}N-enriched body protein stores to fuel gluconeogenesis. In contrast, lower $\delta^{15}N$ values of alanine during fasting were hypothesized to be related to the importance of the Cahill Cycle, which facilitates the transport of nitrogen from catabolized protein stores to the liver. Likewise, a select suite of AAs (glycine and threonine) showed ^{15}N-enrichment in the baleen plates of a limited number (n=3) of migrating juvenile fin whales (*Balaenoptera physalus*), which may reflect fasting [26].

While recent studies show that AAs $\delta^{15}N$ can be used as a tool to assess protein balance and possibly identify specific metabolic pathways used by fasting animals, there are still gaps in our understanding of how different species and demographic groups of the same species overcome these physiological challenges. We investigated the nutrient allocation strategies and protein balance of SRW using bulk tissue and AAs $\delta^{13}C$ and $\delta^{15}N$ analysis of skin, which is easy to collect from live whales. We hypothesized that AAs $\delta^{13}C$ and $\delta^{15}N$ values among SRW belonging to different demographic groups vary in response to differences in energetic demands associated with reproduction. Specifically, we predicted cows that are actively nursing calves would show unique AAs $\delta^{13}C$ and $\delta^{15}N$ due to modifications in the pathways used to maintain nitrogen balance, produce offspring, and route nutrients for milk production. To evaluate this prediction, we measured the AAs isotope values in skin samples of cow-calf pairs, non-lactating adult females, and adult males from

the Aotearoa New Zealand (hereafter New Zealand) SRW population, as part of one of the longest-term monitoring projects for this circumpolar species [27,28]. Skin integrates up to 3 to 5 months of ecological and eco-physiological information [29] to reflect a combination of the third trimester of gestation and lactation in cows, and primarily the nursing period and some *in utero* for calves.

2. Materials and Methods

(a) Sample Collection

Fieldwork was conducted around mainland New Zealand and in Maungahuka Auckland Islands (electronic supplementary material, figure S1) between 1995 and 2020 [27,28,30–34]. Skin biopsy samples were collected from SRW using small stainless steel biopsy darts deployed from a crossbow [35] or a modified veterinary capture rifle [36]. Cows (lactating females) were identified as whales that were accompanied by a calf, the latter defined as another whale that was half the body length or smaller than an adult whale [30]. Females with estimated ages (via photo-recapture identification) that were observed without a calf were classified as non-lactating adult females. Skin samples were either stored frozen at -20°C or preserved in 70% ethanol in the field for transport to the laboratory.

(b) Genetic and Stable Isotope Analysis

DNA profiles comprising genetic sex and multi-locus microsatellite genotype were constructed for each sample following published protocols [30] and used to identify individuals and confirm maternity in cow-calf pairs. Details on methods used are described in Supplemental methods (electronic supplementary material, supplemental methods). Based on the DNA profile records, we selected 22 cow-calf pairs, 1 calf, 4 cows, 15 non-lactating adult

151 females, and 18 adult males for bulk and amino acid isotope analysis. We measured AAs
152 $\delta^{13}C$ in all these samples, but AAs $\delta^{15}N$ were only measured in 14 cow/calf pairs, 1 calf, 1
153 cow, 13 non-lactating adult females, and 8 adult males, because sample masses were too low
154 for AAs $\delta^{15}N$ analysis in all samples.

155 Skin samples were lipid-extracted with three 24 h rinses of a 2:1 chloroform:methanol
156 solution, washed with deionized water and then dried in an oven at 40°C. We only used the
157 intermediate section of the skin (stratum intermedium) to ensure that each sample reflected a
158 similar temporal window [29]. Lipid-extracted skin samples were divided into two sub-
159 samples. One portion was pulverized using a mortar and liquid nitrogen, and 0.5 to 0.6 mg of
160 the homogenized powder was weighed into tin capsules for bulk tissue isotope analysis. The
161 other sub-sample ranging in mass from ~2 to ~15 mg depending on tissue availability was
162 weighed into sterilized glass vials for amino acid isotope analysis. These sub-samples were
163 hydrolyzed in 1.0-1.5 ml 6 N hydrochloric acid (HCL) for 20 h at 110°C. Glass vials were
164 flushed with N_2 gas for 30-60 s before sealing to avoid oxidation during hydrolysis. Free
165 amino acids in solution were then transferred to sterilized 4 ml glass vials dried down under
166 N_2 gas for 60 min. During acid hydrolysis, asparagine (Asn) and glutamine (Gln) are
167 converted into aspartic (Asp) and (Glu) glutamic acid, respectively. Therefore, the AA result
168 for Asp is a total of Asp + Asn, and for Glu is Glu + Gln, hereafter referred to as Asx and
169 Glx. Free AAs were derivatized via esterification of the carboxyl terminus of each AA with a
170 4:1 2-propanol:acetyl chloride solution, and subsequent acetylation of the amine terminus
171 with a 1:1 dichloromethane:trifluoroacetic acid anhydride solution [37]. This derivatization
172 method enables the measurement of 14 AAs, including eight that are considered non-essential
173 – Asx, Glx, glycine (Gly), serine (Ser), alanine (Ala), proline (Pro), tyrosine (Tyr), arginine
174 (Arg) — and six considered essential —threonine (Thr), valine (Val), leucine (Leu),
175 isoleucine (Ile), phenylalanine (Phe), lysine (Lys) — for most eukaryotes. In the case $\delta^{15}N$,

AAs are typically classified as "source" or "trophic" reflecting their connection with glutamine/glutamate at the core of nitrogen cycling in organisms [38]. Source AAs typically exhibit minimal trophic discrimination between a consumer and its diet such that they accurately reflect the baseline nitrogen isotope composition of the food web [20,39]. In contrast, trophic AAs generally exhibit extensive trophic discrimination during transamination and/or deamination during metabolism, resulting in higher $\delta^{15}N$ in consumer tissues relative to their diets [38,39]. Here we classify Gly and Ser as "physiological" AAs because recent work shows that the isotopic composition of these compounds are sensitive to physiological status [25].

