Ann. Zool. Fennici 51: 66–72 ISSN 0003-455X (print), ISSN 1797-2450 (online)
Helsinki 7 April 2014

The hair-diet ^{13}C and ^{15}N fractionation in *Chlorocebus aethiops sabaeus* based on a control diet study

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Received 16 Apr. 2013, final version received 15 Nov. 2013, accepted 19 Nov. 2013

Macharia, A. N., Cerling, T. E., Jorgensen, M. J. & Kaplan, J. R. 2014: The hair-diet ^{13}C and ^{15}N fractionation in *Chlorocebus aethiops sabaeus* based on a control diet study. — *Ann. Zool. Fennici* 51: 66–72.

Understanding the ^{13}C and ^{15}N enrichments between primate diet and hair is necessary for reconstructing primate diets and ecology. However, dietary lipids need to be controlled for in order to accurately estimate the hair ^{13}C and ^{15}N enrichment factors (ε^*). We report diet-hair ^{13}C and ^{15}N enrichment factors for vervet monkeys (*Chlorocebus aethiops sabaeus*) based on a controlled diet study in captivity. The vervet monkeys were fed a standard monkey chow exclusively. The $^{13C}\varepsilon^*_{\text{hair-diet}}$ and $^{15N}\varepsilon^*_{\text{hair-diet}}$ values for male adults were 2.8‰ and 2.9‰, respectively; those for lactating females were 2.4‰ and 2.2‰, respectively. The isotope enrichment between hair from infant (ca. 3 months of age) and hair from its mother were $^{13C}\varepsilon^*_{\text{hair(infant-mother)}}$ and $^{15N}\varepsilon^*_{\text{hair(infant-mother)}}$ were 0.7‰ and 1.8‰, respectively.

Introduction

The stable isotope enrichment between diet and tissues is an essential need for understanding the stable isotope ecology and physiology of mammals. Stable isotopes have been shown to be useful in determining diet preferences of different populations of the same species (Codron *et al*. 2006), comparisons of feeding strategies within mammal groups (Sponheimer *et al*. 2003a), comparisons of diets within ecosystems (Koch *et al*. 1991), diet changes related to seasonality (Cerling & Viehl 2004, Cerling *et al*. 2009), animal migration (Hobson 1999, Cerling *et al*. 2006), and wildlife forensics such as illegal trade (Van der Merwe *et al*. 1988, Cerling *et al*. 2007). Hair has proven to be particularly useful because sampling is relatively non-invasive and in some cases it grows continually (Schwertl *et al*. 2003). Besides the applications to ecology, it has also been used to understand tissue turnover and residence times of different physiological pools in mammals (Tieszen 1983, Ayliffe *et al*. 2004, Zazzo *et al*. 2007, 2008).

Stable isotope studies on primates were conducted on some wild populations to understand aspects of diet and ecology (Schoeninger *et al*. 1997, 1998, 1999, Sponheimer *et al*. 2006,

Loudon *et al*. 2007, O'Regan *et al*. 2008, Smith *et al*. 2010, Oelze *et al*. 2011), and Crowley *et al*. (2010) recently compiled the isotope relationships between different tissues of primates. Although these studies estimated a wide range in ^{13}C and ^{15}N enrichment values attributable to dietary or physiological factors, controlled diet experiments on primates to establish the baseline diet-tissue enrichment are needed to understand fundamental diet-tissue isotope relationships. Such diet studies on nonhuman primates are necessary to understand the nutritional ecology of modern primates (Schoeninger *et al*. 1997, Codron *et al*. 2008, Crowley *et al*. 2010) and to provide insight to diets of past human and primate populations (Codron *et al*. 2008).

We investigated isotopic enrichment of ^{13}C and ^{15}N in body hair compared with diet in vervet monkeys (*Chlorocebus aethiops sabaeus*) using a controlled diet. We compared differences in isotope enrichments in hair as compared with diet for adult males and lactating females, and enrichments in the hair of infants as compared with that in their mothers. This study compares whole to delipified food samples in order to evaluate the effects of lipids and effects of food sample treatments while evaluating isotopic enrichment of ^{13}C and ^{15}N in body hair.

Methods

Fifteen vervet/African green monkeys (*Chlorocebus aethiops sabaeus*) housed at the Wake Forest Primate Center (Winston-Salem, NC, USA) were used in this study: 5 males (4.2–8.7 years old), 5 lactating/nursing females (7.4–13.5 years old), and 5 infants (88–140 days old) that were paired with their mothers. The monkeys were fed a commonly used chow diet for a minimum of 1 year (Purina Monkey Chow #5038; Fairbanks *et al*. 2010, Jorgensen *et al*. 2012). Offspring remained in the natal groups with their mothers from birth to sample collection. Hairs were collected from individuals as described in Laudenslager *et al*. (2011).

