Changes in stable isotope compositions during fasting in phocid seals

Sarah Habran[1,2,§], France Damseaux[1,§], Paddy Pomeroy[3], Cathy Debier[4], Daniel Crocker[5], Gilles Lepoint[1], Krishna Das[1*]

[1] Freshwater and Oceanic sciences Unit of reSearch (FOCUS), Laboratory of Oceanology, University of Liège B6c, 11 Allée du 6 Août, 4000 Liège, Belgium

[2] ISSeP, Institut Scientifique de Service Public, 200 rue du Chéra, 4000 Liège, Belgium

[3] Sea Mammal Research Unit, Scottish Oceans Institute, East Sands, University of St Andrews, KY16 8LB, UK

[4] Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud 2/L7.05.08, 1348 Louvain-la-Neuve, Belgium

[5] Department of Biology, Sonoma State University, Rohnert Park, CA 94928, USA

§: These authors contributed equally to the work

*Corresponding author: krishna.das@uliege.be

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/rcm.8308

Abstract

RATIONALE: The grey seal, *Halichoerus grypus* (GS), and the northern elephant seal, *Mirounga angustirostris* (NES), come ashore for reproduction. This period involves intense physiological processes such as lactation in females and a developmental post-weaning fast in juveniles. Previous studies have shown that $\delta^{13}C$ and $\delta^{15}N$ values are affected by starvation, but the precise effects of fasting associated to lactation and post-weaning fast in seals remain poorly understood.

METHODS: To examine the effect of lactation and post-weaning fast on stable isotope ratios in GS and NES, blood and hair were sampled from twenty-one GS mother-pup pairs on the Isle of May and on twenty-two weaned NES pups at Año Nuevo State Reserve during their respective breeding seasons. Milk samples were also collected from GS mothers. Stable isotope measurements were performed with an isotope ratio mass spectrometer coupled to an N-C elemental analyser.

RESULTS: Changes in stable isotope ratios in blood components during fasting were similar and weak between GS and NES mothers especially in blood cells (GS: $\Delta^{15}N$ = 0.05‰, $\Delta^{13}C$ = 0.02‰; NES: $\Delta^{15}N$ = 0.1‰, $\Delta^{13}C$ = 0.1‰). GS showed a ^{15}N discrimination factor between maternal and pup blood cells and milk, but not for ^{13}C. The strongest relationship between the isotopic compositions of the mother and the pup was observed in the blood cells.

CONCLUSIONS: Isotopic consequences of lactation, fasting, and growth seem limited in NES and GS, especially in medium-term integrator tissues of feeding activity such as blood cells. Stable isotope ratios in the blood of pups and mothers are correlated. We observed a subtle mother-to-pup fractionation factor. Our results suggest that pup blood cells are mostly relevant for exploring the ecology of female seals.

Key words: stable isotopes, seal, lactation, post-weaning fast, reproduction

1. Introduction

Pups of pinnipeds are increasingly used as proxies to investigate maternal foraging strategies thanks to stable isotopes analyses. Indeed, pup isotope ratios reflect those of the females [1–3]. To validate the use of these proxies, the stable isotopes ratios in pup tissues must be linearly correlated with those in maternal tissues. Moreover, investigating the foraging ecology of adult females requires consideration of the isotopic fractionation between mothers and pups, and how this fractionation might change during the course of nursing and weaning. Lactating female seals catabolize their tissues to produce milk. Therefore, nursing pups are placed at a trophic level higher than their mother with a consecutive isotope enrichment [1,3].

Phocid seals such as the grey seal, *Halichoerus grypus* (GS), and the northern elephant seal, *Mirounga angustirostris* (NES), undergo periods of prolonged fasting twice a year, during lactation and the annual moult [4–9]. During lactation, mothers fast for several weeks while secreting a fat-rich milk synthesized from their body reserves (duration: 17-23 days for GS and 24-28 days for NES). Seals may lose between 35 and 57% of stored body reserves during each of these periods [10]. Pups are weaned abruptly and undergo a prolonged land-based post-weaning fast (~1-4 weeks for GS and ~8-10 weeks for NES) before departing to sea and initiating foraging [11–14]. This post-weaning fast is an important developmental time relative to the diving physiology of the pups [6,10]. The metabolic constraints experienced by the animal's body during the breeding season are extreme and can trigger a remobilization of carbon and nitrogen in tissues [15].

Stable carbon and nitrogen isotope ratios (δ^{13}C and δ^{15}N values) have been used widely to study marine mammal ecology [16]. This technique is based on the idea that the isotopic composition of a consumer is a proportional mixing of the isotopic composition of its prey, after accounting for isotopic fractionation in the digestion and assimilation process [17,18]. This fractionation typically results in enrichment in the heavier isotopes (^{13}C and ^{15}N). The variation of the δ^{13}C value between the predator and the prey is usually low. These values are therefore close to that of the diet and are indicators of the primary production supporting the consumer, indicating, for example, the aquatic vs terrestrial, inshore vs offshore, or pelagic vs benthic contribution to food intake [19,20]. At the opposite and of the spectrum, variation of the δ^{15}N value between different trophic levels is typically more marked than for the δ^{13}C value, leading to a predictable increase with trophic level [18,21]. δ^{13}C and δ^{15}N values are also increasingly used in physiology studies of lactation [1,22–25], of energy acquisition [26], or of isotopic relationship between the mother and her offspring [1–3]. In the case of phocids, it is important to consider the effect of fasting periods on their body condition before using pup stable isotopes ratios as proxies of the

foraging behaviour of the mothers. Information regarding the isotopic consequences of fasting during lactation and post-weaning fast is limited, especially in medium-term integrator tissues of feeding activity, such as blood cells. Pinnipeds, which rely on their stored blubber during fasting, should have lower $\delta^{13}C$ values, since lipids are known to be ^{13}C-depleted during biochemical fractionation [17,27]. Many marine mammals also catabolize significant amounts of tissue proteins during lactation and fasting [16,25]. Fasting phocids enrich their tissues in ^{15}N and their blood urea in ^{14}N because of the net catabolic state [8,21,28,29]. In contrast, the anabolic state associated with the protein synthesis [30] can reduce the ^{15}N in the maternal tissues [8] during gestation [32] or lactation [34].

