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Milk isotopic values demonstrate that nursing fur seal pups are a full trophic level higher than their mothers†

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RATIONALE: In mammals including humans, mother-to-offspring transfer of nutrients has been the focus of several isotopic studies. Measurement of $\delta^{13}C$ and $\delta^{15}N$ values were mainly conducted on easily sampled tissues such as blood and hair that allow the calculation of apparent discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) between offspring and maternal tissues. Quantifying real $\Delta^{13}C$ and $\Delta^{15}N$ values requires the measurement of the $\delta^{13}C$ and $\delta^{15}N$ values of milk, the exclusive food of newborns. Surprisingly, little isotopic information is available on milk and its biochemical components (lipids and proteins).
METHODS: Paired blood and milk samples from 10 lactating females and their pups were collected from two otariid species, the Antarctic and subantarctic fur seals. Tissue $\delta^{13}C$ and $\delta^{15}N$ values were measured using continuous-flow isotope ratio mass spectrometry (CFIRMS) on maternal and offspring blood, and on whole milk, lipid-free milk and milk lipids, thus allowing the calculation and comparison of apparent (maternal blood to offspring blood) and real (lipid-free milk to offspring blood) $\Delta^{13}C$ and $\Delta^{15}N$ values.
RESULTS: In both fur seal species, the apparent $\Delta^{13}C$ values averaged ~0.0 ‰. Lipid-free milk was slightly ^{13}C-depleted compared with both maternal and pup blood and it was strongly ^{13}C-enriched (~6.3 ‰) compared with milk lipids. In contrast, the apparent and real $\Delta^{15}N$ values averaged 1.2–1.4 and 2.6–3.0 ‰, respectively, the differences being explained by the ~1.5 ‰ lower milk $\delta^{15}N$ values than those of maternal blood.
CONCLUSIONS: In fur seals, the low apparent $\Delta^{15}N$ translated into a higher real $\Delta^{15}N$ value, amounting to a full trophic level, which is in agreement with the almost never verified hypothesis that ^{15}N differences between mothers and their offsprings should reflect one complete trophic level. The study highlights the need to measure milk isotopic values to disentangle the nutritional mother-to-offspring relationships. Copyright © 2015 John Wiley & Sons, Ltd.

In mammals, newborns are dependent on their mothers for their nutrition, with milk being the unique source of energy during a period that varies in duration according to the species. Following peak lactation, offspring are abruptly or progressively weaned and switch to a diet of solid food. Mother-to-offspring transfer of nutrients has been the focus of isotopic studies, including primarily the stable isotope ratios of carbon ($\delta^{13}C$ values) and nitrogen ($\delta^{15}N$ values).[1–3] The rationale is that since lactating females catabolize their tissues to produce milk, nursing offspring are feeding at a trophic level higher than their mothers. Hence, nursing offspring should be ^{15}N-enriched, because $\delta^{15}N$ values increase in a stepwise fashion with consumers' trophic level.[1,4] High $\delta^{15}N$ values early in life with a subsequent decrease during weaning have been described in many mammals, including humans.[5–7] Using ontogenetic series of various tissues, the isotopic method has proven to be effective for investigating maternal and juvenile strategies in different populations and species, including fossil mammals and some cryptic animals such as cetaceans.[6–9]

Pups of pinnipeds are increasingly used as proxies for maternal foraging strategies with their tissue $\delta^{13}C$ and $\delta^{15}N$ values reflecting the female foraging habitats and trophic levels, respectively.[2,10,11] First, pup tissues have an isotopic composition that is related to female feeding ecology, because the exclusive diet of a nursing pup is its mother's milk. Secondly, pups are easy to access and manipulate, thus overcoming the dangerous and arduous work of capturing and handling adult female seals. The use of offspring isotopic values to study maternal foraging ecology was validated in elephant seals by demonstrating that blood $\delta^{13}C$ and $\delta^{15}N$ values of pups and their mothers were positively and linearly correlated. Hence, maternal isotopic values can be estimated by correcting the blood $\delta^{13}C$ and $\delta^{15}N$ values of their pups.[2,12,13] Blood $\delta^{13}C$ values varied little between elephant seal females and the weaned pups. In contrast, blood $\delta^{15}N$ values were higher in young than in their mothers, which is in agreement with the expected trophic $\delta^{15}N$ increase,[2,13] but the ^{15}N discrimination factor ($\Delta^{15}N$) between pup and maternal blood was smaller (1.3 ‰) than expected (~3 ‰[4]). However, pups do not feed on their mother's blood, but on milk, and the mismatch between the apparent (pup blood – mother blood) and real (pup blood – milk) $\Delta^{15}N$ could result from different tissue-specific $\delta^{15}N$ values of maternal blood