All isotope analyses were completed at the University of New Mexico Center for Stable Isotopes (UNM-CSI; Albuquerque, NM). Bulk skin $\delta^{13}C$ and $\delta^{15}N$ values were measured with a Costech 4010 elemental analyzer (EA) coupled to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer. $\delta^{13}C$ and $\delta^{15}N$ of derivatized AAs were measured with a Thermo Scientific Trace 1310 gas chromatograph containing a BPx5 60 m column (ID 0.32 mm, film thickness 1.0 μm) coupled to an IsoLink II combustion interface and Thermo Scientific Delta V Plus isotope ratio mass spectrometer. Isotope data are reported in delta (δ) notation where $\delta^{13}C$ or $\delta^{15}N = 1000\ [(R_{sample}/R_{standard}) - 1]$, and $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$ ratio of sample and standard with units of parts per thousand or per mil (‰). The internationally accepted standards are atmospheric N_2 for $\delta^{15}N$ and Vienna-Pee Dee Belemnite limestone for $\delta^{13}C$ [40]. Within-run analytical precision (SD) for bulk tissue $\delta^{13}C$ and $\delta^{15}N$ analysis was calculated via measurement of two proteinaceous internal reference materials (casein and tuna muscle), which were calibrated against IAEA N1, IAEA N2 and USGS 43 for $\delta^{15}N$ and NBS 21, NBS 22 and USGS 24 for $\delta^{13}C$. The SD was ±0.2‰ for both isotope systems.

In the case of AAs isotope analysis, derivatization adds carbon but no nitrogen to the compound so $\delta^{13}C$ values need to be corrected to remove the isotopic effects of

derivatization. The equations applied to correct for carbon added during derivatization are described in O'Brien et al. (2002) [41]. An internal laboratory reference material consisting of a suited of powdered AAs purchased from Sigma Aldrich (Saint Louis, MO) was derivatized alongside each batch of samples (electronic supplementary material, table S1). This in-house reference material contains a known concentration of AAs for which $\delta^{13}C$ and $\delta^{15}N$ were measured with a Costech 4010 elemental analyser (EA) coupled to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer at UNM-CSI (electronic supplementary material, table S1). All samples for AAs isotope analysis were measured in duplicate and bracketed with the laboratory reference material. Duplicate samples that exhibited standard deviations higher than 0.8‰ were re-analyzed. The within-run SD of $\delta^{13}C$ and $\delta^{15}N$ of the AAs in the reference material averaged 0.4‰ (electronic supplementary material, table S1). We did not correct bulk tissue and AAs $\delta^{13}C$ data for the Suess effect given that the $\delta^{13}C$ decrease in the Southern Ocean has been consistently weaker over the last decade [42,43].

(c) Statistical Analysis

All statistical analysis were executed in R language v.4.1.3 [44]. To compare the trends in mean $\delta^{13}C$ and $\delta^{15}N$ values of bulk skin and associated AAs among demographic groups, we used a one-way Bayesian analysis of variance ($ANOVA_B$) [45]. $ANOVA_B$ was assessed separately for bulk skin tissue values, each individual AA, and for each isotope system ($\delta^{13}C$ and $\delta^{15}N$). For the $ANOVA_B$ analysis, we tested the normal distribution (likelihood) for SRW skin $\delta^{13}C$ and $\delta^{15}N$ using Bayesian p-values, which are typically used to assess goodness of fit of a model using simulation techniques like Markov Chain Monte-Carlo (MCMC) [45]. A well fit model has a Bayesian p-value close to 0.5, and values close to 0 or 1 suggest doubtful fit of the model. Our $ANOVA_B$ models using normal distribution exhibited a p-value of 0.48,

indicating a good fit for this distribution. $ANOVA_B$ models were fitted using non-informative priors and posterior distributions were generated with a MCMC set as follows: chains = 5, chain length = 1,000,000 iterations, burn-in phase = 300,000 iterations, thinning = one iteration retained each 50. We used the Potential Scale Reduction Factor or Rhat convergence criterion using Just Another Gibbs Sampler to test for chain convergence; values for Rhat near 1 indicate convergence [46]. $ANOVA_B$ estimates the posterior distributions of the means from normal likelihoods. We then estimated the posterior distributions of the differences in per mil units between all demographic groups by subtracting the posterior means in pairwise comparisons. Subsequently, we calculated the proportion of iterations below and above zero from the posterior distributions representing negative and positive differences of the pairwise differences between demographic groups, including cows-adult females, cows-adult males, cows-calves, non-lactating adult females-adult males, non-lactating adult females-calves, and adult males-calves. The highest of those two represents the probability that a given demographic pair is different. Observed differences between demographic groups that were below 0.2‰ for bulk tissue and 0.6‰ for individual AAs were not considered further due to falling within measurement precision (electronic supplementary material, table S1).