To elucidate changes in isotopic composition during fasting in phocids, we investigated carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$ values) isotopic dynamics in blood cells, serum, milk and hair of GS and NES during lactation and/or post-weaning fast.

2. Material and methods

(a) Sampling

Two longitudinal studies were performed in the grey seal, *Halichoerus grypus* (Fabricius 1791), and the northern elephant seal, *Mirounga angustirostris* (Gill, 1866), during lactation and/or post-weaning fast.

The GS were sampled on the Isle of May, UK(56°11'N, 2°33'W), during the breeding season (October-December 2008). Twenty-one GS mother-pup pairs were captured in early lactation (T1: at 2-4 days postpartum) and recaptured 12-14 days later in late lactation (T2: at 15-17 days postpartum). Maternal and pup blood samples and mother's milk samples were collected at each capture (**Table S1, supporting information**). Whole blood samples were collected from the extradural vein in BD Vacutainer™ red top serum tubes including a silicone-coated interior and increased silica act clot activator. Maternal hair and lanugo were collected only during the second lactation (T2). Sixteen of the 21 pups were recaptured once or twice after weaning (T3 and T4: day 19 and day 30 postpartum, respectively) to collect blood samples. The animal handling and sample collection methods have been described previously [35]. All GS captures and sampling were performed under UK Home Office licence.

Northern elephant seals (NES) were sampled at Año Nuevo State Reserve, CA, USA (37°06'30''N, 122°20'10''W), after the breeding season (February-April 2010). Twenty-two NES weaned pups were captured three times throughout the post-weaning fast period. The captures occurred at week 1 (~35 days postpartum), week 4 (~56 days postpartum) and week 7 (~81 days postpartum) of the post-weaning fast. Whole blood samples were collected from the extradural vein in Vacutainer™ red top serum tubes. Lanugo and new hair were collected at week 1 and week 4, respectively. Extra blood samples from 14 of the 22 weaned pups were collected once more at the very end of the post-weaning fast (week 9, ~92 days postpartum). The animal-handling and sample-collection methods have been described previously [36].

GS and NES females and pups were weighed to the nearest 0.2 kg and 0.1 kg, respectively. The length and axial girth of adult females and weaned pups were measured. Pups were sexed. The GS and NES biometric data are summarized in **Tables S1** and **S2** (supporting information).

All samples were kept on ice in the field (at 4°C). At the end of each day, whole blood samples were centrifuged for 20 min and the cellular component was harvested for analysis. Serum was aliquoted into 5-mL plastic tubes and all samples were stored at -20 °C in the laboratory until analysed.

(b) Sample preparation

Prior to stable isotope analysis, blood cell and serum samples were freeze-dried, and ground with a mortar and pestle into powder. After thawing, hair and lanugo were washed ultrasonically with reagent grade acetone (acetone for analysis, EMSURE®, Merck, Darmstadt, Germany) and were rinsed repeatedly with 18.2 MΩ-cm deionized water to remove exogenous contaminants, according to the method recommended by the International Atomic Energy Agency [37]. Hair samples were then freeze-dried for 24 h. Whole blood (containing Red Blood Cells, RBCs) and metabolically inert tissues constructed of keratin such as hair do not require lipid extraction because they contain only low levels of lipids. The major carrier of fatty acids in these tissues is actually serum albumin, meaning that the serum contains higher levels of lipids than the RBCs [34,38].

We investigated possible effects of lipid removal in serum on its isotopic composition. A randomly chosen subset of freeze-dried serum samples (from grey seal mothers and pups, n = 10) were lipid-extracted using three repeated rinses with 2:1 chloroform:methanol for 3 minutes prior to analysis. These preliminary tests showed that lipid removal did not significantly affect the $\delta^{13}C$ values of serum (data not shown).

(c) Stable isotope ratio analysis

Approximately 1.0 -2.0 mg of freeze-dried blood cells and serum, and 1 mg of hair and lanugo were weighed and loaded into tin boats. Approximately 4-5 mg and 1.5 mg of dried full milk were weighed to obtain nitrogen and carbon isotopic compositions, respectively. All dried masses were measured to the nearest 0.01 mg. Stable isotope measurements were performed with an isotope ratio mass spectrometer (VG Optima – Micromass, Middlewich, UK or IsoPrime100, Elementar, Cheadle Hulme, UK) coupled to an N-C-S elemental analyser (Carlo Erba, Milan Italy or Vario MICRO cube, Elementar) for automated analyses.

The SI ratios are expressed in delta (δ) notation as the deviation from standards in parts per thousand (‰) according to the following equation:

$$\delta X = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right)$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$. In this study, δ values are multiplied by 1000 for easier understanding. Standard values were based on the Vienna PeeDee Belemnite (VPDB) for $\delta^{13}C$ measurements and atmospheric nitrogen for $\delta^{15}N$ measurements. Reference materials were IAEA-N1 ($\delta^{15}N$ = 0.4 ± 0.2‰) and IAEA CH-6 (sucrose) ($\delta^{13}C$ = −10.4 ± 0.2‰). Internal standards (glycine) and replicates were inserted into all runs after every 12 samples to calibrate the system and to assess drift over time. The standard deviations of internal standard replicates were 0.1‰ and 0.3‰ for carbon and nitrogen, respectively.

(d) Statistical analyses

Our data were compiled with in-house data (**Figure 3, Table 3**) previously measured in the blood and milk of ten NES females and their pups during lactation (~ day 5 and day 22 post-partum) [39].

The normal distribution of the data was checked with a Shapiro test and the homogeneity of variances with a Barlett test. Because the data were normally distributed and respected the homogeneity of variances, parametric tests were used for statistical analyses. Statistical significance was determined when $p<0.05$. To evaluate changes in stable isotope and elemental ratios in the different tissues and the different sampling times, analysis of variance (ANOVA) with repeated measures and Scheffé post-hoc test were used. In the case of only two tissues or two sampling times, paired t-tests were used to compare means. The results are presented as mean ± standard deviation (SD). The differences observed between isotopic ratios in different periods or between mother and pup are expressed in uppercase delta notation (Δ).

