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ORIGINAL ARTICLE

Human baby hair amino acid natural abundance ^{15}N-isotope values are not related to the ^{15}N-isotope values of amino acids in mother's breast milk protein

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Abstract Since exclusively breast-suckled infants obtain their nutrient only from their mother's milk, it might be anticipated that a correlation will exist between the ^{15}N/^{14}N isotope ratios of amino acids of protein of young infants and those supplied by their mother. The work presented here aimed to determine whether amino nitrogen transfer from human milk to infant hair protein synthesized within the first month of life conserves the maternal isotopic signature or whether post-ingestion fractionation dominates the nitrogen isotope spectrum. The study was conducted at 1 month post-birth on 100 mother–infant pairs. Isotope ratios ^{15}N/^{14}N and ^{13}C/^{12}C were measured using isotope ratio measurement by Mass Spectrometry (irm-MS) for whole maternal milk, and infant hair and ^{15}N/^{14}N ratios were also measured by GC-irm-MS for the *N*-pivaloyl-*O*-isopropyl esters of amino acids obtained from the hydrolysis of milk and hair proteins. The δ^{15}N and δ^{13}C (‰) were found to be significantly higher in infant hair than in breast milk (δ^{15}N, $P < 0.001$; δ^{13}C, $P < 0.001$). Furthermore, the δ^{15}N (‰) of individual amino acids in infant hair was also significantly higher than that in maternal milk ($P < 0.001$). By calculation, the observed shift in isotope ratio was shown not to be accounted for by the amino acid composition of hair and milk proteins, indicating that it is not simply due to differences in the composition in the proteins present. Rather, it would appear that each pool—mother and infant—turns over independently, and that fractionation in infant N-metabolism even in the first month of life dominates over the nutrient N-content.

Keywords Natural abundance ^{15}N/^{14}N ratio · Milk protein · Hair protein · Isotope ratio measurement by mass spectrometry · Isotope fractionation

Abbreviations

EA	Elemental analyser
GA	Gestational age
irm	Isotope ratio measurement
MS	Mass spectrometry
Nleu	Norleucine
Vs	'Area All' output of the mass spectrometer
Xle	Mixture of leucine and isoleucine

Introduction

In the exclusively breast-suckled infant, nitrogenous nutrition is obtained only from the milk of the mother. A balanced protein intake during intra-uterine growth and in early infancy is an important determinant of fetal and post-natal growth. Insufficient protein in utero is linked with,

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amongst other causes, perturbed metabolite supply in the umbilical cord blood (Tea et al. 2012), which tends to result in low birth weight (Ravelli et al. 1998). Similarly, insufficient protein intake after birth can lead to post-natal growth retardation (Koletzko et al. 2009). On the other hand, surplus protein intake by the young infant may cause excess fat mass in childhood and increase the risk of vascular events later in life (Barker et al. 1989, 2005; Rolland Cachera et al. 1995). Hence, understanding the underlying causes is critical, since growth within the very first month is one factor that determines corpulence later in life (Reilly et al. 2005; Botton et al. 2008). A better understanding of the transfer of amino acids from mother to infant and the metabolism of these within the young infant is central to the need to assess nitrogen turnover and correct for any deficiency in the infant diet.

Nonetheless, knowledge of the extent to which amino acid metabolism in the young infant is directly linked to the amino acid supply from the mother is insufficient. This in part reflects the difficulty of conducting experiments involving pre-natal or just post-natal infants. One promising approach is to observe the $^{15}N/^{14}N$ isotope ratios in tissue that can be taken from the infant non-invasively. In the context of probing amino acid metabolism, the isotopic content of hair is a promising marker, since it has been shown to reflect both protein source (animal or vegetable) and intake (Petzke et al. 2005; Petzke and Lemke 2009). Correlations between maternal and fetal nitrogen status have been shown both in utero (Petzke et al. 2010; de Luca et al. 2012) and post-parturition (Fuller et al. 2006). In the case of exclusively suckled babies, the major source of amino-nitrogen is the milk proteins (Carratù et al. 2003), and therefore this might be expected to determine input into the infant amino acid nitrogen pool.

There are several advantages of studying infants who are exclusively breast-fed from birth. First, they have a relatively large nitrogen intake relative to their extant protein. Second, their background nitrogen isotopic profile was determined entirely by the maternal nitrogen isotopic profile and, thirdly, they obtain all their nutrient from a single source. Hence, any correlation between the $^{15}N/^{14}N$ ratios of milk proteins and proteins of the infant might be predicted to be most marked in the first few months of extra-utero life.