We used Linear Discriminant Analysis (LDA) via the package Mass [47] for R language, to determine if the patterns in AAs isotope values were a useful tool to classify the whales correctly into their demographic classes. LDA finds linear combinations of features that separate groups and uses the inputted data as a training set to make predictions by calculating the probability that a new set of inputs belong to each demographic group. Use of LDA allowed us to predict the global probability of correctly classifying all demographic groups, as well as the correct classification probability for each group [47]. We only used the two primary linear discriminants axes (LD1 and LD2) that explained the most variation for the classification analysis. To explore LDA classification probabilities we designed three

LDAs: Code A, an LDA that included all AAs $\delta^{13}C$ values; Code B, an LDA that included all AAs $\delta^{15}N$ values; and Code C, an LDA where SRW adult males and non-lactating adult females were combined into a single adult demographic group and was built only using the $\delta^{13}C$ and $\delta^{15}N$ values of AAs that had the highest contributions for group separation for LDAs used in Code A and B. The grouping of SRW into "adults" was based on the high group overlap between adult males and non-lactating adult females, and similarity in most of the posterior means estimates for bulk skin tissue and constituent AAs. The error rate of the dataset was calculated using a (leave one out) cross validation method.

3. Results

(a) $\delta^{13}C$ and $\delta^{15}N$ Patterns in Bulk Skin and Individual Amino Acids

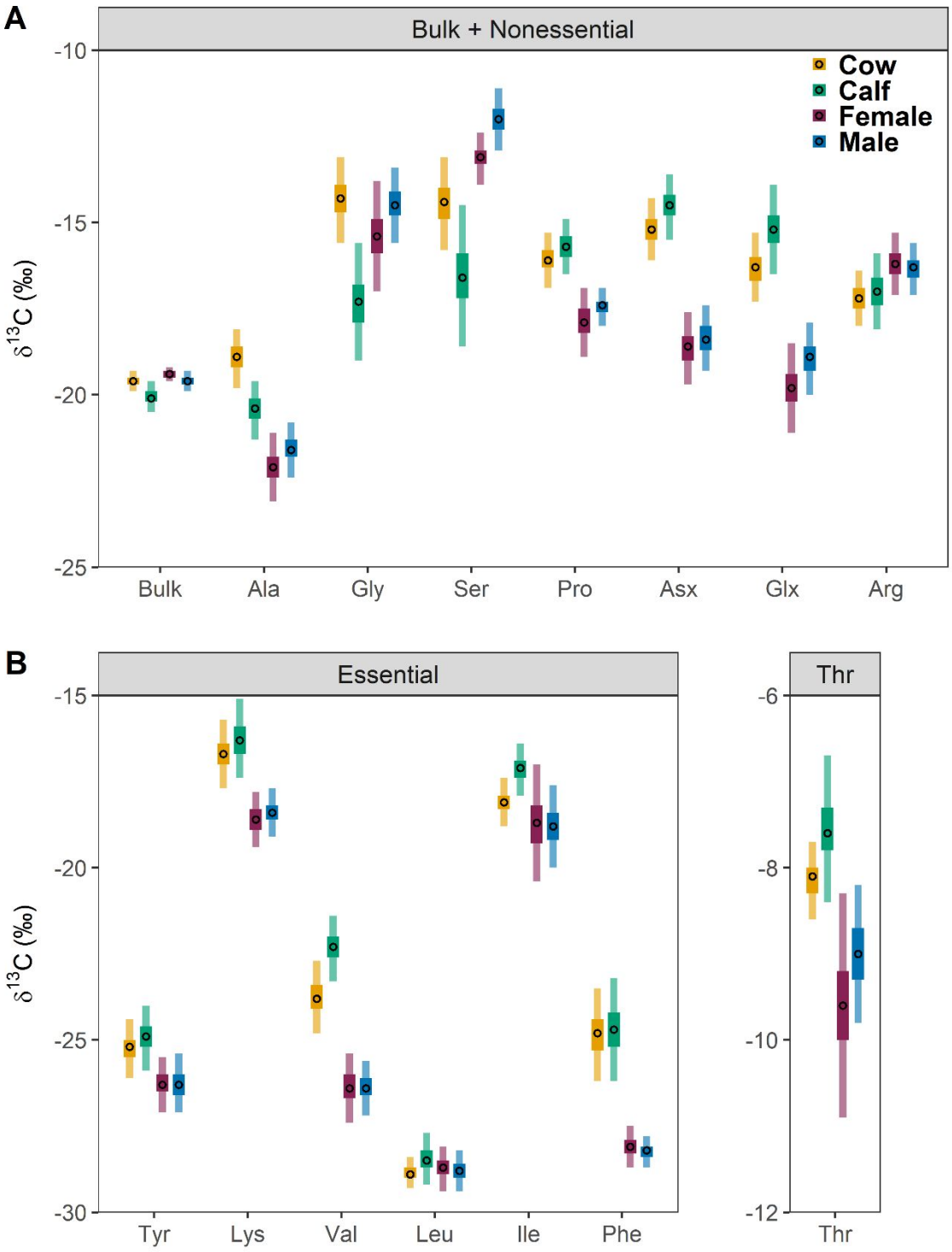
The posterior mean, standard deviation, and 95% credible intervals for $\delta^{13}C$ and $\delta^{15}N$ in SRW bulk skin and associated AAs of different demographic groups are shown in figure 1, figure 2, and electronic supplementary material table S2 and table S3. The posterior estimated differences among demographic groups for all isotope measurements are shown in electronic supplementary material table S4 and table S5.