3. Results

3.1. Tissue variation of $\delta^{13}C$ and $\delta^{15}N$ values in grey seals

In females, the lowest mean $\delta^{15}N$ value was observed in blood cells (14.1‰, at both T1 and T2) and the highest mean value in milk (17.7‰, at T1). The maternal $\delta^{15}N$ values differed between blood cells, serum and milk ($F_{2,80} = 202.4$, $p < 0.001$ at T1 and $F_{2,80} = 104.3$, $p < 0.001$ at T2, ANOVA with repeated measures) while the $\delta^{15}N$ values in serum and milk at T2 were similar (15.0‰ vs. 15.0‰, $p = 1.00$, Scheffé *post-hoc* test). The $\delta^{15}N$ pup values differed between lanugo (only in T2), blood cells and serum at T1, T2, T3, and T4 ($F_{1,59} = 115.3$, $p < 0.001$ at T1, $F_{2,59} = 152.8$, $p < 0.001$ at T2, $F_{1,49} = 168.5$, $p < 0.001$ at T3 and $F_{1,41} = 141.8$, $p < 0.001$ at T4, ANOVA with repeated measures, **Table 1**) but were not statistically different between blood cells and serum ($F_{1,59} = 115.3$, $p = 1.00$ at T1, $F_{1,59} = 152.8$, $p = 1.00$ at T2, $F_{1,49} = 168.5$, $p = 1.00$ at T3 and $F_{1,41} = 141.8$, $p = 1.00$ at T4, ANOVA with repeated measures, **Table 1**).

The lowest $\delta^{13}C$ value was observed in milk (-23.6‰) and the highest in maternal hair (-15.3‰) (**Table 1**). The $\delta^{13}C$ values measured in maternal tissues differed significantly between blood cells, milk, and hair (only at T2) ($F_{1,80} = 202.4$, $p < 0.001$ at T1 and $F_{2,80} = 104.3$, $p < 0.001$ at T2, ANOVA with repeated measures), but were similar between blood cells and serum ($F_{1,80} = 202.4$, $p = 0.285$ at T1 and $F_{1,80} = 104.3$, $p = 0.999$ at T2, ANOVA with repeated measures). The mean $\delta^{13}C$ values differed between pup tissues, i.e. blood cells, serum, and lanugo (only at T2) at T1, T2, T3, and T4 ($F_{1,59} = 115.3$, $p < 0.001$ at T1, $F_{2,59} = 152.8$, $p < 0.001$ at T2, $F_{1,49} = 168.5$, $p < 0.001$ at T3 and $F_{1,41} = 141.8$, $p < 0.001$ at T4, ANOVA with repeated measures).

3.2. Tissue variation of $\delta^{13}C$ and $\delta^{15}N$ values in northern elephant seals

The mean $\delta^{15}N$ values ranged from 15.9‰ in lanugo at week 1 to 18.5‰ in serum at week 9 (**Table 2**). The weaned pup $\delta^{15}N$ values differed between blood cells, serum, new hair (week 1) and lanugo (week 4) at weeks 1, 4, 7 and 9 ($F_{2,84} = 199.7$, $p < 0.001$, $F_{2,84} = 220.8$, $p < 0.001$, $F_{1,84} = 247.6$, $p < 0.001$ and $F_{1,84} = 174.2$, $p < 0.001$ respectively, ANOVA with repeated measures) but they remained similar between serum and new hair at week 1 ($F_{1,84} = 199.7$, $p = 0.814$, ANOVA with repeated measures) and between blood cells and lanugo at week 4 ($F_{1,84} = 220.8$, $p = 0.281$, ANOVA with repeated measures) (**Table 2**).

The NES $\delta^{13}C$ values ranged from -21.0‰ in serum at week 7 to -18.1‰ in new hair at week 1 (**Table 2**). The $\delta^{13}C$ values measured in weaned pup tissue differed significantly between blood cells, serum, new hair (week 1)

and lanugo (week 4) at weeks 1, 4, 7 and 9 ($F_{2,84}$ = 199.7, p < 0.001, $F_{2,84}$ = 220.8, p < 0.001, $F_{1,84}$ = 247.6, p < 0.001 and $F_{1,84}$ = 174.2, p < 0.001, respectively, ANOVA with repeated measures).

3.3. Stable isotope discrimination between grey seal mothers and their pup

In GS, the δ^{15}N values in blood cells were significantly higher in pups than in mothers (Δ^{15}N = + 2.4‰ in T1 and + 2.6‰ in T2, for both p < 0.001, paired t test, **Tables 1 and 5, Figure 1A**). The δ^{15}N values in serum were significantly higher in pups than in mothers (Δ^{15}N = + 1.3‰ in T1 and + 1.8‰ in T2, for both p < 0.001, paired t test, **Tables 1 and 5**). The δ^{15}N value in lanugo was lower than that in maternal hair (Δ^{15}N = 1.4‰, p < 0.001, paired t test, **Tables 1 and 5, Figure 2A**).

The δ^{13}C values differed significantly between mother and pups in blood cells (Δ^{13}C = 0.08‰, p = 0.015 in T1 and Δ^{13}C = 0.2‰, p < 0.001 in T2, paired t test, **Tables 1 and 5, Figure 1B**), in serum (Δ^{13}C = 0.7‰ in T1 and 1.2‰ in T2, for both p < 0.001, paired t test, **Tables 1 and 5**) and in lanugo and maternal hair (Δ^{13}C = 1.5‰, p < 0.001, paired t test, **Tables 1 and 5, Figure 2B**).

3.4. Changes of δ^{13}C and δ^{15}N values during fasting

Grey seal females. Stable isotope ratios did not differ between T1 and T2 in blood cells (Δ^{15}N = 0.05‰, p = 0.244, Δ^{13}C = 0.02‰, p = 0.361, paired t test) but were significantly different in maternal serum (Δ^{15}N = 0.2‰, p < 0.001, Δ^{13}C = 0.3‰, p < 0.001, paired t test) and in milk (Δ^{15}N = -2.7‰, p < 0.001, paired t test; Δ^{13}C = +1.1‰, p < 0.001, paired t test, **Table 1**).

Grey seal pups. Stable isotope ratios in blood cells and in serum did not differ significantly between T1, T2, T3 and T4 (For both δ^{13}C and δ^{15}N values: $F_{3,66}$ = 6.3, p > 0.05; $F_{3,66}$ = 4.0, p > 0.05; ANOVA with repeated measures).

