Fasting affects amino acid nitrogen isotope values: a new tool for identifying nitrogen balance of free-ranging mammals

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Abstract

Changes in the nutritional status of free-ranging animals have a strong influence on individual fitness, yet it remains challenging to monitor longitudinally. Nitrogen (δ^{15}N) and carbon (δ^{13}C) isotope values measured chronologically along the length of metabolically inert keratinous tissues can be used as a nutritional biomarker to retrospectively reconstruct the foraging ecology and eco-physiology of the consumer. We quantitatively describe the physiological effects of fasting on amino acid metabolism using sequentially measured bulk tissue and amino acid δ^{15}N values along the length of whiskers sampled from free-ranging juvenile, subadults, adult female, and male southern elephant seals (SES; *Mirounga leonina*) on Marion Island in the Southern Ocean. For both juveniles and adult females, whisker segments representing fasting had significantly higher bulk tissue δ^{15}N values of 0.6‰ and 1.3–1.8‰, respectively, in comparison to segments unaffected by fasting. We also found a large increase (2–6‰) in δ^{15}N values for most glucogenic amino acids and a simultaneous depletion (2–3‰) of alanine in segments reflecting fasting, which enabled us to accurately predict (74%) the nutritional status of our model species. We hypothesize that the glucose-alanine cycle is the mechanism driving the observed depletion of alanine δ^{15}N values during fasting. We demonstrated that keratinaceous tissues can be used as a longitudinal nutritional biomarker to detect changes in the nitrogen balance of an individual. Moreover, it is evident that physiological factors have an important influence on tissue δ^{15}N values and can lead to erroneous bulk tissue or amino acid isotope-based reconstructions of foraging habits.

Keywords

Introduction

Animals often experience prolonged fasts during which metabolic processes regulate changes in the utilization of stored endogenous substrates such as skeletal muscle (proteins) and adipose tissue (lipids) (Raubenheimer et al. 2009; Fontana and Partridge 2015; Secor and Carey 2011; Vidal et al. 2019). Ultimately, the integrative physiological response to extended fasting bouts can have strong impacts on individual fitness, and are thus intrinsically linked to their evolution (Secor and Carey 2011). Yet, assessing the relative importance of various biochemical and physiological mechanisms that enable organisms to maintain physiological homeostasis while solely relying on endogenous resources remains a contentious topic (Secor and Carey 2011; Lee et al. 2012). Furthermore, our understanding of such mechanisms that enable free-ranging animals to withstand and recover from extended fasts are often hampered by the challenge of building longitudinal (temporal) records of their nutritional status.

Biomarkers can reflect changes in how animals catabolize or synthesize molecules during fasting (Petzke et al. 2010), and chemical analysis of such compounds can provide valuable insights on the relationship between an organism's physiology, diet, and environmental conditions (Fuller et al. 2005; Mekota et al. 2009; Hatch et al. 2012; Ohkouchi et al. 2017). For example, nitrogen (δ^{15}N) and carbon (δ^{13}C) isotope analysis of tissues provide information about resource and habitat use, as well as physiological status (Jaeger et al. 2010; Newsome et al. 2010; O'Brien 2015). While δ^{13}C values are often used to infer sources of primary production that fuel the food webs that animals rely on, δ^{15}N values are commonly used to estimate trophic level (Peterson and Fry 1987). The pattern of trophic enrichment in tissue δ^{15}N is a result of fractionation among the amino radicals (-NH$_2$) that are produced during amino acid catabolism; the isotopically depleted nitrogen (^{14}N) is preferentially excreted in the form of ammonia, urea,

3

or uric acid (Peterson and Fry 1987) and the retained ^{15}N is then incorporated into body nitrogen pools and used to build and maintain tissues. Offsets in δ^{15}N values between animals and their food are referred to as trophic discrimination factors (TDF; Peterson and Fry 1987), which vary depending on the type of nitrogenous waste compound produced (Vanderklift and Ponsard 2003), as well as the quantity and quality of protein in their diets (Hughes et al. 2018).

In addition to diet composition, physiological processes such as starvation or gestation can also affect the isotopic composition of tissues (Hobson et al. 1993; Fuller et al. 2004, 2005). Recent meta-analyses demonstrated that fasting causes a small yet measurable increase in bulk tissue mean ($\pm$SD) δ^{15}N values of +0.5$\pm$0.2‰ (McCue et al. 2010; Hertz et al. 2015) as the individual essentially 'consumes' itself by catabolizing its own protein- (skeletal muscle) and lipid-rich (adipose) tissues to fuel metabolism (Fuller et al. 2005). Theoretically, isotopic analysis of individual compounds may provide additional information about the biochemical mechanisms that enable animals to fast for prolonged periods (Ohkouchi et al. 2017; Whiteman et al. 2019). For animals that do not consume appreciable amounts of glucose (e.g., carnivores), amino acids are catabolized for glucose synthesis via gluconeogenesis to fuel activity, which results in the preferential deamination and excretion of ^{14}N and yields the measurable TDFs described above. During fasting, endogenous protein (i.e., skeletal muscle), which is ^{15}N-enriched relative to exogenous dietary protein, becomes the primary substrate for gluconeogenesis (Krebs 1964) and consequently enriches the δ^{15}N of the remaining amino acids in the body pool that are used to synthesize tissues. Specifically, "trophic" amino acids (e.g., glutamic acid) that are closely linked to nitrogen cycling in animals are subject to more deamination (O'Connell 2017) should show increases in δ^{15}N values in tissues synthesized during fasting. In contrast, we expect that "source" amino acids (i.e., those that are not closely

4

associated with nitrogen cycling and cannot easily be deaminated without catabolizing the entire compound) may not be subject to extensive catabolism during fasting and thus will show minimal to no change in $\delta^{15}N$. In summary, we predict that the offsets between the $\delta^{15}N$ of trophic and source amino acids in fasting animals are expected to be larger than in animals that are consuming exogenous resources.

Adult southern elephant seals (SES; *Mirounga leonina*) experience two distinct prolonged fasting periods during their annual life cycle (Fig. 1S; Le Boeuf and Laws 1994). One occurs during the breeding season (Oct.–Nov.) which ends with a brief foraging bout, and the other during the annual obligatory molt (Dec.–Jan.). Tissues synthesized while fasting provide a means to examine the effects of protein catabolism on nitrogen balance and by extension on $\delta^{15}N$ values of the consumer. After birth, juvenile SES pups suckle for 22–23 days before undergoing a post-weaning fast that lasts ~40–60 days (Wilkinson and Bester 1990). During this fast, recently-weaned pups lose up to ~30% of their original weaning body mass (Wilkinson and Bester 1990; Carlini et al. 2001) before departing on their first foraging trip; the mass lost consists of 39% water, 47% fat, and 12% protein. Using estimates of whisker growth rates (Lubcker et al. 2016), we showed that $\delta^{15}N$ values of longitudinally sub-sampled whisker segments from recently-weaned SES pups showed enrichment of ~1.0‰ while fasting (Lübcker et al. 2017).