In a pioneering study of isotopic fractionation in protein metabolism, Fuller et al. (2006) demonstrated that the $\delta^{15}N$ values of hair and nails from breast-fed infants could be as much as 2–3 ‰ higher than the equivalent tissue of the mother. In the simple hypothesis that the infant $^{15}N/^{14}N$ ratios will reflect the nutrient source, then the implication is that the milk will be enriched in ^{15}N relative to the mother. With this in mind, we have recently investigated the $\delta^{15}N$ values of breast milk and infant hair at 1 month from birth. In addition, we have determined the $^{15}N/^{14}N$ ratios for a number of amino acids of milk proteins. These analyses showed that, contrary to the above hypothesis, milk $\delta^{15}N$ values are 3–4 ‰ lower than both average maternal hair values and hair from the suckling infant (Tea et al. 2013). These data indicate that the isotopic relationship of mother–infant nitrogen transfer is much more complex than might be thought. In the present communication, we probe this relationship, further exploiting the $^{15}N/^{14}N$ ratios for a number of amino acids of milk and hair protein. We show that no direct correlation exists and discussed reasons for this surprising finding.

Experimental

Participants

A population of 100 mother–infant pairs was recruited at the University maternity hospital of Poitiers (France) and the Chatellerault maternity hospital (France), between 22 February 2010 and 10 September 2012. Only infants exclusively breast-fed and their mothers participated in the study. The population was characterized by an average age of 30.7 ± 4.6 (IC_{95} 29.8–31.6) years, an average pre-gestational BMI (kg m^{-2}) of 27.8 ± 6.9 (IC_{95} 26.5–29.2), and an average gestational age (GA) of 40.0 ± 1.1 (IC_{95} 39.8–40.3) weeks. Mothers were excluded for the following reasons: chronic or gestational diseases, smoking during pregnancy, and child born prematurely (GA < 37 weeks) or with a low birth weight [birth weight < 3rd centile for GA according to French references AUDIPOG (Mamelle et al. 1996)]; hospitalized in the neonatal period.

Procedures for sampling

This study was conducted according to the principles expressed in the 1964 Declaration of Helsinki. The Ouest III Ethics Committee approved the protocol on 7 September 2009. After explaining the study protocol to the participating mother, written consent was obtained. This trial was registered as part of the EudraCTClinical Trials Registry (eudract.ema.europa.eu) as 2009-A00912-55.

Participating mothers and their child were investigated at 1 month at the clinical investigation unit (Inserm CIC 0802) of the University hospital of Poitiers. The visits were always programmed between 9 and 11 in the morning. Infant and mother were clinically examined, and the breast milk was collected on the breast opposite to the baby using a breast pump. Breast milk was distributed into aliquots and frozen at −80 °C. A tuft of hair was cut in the occipital area of the infant as close as possible to the scalp. The

longest hair present was selected and the hair root was not included. The cut end was attached to a piece of paper by adhesive tape to maintain orientation and the sample stored dry at room temperature. Samples were blind-coded with a same identification number for each pair.

Procedures for sample preparation for isotope analysis

All milk and hair samples were from mother–infant pairs. Of these, 96 milk and 73 hair samples were suitable for analysis of the δ^{15}N values (a number of the hair samples contained insufficient tissue). Of these samples, 30 milk and 15 hair samples were also analysed for their amino acid profiles: within these were included seven samples where both milk and hair from the same mother–infant pair were analysed.

Breast milk

An aliquot (4 mL) was thawed, homogenized by vigorous vortex mixing and a sample ($\sim$1.5 mL) lyophilized to give a fine powder, which could satisfactorily be weighed into capsules. Tests showed that direct transfer of milk to the capsules gave very high variability in the results, presumably due to lack of homogeneity (data not shown).

From each sample $\sim$2.5 mg (giving $\sim$80 µg N) was weighed with 10^{-6} g precision (balance, Ohaus Discovery DV215CD, Pine Brook, New Jersey, USA) into each of two tin capsules (solids "light" 5 × 9 mm, Thermo Fisher Scientific, www.thermo.com) for isotope analysis.