Northern elephant seals. δ^{15}N and δ^{13}C values in blood cells did not vary throughout the post-weaning fast ($F_{3,84}$ = 1.00, p = 0.154 for δ^{15}N and δ^{13}C, ANOVA with repeated measures, **Table 2**). The δ^{15}N values in serum remained stable at the beginning of the fast ($F_{3,84}$ = 11.1, p > 0.05, ANOVA with repeated measures) and then increased at week 7 ($F_{3,84}$ = 11.1, p < 0.001, ANOVA with repeated measures). The δ^{13}C values in serum decreased at the beginning of the fast ($F_{3,84}$ = 11.1, p < 0.001, ANOVA with repeated measures), then remained stable ($F_{3,84}$ = 11.1, p > 0.05, ANOVA with repeated measures). The δ^{15}N and δ^{13}C values in new hair were greater than in lanugo (Δ^{15}N = 1.9‰, p < 0.001, paired t test; Δ^{13}C = 0.17‰, p < 0.030, paired t test, **Table 2**).

4. Discussion

The aim of this study was to elucidate changes in isotopic composition during fasting in phocids. In that goal, we investigated carbon and nitrogen (δ^{13}C and δ^{15}N) isotopic dynamics in blood cells, serum, milk and hair of GS and NES during lactation and/or post-weaning fast.

We demonstrated that changes in the δ^{13}C and δ^{15}N values during lactation and post-weaning fast were similar between the grey seal and the northern elephant seal. Both species showed a nitrogen isotope fractionation between mother and offspring, but the amplitude of this fractionation was tissue-, time-, and species-specific. Specifically, in contrast to the generally described enrichment of δ^{15}N values in pups relative to maternal tissues [22,23,26,40,41], the δ^{15}N values in the lanugo were lower than those in maternal hair (15.1‰ in pups *vs* 16.5‰ in mothers. The two species displayed a similar time-trend profile in the stable isotope ratios during fasting associated with the lactation and the post-weaning period (**Figure 3**). The main results of this study is that isotope ratios in blood cells from pups strongly reflected those of their mothers (**Figure 1**), confirming the idea of using pups as proxies to investigate maternal foraging strategies, habits and places.

4.1. Fractionation between mothers and their pups

In the present study, the stable isotope ratios measured in the blood cells of the pups reflected those of their mothers in a predictable way (r = 0.96, **Figure 1A**). Both phocid species showed a ^{15}N fractionation between maternal and pup blood cells (from 0.6‰ to 2.6‰, **Table 3**). This fractionation is even more important in milk, consistent with the fact that pups do not feed on their mother's blood but on milk: a Δ^{15}N of 1.7‰ was calculated between milk and pups (serum and blood cells) in grey seal pup at the end of their lactation period. A Δ^{15}N of 1.0‰ has previously been described between milk and serum in elephant seal pups at the end of their lactation period [39]. Whole milk is often ^{15}N-depleted compared with other maternal tissues [1]. In grey seal our data suggest higher or similar δ^{15}N values in milk compared with blood cells and serum at the beginning and end of lactation (**Table 2**). Ducatez et al [2] showed similar results in whole blood of southern elephant seals (**Table 3**). No fractionation was observed for ^{13}C in NES and GS (**Table 3).**

4.2. Fasting and stable isotope ratios

Lactation: The δ^{15}N and δ^{13}C values in female were similar between the beginning and the end of the lactation (~ 2 weeks) for both the GS and the NES. Lactating females catabolize their tissues (blubber, muscle) to produce milk without any food intake and therefore an increase of stable isotope ratios was expected [15,42]. A

previous meta-analysis showed large variations in the $\delta^{13}C$ and $\delta^{15}N$ values of various consumer species in relation to starvation [15]. During starvation, the N and C uptake is near zero, but N and C loss by excretion and respiration remains, although at a low rate [43]. Fasting causes an average increase in the $\delta^{15}N$ values of organisms of 0.5‰, depending on the tissue type [44] and fasting duration [45]. $\delta^{15}N$ values are often seen to increase with fasting once an organism begins to catabolize tissues [46,47]. During fasting, an organism first catabolizes its lipid reserves, before switching to catabolize proteins [47,48]. However, this enrichment in ^{15}N occurs when fasting or starvation is severe enough to cause protein, rather than lipid catabolism [45,47]. In grey seals and elephant seals, fasting is associated with an very intense lactation process and the maternal mass and fat content strongly influence the maternal investment in pinnipeds [49]. This rate of lipid energy output in phocids requires extensive lipid mobilization, mainly from blubber [50]. The organs that will become ^{15}N-enriched during a fast are those that maintain significant synthesis which might not be the case for lactating females [51].

Post-weaning fast: Stable isotope ratios in NES weaned pups (present study) are similar to those observed in suckling pups at the end of their lactation period [39]. The $\delta^{15}N$ and $\delta^{13}C$ values in blood cells of both species did not vary significantly during this period. In contrast, NES serum was progressively enriched in ^{15}N during the fast ($\Delta^{15}N$ = +0.9‰), while it was slightly depleted in ^{13}C ($\Delta^{13}C$ = -0.4‰). $\Delta^{15}N$ values increase with fasting time [45] which was longer in NES (9 weeks) than in GS (less than 2 weeks). These changes in the serum were expected since catabolism of protein from lean tissues (*e.g.*, muscle) during periods of nutritional stress may cause an increase of $\delta^{15}N$ values and a decrease of $\delta^{13}C$ value because of the production of energy compounds (e.g. body proteins synthesis) [23,33,46]. Changes were detectable in serum as it is a short-term integrator of diet [52,53]. In contrast, blood cells integrate a period of several months in large mammals [54,55] as the half-life is estimated to be 35 days in mammal blood [38], and thus buffer the short-term fasting effect found in tissues with high turnover rates.

4.3. Special insight on hair tissue

In contrast to the enrichment in ^{15}N of pup tissues relative to maternal tissues usually described [22,23,26,40,57], the lanugo of GS pups showed $\delta^{15}N$ values lower than those of maternal hair ($\Delta^{15}N$ = 1.4‰ ± 0.7 and ± 0.5, respectively; **Table 1**).