Here we used a combined bulk tissue and compound-specific isotope approach to advance our understanding of physiological changes in amino acid metabolism that occur during fasting, and potentially establish amino acid $\delta^{15}N$ analysis as a robust proxy for identifying the use of endogenous versus exogenous resources. We first compared how the $\delta^{15}N$ of amino acids changed during known catabolic and anabolic states that were identified using bulk tissue $\delta^{15}N$

data measured chronologically along the length of juvenile SES whiskers with known whisker growth histories. We then used these data to determine if fasting influenced the δ^{15}N values measured along the length of whiskers sampled from free-ranging adult SES with unknown whisker growth histories. Although we have previously described the growth rate and shedding phenology of SES whiskers (Lübcker et al. 2016), it is still challenging to ascertain when the whisker growth of free-ranging seals commences (McHuron et al. 2019). Identifying the segment(s) of the whisker grown while fasting on land could clarify the whisker growth histories and refine the use of this tissue to provide longitudinal physiological and ecological information (McHuron et al. 2019). Lastly, by assessing how δ^{15}N values of individual amino acids respond during periods of known fasting, we aimed to advance the use of this approach to assess the nitrogen-balance of free-ranging animals and to provide insights into the biochemical mechanisms that lead to δ^{15}N enrichment of tissues during fasting.

Methods

Sample Collection and Whisker Subsampling.
Sample collection occurred at Marion Island (46.88° S, 37.87° E). We sampled a single whisker by cutting as close to the skin as possible from SES pups < 2 days after they were weaned (21–23 days after birth) during 2012 and 2013. At ~362 days after the initial sampling, we sampled the regrown whisker from the returning juveniles (e.g., Fig. 1S). The mean (±SD) length of these regrown whiskers was 68.6 ± 13.8 mm (n = 17; 6 females and 11 males). Thus, isotopic information in the regrown whiskers encompasses the post-weaning fast (catabolic state) followed by independent foraging at sea (anabolic state). Whisker regrowths of n = 5 of the individuals used for the bulk δ^{15}N analysis were also used for the amino acid δ^{15}N analysis.

6

Further details on how these whiskers were sub-sampled for isotope analysis are provided in the Supplementary Material and in Lübcker et al. (2017).

We also collected multiple fully-grown whiskers with unknown growth histories from 23 individuals representing subadult (2 males, 1 female), adult female ($n = 17$), and adult male SES ($n = 3$) ranging from 2 to 13-years old (median 5-years). Sampling occurred between 2009 and 2016 during satellite telemetry tag deployments. The mean ($\pm$ SD) length of these whiskers was 116.4 $\pm$ 19.1 mm (range: 79.0–165.0 mm long) and we noted cases where whiskers were plucked instead of cut. Although we considered the growth history as unknown, the growth of the sampled whiskers most likely commenced during the molt (Lübcker et al. 2016; McHuron et al. 2019). A second whisker from adult females ($n = 10$) used for the bulk $\delta^{15}N$ analysis was also used for the amino acid $\delta^{15}N$ analysis. We used the whiskers sampled from one adult male SES to demonstrate how the whisker subsampling for the amino acid $\delta^{15}N$ analysis was achieved (Fig. 2S). Further details on how these whiskers were sub-sampled for isotope analysis are provided in the Supplementary Material.

Stable Isotope Analysis.
Whiskers were pre-treated following the protocol reported in Lübcker et al. (2017). Samples were scrubbed with deionized (DI) water and rinsed with a 2:1 chloroform:methanol solvent solution to remove surface contaminants. Samples were then rinsed twice with the chloroform:methanol solution and subsequently rinsed thoroughly with DI and dried prior to analysis. All whiskers were subsampled sequentially into ~2.1 $\pm$ 0.4 mm segments for bulk tissue isotope analysis. Because the distal end (tip) of the fully-grown whiskers was not as thick as the base, segments of 6–16 mm were required to obtain the desired 0.5–0.6 mg sample mass. Segments were weighed into tin capsules and $\delta^{15}N$ values were measured with a Thermo

Scientific Flash 1112 Series elemental analyzer coupled to a Thermo Scientific Delta V Plus isotope ratio mass spectrometer (EA-IRMS) in the Stable Isotope Laboratory at the University of Pretoria (Pretoria, South Africa). Isotope results are expressed as delta (δ) values in parts per mil (‰) relative to the international standard atmospheric N^2. Two internal standards (Merck gel and DL-Alanine) were used to assess analytical precision (SD), which was ±0.3‰ for $\delta^{15}N$.

For amino acid $\delta^{15}N$ analysis, juvenile whisker regrowth segments with a mean (± SD) sample mass of 8.2 ± 2.0 mg (minimum: 5.6 mg; $n = 10$ samples) and adult female whisker segments with a mass of 9.9 ± 2.9 mg ($n = 30$ samples), were hydrolyzed in 1 ml 6N hydrochloric acid (HCl) for 20 hours at 110°C. During the hydrolysis, asparagine is converted to aspartic acid and glutamine to glutamic acid (Whiteman et al. 2019). Amino acids were subsequently derivatized with 2-isopropanol and N-TFAA (Fantle et al. 1999) and then analyzed for $\delta^{15}N$ after separation on a 60m DB-5 column (SGE Analytical Science) in a Thermo Scientific Trace 1310 gas chromatographer coupled to a Isolink II and Thermo Scientific Delta V Plus IRMS at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM, USA). All samples were analyzed in duplicate and were bracketed by an internal stock standard consisting of pure (powdered) amino acids (Sigma-Aldrich Co.) that were derivatized alongside batches of unknown samples; $\delta^{15}N$ values of the pure amino acids were measured with EA-IRMS. This method provided $\delta^{15}N$ measurements of 13 amino acids: alanine (Ala), isoleucine (Iso), leucine (Leu), valine (Val), proline (Pro), glycine (Gly), serine (Ser), phenylalanine (Phe), lysine (Lys), tyrosine (Tyr), threonine (Thr), glutamic acid (Glu) and aspartic acid (Asp). Within-run precision of amino acid $\delta^{15}N$ measurements was estimated by calculating SD of duplicate samples analyzed with bracketing standards; the mean SD for all amino acids was 0.4‰ and ranged from 0.3‰ for lysine and 0.5‰ for threonine.

Statistical Analyses.

A Piecewise Linear Regression Model was used to characterize isotopic variation along the length of the whisker corresponding to specific life-history events (R package *segmented*; Muggeo 2008). This was done after estimating the breakpoints by visual inspection of the plotted data and the fitted Loess smoothing polynomial regression. In all cases, normality of the data and model residuals was assessed using a Shapiro-Wilk normality test before applying appropriate parametric or non-parametric statistical analyses. For bulk tissue $\delta^{15}N$ results, we reported the mean and standard deviation (SD). For amino acid $\delta^{15}N$ results, we reported medians and associated upper and lower 95% confidence intervals. We used Linear Discriminant Analysis (LDA) to classify the amino acid $\delta^{15}N$ values as being synthesized during fasting or active foraging (*MASS* package; Venables and Ripley 2002); details are in the Supplementary Material. The amino acid $\delta^{15}N$ values were divided for model training ($n = 24$ data points, 60% of data) and testing ($n = 19$ data points, 40% of data) and multivariate normality was confirmed using the *ICS* package in R (Nordhausen et al. 2008). Statistical computations were performed using R for statistical computing (R Development Core Team 18, version 3.4.4) coupled with the RStudio interface (version 1.0.153).

Results

Effects of Known Fasting on Bulk Tissue $\delta^{15}N$ Values.

A total of 560 chronologically subsampled whisker regrowth segments from juvenile SES (32.9 $\pm$ 6.6 segments per individual) were analyzed for bulk tissue $\delta^{15}N$. The mean $\delta^{15}N$ values were higher (Kruskal-Wallis $\chi^2 = 351.75$, $df = 4$, $P < 0.001$) for whisker segments grown during the catabolic, post-weaning fast as compared to segments of the whisker grown *in utero* and while nursing. The fasting-associated $\delta^{15}N$ values were also higher than the transition from milk to

Table 1: Bulk tissue $\delta^{15}N$ values reflecting different life-history events captured along the length of whiskers sampled for juvenile ($n = 17$), male and female subadult ($n = 3$), adult male ($n = 3$) and breeding adult female ($n = 17$) southern elephant seals (*Mirounga leonina*; mean ± SD, expressed as parts per mil (‰)).