Most AA $\delta^{13}C$ (12/14) values were higher (positive differences) in cows in comparison to adult males and non-lactating adult females. Mean positive offsets between cows and other demographic groups ranged between 0.7 and 3.5‰ (figure 1, electronic supplementary material, table S4), and the probability of these differences was 84 to 100% (electronic supplementary material, table S4). Ala, Asx, Glx, Phe and Val exhibited the highest positive offset between cows and adult males or non-lactating adult females (figure 1, electronic supplementary material, table S4). In contrast, $\delta^{13}C$ values of bulk tissue, Ser, and Arg were 0.3‰ to 2.4‰ higher in adult males and non-lactating adult females in comparison

to cows (figure 1A, electronic supplementary material, table S4). Cows also had higher $\delta^{13}C$ values for bulk tissue, Ala, Gly, and Ser but lower Glx, Asx, Val, Ile, and Thr in comparison to calves (figure 1, electronic supplementary material, table S4); values for Arg, Pro, Leu, Phe, Lys, Tyr were similar between cows and calves (figure 1, electronic supplementary material, table S4). Several AAs in calves had higher mean $\delta^{13}C$ values ranging between 1.2‰ and 4.6‰ in comparison to adult males (Ala, Pro, Asx, Glx, Tyr, Lys, Thr, Val, Ile, Phe) and non-lactating adult females (Ala, Pro, Asx, Glx, Tyr, Lys, Thr, Val, Ile, Phe) (figure 1, electronic supplementary material, table S4). Bulk tissue, Ala, Gly, Ser, Arg $\delta^{13}C$ values were lower (0.5‰ to 3.0‰) in calves compared to cows and non-lactating adult females (figure 1, electronic supplementary material, table S4). Calves had lower bulk tissue, Gly, Ser Arg $\delta^{13}C$ values compared to adult males by 0.7 to 4.6‰ (figure 1, electronic supplementary material, table S4). Adult males and non-lactating adult females had near identical $\delta^{13}C$ values except for Gly, Ser, Glx and Thr (figure 1, electronic supplementary material, table S4).

Bulk tissue and most (10/14) AAs in cows had lower $\delta^{15}N$ values by 0.6 to 2.6‰ in comparison to adult males or non-lactating adult females (figure 2, electronic supplementary material, table S5). $\delta^{15}N$ values of only a few Aas were similar between cows and adult males (Val) and non-lactating adult females (Val, Ile, Tyr) (figure 2, electronic supplementary material, table S5). Calves had higher bulk tissue, Pro, Arg, Asx, Phe, Tyr, Lys, and Gly $\delta^{15}N$ values in comparison to cows, but similar Ala, Glx, Val, Leu, Ile, and Ser (figure 2, electronic supplementary material, table S5). In contrast, bulk tissue and AA $\delta^{15}N$ values of adult males (bulk tissue, Ala, Asx, Glx, Val, Leu, Ile, Pro, Ser, Gly) and non-lactating adult females (bulk tissue, Ala, Glx, Val, Leu, Pro, Ser, Gly; adult males: bulk tissue, Ala, Asx, Glx, Val, Leu, Ile, Pro, Ser, Gly) were higher in comparison to calves. A notable pattern in calves was Thr $\delta^{15}N$ values, which were 3.4‰, 6.4‰, and 4.6‰ lower in comparison to cows, adult males,

301 and non-lactating adult females, respectively; the probability of these differences was 100%
302 (figure 2, electronic supplementary material, table S5). $\delta^{15}N$ values between adult males and
303 non-lactating adult females were similar except for Ala, Ile, and Thr (figure 2, electronic
304 supplementary material, table S5).
305



306

307 **Figure 1.** Posterior distribution estimates of mean $\delta^{13}C$ values in SWR skin. (A) bulk skin
308 tissue and non-essential AAs and (B) essential AAs (including Thr). Demographic groups
309 include cows (yellow), calves (green), non-lactating (adult) females (purple), and adult males
310 (blue). Each plot shows the mean (circle) and shaded boxes represent the 50% and 95%
311 credible intervals from darker to lighter color. The probability of the differences observed
312 among demographic groups range from 52% to 100% (electronic supplementary material,
313 table S4).