This highlights again that the isotopic fractionation between offspring and mother is tissue-specific and no generalizations can be made across multiple tissues. Moreover, the relationship between δ^{15}N values in lanugo and maternal hair in the GS (**Figure 2**) was weaker than in RBC (**Figure 1**). Various studies have already used pup hair as a proxy to investigate the foraging ecology of adult females [3,54], but the key difference between these studies and the present one is the hair physiology of the different mammal species. Dalerum et al, for example, collected hair from meerkat pups and their mothers but the hair of mother and pups is synthesised during the same period and is thus comparable [26]. Porras-Peters analysed fur from suckling California sea lion pups, assuming that they would accurately record differences in the foraging patterns in their mothers [54]. However, the growth histories and shedding phenologies of hair of sea lions (otarids) and GS/NES are different, with the NES and GS undergoing moults during a different period from when the pup's lanugo is synthesised. Our study showed that using pup hair as a proxy to investigate the foraging ecology of adult females was not appropriate in these two specific species (GS and NES), as the hair physiology is very different from one mammal to another**.** In the present study, we observed a negative fractionation between maternal hair and lanugo and no correlation between the δ^{15}N values of the maternal hair and the lanugo. This may be explained by the fact that the active hair growth of phocids begins at the annual moult for ~12 weeks [55], meaning that the isotopic composition of the hair after the moult and thus before the lactation represents prior moult foraging activities. On the other hand, the lanugo is synthesized during gestation and thus according to the post moult foraging activities, meaning that maternal hair and lanugo represent different period of feeding. Lanugo may not be used as a proxy of the foraging habit of the mother as shown by our results. Considerable caution should be taken before comparing hair and lanugo in GS and NES, as this assumes that the adult female uses the same foraging habitat and resources before and after the moult and that there is no change of baseline isotopic ratios during these two periods [56].

The δ^{15}N and δ^{13}C values observed in the lanugo of NES (15.9 ± 0.9‰ and −18.3 ± 0.3‰, respectively) were similar to those reported previously in the lanugo of NES suckling pups from Año Nuevo [3], with values of 15.6 ± 1.0‰ and −17.6 ± 0.4‰, respectively. In the present study the δ^{15}N values in NES pup hair differed between lanugo and the new hair of weaned pups (Δ1.9‰ ± 0.9 and ± 0.6, respectively; **Table 2**). Lanugo is produced during gestation and nutrients required for its growth were directly transferred from the maternal blood. In contrast, the new hair of weaned pups is probably produced at the end of lactation and at the beginning of the post-weaning fast; the protein required was thus obtained from the milk or from mobilization of pup tissue stores, meaning that hair is protein. In addition to the distinct nutrient source and period of growth (gestation vs

lactation/fast), a likely different composition of hair might also influence the isotopic composition. Indeed, the lanugo is composed of long, thin, woolly hair whereas the new hair of weaned pups looks like adult hair, which is short, dense, and thick. Consequently, it is essential to clearly define the hair type of pups (lanugo or new hair) in ecological studies using stable isotope analysis [3,54].

Although hair is a metabolically inert tissue, it would also be interesting to confirm the absence of changes in isotopic composition through time, i.e. over the whole year, between new hair collected in a season and moult hair in the following season (thus the same hair collected from a same animal). These changes might be due to potential alteration, depigmentation, or exogenous deposit on hair surface. Once the methodology is validated, monitoring of annual fluctuations in the isotopic signature of hair could be performed.

Conclusion

Many marine mammals, such as phocids, experience seasonal cycles in food intake and energy demands that may impact the physiological processes governing isotopic fractionation during metabolism and tissue synthesis [57]. In the light of our findings, isotopic consequences of lactation, fasting, and growth seem limited in NES and GS, especially in medium-term integrator tissues of feeding activity such as blood cells. In addition, the pup blood reflects the isotopic composition of maternal blood, supplemented by a subtle mother-to-pup fractionation factor. Our results suggest that pup blood cells are mostly relevant for exploring the ecology of adult mammal populations.

Acknowledgements

The authors are grateful to R. Biondo for his technical assistance. The authors would also like to thank M. Tift (UC, San Diego) C. Champagne and M. Fowler for their assistance with sample collection and the park rangers of Año Nuevo (University of California Natural Reserve system) for their cooperation in this study. We would additionally like to acknowledge the Clairol Corporation for providing hair dye that was used as a marking solution. The authors thank S. Moss, P. Reimann, C. Morris, W. Paterson, A. Hall, and N. Hanson for their assistance with sample collection on the Isle of May. All capture and handling procedures were performed under UK Home Office project licence #60/3303 and conformed to the Animals (Scientific Procedures) Act 1986. K.D. G.L, S.H and F.D are supported by F.R.S.-FNRS. This study was supported by NSF grant #0213095 and

by FRFC grant #2.4502.07 (F.R.S.-FNRS). Research was conducted under National Marine Fisheries Service marine mammal research permit #786-1463. All animal handling procedures were approved by the Institutional Animal Care and Utilization Committee of the University of California, Santa Cruz.

This paper is MARE publication XXX.

References

1. Cherel Y, Hobson KA, Guinet C. Milk isotopic values demonstrate that nursing fur seal pups are a full trophic level higher than their mothers. *Rapid Commun Mass Spectrom*. 2015;29(16):1485-1490. doi:10.1002/rcm.7243

2. Ducatez S, Dalloyau S, Richard P, Guinet C, Cherel Y. Stable isotopes document winter trophic ecology and maternal investment of adult female southern elephant seals (Mirounga leonina) breeding at the Kerguelen Islands. *Mar Biol*. 2008;155(4):413-420. doi:10.1007/s00227-008-1039-3

3. Aurioles D, Koch PL, Le Boeuf BJ. Differences in foraging location of Mexican and California elephant seals: Evidence from stable isotopes in pups. *Mar Mammal Sci*. 2006;22:326-338.

4. Costa D, Ortiz C. Blood chemistry homeostasis during prolonged fasting in the northern elephant seal. *Am J Physiol*. 1982;242(5):R591-5.