Infant hair

The infant hair samples were divided into three groups based on the mass present: large, medium, and small. Each sample of the large group ($N = 18$) was analysed in two parts: the basal $\sim$10 mm (representing post-parturition growth) and the tip section (representing intra-uterine growth). For samples classified as 'medium' ($N = 55$), the entire sample was used. Samples in the 'small' group were discarded (insufficient mass). The hair was removed from the adhesive tape, carefully transferred into a pre-weighed 4-mL glass vial, and the mass determined. The samples were washed in ethyl acetate (2 mL, 30 min) to remove traces of adhesive and polar compounds then, following aspiration to remove solvent, in hexane (2 x 2 mL 30 min) to remove sebum (lipids) and residue (e.g., shampoo). Remaining traces of solvent were removed by evaporation at 45 °C under a stream of pure N_2 gas until completely dry. Samples were then chopped manually into very fine pieces.

From each sample $\sim$0.6 mg (giving $\sim$80 µg N) was weighed into each of two tin capsules as described above. Each hair sample was analysed in duplicate (with a few exceptions where the infant sample mass was insufficient to prepare two capsules). Samples falling outside the defined acceptable difference between two measurements were re-analysed or discarded if insufficient material remained.

Procedures for analysis of total δ^{13}C and δ^{15}N values of milk and hair samples

The ^{13}C/^{12}C ($\delta^{13}C_g$) and ^{15}N/^{14}N ($\delta^{15}N_g$) isotope ratios were obtained by isotope ratio measurement by mass spectrometry (irm-MS) using an Integra2 spectrometer (Sercon Instruments, www.sercongroup.com) linked to a Sercon elemental analyser. Isotope ratios [δ^{13}C and δ^{15}N (‰)] were expressed relative to the international references using the equation $\delta(‰) = \left(\frac{R}{R_{std}} - 1\right) \times 1,000$ where for δ^{13}C the reference (R_{std}) is Vienna-Pee Dee Belemnite (V-PDB) and for δ^{15}N it is atmospheric N_2. The calibrated international reference materials NBS-22, SUCROSE-C6, and PEF-1 (IAEA, Vienna, Austria) were used for δ^{13}C calibrations, via a laboratory standard of glutamic acid. The calibrated international reference materials IAEA-N1 or IAEA-N2 (IAEA, Vienna, Austria) were used for δ^{15}N calibrations, via a laboratory standard of glutamic acid.

Procedures for analysis of δ^{15}N values and the quantities of amino acids present in milk and hair proteins

Isotope values for individual amino acids from milk and hair proteins were determined as described previously (Tea et al. 2013). Briefly, protein was obtained from homogenized milk (1 mL) by precipitation. Finely chopped hair was used directly. The amino acids were recovered from proteins by acid hydrolysis (6 M HCl, 110 °C), derivatized as their *N*-pivaloyl-*O*-isopropyl esters, and the esters separated by gas chromatography (60 m ZB-WAX column) linked via a combustion interface to the irm-MS. The ^{15}N/^{14}N ratios were obtained from the *m/z* 28, 29 and 30 peak intensities and the quantities from the 'Area All' (Vs) integrated peak surface areas.

Calculations

Isotope ratios

In order to relate different sets of data to a standard set of conditions, values of $\delta^{15}N_{GC\text{-}A}$ for analytes were normalized, first with a correction factor (F) that relates the measured value ($\delta^{15}N_{GC\text{-}N}$) of the internal reference standard amino acid Nleu in the given sample to that measured by elemental analysis coupled to irm-MS (EA-irm-MS)

($\delta^{15}N_{EA\text{-}N}$). This correction factor is described by $F = \delta^{15}N_{EA-N} - \delta^{15}N_{GC-N}$ where $\delta^{15}N_{EA\text{-}N}$ is the mean value for non-derivatized Nleu measured by EA-irm-MS, and $\delta^{15}N_{GC\text{-}N}$ is the measured value for Nleu in the sample. Each amino acid is then corrected by $\delta^{15}N_A = F + \delta^{15}N_{GC-A}$. To make data fully comparable between EA-irm-MS and GC-irm-MS, a further correction factor is applied, to take into account the difference in the measured value for standards of the target amino acid run in sequence with the unknown samples ($\delta^{15}N_{GC\text{-}Acor}$) and the mean value for non-derivatized target amino acid measured by EA-irm-MS ($\delta^{15}N_{EA\text{-}N}$): $\delta^{15}N_{GC-Acor} = \delta^{15}N_{GC-A} + \delta^{15}N_{EA-A}$.

