Short title: Towards the development of a nutritional biomarker.

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

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Supplementary Material

Sample Collection and Whisker Subsampling.

Regrown whiskers sampled from juvenile SES were first carefully split in half longitudinally along the midline of the whiskers using a scalpel. One half was used for bulk isotope analysis; then, based on the obtained $\delta^{15}N$ data, we subsampled the other half in two subsegments per whisker for the amino acid $\delta^{15}N$ analysis (n = 5 individuals). Following this approach, we ensured that we only included the segments of each whisker regrowth synthesized either during the post-weaning fast (catabolic state) or while foraging at sea (an anabolic state) (e.g., Fig. 1a).

Similar to using the bulk tissue $\delta^{15}N$ data of the juvenile whiskers to direct the subsampling for amino acid $\delta^{15}N$ analysis, we used one of two whiskers of equal length, sampled from the same adult female, to obtain the bulk tissue $\delta^{15}N$ values; this was then used to inform the subsampling approach of the adult female amino acid $\delta^{15}N$ analysis (e.g., Fig. 2S). The 118.3 ± 12.7 mm long whiskers of the adult females (n = 10 individuals) were subsampled into three segments (base, middle, and tip). The basal segment represented the proximal 2.6–18.9 mm of the whisker (base), while the middle section spanned from 41.0–55.4 mm from the base of the whisker. The distal segment represented the finer tip of the whisker that had a mean (±SD) length of 34.1 ± 12.3 mm. We assumed that the two near-equal length whiskers sampled from the same individual grew synchronously, as previously demonstrated (Lübcker et al. 2016). We did not include any subadult whiskers for the amino acid $\delta^{15}N$ analysis.

Additional Sampling Considerations.

The bulk tissue $\delta^{15}N$ values of the female's whiskers were used to infer the subsampling of the sections used for amino acid-specific $\delta^{15}N$ values. The fasting signature is captured in the distal 38.4 ± 12.0 mm (18–60.6 mm) of the whisker, which starts growing at the end of the molt. Yet,

to obtain the 7.7 ± 2.8 mg (mean ± SD) required for the amino acid-specific $\delta^{15}N$ analysis, we included the distal 34.1 ± 12.3 mm (11.0–57.0 mm) of the whiskers. Considering that the $\delta^{15}N$ enrichment became progressively more enriched closer to 38.4 mm, we inevitably included segments of the tip of the whiskers not yet affected by the fast (ca. first 15 mm of whisker), despite being careful (Fig. 2S). This would diminish some of the differences observed between fasting and foraging. However, this was not an issue when using the thicker whisker regrowths of the juvenile SES.

Statistical Analyses.

A Linear Discriminant Analysis (LDA) was used to classify the amino acid $\delta^{15}N$ values as either being synthesized during fasting or active foraging using the *MASS* package in R (Venables and Ripley 2002); seed was set to 123. The amino acid $\delta^{15}N$ values during fasting (n = 16 data points) and foraging (n = 27 data points) in the whiskers of juvenile, breeding adult females, and one adult male SES were divided for model training (n = 24 data points, 60% of data) and testing (n = 19 data points, 40% of data). Also, when comparing longitudinal isotope data of animals like SES that forage over larger geographical ranges (Tosh et al. 2012), it is important to consider that the $\delta^{15}N$ at the base of the food web might also change. One advantage of amino acid $\delta^{15}N$ analysis is that the isotopic composition of source amino acids (e.g., Phe or Lys) can be used to assess shifts in isotopic baselines (Ohkouchi et al. 2017). We accounted for any baseline shifts in $\delta^{15}N$ by using the $\Delta^{15}N_{X\text{-}Phe}$ (where X is a trophic amino acid) offsets to construct a second comparable model. Multivariate normality was confirmed using the *ICS* package in R (Nordhausen et al. 2008). Model evaluation was done by comparing the accuracy of the testing and training datasets, as well as by using a leave-one-out cross-validation

procedure performed using the *Displayr/flipMultivariates* package in R (https://rdrr.io/github/Displayr/flipMultivariates/, *accessed* 2019/02/10).

The LDA including all 43 data points, validated using a cross-validation approach and building the LDA model in the *flipMultivariates* R package, had an accuracy of 95.4% (fasting: 87.5%; foraging: 100%) (Table 2S). The $\delta^{15}N$ of glycine ($R^2 = 0.53$), serine ($R^2 = 0.29$), alanine ($R^2 = 0.29$), and proline ($R^2 = 0.27$), where the most significant predictors ($P < 0.001$) separating foraging and fasting.