5. Reiter J, Stinson NL, Le Boeuf BJ. Northern elephant seal development: The transition from weaning to nutritional independence. *Behav Ecol Sociobiol*. 1978;3(4):337-367. doi:10.1007/BF00303199

6. Hall A, Thompson D. Gray Seal (Halichoerus grypus). *Encycl Mar Mamm*. 2009;35:412-413. doi:10.1578/AM.35.3.2009.412

7. Hindell MA. Elephant Seals. In: *Encyclopedia of Marine Mammals*. Elsevier; 2018:303-307. doi:10.1016/B978-0-12-804327-1.00115-1

8. Champagne CD, Crocker DE, Fowler MA, Houser DS. Fasting Physiology of the Pinnipeds: The Challenges of Fasting While Maintaining High Energy Expenditure and Nutrient Delivery for Lactation. In: McCue MD, ed. *Comparative Physiology of Fasting, Starvation, and Food Limitation*. Springer Berlin Heidelberg; 2012:309-336. doi:10.1007/978-3-642-29056-5_19

9. Rosen D, Hindle A. Fasting. In: Castellini MA, Mellish J-A, eds. *Marine Mammal Physiology*. Boca Raton, FL, USA: CRC Press; 2016:169-191.

10. Crocker DE, Costa DP. Pinniped physiology. In: Perrin WE, Würisg B, Thewissen JGM, eds. *Encyclopedia of Marine Mammals*. San Diego: Academic Press; 2002:935-937.

11. Fedak MA, Anderson SS. The energetics of lactation: accurate measurements from a large wild mammal, the grey seal (Halichoerus grypus). *J Zool*. 1982;198:473–479.

12. Noren SR, Boness DJ, Iverson SJ, McMillan J, Bowen WD. Body Condition at Weaning Affects the Duration of the Postweaning Fast in Gray Seal Pups (Halichoerus grypus). *Physiol Biochem Zool*. 2008;81(3):269-277. doi:10.1086/528777

13. Perrin WF, Würsig B, Thewissen JGM. *Encyclopedia of Marine Mammals (Second Edition)*. London: Academic Press; 2009. doi:http://dx.doi.org/10.1016/B978-0-12-373553-9.00153-X

14. Boness DJ, Bowen WD. The Evolution of Maternal Care in Pinnipeds. *Bioscience*. 1996;46:645-654. doi:10.2307/1312894

15. Doi H, Akamatsu F, González AL. Starvation effects on nitrogen and carbon stable isotopes of animals:

an insight from meta-analysis of fasting experiments. *R Soc Open Sci*. 2017;4(8):170633. doi:10.1098/rsos.170633

16. Newsome SDDSD, Clementz MTTMT, Koch PLLPL. Using stable isotope biogeochemistry to study marine mammal ecology. *Mar Mammal Sci*. 2010;26(3):509-572.

17. DeNiro MJMJMJMJMJ, Epstein S. Influence of diet on the distribution of carbon isotopes in animals. *Geochim Cosmochim Acta*. 1978;42:495-506. doi:10.1016/0016-7037(78)90199-0

18. DeNiro MJ, Epstein S. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim Cosmochim Acta*. 1981;45:341-351.

19. Louis C, Dirtu AC, Stas M, et al. Mobilisation of lipophilic pollutants from blubber in northern elephant seal pups (Mirounga angustirostris) during the post-weaning fast. *Environ Res*. 2014. doi:10.1016/j.envres.2014.04.016

20. Smith RJ, Hobson KA, Koopman NH, Lavigne DM. Distinguishing between populations of fresh- and salt-water harbour seals (Phoca vitulina) using stable isotope ratios and fatty acid profiles. *Can J Fish Aquac Sci*. 1996;53:272-279.

21. Minagawa M, Wada E. Stepwise enrichment of 15N along food chains: further evidence and the relation between d15 N and animal age. *Geochim Cosmochim Acta*. 1984;48:1135-1140.

22. Nelson DE, Angerbjörn A, Lidén K, Turk I. Stable isotopes and the metabolism of the European cave bear. *Oecologia*. 1998;116:177-181.

23. Polischuk SC, Hobson KA, Ramsay MA. Use of stable-carbon and -nitrogen isotopes to assess weaning and fasting in female polar bears and their cubs. *Can J Zool*. 2001;79:499-511.

24. Sare DTJ, Millar JS, Longstaffe FJ. Nitrogen- and carbon-isotope fractionation between mothers and offspring in red-backed voles (Clethrionomys gapperi). *Can J Zool*. 2005;83:712-716.

25. Newsome SD, Koch PL, Etnier MA, Aurioles-Gamboa D. Using carbon and nitrogen isotope values to investigate maternal strategies in northeast pacific otariids. *Mar Mammal Sci*. 2006;22(556-572).

26. Dalerum F, Bennett NC, Clutton-Brock TH. Longitudinal differences in 15N between mothers and offspring during and after weaning in a small cooperative mammal, the meerkat (Suricata suricatta). *Rapid Commun Mass Spectrom*. 2007;21:1889-1892.

27. Tieszen LLL, Boutton TWW, Tesdahl KGG, Slade NA a. Fractionation and turnover of stable carbon isotopes in animal tissues: Implications for d13C analysis of diet. *Oecologia*. 1983;57(1):32-37. doi:10.1007/bf00379558

28. Kelly JF. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. *Can J Zool*. 2000;78(1):1-27.

29. Fuller BT, Fuller JL, Sage NE, Harris DA, O'Connell TC, Hedges REM. Nitrogen balance and d15N: Why you're not what you eat during nutritional stress. *Rapid Commun Mass Spectrom*. 2005;18:2481–2728.

30. Cherel Y, Hobson KA, Bailleul F, Groscolas R. Nutrition, physiology, and stable isotopes: new information from fasting and molting penguins. *Ecology*. 2005;86:2881-2888.

31. Kurle CM. Stable-isotope ratios of blood components from captive northern fur seals (Callorhinus ursinus) and their diet:applications for studying the foraging ecology of wild otariids. *Can J Zool*. 2002;80:902-909.