314

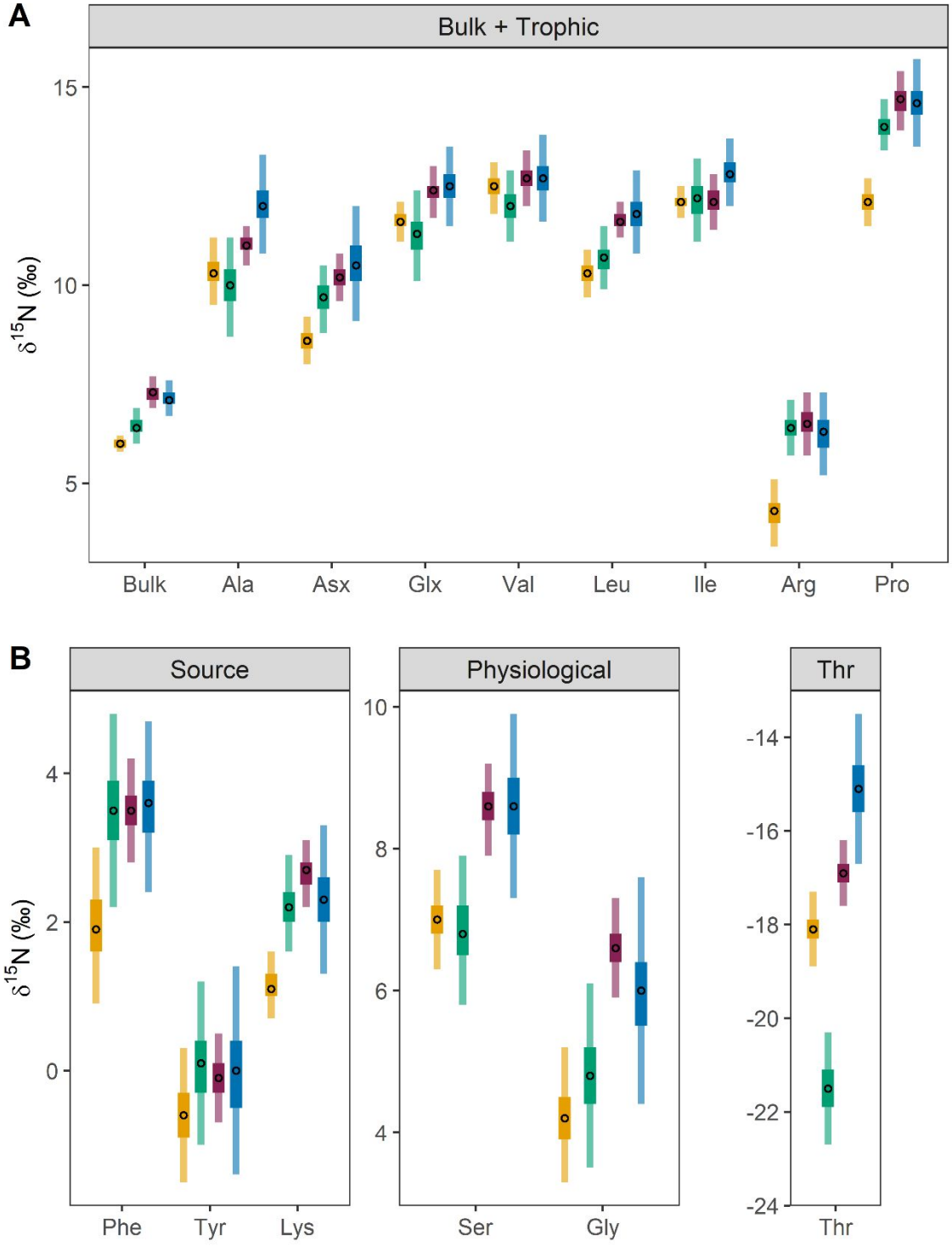


Figure 2. Posterior distribution estimates of mean $\delta^{15}N$ values in SWR skin. (A) bulk skin tissue and trophic AAs, and (B) Source AAs, Physiological AAs, and Thr. Demographic groups include cows (yellow), calves (green), non-lactating (adult) females (purple), and adult males (blue). Each plot shows the mean (circle) and shaded boxes represent the 50% and 95% credible intervals from darker to lighter color. The probability of the differences observed among demographic groups range from 53% to 100% (electronic supplementary material, table S5).

(b) Linear Discriminant Analysis (LDA)

Classification probabilities for the three LDA (Codes A, B, and C) are presented in table 1 and the variables (AAs) that contributed the highest loadings for SRW demographic group separation, for are presented in electronic supplementary material table S6 and table S7, for LD1. The LDA that included $\delta^{13}C$ values for all AA (Code A) had a global classification probability of 61%, and the specific demographic classification probabilities are show in table 1 and figure 3A, and the rate of classification is reported in electronic supplementary material table S8 (Code A). The LDAs that included $\delta^{15}N$ values for all AA (Code B) and both $\delta^{13}C$ and $\delta^{15}N$ values for all AAs (Code C) had higher global classification probability (figure 3B, C, table 1). The demographic group classification probability was also higher in LDA Code B and C compared to Code A (table 1); the rate of correctly classified groups is reported in in electronic supplementary material table S8. Cows and calves had the highest classification probability for each LDA compared to adult males and non-lactating adult females, or adults (table 1). The loadings for LD2 for the three LDAs (Code A, B and C) are reported in electronic supplementary material table S9.