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and milk protein. Indeed, milk of terrestrial mammals appears to be depleted in ^{15}N relative to maternal plasma, thus lowering apparent $\Delta^{15}N$ values between the plasma of nursing offspring and their mothers.[1] The latter investigation is the largest one (10 species) among the few that quantify and compare real and apparent $\Delta^{15}N$ (and $\Delta^{13}C$) values in animals held in controlled conditions (see also[14,15]). A single study conducted in the wild showed little ^{15}N enrichment between milk and maternal serum (0.5 ‰) and slightly higher ^{15}N enrichment between milk and pup serum (1.0 ‰[13]).

The present work adds new information on apparent (maternal tissue to offspring tissue, here blood) and real (milk to offspring blood) isotopic discrimination factors by collecting paired blood and milk samples from mothers and offspring of two species of pinnipeds, the Antarctic (AFS, *Arctocephalus gazella*) and subantarctic (SAFS, *A. tropicalis*) fur seals. Fur seals were chosen as model animals because first preliminary data on serially sampled whiskers showed a well-defined isotopic pup stage marked by the highest $\delta^{15}N$ values along the whiskers.[16] Secondly, adult AFS and SAFS females from our study colonies forage consistently within the same water mass where they feed on the same prey all year long,[16] thus minimizing temporal dietary shifts and the corresponding isotopic changes. Finally, female fur seals are income breeders and not capital breeders as true seals are; hence, they do not use during lactation energy stores built up during extensive previous migrations. During the study period, both AFS and SAFS preyed almost exclusively on protein- and lipid-riched lanternfish.[17,18] Since blood and keratinous tissues (hair, whiskers) can be sampled easily and non-destructively in the field, they are the main targeted tissue when using offspring isotopic values to study female foraging ecology.[2,10,11] Here, $\delta^{13}C$ and $\delta^{15}N$ values were measured on pup and maternal whole blood and on milk components, namely lipid-free milk (corresponding to milk proteins) and milk lipids of both AFS and SAFS. Pinniped milk has a high concentration of lipids[19,20] that are likely to be ^{13}C-depleted, thus complicating prediction about apparent and real $\Delta^{13}C$. In contrast, the main driving hypothesis is that the apparent $\Delta^{15}N$ value should be lower than the real $\Delta^{15}N$ value, with the latter reflecting a full trophic level between pups and their mothers.

EXPERIMENTAL

The study was conducted at La Mare aux Elephants (46°22′S, 51°40′E) located on Possession Island, Crozet Archipelago (southern Indian Ocean). AFS and SAFS breed sympatrically at this site, with a pup production of 164 AFS and 80 SAFS during the study period.[21] The study was part of a larger scientific investigation looking at the foraging strategies of the two closely related species of fur seals that included detailed analyses of the $\delta^{13}C$ and $\delta^{15}N$ values of adult males and females over both the medium (blood[17]) and long term (whiskers[16,22,23]). Randomly selected lactating females of unknown age and their pups (n = 10) were sampled in January 2002, meaning that the pups are more than 1 month old. Females were captured using a hoop net and placed on a restraint board. Mother and pup blood was collected into a heparinized syringe by venipuncture of an inter-digital vein in the hind-flipper. A minute amount of sodium heparin has no measurable effect on the isotopic values of blood.[24] Milk collection was facilitated by an intramuscular injection of oxytocin (1 mL, 10 IU/mL; Heriot Agvet, Melbourne, Australia) into the mother upon capture. The milk was then manually extracted into vials. Ethanol (70%) was added to whole blood and milk, because freezing was not possible in the field and storage in 70% ethanol does not alter the isotopic composition of tissues.[25] Before isotopic analysis, the milk samples were decanted thus inducing a significant loss of milk lipids that was indicated by low C:N mass ratio of the whole milk (4.06 ± 0.25 and 4.63 ± 0.67 for AFS and SAFS, respectively). Hence, the $\delta^{13}C$ values of whole milk were discarded. Whole blood and milk were dried in an oven at +60°C. Subsamples of whole milk were treated with a 2:1 chloroform/methanol solution to extract lipids, thus allowing the measurement of the $\delta^{13}C$ and $\delta^{15}N$ values of lipid-free milk (corresponding mainly to bulk proteins) and the $\delta^{13}C$ values of milk lipids (neutral lipids do not contain N atoms). Whole blood has a low lipid content that does not necessitate lipid extraction,[26] as verified here by the consistently low values of blood C:N mass ratio (<4.0; Table 1).