Quantity

The Area All (V_s) peak surface measurement output of the ISODAT software (Thermo Scientific), which is the sum of the integrated peak areas (V_s) for the ion currents at *m/z* 28, *m/z* 29 and *m/z* 30, was used to calculate the amount of amino acid present, as described previously (Tea et al. 2013). The Area All (V_s) value of each amino acid was corrected by reference to the Area All value obtained for a known quantity of the same amino acid in a standard mixture run by GC-irm-MS concurrently.

Statistical analysis

Data are expressed as mean ± SD. Student *t* tests were performed within the Excel. 2010 software.

Results

Isotope fractionation in the transfer of bulk nitrogenous nutrients from mother to infant

At 1 month after birth, natural abundance ^{15}N and ^{13}C content in the infant's hair and in whole mother's milk (lyophilized) were analysed by EA-irm-MS for $\delta^{13}C_g$ and $\delta^{15}N_g$ values (Fig. 1; Table SI1). It was found that the values for the milk and hair samples cluster as two distinct groups separated from each other by mean values of 3.5 ‰ on the $\delta^{15}N$ axis and 4.5 ‰ on the $\delta^{13}C$ axis. The infant hair group is richer in heavy isotope for both elements ($\delta^{15}N$, $P < 0.001$; $\delta^{13}C$, $P < 0.001$).

Isotope fractionation in the transfer of individual amino acids from mother to infant

The amino acids were obtained and analysed from a selection of samples from the population of 96 milk and 76 hair samples. The insufficient mass of hair samples available limited the number of samples that could be analysed for amino acid $^{15}N/^{14}N$ ratios. Overall, the $\delta^{15}N$ values for 30 samples of milk and 14 of hair were measured (Table 1). Within this data set are included seven mother–infant pairs for which the $\delta^{15}N$ values of the amino acids of both milk and hair were obtained (see below). As can be seen from Table 1, the different amino

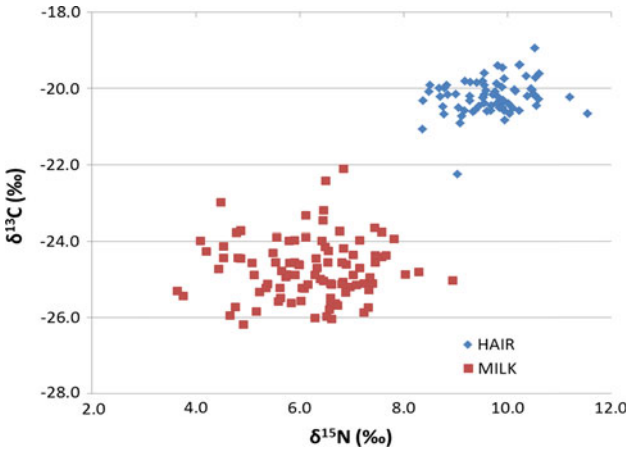

Fig. 1 2D isotope plot for $\delta^{13}C_g$ and $\delta^{15}N_g$ (‰) values for total hair and total milk solids. Each data point represents one mother–infant pair, analysed by EA-irm-MS

Table 1 Variability of $\delta^{15}N$ values (‰) of amino acids recovered from the hydrolysis of milk proteins (15 h) and hair proteins (72 h)

Amino acid[a]	Milk			Hair			H-M
	$\delta^{15}N_M$ (‰)			$\delta^{15}N_H$ (‰)			$\Delta\delta^{15}N$ (‰)[c]
	N[b]	Mean	SD	N[b]	Mean	SD	
Ala	26	11.52	1.46	12	9.85	1.9	−1.67
Val	26	12.02	1.32	10	10.3	2.29	−1.72
Xle	30	12.99	2.62	13	13.07	6.12	0.08
Gly	27	6.26	3.75	14	6.22	2.49	−0.04
Thr	28	−10.36	1.97	4	−7.37	2.16	2.99
Pro	30	14.47	1	14	17.72	2.01	3.25
Ser	29	7.33	4.89	10	10.39	1.8	3.06
Asx	30	11.48	1.43	14	10.13	1.9	−1.35
Met	6	3.22	1.35				
Glx	30	14.54	1.14	13	16.03	1.68	1.49
Phe	30	7.23	2.02				
Lys	30	0.94	2.12	3	−0.18	3.82	−1.12