Age	*In utero*	Lactation	Fasting (*catabolic*)	Transition	Foraging (*anabolic*)
Juvenile	10.9 ± 0.5	11.3 ± 0.8	11.9 ± 0.7	10.3 ± 1.3	8.7 ± 0.5
Subadult	-	-	11.7 ± 0.3	11.3 ± 0.6	10.9 ± 0.4
Adult female[*]	-	-	11.6 ± 0.8	11.2 ± 0.8	10.0 ± 0.7
Adult male	-	-	12.9 ± 0.6	12.5 ± 0.8	11.6 ± 0.6

[*]Excludes plucked whisker segments.

independent foraging (Table 1), and higher than independent foraging ($P < 0.001$). A Piecewise Linear Regression Model (*Adj. R^2* = 0.79) was used to define the changes that occurred in the δ^{15}N values captured along the length of the whisker regrowths (Table 2). The δ^{15}N values increased by 0.6‰ during the post-weaning fast from post- lactation δ^{15}N values; an average increase of 1.2 ± 0.6‰ (max = 2.4‰) from pre-lactation δ^{15}N values. The transition from fasting to independent foraging at sea (an anabolic state), depleted the δ^{15}N values by 3.7‰, whereafter the δ^{15}N values equilibrated to the new diet and remained low (8.7 ± 0.5‰, mean ± SD).

Identifying Unknown Fasting using Bulk Tissue δ^{15}N Values.
To determine if we could detect fasting-related δ^{15}N changes along the length of the whiskers with unknown growth histories, we analyzed a total of 1136 whisker segments (49.4 ± 10.6 segments per individual) collected from subadult ($n = 3$) and adult male ($n = 3$) and adult female ($n = 17$) SES. The distinctive slope (*b*) and positive change in δ^{15}N values observed during the catabolic state of the post-weaning fast ($b = 0.027$; Table 2) did not differ significantly from the change in δ^{15}N values observed at the tip of the whiskers of subadult ($b = 0.023$), adult female ($b = 0.035$), and adult male SES ($b = 0.030$) (Kruskal-Wallis $\chi^2 = 3$, $df = 3$, $P = 0.391$), suggesting that the whisker tips were grown while fasting. The trends in the δ^{15}N values measured along the length of whiskers sampled from male and female SES of various ages were comparable (Fig. 1b, c, d). The presumed fasting-associated changes in δ^{15}N of subadult and adult male SES included increases of 1.3‰ (Kruskal-Wallis $\chi^2 = 53.40$, $df = 2$, $P < 0.001$) and 1.6‰ (Kruskal-Wallis $\chi^2 = 65.12$, $df = 2$, $P < 0.001$) from the start to end of the fast, respectively (Table 2). The largest predicted enrichment occurred in adult females (1.8‰). Thereafter, the whisker δ^{15}N of subadult and adult females declined by 1.4‰, and by 1.6‰ for adult males when the bulk tissue δ^{15}N equilibrated to the new δ^{15}N values reflecting foraging at sea.
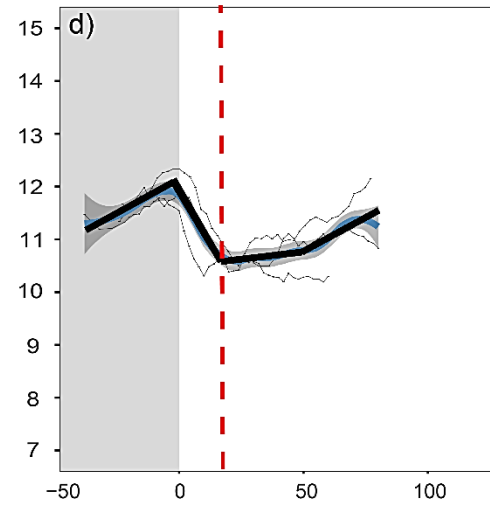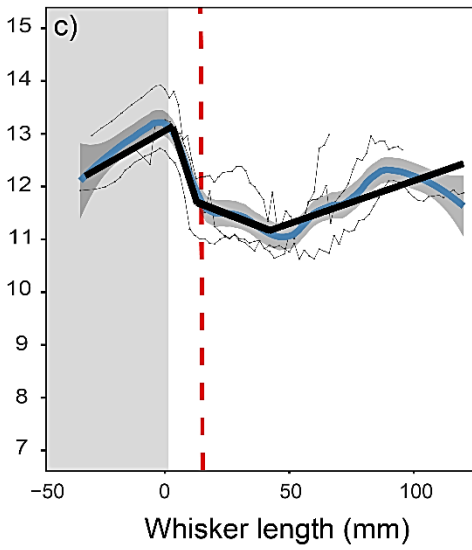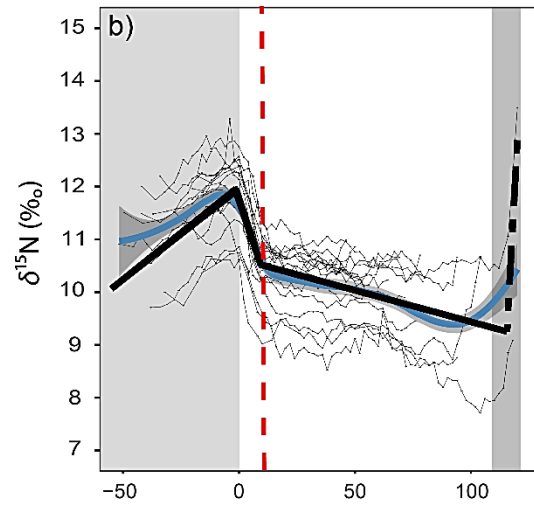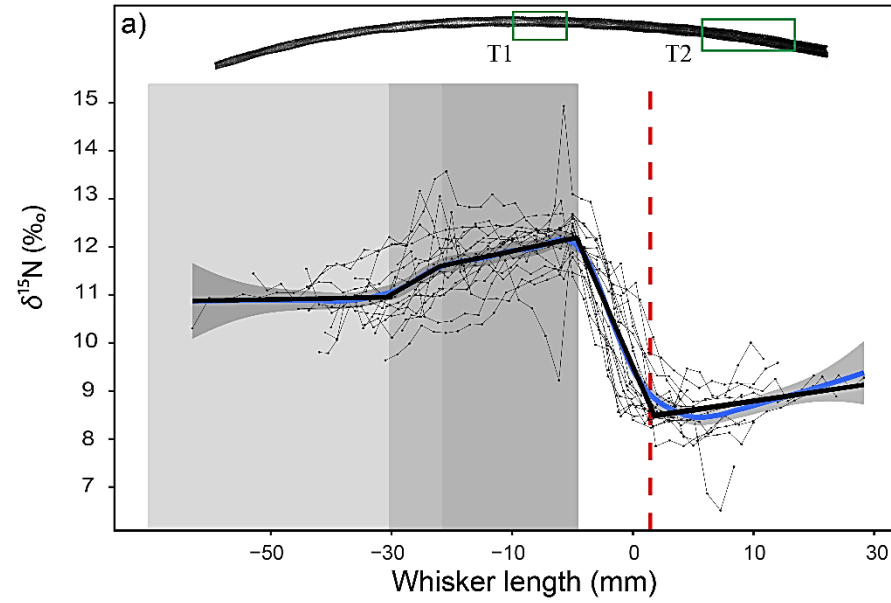
Figure 1: Bulk (whole sample) tissue $\delta^{15}N$ values measured along the length of whiskers sampled from (a) juvenile ($n = 17$), (b) adult female ($n = 17$), (c) adult males ($n = 3$), and (d) male and female subadult ($n = 3$) southern elephant seals (*Mirounga leonina*). The plot position (x-axis) was set to zero at the onset of data reflecting independent foraging and the red vertical lines indicate the predicted position along the length of the whiskers where the isotope values are no longer affected by preceding fast. The blue lines with grey bars (standard error) represent the Loess smoother, while the black line represents the fitted Piecewise Linear Regression Models. For panel (a), the lightest grey area (far left) reflects the segment of the whisker that was grown *in utero* (10.0 ± 8.2 mm at the tip of whisker), followed by segments grown during lactation (darker gray; 6.6 ± 1.4 mm segment), the post-weaning fast (darkest grey; 20.5 ± 1.1 mm), and independent foraging (white). The green boxes on the whisker schematic (top) indicate the position on the whisker where subsamples were collected for AA-CSIA. Samples representing (T1) 'fasting' was recorded 35.6 mm from the tip of the whisker regrowths (13.6 ± 2.9 mm long segment), while samples representing (T2) 'foraging' were subsampled on average 7.2 mm from the whisker base. For panel (b–d), the light grey boxes (far left) indicate the segment of whisker grown on land while fasting. The darker grey bar at the base of the whiskers in panel (b) reflects the embedded segment of three whiskers that was plucked and excluded from further analyses.