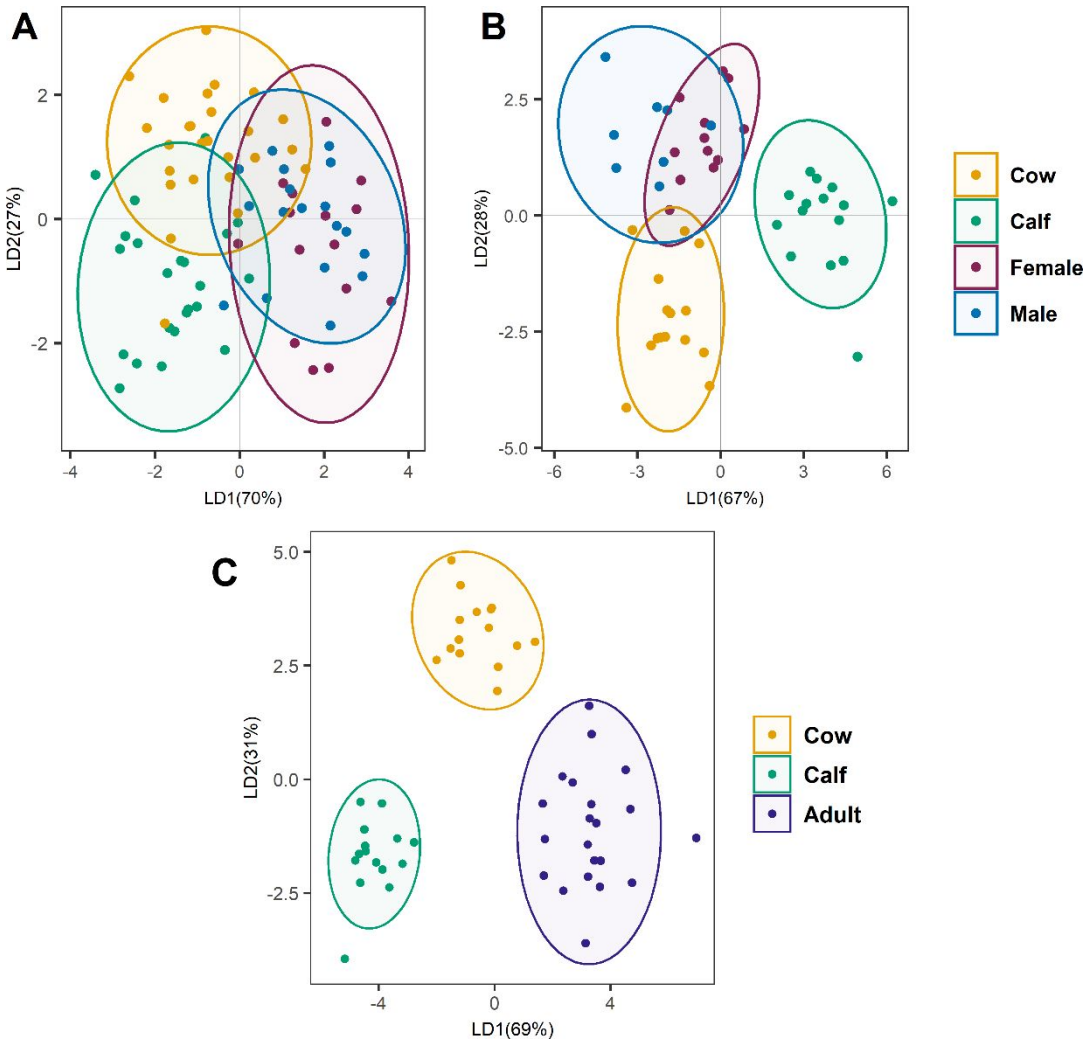


Figure 3. Linear discriminant analysis including (**A**) all AAs $\delta^{13}C$ (Code A); (**B**) all AAs $\delta^{15}N$ (Code B); (**C**) $\delta^{13}C$ and $\delta^{15}N$ values of AAs that had the highest loadings for LD1 for Code A analysis shown in panel A (Ala, Thr, Val, Leu, Pro, Asx, Glx, Lys) and Code B analysis shown in panel B (Ala, Thr, Val, Leu, Ile, Pro, Asx, Lys); non-lactating adult females and males are grouped as adults for the combined LDA shown in Panel C (Code C). See in electronic supplementary material table table S6 for AAs loadings and table S7 for a list of the AAs used for the combined analysis shown in Panel C. All loadings for LD1 and LD2 are presented in electronic supplementary material table S6, table S7 and table S9. The proportion of variance explained by each axis is provided in parentheses for each LDA.

Table 1. Classification probabilities of SRW demographic groups using three different Linear Discrimination Analysis (LDA) approaches (Code A-C): (Code A) LDA including all AA $\delta^{13}C$; (Code B) LDA including all AA $\delta^{15}N$; (Code C) LDA including $\delta^{13}C$ and $\delta^{15}N$ values of AAs that had the highest loadings for LD1 when using Code A and B (electronic supplementary material, table S6); adult males and non-lactating adult females are grouped as adults. GCP, Global Classification Probability.

LDA	GCP	Demographic Group Classification Probability			
		Cows	Calves	Non-lactating Adult Females	Adult Males
Code A	61%	77%	70%	47%	39%
Code B	76%	80%	87%	69%	62%
Code C	96%	100%	93%	95% (Adults)	

4. Discussion

Our results show that SRW demographic groups exhibit distinct AA $\delta^{13}C$ and $\delta^{15}N$ values in skin that reflect differential nutrient allocation strategies related to reproductive and metabolic status (figures 1, 2 and 3, table 1, electronic supplementary material, tables S2–S8). The patterns among key AAs in SRW skin, identified using different LDAs, enabled correction classification of cows with a probability of 77% to 100% (table 1). This novel result indicates that AA isotope analysis can be used to identify female reproductive events in SRW and the impacts of gestation and lactation on nitrogen balance. As described in earlier sections, complete isotopic turnover in cetacean skin occurs over 3 to 5 months (Busquets-Vass et al., 2017; Giménez et al., 2016), therefore the time period captured in samples collected from cows reflects lactation and the last trimester of gestation that collectively represent the highest energetic cost for female SRW reproduction (Christiansen et al., 2022). Skin collected from calves reflect nursing and to a lesser extent the *in utero* period. The trends in AA isotope composition observed in SRW adults likely represent the physiological status of healthy fasting whales given that no signs of nutritional stress were reported during field sampling. In the following sections we discuss the underlying physiological mechanisms

383 that are most likely driving the isotope patterns observed among these demographic groups
384 and address the potential metabolic pathways that whales use to support reproduction and
385 maintain homeostasis.