References.

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Supplementary Tables.

Table 1S: Differences (Δ*)* in the median $\delta^{15}N$ values of source (S) or trophic (T) amino acids reflecting different life-history events captured along the length of whiskers sampled from juvenile (n = 5) and breeding adult female (n = 10) southern elephant seals (*Mirounga leonina*). The adult female whiskers were sectioned into n = 3 segments, with the tip of the whisker representing fasting on land, while the middle and base of the whisker represented active foraging at sea. Threonine is considered a metabolic amino acid (M), whilst serine and glycine can be synthesized *de novo* and are either source (S) or trophic amino acid (T), depending on the diet and nutritional requirements of the organism. The amino acid $\delta^{15}N$ values are expressed as median and 95% confidence intervals in parts per mil (‰).

Group	Amino acid	Juveniles (Whisker Regrowths)			Adult Females				
		Fasting (*Catabolism*)	Foraging (*Anabolism*)	$\Delta^{15}N_{Fast\text{-}Foraging}$	Fasting (*Catabolism*)	Foraging (Middle)	$\Delta^{15}N_{Fast\text{-}Middle}$	Foraging (Base)	$\Delta^{15}N_{Fast\text{-}Base}$
M	Thr	-35.3 (-35.8; -34.0)	-29.9 (-30.2; -29.7)	+5.4**	-31.3 (-33.8; -30.2)	-30.8 (-33.4; -29.4)	+0.5	-30.6 (-32.8; -29.3)	+0.7
S	Lys	7.8 (6.1; 9.0)	4.8 (3.6; 6.2)	-3.0*	5.9 (2.4; 6.7)	5.8 (4.2; 5.9)	-0.1	5.1 (4.2; 6.4)	-0.8
S	Phe	7.2 (5.7; 9.0)	4.3 (2.8; 5.9)	-2.8*	6.5 (4.8; 8.1)	5.3 (4.5; 7.3)	-1.2	5.2 (3.6; 6.7)	-1.3
S	Tyr	9.9 (8.9; 10.7)	7.9 (7.7; 9.0)	-2.1*	8.6 (7.9; 10.3)	8.5 (7.8; 9.0)	-0.1	8.1 (6.8; 9.9)	-0.5
T	Ile	20.4 (19.6; 21.7)	20.3 (18.2; 21.9)	-0.1	23.2 (21.5; 24.4)	23.2 (21.3; 24.2)	0.0	21.9 (19.6; 24.0)	-1.3
T	Val	18.9 (18.1; 20.8)	19.1 (17.9; 19.7)	-0.2	21.0 (19.2; 24.1)	21.3 (18.6; 23.1)	+0.3	20.5 (18.5; 22.8)	-0.5
T	Leu	20.2 (19.4; 21.1)	20.5 (20.0; 21.0)	-0.3	21.9 (21.0; 24.0)	22.7 (21.5; 23.3)	+0.8	21.4 (20.9; 23.6)	-0.5
T	Asp	16.2 (15.9; 17.4)	14.3 (13.8; 15.7)	-1.9**	14.6 (13.4; 15.9)	14.0 (13.3; 15.5)	-0.6	13.8 (12.3; 15.0)	-0.8
T	Glu	19.7 (19.0; 20.7)	18.5 (17.7; 20.9)	-1.2	21.5 (19.6; 22.3)	22.0 (20.2; 22.4)	+0.5	20.8 (19.0; 23.4)	-0.7
T	Ala	16.0 (15.7; 18.5)	18.4 (17.5; 21.0)	+2.4*	18.6 (16.5; 20.9)	20.7 (18.6; 22.1)	+2.1	20.4 (18.6; 21.7)	$+1.8^{\dagger\dagger}$
T	Pro	23.6 (23.0; 23.9)	18.6 (17.8; 18.9)	-4.9**	21.5 (19.6; 24.2)	20.7 (19.6; 22.5)	-0.8	21.1 (19.5; 21.5)	-0.4
T/S	Ser	10.4 (6.9; 11.6)	4.1 (2.2; 5.0)	-6.3**	6.5 (3.2; 8.2)	4.4 (2.5; 7.8)	-2.1	3.8 (1.6; 5.9)	$-2.7^{\dagger}$
T/S	Gly	6.5 (5.5; 8.4)	0.2 (-1.1; 3.4)	-6.3**	3.4 (1.1; 5.2)	1.0 (-0.8; 2.8)	-2.4	-0.1 (-1.7; 2.7)	-3.5**

*$P < 0.05$, **$P < 0.01$, $^{\dagger}P = 0.099$, $^{\dagger\dagger}P = 0.059$.