Table 2: Piecewise Linear Regression Models ($y = a + bx$) characterizing the changes observed in the bulk $\delta^{15}N$ values during different life-history events measured sequentially along the length of whiskers sampled from juvenile ($n = 560$ whisker segments), male and female subadult ($n = 141$ segments), adult male ($n = 174$ segments) and breeding adult female ($n = 821$ segments) southern elephant seals (*Mirounga leonina*). We report the standard error (SE) with lower and upper 95% confidence intervals in brackets.

Period	Segments	Slope (*b*)		y-axis intercept (*a*)		*Predicted* $\Delta^{15}N_{Start-End}$
	n	Estimate	SE (95% CI)	Estimate	SE	
Juvenile[1]						
In utero growth	77	0.002	0.007(-0.023; 0.025)	10.9	0.025	<0.1‰
Lactation	73	0.062	0.022 (0.019; 0.105)	12.9	0.023	+0.7‰
Fasting	186	0.027	0.007 (0.012; 0.042)	12.2	0.019	+0.6‰
Transition	105	-0.287	0.018 (-0.322; -0.252)	12.4	0.019	-3.7‰
Foraging	119	0.018	0.007 (0.004; 0.032)	8.3	0.186	+0.9‰
Subadult[2]						
Fasting	30	0.023	0.008 (0.008; 0.038)	12.0	0.014	+1.3‰
Transition	29	-0.073	0.012 (-0.097; -0.050)	11.8	0.013	-1.4‰
Foraging	82	0.005	0.006 (-0.006; 0.016)	10.5	0.009	+0.2‰
Foraging[†]		0.025	0.007 (0.012; 0.038)	9.5	0.007	+1.0‰
Adult female[3]						
Fasting	170	0.035	0.003 (0.029; 0.042)	12.0	0.028	+1.8‰
Transition	158	-0.135	0.027 (-0.187; -0.082)	11.8	0.027	-1.4‰
Foraging	493	-0.012	0.001 (-0.014; -0.009)	10.6	0.22	-1.3‰
Adult male[4]						
Fasting	23	0.030	0.010 (0.010; 0.049)	13.2	0.049	+1.6‰
Transition	31	-0.165	0.048 (-0.259; -0.070)	13.8	0.049	-1.6‰
Foraging	120	-0.018	0.010 (-0.039; 0.002)	11.9	0.011	-0.5‰
Foraging[†]		0.016	0.003 (0.010; 0.022)	10.5	0.003	+1.3‰

[1]Piecewise Linear Regression Model standard error = 0.656; $P < 0.001$; *Adj.* R^2 = 0.79 (*df* = 550); *x*-axis estimated break-points on set plot position (±SE) = 13.5 ± 0.5 mm, 0.7 ± 0.5 mm, -21.4 ± 4.8 mm, and -32.6 ± 3.1 mm.

[2] Piecewise Linear Regression Model standard error = 0.526; $P < 0.001$; *Adj. R^2* = 0.15 (*df* = 139); *x*-axis estimated break-points (±SE) = -2.2 ± 1.8 mm, 16.9 ± 2.2 mm, and 48.2 ± 7.6 mm.

[3] Piecewise Linear Regression Model standard error = 0.724; $P < 0.001$; *Adj. R^2* = 0.48 (*df* = 799); *x*-axis estimated break-points (±SE) = -1.3 ± 1.1 mm, 9.4 ± 1.4 mm, and 115.5 ± 0.7 mm. Plucked whisker values excluded.

[4] Piecewise Linear Regression Model standard error = 0.335; $P < 0.001$; *Adj. R^2* = 0.09 (*df* = 172); *x*-axis estimated break-points (±SE) = 3.1 ± 1.7 mm, 12.9 ± 2.2 mm, and 42.0 ± 6.0 mm.

[†]Breakpoint during foraging

Effects of Fasting on Amino Acid δ^{15}N Values.

We observed both increases and decreases in the δ^{15}N values of individual amino acids measured along the length of the whisker regrowth segments synthesized during a known catabolic and anabolic period, respectively ($n = 5$ juveniles; $n = 10$ samples; Fig. 2). A paired Wilcoxon Signed-Rank Test indicated that the rank sum of the δ^{15}N of the trophic amino acids, aspartic acid and proline ($Z = 2.611$, $P < 0.01$), were higher during the post-weaning fast as compared to the independent foraging period (Table 1S). The δ^{15}N values of the branched-chain amino acids, leucine, isoleucine, and valine remained unchanged. The δ^{15}N values of several source amino acids were > 2‰ higher during fasting, including lysine ($Z = 2.193$, $P < 0.05$), phenylalanine ($Z = 2.402$, $P < 0.05$), and tyrosine ($Z = 2.402$, $P < 0.05$; Table 1S). The δ^{15}N of threonine was lower when fasting ($Z = 2.611$, $P < 0.01$), increasing by 5.0‰ during active foraging. Glycine and serine were both ($Z = 2.611$, $P < 0.01$) enriched by 6.3‰ during fasting as compared to foraging. Surprisingly, the δ^{15}N value of the trophic amino acid alanine was 2.4‰ lower during the fast ($Z = -2.311$, $P < 0.05$) than during foraging.