386

387 **(a) Cows**

388 $\delta^{13}C$ values of nearly all AAs (12/14) were higher in cows than adult males and non-lactating
389 adult females (figure 1, electronic supplementary material, table S4), which may suggest that
390 cows are catabolizing endogenous AAs to a greater degree than other age/sex classes to
391 maintain homeostasis and support reproduction. Metabolic processes like gluconeogenesis,
392 which carnivores like cetaceans use to synthesize glucose [48,49] requires the catabolism of
393 proteins and subsequent deamination of AAs to intermediaries in the tricarboxylic acid cycle
394 and glycolysis. While carbon isotopes are not likely fractionated during AA catabolism [50],
395 a switch from exogenous (prey) to endogenous (muscle) stores to fuel gluconeogenesis would
396 yield higher $\delta^{13}C$ values in fasting cows relative to adult males or non-lactating adult females
397 that do not have the same physiological demands associated with reproduction. In contrast,
398 the use of stored lipids (blubber) to fuel gluconeogenesis would yield lower $\delta^{13}C$ values in
399 fasting whales because lipids are ^{13}C-depleted relative to co-occurring proteins. Our data
400 show that cows are not using ^{13}C-depleted lipids catabolized from blubber to fuel non-
401 essential amino acid synthesis, and instead may be routing lipid stores to produce milk for
402 their calves [51], which has an extremely high fat content (30-50%) [52,53]. Instead, healthy
403 fasting cows sampled in this study are using skeletal muscle or other proteinaceous reserves
404 to maintain their skin tissue, resulting in higher AAs $\delta^{13}C$.

405 An exception to this pattern was Ser, a non-essential AA that had lower $\delta^{13}C$ by 1.3‰
406 and 2.4‰ in cows in comparison to non-lactating adult females and males, respectively

(figure 1, electronic supplementary material, table S2 and table S4). Ser is a major contributor of one carbon metabolism used to synthesize other amino acids (Gly, cysteine, taurine) and phospholipids [54,55]. During pregnancy in humans, the concentration and turnover of Ser decreases in blood plasma, likely as a mechanism to conserve nitrogen to fuel foetal tissue synthesis [56,57]. SRW cows could be using a similar strategy for sparing nitrogen during lactation and the last trimester of gestation, resulting in less catabolism and lower $\delta^{13}C$ values in Ser compared to adult whales.

In contrast to the patterns observed in carbon isotopes, $\delta^{15}N$ values of bulk skin tissue and most (10/14) AAs were lower in SRW cows compared to other adult whales (figure 2, electronic supplementary material, table S5). We expected cows to have similar $\delta^{15}N$ values as non-lactating adult females and adult males because all three demographic groups fast and are in catabolic state when on the breeding grounds during winter. However, several studies have reported lower $\delta^{15}N$ values of bulk tissues (hair and blood) in lactating mammals [58,59]. For example, the magnitude of declines in bulk human hair $\delta^{15}N$ values collected from pregnant women was inversely correlated to weight gain during pregnancy [60,61], a pattern hypothesized to result from a decrease in nitrogen excretion required to enhance protein synthesis for foetal development [60]. Similarly, a decline in $\delta^{15}N$ values was also observed in gestating elephant seals, offset by a concurrent increase in the foetal $\delta^{15}N$ values [62]. This protein sparing mechanism might be enhanced in capital breeding cetaceans and other marine mammals that are simultaneously fasting and producing milk with an extraordinarily high lipid content (30-50%), but lower amounts of protein (9-13%) and water content (40-50%) [52]. In such scenarios, females may primarily rely on the products of lipid (blubber) catabolism rather than protein catabolism for milk production [52,63], a strategy that potentially enhances protein sparing associated with lower apparent $\delta^{15}N$ isotopic discrimination [62].

Another potential mechanism that would lower tissue $\delta^{15}N$ values in fasting cows is the conversion of ^{15}N-depleted urea into ammonia by microflora in the gastrointestinal tract, which can be used as a nitrogen source to synthesize amino acids and peptides [64,65]. Like ruminants, baleen whales have multichambered stomachs and preliminary results suggest that microbial fermentation occurs in the forestomach [64]. Urea is ^{15}N-depleted compared to exogenous (dietary) or endogenous protein [66], thus the recycling of isotopically light nitrogen to the central nitrogen pool for protein synthesis would also result in a decrease in $\delta^{15}N$ of lactating SRW females.

We hypothesize that AAs in higher demand during gestation and lactation have lower $\delta^{15}N$ values (figure 2, electronic supplementary material, table S3 and table S5), which results in the unique AAs isotope fingerprints for cows in comparison to adult males and non-lactating adult females. The two AAs that showed the lowest $\delta^{15}N$ values in cows compared to the rest of the demographic groups were Arg and Pro (figure 2), with the latter compound having some of the highest positive loadings in all LDAs (table 1, electronic supplementary material, table S6 and table S7) and contributing considerably to demographic group separation. Pro is one of the most abundant AAs in the protein casein [67], a major component of whale milk [53], and is often considered a conditionally essential AA because dietary concentrations are typically not high enough to fuel demand during lactation. In addition, Pro can be synthesized from Arg [68], which is probably why they show similar $\delta^{15}N$ (and $\delta^{13}C$) patterns. Arg is one of the only AA that contains two nitrogen atoms, at least one of which is derived from ^{15}N-depleted ammonia transaminated from the central nitrogen pool, resulting in Arg having lower $\delta^{15}N$ values compared to other trophic AAs.