The relative abundances of ^{13}C and ^{15}N were determined by continuous-flow isotope-ratio mass spectrometry (CFIRMS) as described previously.[27] In brief, stable carbon and nitrogen isotope assays were performed on 1 mg subsamples of homogenized materials by loading them into tin cups and combusting at 1200°C in a Robo-Prep elemental analyzer (Sercon Ltd, Crewe, UK). The resultant CO_2 and N_2 gases were then analyzed using an interfaced Europa 20:20 continuous-flow isotope ratio mass spectrometer (Sercon Ltd). The results are presented in the usual δ notation relative to Vienna PeeDee Belemnite and atmospheric N_2 (Air) for $\delta^{13}C$ and $\delta^{15}N$ values, respectively. Within-run replicate measurements of internal laboratory standards (egg albumin, bowhead whale keratin) placed after every five unknowns indicate measurement errors of ±0.1 ‰ and ±0.3 ‰ for $\delta^{13}C$ and $\delta^{15}N$ values, respectively. The isotopic values of whole blood were considered to be representative of the feeding ecology of fur seals during the weeks preceding sampling,[17] thus corresponding to the beginning of the breeding cycle of females who arrive in the colony to breed in late November, and to the first month of the nursing period for pups. It is thus likely that the blood of fast-growing pups was in isotopic equilibrium with their diet after a period of more than 1 month during which they fed exclusively on maternal milk. Data were statistically analyzed using SYSTAT 13 for WINDOWS (Systat Software, Chicago, IL, USA). Values are means ± standard deviation (SD).

RESULTS

In both fur seal species, the blood $\delta^{13}C$ values of pups and their mothers were not statistically different, with pup-mother blood $\delta^{13}C$ differences (apparent $\Delta^{13}C$) averaging –0.1 ± 0.5 and 0.0 ± 0.2 ‰ (n = 10) in AFS and SAFS, respectively (Table 1, Figs. 1 and 2). In contrast, the blood $\delta^{15}N$ values were signicantly higher in pups than in the corresponding lactating females (paired t-tests). The apparent $\Delta^{15}N$ values averaged 1.4 ± 0.2 and 1.2 ± 0.1 ‰ in AFS and SAFS, respectively, and they were marginally statistically significant (two-sample t-test, t = 2.45, p = 0.025).

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Table 1. $\delta^{13}C$ and $\delta^{15}N$ values of lactating females, milk and pups of Antarctic and subantarctic fur seals, and differences between female and pup blood and other groups. Values are means ± SD. Differences in bold mean that isotopic values were significant at $p < 0.05$ (*), $p \leq 0.01$ (**) and $p < 0.0001$ (***) using paired t-tests

Groups	n	$\delta^{13}C$ (‰)	$\delta^{13}C$ differences (‰)		$\delta^{15}N$ (‰)	$\delta^{15}N$ differences (‰)		C:N mass ratio
			Female - groups	Pup - groups		Female - groups	Pup - groups	
Antarctic fur seal								
Female blood	10	–20.61 ± 0.77	–	–0.05 ± 0.46	10.99 ± 0.19		**1.41 ± 0.24****	3.60 ± 0.12
Whole milk	10				9.38 ± 0.53	**1.61 ± 0.62****	**3.02 ± 0.67****	
Lipid-free milk	10	–19.45 ± 1.04	**–1.16 ± 1.51***	**–1.21 ± 1.25***	9.36 ± 0.61	**1.63 ± 0.64****	**3.04 ± 0.72****	3.56 ± 0.15
Milk lipids	10	–25.46 ± 0.88	**4.85 ± 1.33*****	**4.80 ± 1.10*****				
Pup blood	10	–20.66 ± 0.55	0.05 ± 0.46	–	12.40 ± 0.24	**–1.41 ± 0.24****	–	3.73 ± 0.21
Subantarctic fur seal								
Female blood	10	–19.37 ± 0.19	–	0.02 ± 0.20	10.79 ± 0.29		**1.19 ± 0.12****	3.52 ± 0.05
Whole milk	6				9.25 ± 0.50	**1.48 ± 0.64****	**2.63 ± 0.59*****	
Lipid-free milk	6	–18.78 ± 1.14	–0.22 ± 0.99	–0.25 ± 0.93	9.27 ± 0.49	**1.47 ± 0.68****	**2.61 ± 0.65*****	3.51 ± 0.29
Milk lipids	10	–25.50 ± 0.87	**6.12 ± 1.08*****	**6.09 ± 1.10*****				
Pup blood	10	–19.38 ± 0.18	–0.02 ± 0.20	–	11.98 ± 0.29	**–1.19 ± 0.12****	–	3.56 ± 0.05