Table 2S: Linear Discriminate Analyses model that included all 43 $\delta^{15}N$ amino acid measures and used a cross-validation approach predicted fasting with an accuracy of 95.4% (fasting: 87.5%; foraging: 100%). The median $\delta^{15}N$ values of source (S) or trophic (T) amino acids contrasting a catabolic (fasting) and anabolic state (foraging) captured along the length of whiskers sampled from juvenile (n = 5), breeding adult female (n = 10), and one adult male southern elephant seal (*Mirounga leonina*) are shown. Threonine is considered a metabolic amino acid (M), whilst serine and glycine can be synthesized *de novo* and are either source (S) or trophic amino acid (T), depending on the diet and nutritional requirements of the organism. The median amino acid $\delta^{15}N$ values are expressed in parts per mil (‰). The $\delta^{15}N$ values of serine, glycine, proline, phenylalanine, and alanine were the most significant predictors ($p <$ 0.001) separating foraging and fasting. The correlation coefficients (R^2 values) are comparable to the requirements to meet the criteria for biomarker evaluation, despite not being a controlled feeding study and limited sample size.

Group	Amino acid	Fasting (n = 16) (*Catabolism*)	Foraging (n = 27) (*Anabolism*)	R^2 *values*	*P* values
M	Threonine	-32.7	-31.0	0.15	**0.018****
S	Lysine	6.1	5.3	0.10	0.069
S	Phenylalanine	6.8	5.3	0.23	**0.002****
S	Tyrosine	9.2	8.5	0.13	**0.029***
T	Isoleucine	22.1	22.2	0.00	1.000
T	Valine	20.5	20.7	0.00	1.000
T	Leucine	21.6	22.0	0.02	0.528
T	Aspartic Acid	15.4	14.2	0.19	**0.006****
T	Glutamic Acid	20.8	21.1	0.01	0.872
T	Alanine	18.1	20.3	0.29	**<0.001*****
T	Proline	22.3	20.5	0.27	**<0.001*****
T/S	Serine	7.2	4.3	0.29	**<0.001*****
T/S	Glycine	4.5	0.6	0.53	**<0.001*****

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Supplementary Figure Legends.

Figure 1S: Life cycle of a) juvenile and b) adult southern elephant seals (SES; *Mirounga leonina*) corresponding to the whisker sampling design. We sampled (by cutting) whiskers from SES pups direct after being weaned during the post-weaning fast. These *in utero* grown whiskers represent the gestation period (green). After spending a year foraging at sea (anabolic states in blue), we resampled the resulting whisker regrowth during their first obligatory pelage molt in December (red; catabolic states). The whisker growth of adult female SES (b) commences at the end of the annual pelage molt (Jan.) when their whiskers are likely shed (red; Lübcker et al., 2016). The delayed blastocyst implantation occurs at the end of the molt and the growth of these whiskers represents the entire gestation period. The adult female whiskers were collected once-off during the subsequent breeding season in October. Solid lines represent the portion of the SES life cycle reflected by the whiskers included herein

Figure 2S: Comparison between sampling approach used to measure the bulk (whole sample) $\delta^{15}N$ values (top) and $\delta^{15}N$ amino acid analysis (bottom) captured in the α-keratin along the length of whisker sampled from a single adult male southern elephant seal (*Mirounga leonina*). The whisker was subsampled into 46 segments for the bulk $\delta^{15}N$ analyses and $n = 3$ segments for the $\delta^{15}N$ amino acid analysis. The bulk tissue $\delta^{15}N$ values measured in the tip 42 mm of the 105 mm long whisker while fasting (on land) during the annual pelage molt are bounded by the light grey box (left). Thereafter, the $\delta^{15}N$ values equilibrate to the new diet during the post-molt foraging trip, depleting the $\delta^{15}N$ by 1.7‰ over 12 mm. The red vertical line indicates the onset of the whisker isotopic data unaffected by fasting (51–86 mm from the tip of the whisker). Data points in the dark grey box measured at the base of the whisker (proximal 16 mm) are again likely affected by fasting associated with the breeding season and the $\delta^{15}N$ enriched by 2.1‰.