The δ^{15}N of the amino acids measured along the length of the whiskers of the breeding adult females ($n = 10$ individuals; $n = 30$ samples; Fig. 3) and the one adult male (Fig. 2S) showed a similar pattern of increases and decreases as observed in juvenile SES (Fig. 2). The glycine δ^{15}N value in the tip of adult female whiskers, likely reflecting fasting on land, was 2.4‰ higher than in the middle and basal segments (Kruskal-Wallis $\chi^2 = 12.963$, $df = 2$, $P < 0.001$) that represented at-sea foraging. The serine δ^{15}N value was 2.1‰ higher (Kruskal-Wallis $\chi^2 = 4.560$, $df = 2$, $P = 0.099$) during fasting compared to when foraging (Table 1S). The δ^{15}N value of alanine was 2.1‰ lower (Kruskal-Wallis $\chi^2 = 5.546$, $df = 2$, $P = 0.059$) during fasting as compared to the middle of the whisker when at-sea foraging commenced (Table 1S). There were
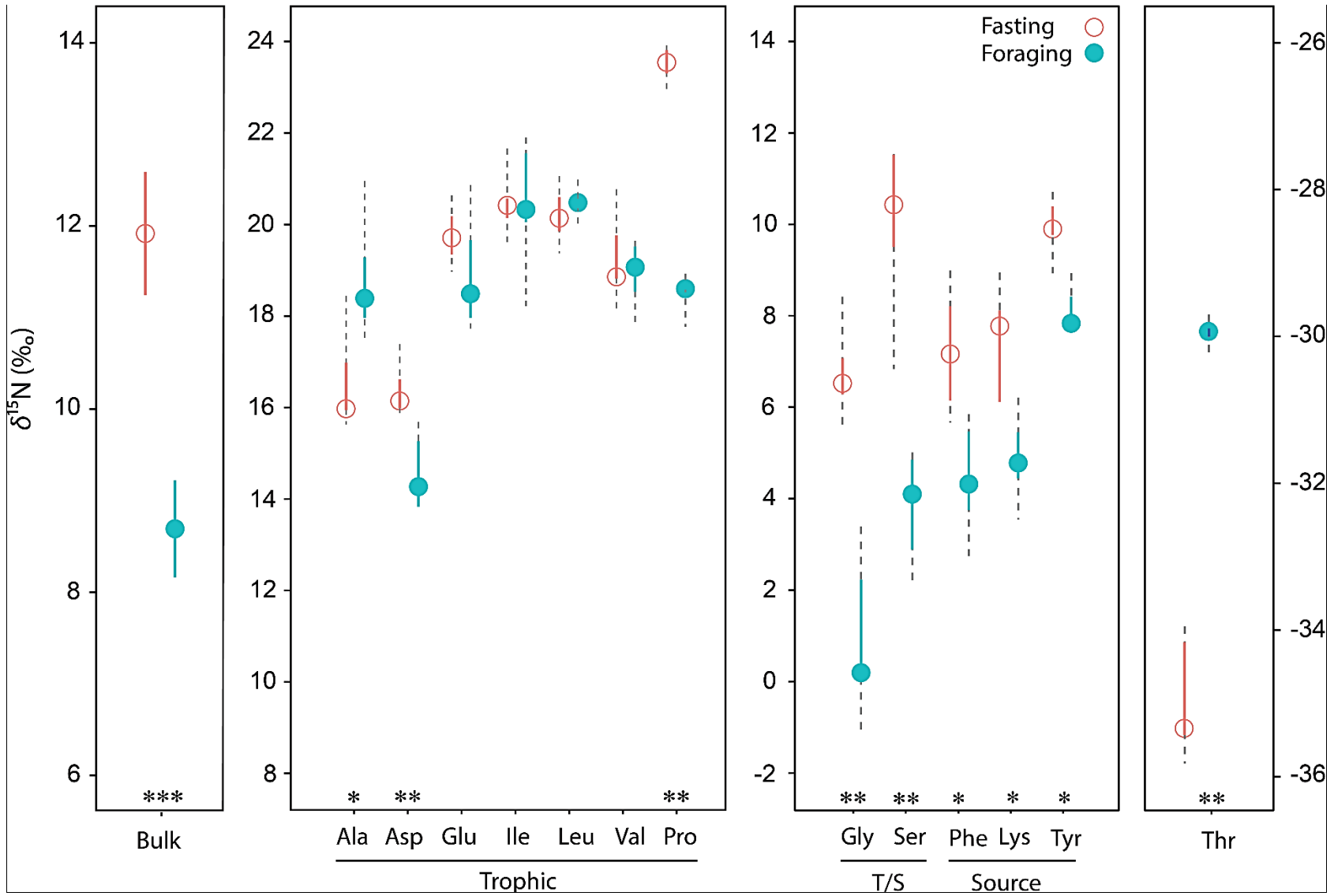
Figure 2: Comparison of the mean ($\pm$SD) bulk tissue δ^{15}N values (left panel) and median amino acid δ^{15}N values of juvenile southern elephant seals ($n = 5$) whiskers reflecting the post-weaning fast (red open symbols) and foraging at-sea (blue open symbols). Solid error bars reflect the 25% and 75% confidence intervals (CI) around the median δ^{15}N values, while the dashed lines reflect the 5% and 95% CI. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Amino acid abbreviations are defined in Methods and Materials.
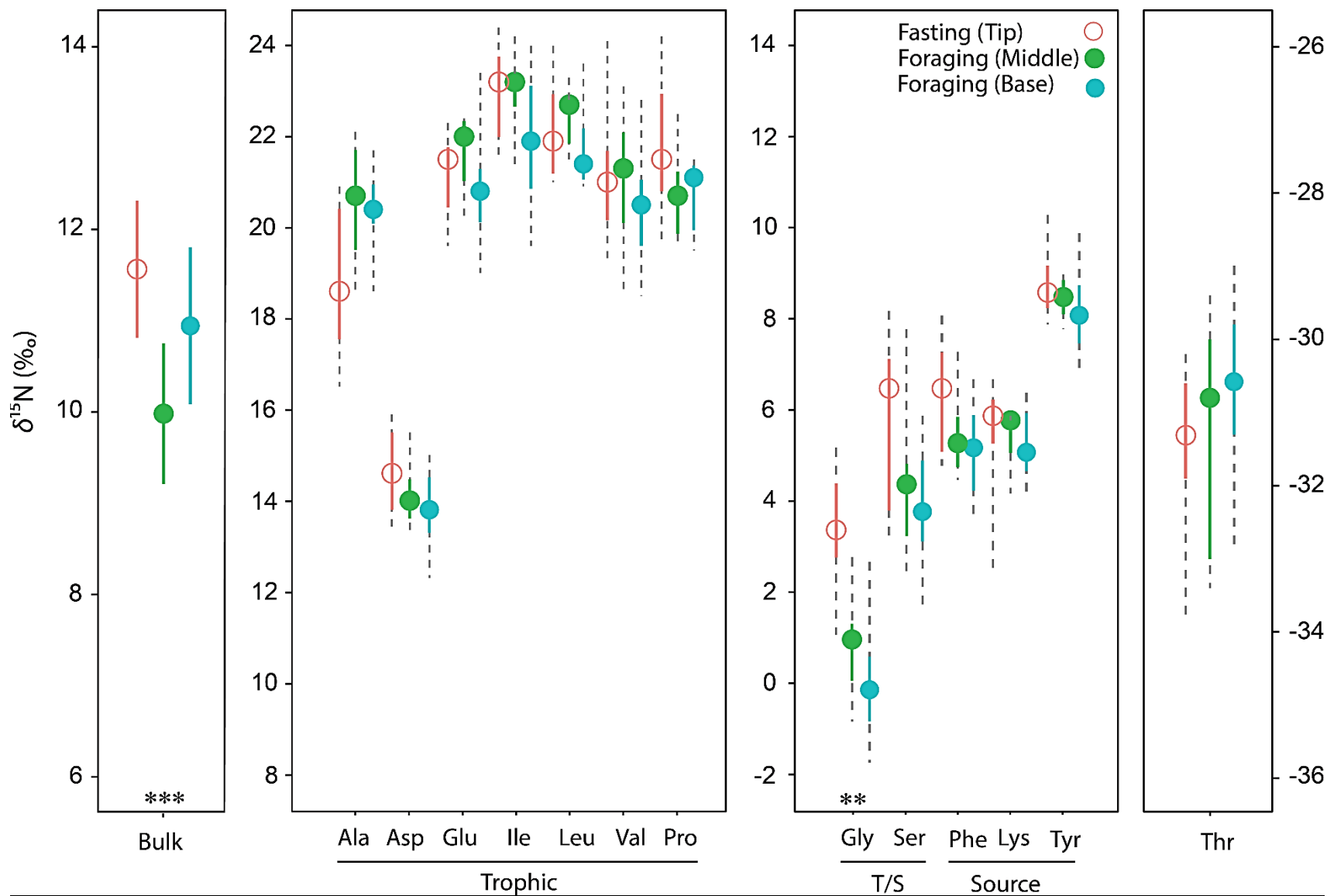
Figure 3: Mean (±SD) bulk tissue $\delta^{15}N$ values (left panel) and median amino acid $\delta^{15}N$ values measured in the tip (red), middle (green) and basal (blue) whisker segments from breeding adult female ($n = 10$) southern elephant seals. Solid error bars reflect the 25% and 75% confidence intervals (CI) around the median $\delta^{15}N$ values and the dashed lines reflect the 5% and 95% CI. **$P < 0.01$. Amino acid abbreviations are defined in the Methods and Materials.