32. Habran S, Pomeroy PP, Debier C, Das K. Changes in trace elements during lactation in a marine top predator, the grey seal. *Aquat Toxicol*. 2013;126:455-466. doi:10.1016/j.aquatox.2012.08.011

33. Habran S, Crocker DE, Debier C, Das K. How are trace elements mobilized during the postweaning fast in Northern elephant seals? *Environ Toxicol Chem*. 2012;31(10):2354-2365. doi:10.1002/etc.1960

34. Chatt A, Katz S. *Hair Analysis. Applications in the Biomedical and Environmental Sciences.* New York: VCH Publisher; 1988.

35. Lehninger A, Nelson D, Cox M. *Lehninger Principles of Biochemistry*.; 2008.

36. Habran S, Debier C, Crocker DE, et al. Assessment of gestation, lactation and fasting on stable isotope ratios in northern elephant seals (Mirounga angustirostris). *Mar Mammal Sci*. 2010;4(4):26-880. doi:10.1111/j.1748-7692.2010.00372.x

37. Hobson KA, Sease JL. Stable isotope analyses of tooth annuli reveal temporal dietary records: An example using Steller sea lions. *Mar Mammal Sci*. 1998;14:116-129.

38. Jenkins SGG, Partridge ST, Stephenson TR, et al. Nitrogen and carbon isotope fractionation between mothers, neonates, and nursing offspring. *Oecologia*. 2001;129:336-341.

39. Plotnikoff NP. Prolyl-Leucyl-Glycine Amide (PLG) and Thyrotropin-Releasing Hormone (TRH): DOPA Potentiation and Biogenic Amine Studies. In: *Progress in Brain Research*. Vol 42. ; 1975:11-23. doi:10.1016/S0079-6123(08)63637-7

40. Lauff RF, Wood CM. Respiratory gas exchange, nitrogenous waste excretion, and fuel usage during starvation in juvenile rainbow trout, Oncorhynchus mykiss. *J Comp Physiol B*. 1996;165(7):542-551. doi:10.1007/BF00387515

41. Hertz E, Trudel M, Cox MK, Mazumder A. Effects of fasting and nutritional restriction on the isotopic ratios of nitrogen and carbon: A meta-analysis. *Ecol Evol*. 2015;5:4829-4839. doi:10.1002/ece3.1738

42. Martínez del Rio CM, Wolf B. Mass balance models for animal isotopic ecology. In: T.Wang, M.A.Starck, eds. *Physiological and Ecological Adaptations to Feeding in Vertebrates*. Science Publishers, Enfield, New Hampshire; 2005:141-174.

43. Hobson KA, Alisauska RT, Clark RG. Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: Implications for isotopic analyses of diet. *Condor*. 1993;95:388.

44. Hatch KA. The Use and Application of Stable Isotope Analysis to the Study of Starvation, Fasting, and Nutritional Stress in Animals BT - Comparative Physiology of Fasting, Starvation, and Food Limitation. In: McCue MD, ed. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:337-364. doi:10.1007/978-3-642-29056-5_20

45. Doucett RR, Booth RK, Power G, McKinley RS. Effects of the spawning migration on the nutritional status of anadromous Atlantic salmon (Salmo salar): insights from stable-isotope analysis. *Can J Fish Aquat Sci*. 1999;56(11):2172-2180. doi:10.1139/f99-147

46. Crocker DE, McDonald BI. Chapter 10: Post-partum. In: Castellini MA, Mellish J.-A., eds. *Marine Mammal Physiology. Requisite for Ocean Living*. Boca Raton, FL: CRC Press; 2016:219-241.

47. Iverson SJ, Oftedal OT, Bowen WD, Boness DJ, Sampugna J. Prenatal and postnatal transfer of fatty acids from mother to pup in the hooded seal. *J Comp Physiol B*. 1995;165:1-12. doi:10.1007/BF00264680

48. Wolf N, Carleton SA, Martínez del Rio C. Ten years of experimental animal isotopes ecology. *Funct Ecol*. 2009;23(1):17-26. doi:10.1111/j.1365-2435.2008.01529.x

49. Hobson KA, Clark RG. Turnover of 13C in cellular and plasma fractions of blood: implications for nondestructive sampling in avian dietary studies. *Auk*. 1993;110:638-641.

50. Hobson KA, Clark RG. Assessing avian diets using stable isotopes I: turnover of 13C in tissues. *Condor*. 1992;94:181-188. doi:10.2307/1368807

51. Hilderbrand GV, Farley SD, Robbins CT, Hanley TA, Titus K, Servheen C. Use of stable isotopes to determine diets of living and extinct bears. *Can J Zool*. 1996;74:2080-2088.

52. Caut S p., Laran S, Garcia-Hartmann E, Das K. Stable isotopes of captive cetaceans (killer whales and bottlenose dolphins). *J Exp Biol*. 2011;214(4):538-545.

53. Jenkins SG, Partridge ST, Stephenson TR, Farley SD, Robbins CT. Nitrogen and carbon isotope fractionation between mothers, neonates, and nursing offspring. *Oecologia*. 2001;129:336-341.

54. Porras-Peters H, Aurioles-Gamboa D, Cruz-Escalona VH, Koch PL. Trophic level and overlap of sea lions (Zalophus californianus) in the Gulf of California, Mexico. *Mar Mammal Sci*. 2008;24:554-576.

55. K. Ling J. *The Skin and Hair of the Southern Elephant Seal, Mirounga Leonina (Linn.). IV. Annual Cycle of Pelage Follicle Activity and Moult*. Vol 60.; 2012. doi:10.1071/ZO12049

56. Rita D, Drago M, Galimberti F, Cardona L. Temporal consistency of individual trophic specialization in southern elephant seals Mirounga leonina. *Mar Ecol Prog Ser*. 2017;585:229-242. doi:10.3354/meps12411

57. Newsome SD, Clementz MT, Koch PL. Using stable isotope biogeochemistry to study marine mammal ecology. *Mar Mammal Sci*. 2010.

Table.1. Mean (± SD) $\delta^{15}N$ and $\delta^{13}C$ values (‰) in blood cells, serum, milk, and hair of grey seals (*Halichoerus grypus*). Mother-pup pairs (n = 21) were repeatedly captured during lactation and post-weaning fast (T1: early lactation, T2: late lactation, T3: early post-weaning fast, T4: middle post-weaning fast). Different letters (A, B for mothers and a, b, c, d for pups) indicate significant difference in values between T1, T2, T3, and T4 for each tissue (ANOVA with repeated measures, paired t-test and Scheffé *post*-hoc test).