Isotope ($\delta^{15}N$ ‰) values are corrected relative to values from EA-irm-MS ($\delta^{15}N_{EA}$)

[a] Essential amino acids in italics and amino acids that do not undergo transamination are bold

[b] *N* number of samples

[c] $\Delta\delta^{15}N = (\delta^{15}N_H - \delta^{15}N_M)$

Table 2 Comparison of (a) δ^{15}N values obtained by EA with those obtained by calculation based on the individual δ^{15}N and quantities from irm-MS; (b) δ^{15}N values from milk and hair from the same mother–infant pairs

Pair no.	MILK			HAIR			HAIR–MILK	
	Calc δ^{15}N (‰)	EA δ^{15}N (‰)	Δ (C-E)	Calc δ^{15}N (‰)	EA δ^{15}N (‰)	Δ (C-E)	ΔCalcδ^{15}N (H-M) (‰)	ΔEAδ^{15}N (H-M) (‰)
(a) Full data set for both hair and milk samples								
59	5.58	5.22	−0.37	9.42	9.12	0.30	4.57	3.90
63	6.62	4.92	1.70	11.82	7.09	4.73	5.20	2.17
72	4.91	4.76	0.15	9.29	6.86	2.43	4.38	2.10
74	7.50	7.40	0.10	11.96	8.38	3.58	4.46	0.98
81	6.05	6.00	0.05	9.23	9.53	−0.30	3.18	3.54
91	7.75	4.44	3.31	9.70	9.83	−0.13	1.95	5.39
108	6.58	4.86	1.72	8.01	9.61	−1.60	1.43	4.75
N	7	7	7	7	7	7	7	7
Mean	6.43	5.37	0.95	9.92	8.63	1.29	3.59	3.26
SD	1.01	1.02	1.33	1.45	1.23	2.32	1.44	1.58
(b) Partial data sets only for either hair or milk samples (see Table SI4)								
N	41	45	40	13	33	8	11	19
Mean	7.55	5.91	1.51	10.37	9.46	1.41	3.22	3.58
SD	1.25	1.09	1.16	1.39	1.05	2.18	1.36	1.15

acids present show a considerable range of δ^{15}N (‰) values, falling into three broad categories: those with hair values < the milk values (Ala, Val, Asx, Lys); those with hair values ≈ milk values (Gly, Xle (note Xle is a mixture of Leu and Ile); those with hair values > milk values (Thr, Pro, Ser, Glx).

Correlation of the isotopic composition of individual amino acids in a mother–infant population

No correlation was observed between the infant hair $\delta^{15}N_g$ values and the mother milk $\delta^{15}N_g$ values ($R^2 = 0.108$, $N = 73$, $P > 0.05$) or between the infant hair δ^{13}C values and the mother milk δ^{13}C values ($R^2 = 0.012$, $N = 73$) (Fig. SI1). Furthermore, when individual amino acids are examined, there is no significant correlation between hair and milk values (Table SI2).

The amino acid compositions of hair and milk protein, determined by GC-irm-MS (Tea et al. 2013) are reported in Table SI3. The δ^{15}N data for each sample (Table 1) can be used in combination with the percentage composition of each sample (Table SI3) to calculate the overall δ^{15}N for each sample of hair and milk from the same mother–infant pair. This can then be compared with the data obtained by direct measurement with EA-irm-MS data. The analysis for the full mother–infant pairs is presented in Table 2 and for the complete data set in Table SI4.

Discussion

Values of natural abundance ^{15}N ($\delta^{15}N_g$ ‰) and ^{13}C ($\delta^{13}C_g$ ‰) supplied in milk nutrients and in storage protein synthesized in the infant show a common trend: the infant hair values are significantly richer in heavy isotope than is the nutrient source. Although such an isotopic fractionation is a well-established relationship for many source–sink systems, relatively little direct data are available to explain its underlying cause(s). Studying the mother–infant relationship has allowed us to focus on the isotope profiles of amino acids in proteins from the sole nutrient source (breast milk) and compare these with those from infant hair. Because the source of nutrient is fully known, is relatively consistent in composition, and can be characterized, we can ask the question "Does the compositional differences of hair and milk protein account for the differences in the isotope values observed?" If this is so, then the difference is not an isotopic fractionation event per se. As we shall argue, differences in protein composition do not appear to account for the isotopic shift.