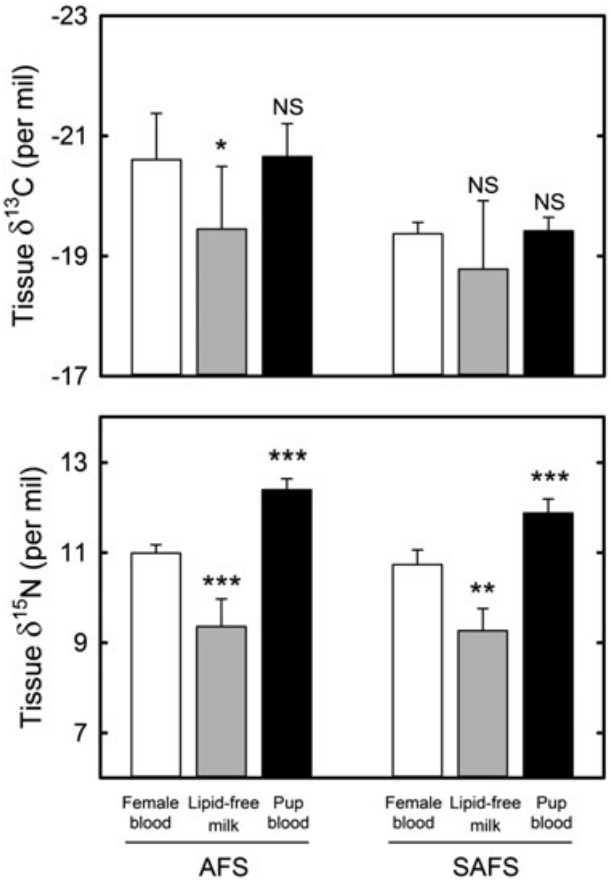

Figure 1. Blood and lipid-free milk $\delta^{13}C$ and $\delta^{15}N$ values of Antarctic (AFS) and subantarctic (SAFS) fur seals from the Crozet Islands. Values are means ± SD. Asterisks mean that isotopic values were significant at $p < 0.05$ (*), $p \leq 0.01$ (**) and $p < 0.0001$ (***) compared with female blood, using paired t-tests; NS: not significant.

Milk lipids had the expected negative $\delta^{13}C$ values, much lower than the corresponding lipid-free milk $\delta^{13}C$ values (paired t-tests, t = 20.12 and 10.12 in AFS and SAFS, respectively, both $p \leq 0.001$). The lipid-free milk to milk lipids $\delta^{13}C$ differences averaged 6.0 ± 0.9 and 6.3 ± 1.4 ‰ in AFS and SAFS, respectively, the two values not being significantly different (two-sample t-test, t = 0.55, $p = 0.595$). Finally, lipid removal did not affect the fur seal milk $\delta^{15}N$ values (paired t-tests, t = 0.26 and 0.21, $p = 0.798$ and 0.842 for AFS and SAFS, respectively) (Table 1).

Lipid-free milk was marginally ^{13}C-depleted compared with blood from lactating females and pups of both AFS and SAFS (Table 1). The lipid-free milk-pup blood $\delta^{13}C$ differences averaged 1.2 ± 1.3 and 0.2 ± 0.9 ‰ for AFS and SAFS, respectively, with no significant differences between the two values (t = 1.50, $p = 0.16$). Whole milk and lipid-free milk were significantly ^{15}N-depleted compared with lactating female and pup blood (Fig. 1). The lipid-free milk-female blood $\delta^{15}N$ differences averaged 1.6 ± 0.6 and 1.5 ± 0.6 ‰ for AFS and SAFS, respectively, and they were not significantly different among species (two-sample t-test, t = 0.48, $p = 0.637$). The lipid-free milk-pup blood $\delta^{15}N$ differences (real $\Delta^{15}N$) averaged 3.0 ± 0.7 and 2.6 ± 0.6 ‰ for AFS and SAFS, respectively, with no significant differences between the two values (t = 1.18, $p = 0.258$) (Fig. 2).