The whisker schematic (top) indicates the position on the whisker (green boxes) where the corresponding $\delta^{15}N$ amino acid analysis samples were subsampled from. Samples representing 'foraging' were subsampled from the middle (B) and base (C) of the whiskers, while the distal (tip) 40 mm of the whisker represented 'fasting' (A). In this case, only 37% of the data (35 mm of the 107 mm whisker) appeared to be unaffected by physiological factors. It is possible that we included segments of the tip of the whiskers not yet affected by fasting (e.g., distal 10 mm) that might have moderated the observed changes in the $\delta^{15}N$ amino acid values during fasting. The depleted $\delta^{15}N$ of alanine during fasting and concurrent glycine enrichment are comparable to the pattern observed in juveniles (Fig. 2) and adult female SES (Fig. 3)

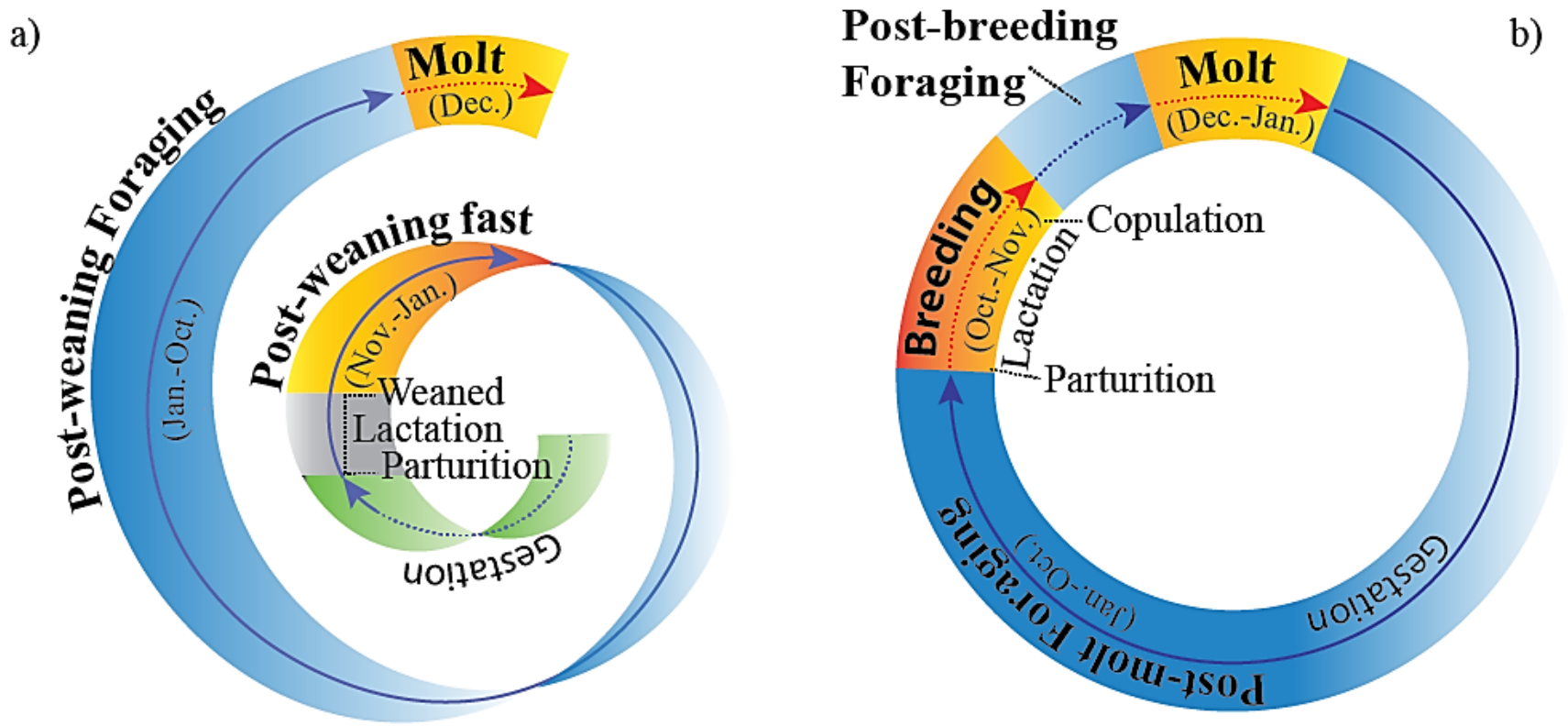
a)
Molt
(Dec.)
Post-weaning Foraging
(Jan.-Oct.)
Post-weaning fast
(Nov.-Jan.)
Weaned
Lactation
Parturition
Gestation
Post-breeding
Foraging
b)
Molt
(Dec.-Jan.)
Breeding
(Oct.-Nov.)
Copulation
Lactation
Parturition
Post-molt Foraging
(Jan.-Oct.)
Gestation

Fig. 1S.