no significant differences between fasting and foraging measured for any of the source amino acids (phenylalanine, tyrosine, lysine), branched-chain amino acids (isoleucine, leucine and valine), other trophic amino acids (proline, aspartic acid, glutamic acid), or threonine.

Identifying Fasting Based on Amino Acid $\delta^{15}N$ values.
The basic LDA models parameterized with the $\delta^{15}N$ of all the amino acids or with the baseline-corrected data ($\Delta^{15}N_{\text{Trophic AA-Phe}}$) produced identical outputs. The training dataset accurately predicted fasting in 96% of the cases, with a single misclassification of actual foraging as fasting. The accuracy of the model based on the testing dataset was 74%, with fasting incorrectly classified as foraging in 12% of the data points. The $\delta^{15}N$ of glycine, serine, alanine and proline were the most significant predictors ($P < 0.001$) for separating foraging and fasting (Table 2S).

Discussion

*Fasting Caused an Enrichment in Whisker Bulk Tissue $\delta^{15}N$ Values.*
Fasting-related enrichment of bulk tissue $\delta^{15}N$ values has been reported in a variety of tissues from various species (Hobson et al. 1993; Fuller et al. 2004; Doi et al. 2017). Here, we observed bulk tissue $\delta^{15}N$ enrichments of 0.8‰ and 1.8‰ in the tip of subadult and adult whiskers with unknown whisker growth histories, respectively (Fig. 1b, c, d), which is comparable to the fasting-related $\delta^{15}N$ enrichment observed in the whiskers segments of juvenile SES that were synthesized during a known catabolic period while fasting on land (Lübcker et al. 2016, 2017). The consistency of the location of the segments exhibiting the fasting-associated ^{15}N-enrichment in the whiskers of SES adults supports that elephant seals shed their whiskers synchronously during the annual pelage molt (Lübcker et al. 2016; Aurioles-Gamboa et al. 2019; McHuron et al. 2019). Thus, the longitudinal record captured in subadult and adult SES whiskers essentially represents the ~8-month period of the foraging trip that commences at the end of the molt and

continues until the onset of the breeding season when the whiskers were collected (Fig. 1S). This enables the identification of segments of the whiskers representing the phase when SES are solely relying on exogenous energy sources, and thus accurate reconstructions of habitat use and diet for phocids that can function as sentinels of ocean health (Fedak 2004).

Fasting Caused an Enrichment in Glucogenic Amino Acid $\delta^{15}N$ Values.
Amino acid nitrogen isotope data show that the fasting-induced ^{15}N-enrichment in bulk whisker tissue is driven by increases in the $\delta^{15}N$ in the pool of amino acids that are readily deaminated during catabolism (O'Connell 2017). Specifically, glycine, serine, proline, and aspartic acid had significantly higher $\delta^{15}N$ values in whisker segments grown during the post-weaning fast in juveniles and molting period in adult females (Fig. 2, 3). These four amino acids serve as primary substrates for gluconeogenesis, a crucial biochemical pathway through which carnivorous mammals convert exogenous (dietary) or endogenous (body) protein into glucose (Krebs 1964). Our findings suggest that after muscle peptides are hydrolyzed into amino acids, those amino acids containing isotopically light amine groups (^{14}N) are then preferentially deaminated in the first step of gluconeogenesis, resulting in the isotopic enrichment of the remaining free amino acids in plasma that is subsequently used to synthesize whiskers during fasting (Fig. 4). In addition, splanchnic *de novo* synthesis of glycine, serine, and proline (Felig et al. 1969; Kalhan and Hanson 2012; Wang et al. 2013) from endogenous pools of nitrogen that are ^{15}N-enriched relative to exogenous sources can also contribute to the observed increase in $\delta^{15}N$ values of these amino acids in whiskers (Fig. 5); this process is known as the anabolic model of nitrogen flux (Lee et al. 2012). Data from humans show support for the anabolic model, where plasma glycine, serine, and proline concentrations increase during fasting (Felig et al. 1969). Thus, our findings suggest that the $\delta^{15}N$ enrichments (0.4–6.3‰; Table 1S) of these three
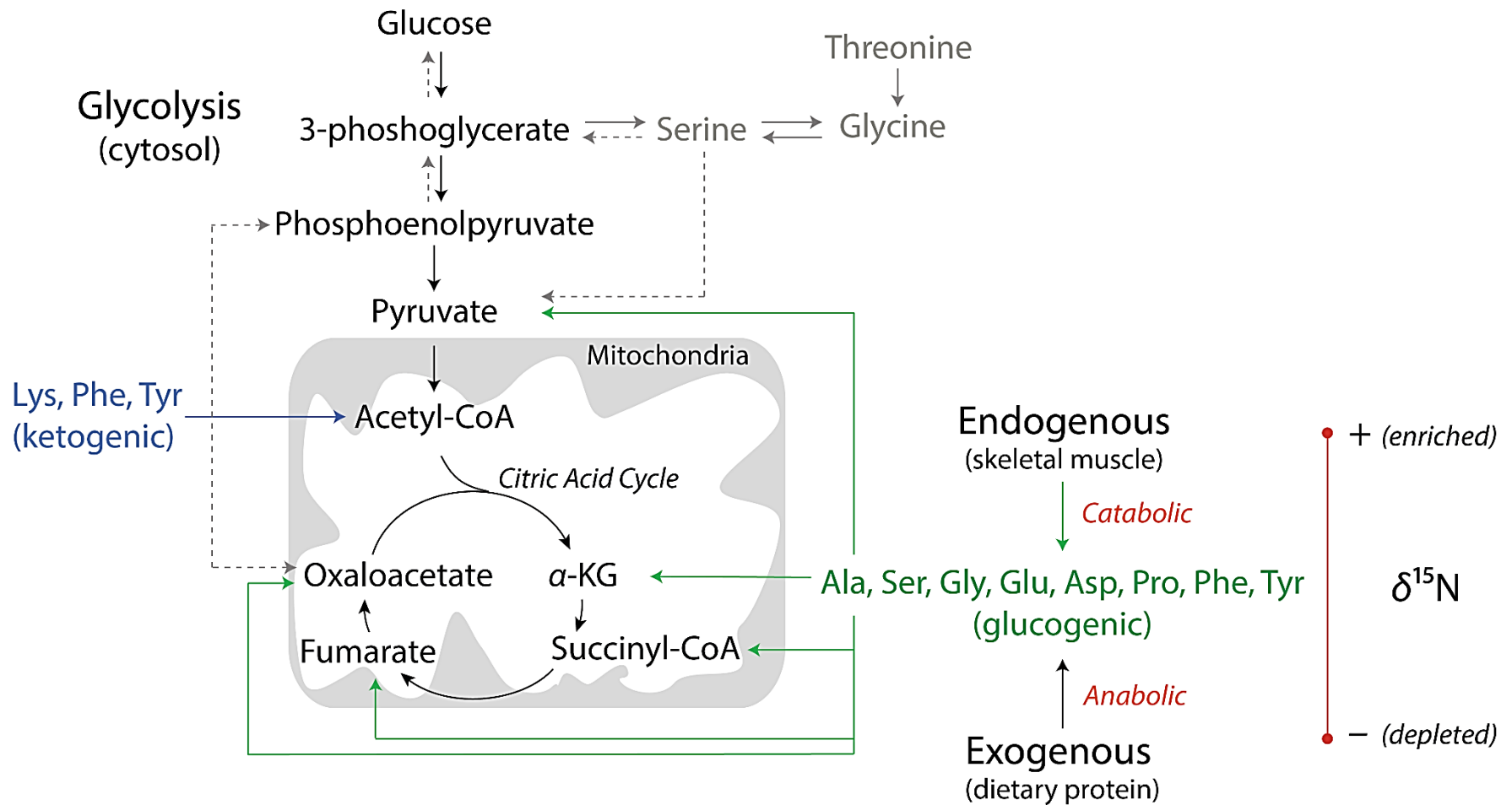
Figure 4: Gluconeogenesis (grey dashed lines) represents a key metabolic pathway that results in nitrogen isotope discrimination when either exogenous (dietary) or endogenous (muscle) protein is catabolized to produce glucose. Both glucogenic amino acids (green) via Citric Acid Cycle intermediaries and ketogenic (blue) amino acids via conversion to acetyl CoA can contribute to gluconeogenesis. Catabolism involves preferential deamination of amino acids containing ^{14}N, which leads to isotopic enrichment of the remaining amino acid pool used to synthesize new tissue (e.g., whiskers).