(b) Calves

Overall, variation in AA $\delta^{13}C$ and $\delta^{15}N$ values of calves allowed us to separate them from other demographic groups with a high percentage of accuracy using LDAs (figure 3). In contrast to adults that were sampled while fasting, calves were rapidly growing in an anabolic state and the isotopic composition of their skin largely reflects the nursing period and to a lesser extent time *in utero*. In general, calf AA $\delta^{13}C$ values were similar to cows but higher than those in non-lactating adult females and adult males (Figure 1). This pattern shows that calves are not using ^{13}C-depleted lipid derived carbon to synthesize most non-essential amino acids while nursing, but instead are acquiring enough protein from milk to fuel tissue synthesis and rapid growth. The only exception to this pattern was Ser and Gly (figure 1), which had lower but more variable $\delta^{13}C$ values in comparison to cows and other adults. We suspect this pattern is the result of *de novo* synthesis of these two non-essential AAs from lipid precursors, specifically glycerol that can be phosphorylated to form 3-phosphoglycerate, an intermediary in glycolysis and the precursor for Ser synthesis. Ser can then be converted to Gly to provide carbon units for one-carbon metabolism to synthesize proteins, lipids, nucleic acids, and other cofactors [55,69], an anabolic pathway that may be crucial for rapidly growing calves that are consuming a (milk) diet rich in lipids.

For AA $\delta^{15}N$, calves also exhibited values that were more like cows than non-lactating adult females and adult males (figure 2, electronic supplementary material, tables S3 and S5). This pattern suggests that calves are directly routing AA from resources acquired while nursing and *in utero* to fuel rapid growth [11]. A notable pattern was that calves had lower Thr $\delta^{15}N$ compared to the other demographic groups (figure 2, electronic supplementary material, table S5), and this AA had the highest loadings for group separation and classification for LDA Code B (electronic supplementary material, table S6). Thr typically exhibits ^{15}N-depletion during trophic transfer [70,71], so lower $\delta^{15}N$ values in calves would indicate they are feeding at a higher trophic level than cows as the milk they are

481 consuming is synthesized from their mothers tissues, which agrees with mechanisms
482 proposed in previous studies focused on isotope analysis of bulk tissues in mammals [72–74].
483 Of the trophic AAs, only Pro and Arg had higher $\delta^{15}N$ values indicative of higher trophic
484 level in calves compared to cows. Given that Arg and Pro $\delta^{15}N$ patterns in cows suggest
485 sparing of these compounds, and Pro is a major component of milk protein (casein), these
486 AAs may be routed into offspring and then deaminated to a greater degree than other trophic
487 AAs by nursing calves as a source of nitrogen to fuel metabolism and growth.

488

489 **(c) Adult Males and Non-Lactating Adult Females**

490 Non-lactating adult females and males had nearly identical bulk tissue and AAs $\delta^{13}C$ and
491 $\delta^{15}N$ values (figure 1, figure 2, and electronic supplementary material, tables S2-S5),
492 resulting in a high degree of overlap in LDA space (figure 3A, B) and suggests that these two
493 demographic groups use similar metabolic pathways to support fasting. The metabolic
494 pathways used by baleen whales during fasting have not been explored in detail, but studies
495 on other taxa show that starvation and fasting can impact bulk tissue and AAs $\delta^{13}C$ and $\delta^{15}N$
496 values [21,26,75–77]. In southern elephant seals, $\delta^{15}N$ values of glucogenic AA (e.g., Gly,
497 Ser, Pro, and Asx) tend to increase during fasting, while Ala $\delta^{15}N$ decreases due to its central
498 role in the Cahill Cycle. $\delta^{15}N$ values of branch-chained AA Val, Ile, and Leu do not change,
499 likely due to protein sparing of these essential AAs [21]. Increases in $\delta^{15}N$ values of Gly and
500 Thr have also been observed in the baleen of fin whales during the phase of migration were
501 fasting likely occurred [26]. Interestingly, most AAs $\delta^{15}N$ values were higher in SRW adult
502 males and non-lactating females compared to actively nursing calves, which suggests that
503 fasting non-breeding whales could exhibit higher AAs $\delta^{15}N$ values compared to non-breeding
504 whales foraging in the summer and fall. However, without comparable data for actively
505 foraging whales in an anabolic state sampled on the foraging grounds, it's difficult to assess

whether fasting produces a unique AAs $\delta^{13}C$ and/or $\delta^{15}N$ fingerprint in SRW. Future research on skin collected in the SRW feeding grounds, and on baleen plates collected from stranded SRW would enable comparison of anabolic versus catabolic periods in the annual life cycle to better understand how bulk tissue and AA isotope patterns are influenced by fasting in large cetaceans.

5. Conclusion

Here we demonstrate that AA isotope analysis of skin, the most accessible and sampled tissue in baleen whales, allows us to identify differential nutrient allocation strategies among SRW demographic groups and make inferences of protein balance and reproductive status. We propose that this approach can be used to identify the metabolic impacts of reproduction (gestation and lactation) in SRW cows, akin to a lactation/pregnancy test that could be applied more broadly across other cetacean species. We also identify the AA isotope patterns of healthy fasting whales that could be used to monitor the physiological status of whale populations in response to short-term environmental perturbations. For $\delta^{13}C$, an increase of endogenous AAs catabolism and routing of lipid stores (blubber) to produce milk are the mechanisms that likely contribute to the higher values in cows relative to other demographic groups. For $\delta^{15}N$, protein sparing and recycling of urea are two potential mechanisms that likely contribute to the general pattern of lower $\delta^{15}N$ values in cows and to a lesser degree calves relative to adult males and non-lactating adult females. We propose this approach can be used in animals with complex annual life history strategies characterized by geographical separation of reproduction and foraging that often requires long-distance migration and a capital breeding strategy. We anticipate this method could be used to identify successful calving events and estimate calving intervals along a chronology of metabolically inert baleen

plates and provide a multi-year record of ecological and physiological information at the individual level. Sub-sampling baleen plates and other metabolically inert but continuously growing mammalian tissues (e.g., vibrissae) could identify successful reproductive events, estimate inter-breeding intervals, and identify periods of fasting (catabolism) versus active foraging (anabolism).

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