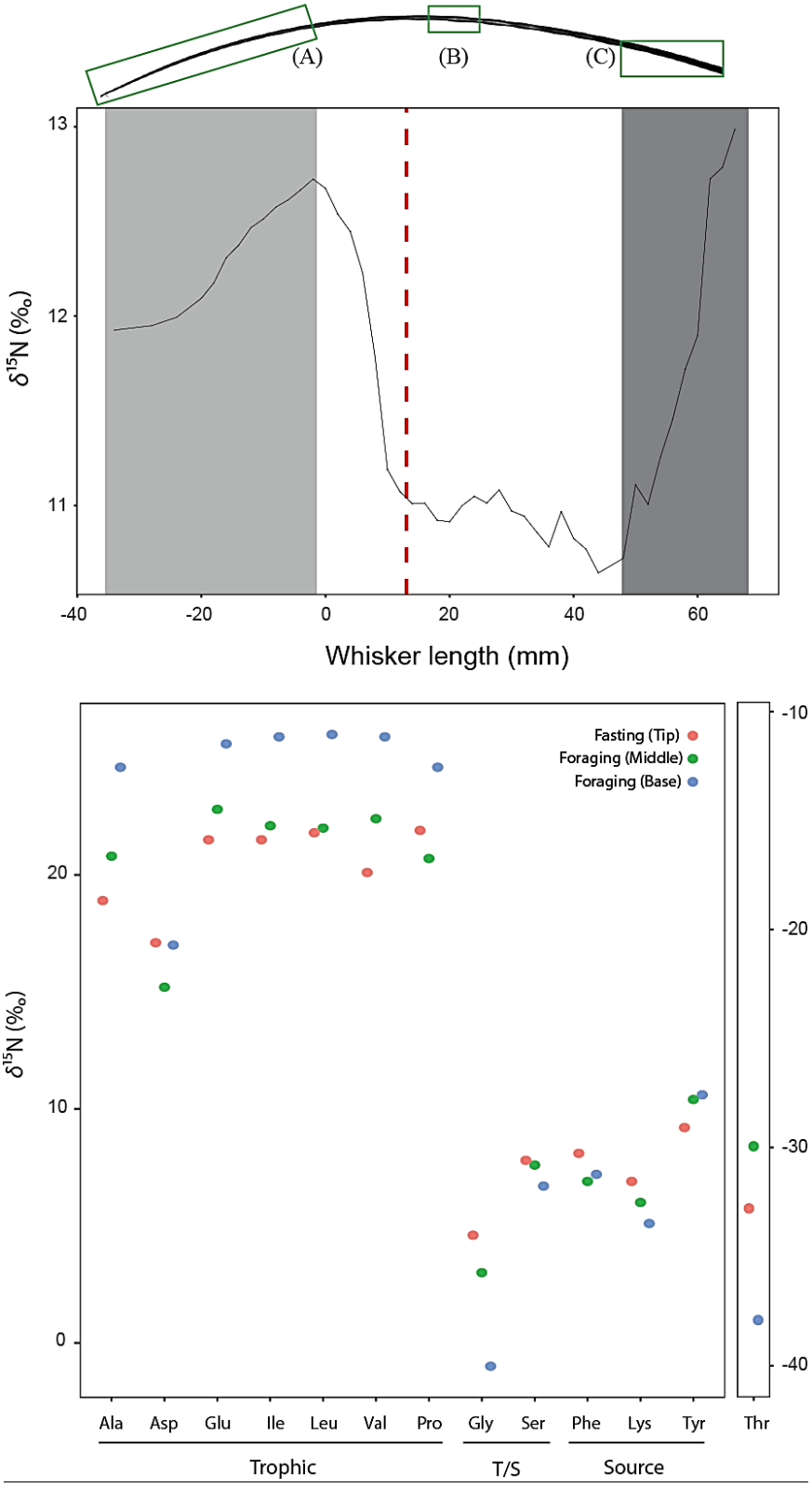
(A)
(B)
(C)
13
12
11
δ15N (‰)
-40
-20
0
20
40
60
Whisker length (mm)
Fasting (Tip)
Foraging (Middle)
Foraging (Base)
20
10
0
δ15N (‰)
-10
-20
-30
-40
Ala
Asp
Glu
Ile
Leu
Val
Pro
Gly
Ser
Phe
Lys
Tyr
Thr
Trophic
T/S
Source

Fig. 2S.