Prior to focussing on the individual amino acids and then the individual mother–infant pairs, it is necessary to discuss to what extent there is variation within the populations. The 96 samples of milk taken from breast-feeding mothers 1 month post-parturition gave a mean value of $\delta^{15}N = 6.2 \pm 1.0$ ‰ with a 75/25 % quartile range of only 1.3 ‰. This indicates that the mothers were eating a regular diet, introducing a low level of variation into the

population. The 73 samples of hair from 1-month-old infants gave a mean value of $\delta^{15}N = 9.7 \pm 0.7$ ‰ with a 75/25 % quartile range of only 0.8 ‰, indicating that fluctuation in the $^{15}N/^{14}N$ ratio was of the same order as in the maternal nutrient supply.

Despite this, the $\delta^{15}N_g$ and $\delta^{13}C_g$ values for the milk samples are dramatically lower than mother's hair and infant hair at birth (de Luca et al. 2012), as well as infant hair at 1-month-old (Table SI1). We found an excellent agreement of the natural $\delta^{15}N$ or $\delta^{13}C$ values in hair (9.7 ± 0.7 ‰ for $\delta^{15}N$, -20.2 ± 0.5 ‰ for $\delta^{13}C$, $N = 73$; see Table SI1) with the values determined in our previous study, in which these parameters were measured at birth in hair taken from newborn infants from the same vicinity (9.7 ± 0.7 ‰ for $\delta^{15}N$ and -20.0 ± 0.4 ‰ for $\delta^{13}C$, $N = 249$) (de Luca et al. 2012). In this same study, maternal hair $\delta^{15}N$ (8.8 ± 0.6 ‰), while always lower than that of the newborn infant for each mother–infant pair, was significantly superior to the milk values presented here (Table SI1). Overall, then, these data indicate that there is neither a dramatic shift in N-metabolism from intra- to exo-uterine status nor a wide variation in the breast-fed population. They also indicate that it is the ^{15}N in milk that is relatively impoverished, rather than that the infant protein is enriched. This might reflect fractionation in ^{15}N of amino acids during the synthesis of milk proteins in lactatory glands, or be due to the non-protein nitrogenous compounds present in milk. Human milk protein content is principally composed of casein (30 %), α-lactalbumin (30 %), lactoferrin (20 %), immunoglobulin IgA (10 %), lysozyme (5 %), and albumin (5 %) (Davis et al. 1994). Human milk also has a variable non-protein nitrogen content, which may contribute up to 30 % of total nitrogen, primarily accounted for by urea, with lesser amounts of uric acid, ammonia, creatinine, and traces of free amino acids and growth factors (Carratù et al. 2003).

Exploiting recently-developed methodology (Tea et al. 2013), we have determined both the $\delta^{15}N$ values and the quantities of amino acids present in hair and milk proteins (Table 2 and Table SI3). From these, it is possible to compare the individual amino acid $\delta^{15}N$ values (Table 1) as well as to estimate a value for the overall $\delta^{15}N$ (Calc $\delta^{15}N$) that can be compared with that measured (EA $\delta^{15}N_g$). For both hair and milk, the calculated value (Calc $\delta^{15}N$) is higher than the measured value (EA $\delta^{15}N_g$) by 1.0 and 1.3 ‰, respectively. Considering that these values are obtained from independent analyses, they are remarkably close. Crucially, the observed shift in $\delta^{15}N$ values is essentially equivalent for the two materials, indicating that it cannot be attributable simply to the presence of the other N-compounds present in the milk. These are not present in the hair sample, which is essentially pure protein. Hence, it can be deduced that the small difference between calculated value (Calc $\delta^{15}N$) and measured value (EA $\delta^{15}N_g$) is most probably due to not all amino acids being taken into account in the calculated values, either due to their degradation in hydrolysis (His, Cys) or poor resolution in the GC-irm-MS (Leu, Ileu, Ser).

A key further observation is that the difference between hair and milk $\delta^{15}N$ values reported in Table 2 and Fig. 1 is maintained both in the calculated [ΔCalcδN^{15} (H-M)] and the measured [ΔEA$\delta^{15}N_g$ (H-M)] pairs: 3.2 ± 1.4 ‰ and 3.6 ± 1.2 ‰, respectively. Thus, the calculated values again compare very favorably with the directly measured difference. Hence, it is apparent that the weighted sum of the amino acid $\delta^{15}N$ values gives the same difference between the value of $\delta^{15}N$ (‰) in hair and milk as does the $\delta^{15}N_g$ (‰) value and that this value is consistently different. Consequently, we can eliminate the hypothesis that the difference is purely an effect of the amino acid composition of the two materials analysed: otherwise, the shift [ΔCalc$\delta^{15}N$ (H-M)] should be negligible.