amino acids could result from their *de novo* synthesis from recycled ^{15}N-enriched nitrogen pools. In regard to proline, its ring structure prevents transamination of its nitrogen (O'Connell 2017), suggesting that the observed increase in δ^{15}N of proline during fasting occurs when ^{15}N-enriched glutamic acid (sourced from ^{15}N-enriched endogenous protein or synthesized *de novo*), is converted into proline (O'Connell 2017). Lastly, aspartic acid is synthesized when the nitrogen of glutamic acid is transaminated to oxaloacetate in the tricarboxylic acid cycle (Macko et al. 1986). The amide transferred from glutamic acid is likely ^{15}N-enriched as a result of gluconeogenesis when fasting. Furthermore, deamination of ^{14}N-containing aspartic acid during the urea cycle and purine synthesis would further enrich the remaining aspartic acid used to synthesize tissues (McMahon and McCarthy 2016).

We observed a decrease in threonine δ^{15}N values during fasting relative to foraging, which may result from selective (enzymatic or microbial) recycling of threonine sourced from intestinal mucin during fasting to maintain amino acid homeostasis (Wallace and Hedges 2016). Although the molecular mechanism responsible for the often-observed negative fractionation of threonine δ^{15}N between consumer and diet is unclear (Whiteman et al. 2019) it nonetheless may be a useful ecological (e.g., trophic level) or physiological (e.g., fasting) biomarker.

Depletion of Alanine δ^{15}N Values During Fasting.
The significant decrease in alanine δ^{15}N values of ~2–3‰ in both juvenile and adult female SES during fasting was unexpected. We suggest that this pattern is driven by isotopic fractionation associated with the glucose-alanine (Cahill) cycle (Fig. 5), which is the dominant metabolic pathway used by vertebrates to (a) transport nitrogen from the muscle to the liver (Felig et al. 1969), and (b) cycle carbon in the form of glucose between the liver and muscle during fasting (Felig et al. 1970). The observed isotopic depletion is likely caused by the transamination of
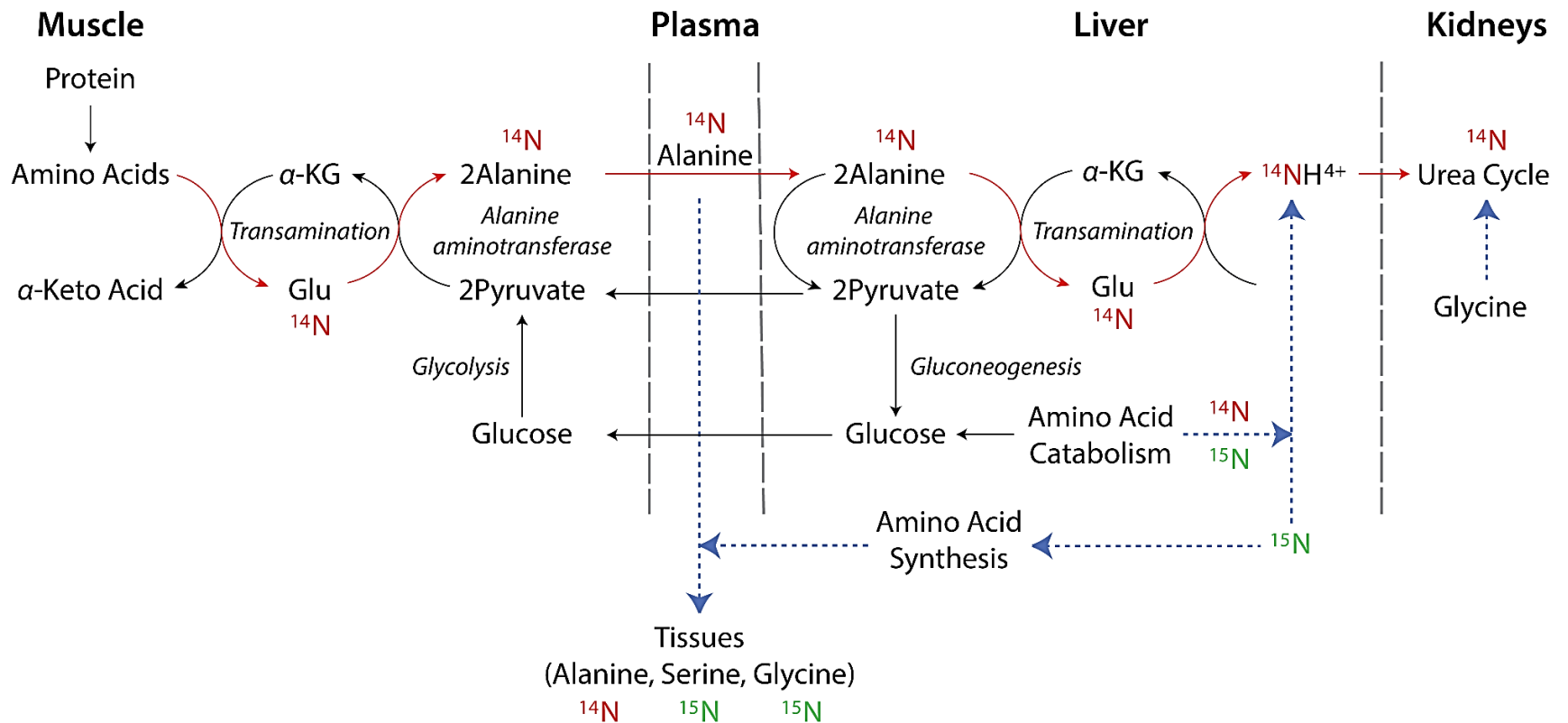
Figure 5: Schematic of the glucose-alanine (Cahill) cycle portraying the mechanism responsible for isotopic depletion of alanine during fasting. Alanine aminotransferase catalyzes the synthesis of alanine from pyruvate, in which isotopically light nitrogen (^{14}N) is transaminated from glutamate. Alanine is then transferred to the liver and deaminated to produce urea while the carbon skeleton is recycled into pyruvate during gluconeogenesis. The pathway by which ^{14}N are delivered from the muscle to the urea cycle is indicated in red. The blue dashed lines depict the pathway by which the ^{14}N-alanine and ^{15}N-enriched gluconic amino acids (glycine and serine) are incorporated into newly synthesized tissues (e.g., whiskers) during fasting.

isotopically light (^{14}N) nitrogen atoms from glutamate to alanine that is transported via blood plasma to the liver where it is deaminated to produce glucose (Krebs 1964; Felig et al. 1970). While in blood circulation, this ^{14}N-alanine can also be used to maintain actively growing tissues like whiskers during fasting.