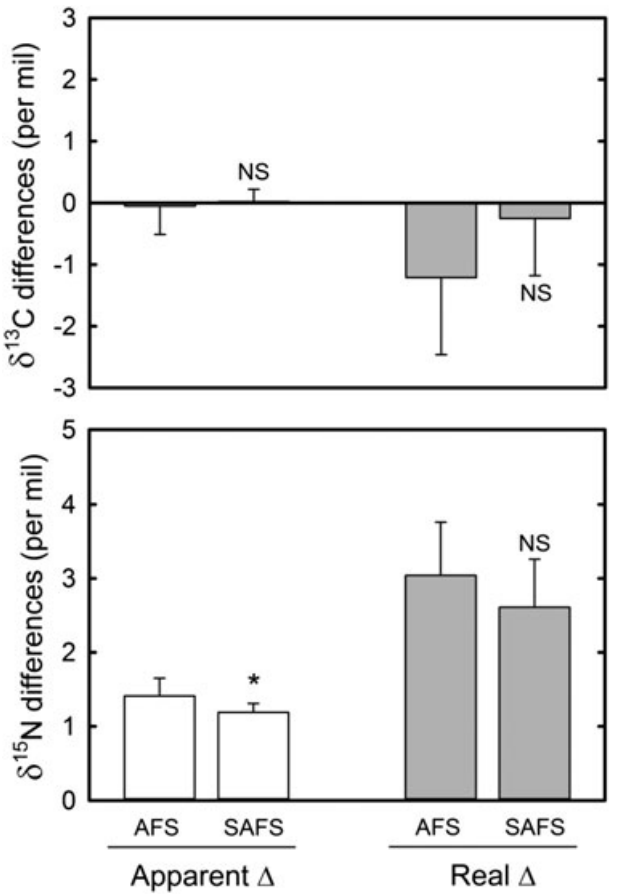

Figure 2. Apparent (maternal blood to offspring blood) and real (lipid-free milk to offspring blood) discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) of Antarctic (AFS) and subantarctic (SAFS) fur seals from the Crozet Islands. Values are means ± SD. Asterisks mean that isotopic values of AFS and SAFS were significantly different at $p < 0.05$ (*), $p \leq 0.01$ (**) and $p < 0.0001$ (***) using two-sample t-tests; NS: not significant.

DISCUSSION

A primary advantage of our approach was that it used simultaneously collected milk and maternal and offspring tissues from wild mammals feeding on their natural diet in their natural habitat to quantify both apparent (maternal blood to offspring blood) and real (milk to offspring blood) isotopic discrimination factors. Ours is the first study, to the best of our knowledge, to measure $\delta^{13}C$ and $\delta^{15}N$ values on whole milk, lipid-free milk (corresponding to milk proteins) and milk lipids to recognize metabolic pathways when deriving pup trophic position. Milk has rarely been analyzed in stable isotope studies investigating maternal-offspring nutrition, the most likely explanation being that collecting milk is not easy because it necessitates capturing and handling adult females. A few investigations focused on whole milk, mainly of terrestrial mammals,[1,13,14] with only one study testing the isotopic effect of lipid removal,[28] and another one measuring $\delta^{13}C$ and $\delta^{15}N$ values from both lipid-free milk and milk lipids.[29] Measuring $\delta^{13}C$ and $\delta^{15}N$ values on milk, in addition to maternal and offspring tissues, is the only way to quantify real $\Delta^{13}C$ and $\Delta^{15}N$ values between offspring and their mothers. The isotopic method can also help to investigate temporal changes in maternal diet,[30] as has already been done using milk lipids as trophic markers.[31] Noticeably, most previous studies have focused on marine mammals,[13,28–30] thus underlining the need for similar investigations on a wider range of species encompassing different taxonomical groups, life-history traits and eco-physiological adaptations.