The range in the actual $\delta^{15}N$ (‰) values of the amino acids, a variation seen in both the milk and hair samples (Table 1), also merits discussion. Similar ranges of $\delta^{15}N$ (‰) values have been found in hair from a number of sources (Petzke et al. 2005, 2010; Petzke and Lemke 2009; Lehn et al. 2012; Petzke and Fuller 2012), although the actual values in $\delta^{15}N$ (‰) reflect differences in sample origin, correction factors applied, and details of the analytical conditions. It has been suggested that this disparity might reflect the essential or non-essential nature of each amino acid (Petzke and Fuller 2012). However, no apparent correlation is seen here between the $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) values which should reflect this distinction and the trophic effect associated with the essential amino acids is no more evident than for the non-essential amino acids (Table 1 and Table SI2). Clearly, this should not be the case if the nitrogen isotope values of the mother's milk were dictating the isotope values for the infant's hair.

A more plausible explanation of the variation in the $\delta^{15}N$ (‰) values of the amino acids is that it is the metabolic origin of the nitrogen in each amino acids that primarily influences the observed $\delta^{15}N$ (‰) values. Those that obtain their α-amino group in one step by the transamination of an immediate precursor α-keto acid tend to have higher values (e.g., Glu, Asp, Val): in contrast, those in which the nitrogen is introduced at an earlier step in the pathway have lower values (e.g., Met, Thr, Lys). The number of steps post-N-incorporation correlates surprisingly well with the $\delta^{15}N$ (‰) values ($R^2 = 0.822$). This will hold true for both maternal and infant N-metabolism. It is noteworthy that both Thr and Lys, neither of which undergoes transamination in humans, have retained their relatively low $\delta^{15}N$ values, whereas both essential and non-

essential amino acids that can be freely transaminated tend to have higher δ^{15}N values.

Overall, it can be concluded from our findings that the δ^{15}N values of the amino acids extracted from infant hair are not closely related to those extracted from the mother's milk. It has long been considered that the isotope values in an organism nourished by another organism should reflect the source of the nutrient: the "you are (isotopically) what you eat, plus a bit" concept. While in many trophic systems this holds true, a number of studies have shown modified maternal N-metabolism during pregnancy, with an overall isotopic enrichment in the infant relative to the mother, both in utero and post-parturition (Petzke et al. 2010; Fuller et al. 2004; Fuller 2003). However, these observations are complicated to interpret, as the δ^{15}N (‰) values are influenced by undefined metabolic changes and by associated nutritional stress, with an overall decrease in ^{15}N/^{14}N ratios of 0.5–1 ‰ (Fuller et al. 2004, 2005). An added complication is that we have found milk proteins to be impoverished by $\sim$3 ‰ relative to hair proteins, indicating a marked isotopic fractionation related to the synthesis of the milk proteins. It should be noted that ^{15}N (‰) values for individual amino acids in maternal plasma closely reflect those from hair (de Luca and authors, unpublished data). In contrast, stressful states such as digestive difficulties (Petzke et al. 2006) and eating disorders (Hatch et al. 2006) can both lead to lower ^{15}N content: milk protein production during the stress of pregnancy may be similarly affected.

What we demonstrate here is that the ^{15}N of proteins in maternal milk and infant hair is adequately explained on the basis of the amino acids available within the relevant partner of the mother–infant pair and is not dictated by the nutrient supply. Therefore, a major shift in isotopic values of the nutrient supply from intra-to extra-utero can be excluded as the cause of the difference in the milk and hair δ^{15}N values. Nor can it be simply due to differences in the amino acid composition of the milk and hair proteins. Hence, we conclude that the metabolic status of the infant has a greater impact on the ^{15}N of the amino acids of the infant than does the ^{15}N of the amino acids of the mother's milk. This strongly suggests that infant N-metabolism dominates over nurture, raising the possibility that hair δ^{15}N may be admissible as an index of whole body protein metabolism in the infant.

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Conflict of interest All authors declare that they have no conflict of interest.

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