Lack of Change in δ^{15}N Values of Branched-Chain Amino Acids and Glutamic Acid.
While the branched-chained amino acids valine, leucine, and isoleucine can be catabolized to fuel for gluconeogenesis, the lack of change in their δ^{15}N value suggests that these amino acids are being spared during fasting. These three amino acids are considered essential for most eukaryotes and cannot be synthesized *de novo*, thus it may be advantageous to spare them and keep their catabolism to a minimum during periods of fasting, a strategy that has been previously shown to occur in nutritionally stressed captive mammals (Adibi 1980; Tom and Nair 2006). In addition, the interchangeability of the amine group between branched-chain amino acids and glutamic acid during amino acid catabolism, anabolism, and urea (^{14}N) nitrogen recycling may reduce isotopic fractionation leading to ^{15}N-enrichment (O'Connell 2017).

We also observed no difference in whisker glutamic acid δ^{15}N values between fasting and foraging for juvenile and adult SES. We suggest that the lack of change is likely the result of the large degree of nitrogen exchange between glutamic acid and other amino acids (O'Connell 2017), combined with the fact that glutamate and glutamine represent the largest nitrogen pool in the body. The insensitivity of glutamic acid nitrogen isotope values to fasting is beneficial for studies of trophic ecology, which commonly use the δ^{15}N value of this amino acid to estimate the trophic position occupied by consumers (Chikaraishi et al. 2009). Yet, when combined with observed patterns in source amino acid δ^{15}N values (see below), this finding contradicts our expectation that the offset between the δ^{15}N values of trophic and source amino acids of animals

should increase during fasting relative to when their energy demands are satisfied by exogenous (dietary) resources.

Fasting Leads to Increases in $\delta^{15}N$ Values of Source Amino Acids.
Source amino acids like phenylalanine and lysine are expected to have unchanged $\delta^{15}N$ values during fasting, similar to branched-chained amino acids. Contrary to expectations, whisker phenylalanine, lysine, and tyrosine $\delta^{15}N$ values, however, significantly increased by ~2–4‰ during fasting in both pups (Fig. 2) and adult females (Fig. 3), suggesting that these amino acids were catabolized to fuel gluconeogenesis; tyrosine is considered to be a conditionally essential amino acid because it can only be synthesized from phenylalanine. Importantly, phenylalanine and lysine are irreversibly lost when catabolized during fasting because they are essential amino acids that cannot be synthesized de novo. The $\delta^{15}N$ difference ($\Delta^{15}N_{Glu-Phe}$) between glutamic acid (trophic) and phenylalanine (source) has been widely used to infer the trophic level (TL) of marine consumers (Chikaraishi et al. 2009; Germain et al. 2013; McMahon and McCarthy 2016). Because $\delta^{15}N$ values of source amino acids are often assumed to be insensitive to TL and physiology (Chikaraishi et al. 2009), the enrichment of phenylalanine has a strong influence of TL estimates for SES. For example, the $\Delta^{15}N_{Glu-Phe}$ of foraging adult female SES during gestation was 16.0‰, which produces a TL estimate of ~3.5. If we assume that nursing pups are feeding approximately one TL above adult females (Jenkins et al. 2001), we would expect to observe $\Delta^{15}N_{Glu-Phe}$ of ~23.6‰ and an associated TL estimate of ~4.5. In contrast, the unexpectedly stable $\delta^{15}N$ values of glutamic acid and higher phenylalanine observed during fasting resulted in these pups having a $\Delta^{15}N_{Glu-Phe}$ of ~12.5‰ and a calculated TP of ~3.1. In other words, the trophic-source offset in nitrogen isotope values placed the pups at roughly half of a trophic level lower than their mothers, rather than at a trophic level higher. This discrepancy, combined with the

observed increase in phenylalanine, lysine, and tyrosine δ^{15}N values during fasting suggests that physiological status must be taken into account when interpreting offsets in amino acid δ^{15}N values as proxies for trophic level (Chikaraishi et al. 2009).

Accuracy of Identification of Fasting Events with Amino Acid δ^{15}N Values.
The various patterns in δ^{15}N enrichment enabled us to identify fasting events with an accuracy of 74%. The small sample size used for model testing and possible inclusion of whisker segments that reflected a mixture of fasting and foraging potentially reduced the predictive power of the models (Fig. 2S). Nevertheless, the correlation coefficients (R^2 values) met the required criteria for biomarker evaluation (Yun et al. 2018), even though our study was focused on free-ranging animals. The bulk whisker tissue δ^{15}N enrichments associated with fasting in juveniles (1.2‰) was similar to that observed in adult females (1.3‰) (Table 2). The mass of protein lost as a percentage of body weight for fasting adult female SES during the molt (14%) is comparable to that lost by juveniles (12–32%) during the post-weaning fast (Boyd et al. 1993; Carlini et al. 2001). The higher body masses and slower tissue turnover rates of adult female SES may be a factor in explaining why the fasting-associated increases in δ^{15}N values for some amino acids were smaller in magnitude (Fig. 3) in comparison to those observed in juveniles (Fig. 2).

Conclusions.
Irrespective of the specific biochemical pathways involved, our study illustrates the utility of using both bulk tissue and amino acid isotope analysis as nutritional biomarkers. Our sampling methodology and analytical approach provide a longitudinal record of nutrient inputs (diet), trophic position, and physiological status (e.g., nitrogen-balance) for consumers (Mekota et al. 2009; McCue et al. 2010). Non-invasively obtaining such information from a single tissue

sample is currently unattainable with other techniques (Reitsema 2013; Ohkouchi et al. 2017; Yun et al. 2018).

Our study establishes that it is feasible to identify periods of fasting by bulk tissue δ^{15}N analysis, while also providing a new compound-specific amino acid framework for assessing nitrogen balance. Specifically, substantial isotopic enrichments (~2–6‰) in glucogenic and even some ketogenic amino acids are likely driven by a switch in the catabolism of exogenous (prey) amino acids during foraging to endogenous (skeletal muscle) amino acids during fasting for gluconeogenesis. The significant depletion in alanine δ^{15}N during fasting is likely associated with the glucose-alanine cycle that shuttles nitrogen from muscle to liver to fuel amino acid metabolism.

Moreover, we can also accurately identify the segment of the whisker grown while free-ranging SES are on land without prior knowledge of whisker growth histories. These findings show that continuously growing, but metabolically inert keratinaceous tissues like whiskers and baleen, can be used to construct a longitudinal dietary and physiological record of an individual that can span months (phocids) to years (mysticetes) depending on the species. Importantly, the isotopic variation associated with physiological challenges like fasting must be taken into consideration when interpreting isotope-based reconstructions of diet. This is especially important for species with complex annual life histories (e.g., marine mammals) that include extended periods of fasting associated with migration and/or reproduction.

Acknowledgements

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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