The apparent $\Delta^{13}C$ values between pup and maternal blood of the two species of fur seals were negligible, being thus in general agreement with the scientific literature on the blood of mammals, including the two elephant seal species.[2,13] Overall, the $\delta^{13}C$ values of exclusively milk-fed human offspring show a ~1 ‰ increase compared with that of the mother, no change for the nonprimate terrestrial mammals, and a slight decrease in some marine mammals (see review by Tsutaya and Yoneda[7]). The present fur seal study also verified that milk lipids were ^{13}C-depleted compared with lipid-free milk (bulk proteins) and with maternal and pup blood. The lipid $\delta^{13}C$ values are 6–8 ‰ lower than those of proteins and carbohydrates, because the lighter carbon isotope is preferentially routed into fatty acids during fatty acid synthesis.[32,33] Accordingly, the $\delta^{13}C$ differences between milk lipids and lipid-free milk in fur seals was 6.0–6.3 ‰ which is comparable with the values obtained with polar bear milk (5.6–7.5 ‰[29]). The lack of $\delta^{13}C$ decrease from maternal to pup blood together with limited $\delta^{13}C$ changes from lipid-free milk to pup blood (real $\Delta^{13}C$) was therefore remarkable. It suggests little incorporation of carbon atoms from milk lipids into the carbon skeleton of amino acids that constitute the blood proteins of fur seal pups, because any significant incorporation of carbon atoms from milk lipids into blood proteins would have lowered the $\delta^{13}C$ values of pup blood. A further step in the quantification of this biochemical pathway would require the use of compound-specific isotope analysis, namely determining and comparing the $\delta^{13}C$ values of non-essential amino acids in maternal milk and offspring tissues.[34]

The small apparent blood $\Delta^{15}N$ values between fur seal females and their pups (1.2–1.4 ‰) is in agreement with the few available studies on the comparative isotopic composition of whole blood and red blood cells (1.3 ‰[2,13]). In plasma[1,13,29] and muscle,[35] offspring-maternal ^{15}N enrichment is lower (≤1 ‰), while both keratinous and collagenous tissues generally showed higher apparent $\Delta^{15}N$ values by 2–3 ‰.[6,36,37] Such tissue-dependent $\Delta^{15}N$ differences are probably explained by tissue-specific amino acid composition, namely the amino acid content of tissue proteins together with $\delta^{15}N$ differences among individual amino acids.[38] It is well known that $\Delta^{15}N$ values between tissue and diet are tissue-dependent with, for example, keratinous and collagenous tissues being ^{15}N-enriched compared with blood.[6,39,40] Whatever the tissue type, however, the apparent $\Delta^{15}N$ between offsprings and their mothers is generally lower than the corresponding $\Delta^{15}N$ values between tissue and diet. Hence, in most cases, the apparent $\Delta^{15}N$ reflects less than an (assumed) full trophic level between offsprings and their mothers.

In all but two of the species so far investigated,[13,29] whole milk was ^{15}N-depleted compared with maternal tissues.[1,13,14] Accordingly, AFS and SAFS milk were ^{15}N-depleted relative to female blood. Taking into account the low milk $\delta^{15}N$ values, the low apparent $\Delta^{15}N$ values between whole blood of fur seal pups and their mothers (1.2—1.4 ‰) translated into a higher real $\Delta^{15}N$ between pup blood and milk, corresponding to a complete trophic level in both AFS and SAFS (2.6–3.0 ‰). Indeed, $\Delta^{15}N$ values between whole blood and red blood cells

of consumers and their diet average 3 ‰ in mammals,[4] including pinnipeds (1.7–4.1 ‰[6]). Surprisingly, however, the results are inconsistent with the real $\Delta^{15}N$ values obtained with other tissues, namely plasma/serum,[1,13,28,29] hair[15] and vibrissae,[28,41] with all the values being less than a full trophic level.[4,6] There is no obvious explanation for this discrepancy. Whatever the real $\Delta^{15}N$ is, however, three preliminary conclusions arise from the few available data: first the apparent $\Delta^{15}N$ is lower than the real $\Delta^{15}N$, secondly in many cases the real $\Delta^{15}N$ is less than a full trophic level, and thirdly the real $\Delta^{15}N$ appears to be species- and tissue-specific.

Correcting pup isotopic values to investigate the food and feeding ecology of their mothers requires only the apparent $\Delta^{15}N$ and $\Delta^{13}C$ to be measured.[2] In contrast, quantification of real $\Delta^{15}N$ and $\Delta^{13}C$ values is of primary importance for a better physiological and biochemical understanding of the isotopic relationships between offsprings and their mothers. Together with the $\delta^{13}C$ and $\delta^{15}N$ values, other isotopic ratios (e.g. $\delta^{2}H$, $\delta^{18}O$ and $\delta^{34}S$ values) are increasingly used in isotopic ecology.[7,42,43] To our knowledge, however, no quantification was available on either their apparent or their real discrimination factors, which therefore need to be quantified on non-destructively sampled tissues (blood, keratinous tissues) and on ontogenetic series of bones and annuli in dentin of sectionned teeth from captive-raised and wild mammals.

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