			Blood cells		Serum		Milk		Hair	
		n	$\partial^{15}N$	$\partial^{13}C$	$\partial^{15}N$	$\partial^{13}C$	$\partial^{15}N$	$\partial^{13}C$	$\partial^{15}N$	$\partial^{13}C$
Mother	T1	21	14.1 ± 0.6 ^{A}	-17.1 ± 0.4 ^{A}	15.1 ± 0.7 ^{A}	-17.4 ± 0.5 ^{A}	17.7 ± 1.0 ^{A}	-23.6 ± 0.5 ^{A}		
	T2	21	14.1 ± 0.6 ^{A}	-17.1 ± 0.4 ^{A}	15.0 ± 0.7 ^{B}	-17.1 ± 0.4 ^{B}	15.0 ± 0.7 ^{B}	-22.6 ± 0.6 ^{B}	16.5 ± 0.5	-15.3 ± 0.6
Pups	T1	21	16.4 ± 0.6 ^{a}	-17.2 ± 0.5 ^{a}	16.4 ± 0.6 ^{a}	-18.2 ± 0.4 ^{a}				
	T2	21	16.7 ± 0.6 ^{a}	-17.3 ± 0.4 ^{a}	16.7 ± 0.6 ^{a}	-18.3 ± 0.4 ^{a}			15.1 ± 0.7	-16.8 ± 0.4
	T3	16	17.0 ± 0.7 ^{a}	-17.2 ± 0.4 ^{a}	17.0 ± 0.7 ^{a}	-18.3 ± 0.4 ^{a}				
	T4	12	16.8 ± 0.5 ^{a}	-17.1 ± 0.4 ^{a}	16.8 ± 0.5 ^{a}	-18.6 ± 0.3 ^{a}				

Note: For $\delta^{15}N$ values in milk: n=21 in T1, n=20 in T2; for $\delta^{13}C$ values in milk: n=18 in T1; n=14 in T2. For $\delta^{15}N$ and $\delta^{13}C$ values in pup hair: n=20.

Table 2. Mean (± SD) $\delta^{15}N$ and $\delta^{13}C$ values (‰) and C:N ratios in blood cells, serum, and hair of northern elephant seal (*Mirounga angustirostris*) at different stages of the post-weaning fast (weeks 1, 4, 7, and 9). Different letters indicate significant difference in values between weeks 1, 4, 7, and 9 for each tissue (ANOVA with repeated measures, paired t-test and Scheffé *post-hoc* test).

		Blood cells		Serum		Hair	
	n	$\partial^{15}N$	$\partial^{13}C$	$\partial^{15}N$	$\partial^{13}C$	$\partial^{15}N$	$\partial^{13}C$
Week 1	22	16.2 ± 0.7^{a}	-19.3 ± 0.1^{a}	15.1 ± 0.7^{A}	-17.4 ± 0.5^{A}	15.9 ± 0.9^{a}	-18.3 ± 0.3^{a}
Week 4	22	16.3 ± 0.6^{a}	-19.2 ± 0.2^{a}	15.0 ± 0.7^{B}	-17.1 ± 0.4^{B}	17.8 ± 0.7^{b}	-18.1 ± 0.3^{b}
Week 7	22	16.2 ± 0.6^{a}	-19.2 ± 0.2^{a}	17.0 ± 0.7^{a}	-18.3 ± 0.4^{a}		
Week 9	14	16.3 ± 0.5^{a}	-19.2 ± 0.1^{a}	16.8 ± 0.5^{a}	-18.6 ± 0.3^{a}		

Note: n = 20 in blood cells at week 1.

Table 3. Relationships of stable isotope ratios between maternal and pup tissues (whole blood, blood cells, serum, and hair). The mother-to-pup isotopic fractionation ($\Delta X_{pup-mother}$) according to the tissue and the time of sampling (T1 vs T2) is also given. GS: grey seal, NES: northern elephant seal, SES: southern elephant seal.
* significant ($p < 0.05$), ** highly significant ($p < 0.001$)

Species	Tissue	Time of sampling		δ^{15}N values	$\Delta^{15}N_{pup-mother}$		δ^{13}C values	$\Delta^{13}C_{pup-mother}$	Reference
SES	Whole blood	Late lactation	*	$r^2 = 0.33$ $p = 0.016$	+ 1.3‰	**	$r^2 = 0.87$ $p < 0.001$	+ 0.3‰	Ducatez et al [2]
NES	Blood cells	Early lactation	*	$r^2 = 0.72$ $p = 0.004$	+ 0.6‰	**	$r^2 = 0.90$ $p < 0.001$	0‰	Habran et al [36]
		Late lactation	*	$r^2 = 0.72$ $p = 0.003$	+ 1.3‰	**	$r^2 = 0.94$ $p < 0.001$	0‰	Habran et al [36]
GS	Blood cells	Early lactation	**	$r^2 = 0.86$ $p < 0.001$	+ 2.4‰	**	$r^2 = 0.92$ $p < 0.001$	– 0.1‰	Present study
		Late lactation	**	$r^2 = 0.92$ $p < 0.001$	+ 2.6‰	**	$r^2 = 0.96$ $p < 0.001$	– 0.2‰	Present study
NES	Serum	Early lactation	*	$r^2 = 0.56$ $p = 0.030$	+ 1.1‰	**	$r^2 = 0.90$ $p < 0.001$	0‰	Habran et al [36]
		Late lactation		$r^2 = 0.03$ $p = 0.683$	+ 0.5‰	*	$r^2 = 0.58$ $p = 0.028$	+ 0.4‰	Habran et al [36]
GS	Serum	Early lactation	**	$r^2 = 0.90$ $p < 0.001$	+ 1.3‰	**	$r^2 = 0.62$ $p < 0.001$	– 0.8‰	Present study
		Late lactation	**	$r^2 = 0.79$ $p < 0.001$	+ 1.8‰	*	$r^2 = 0.38$ $p = 0.003$	– 1.2‰	Present study
GS	Hair – Lanugo			$r^2 = 0.03$ $p = 0.445$	– 1.4‰	*	$r^2 = 0.41$ $p = 0.002$	– 1.6‰	Present study