Food pellets were ground in a Wiley Mill and sieved to < 500 μm, which was further ground in a ball mill to decrease the particle size. Both whole and delipified samples were analyzed for stable isotopes. For delipifications, splits of samples were treated for 48 hours with a 2:1 mixture of chloroform:methanol. A second 48-hour extraction with the mixture was followed by 3X washing in distilled water. The delipified samples were dried at 60 °C.

Individual hair samples were washed with water to remove dirt; oils or grease were removed with a 2:1 mixture of chloroform:methanol. After a second treatment with a 2:1 mixture of chloroform:methanol, samples were repeatedly rinsed with distilled water and then dried at 60 °C. Hair samples were homogenized in a ball-mill.

Powdered food and hair samples were weighed (ca. 500 mg), and analyzed in replicate by combustion in a Costech 4010 Elemental Analyzer at 1650 °C connected to gas chromatograph and a Finnigan MAT 252® Isotope Ratio Mass Spectrometer (IRMS) in continuous flow mode at the SIRFER laboratory at the University of Utah.

The isotope values were calculated as:

$$\delta x = 1000 \times (R_{\text{sample}}/R_{\text{standard}} - 1)\ (‰) \quad (1)$$

where x is either ^{15}N or ^{13}C, while R is the ratio of $^{15}N/^{14}N$ or $^{13}C/^{12}C$, respectively. δx is expressed in per mil (‰) relative to international standards: V-PDB for carbon, and atmosphere (AIR) for nitrogen, respectively. The standard deviations (1 SD) of isotope measurements of $\delta^{13}C$ and $\delta^{15}N$ were ≤ 0.2‰ for standards and 0.1‰ for samples under replicate analyses.

The isotope enrichment between hair and diet was calculated as follows:

$$\varepsilon^*_{\text{hair-5038}} = (R_{\text{hair}}/R_{\text{diet}} - 1) \times 1000 \quad (2)$$

where the asterisk (*) indicates that these are not reversible equilibrium reactions (Cerling & Harris 1999). $^{13C}\varepsilon^*_{\text{hair-5038}}$ and $^{15N}\varepsilon^*_{\text{hair-5038}}$ are the isotope enrichments for ^{13}C and ^{15}N, respectively; the subscript "5038" refers to the formulated diet. We also consider the analogous expressions for isotope enrichment between infant and mother: $^{13C}\varepsilon^*_{\text{hair(infant-mother)}}$ and $^{14N}\varepsilon^*_{\text{hair(infant-mother)}}$, respectively.

Non-parametric statistical tests were performed using the IBM® SPSS® Statistics ver. 20. First, we performed a related sample Kruskal-Wallis test to establish if the distribution and

medians of the whole *versus* delipified food samples were different for $\delta^{13}C$ or $\delta^{15}N$. Second, to establish whether there are significant differences in $\varepsilon^{*13}C_{hair\text{-}5038}$ and $\varepsilon^{*15}N_{hair\text{-}5038}$ between the individual groups, a post-hoc independent samples Kruskal-Wallis test was performed (i.e., infants *vs*. adult nursing females, adult nursing females *vs*. adult males, and infants *vs*. adult males).

Results

Diets: isotope effect of delipification

Delipified diets were slightly lower in %C than the whole diet [41.4 ± 0.9 (n = 10) and 42.9% ± 1.7% (n = 6), respectively]. Mass balance using the lever rule gave a lipid content of 4.2% assuming lipids to be 77% C; this compares well to the crude fat content of 5% (published formulation, Table 1). %N, $\delta^{13}C_{5038}$, and $\delta^{15}N_{5038}$ did not differ significantly between the whole and the delipified diets (Table 1).

Estimated C- and N-isotopic enrichments for infants, nursing females, and adult males

$^{15N}\varepsilon^*_{hair\text{-}5038}$ for adult males and adult lactating females were 2.9 ± 0.2 and 2.2 ± 0.3, respectively (*see* Table 2 and Fig. 1). For infants, $^{15N}\varepsilon^*_{hair\text{-}5038}$ was 4.0‰ ± 0.5‰ and $^{15N}\varepsilon^*_{hair(infant\text{-}mother)}$ was 1.8‰ ± 0.7‰.

$^{13C}\varepsilon^*_{hair\text{-}5038}$ for adult males and adult lactating females were 2.8 ± 0.4 and 2.4 ± 0.4, respectively. For infants $^{13C}\varepsilon^*_{hair\text{-}5038}$ was 3.1‰ ± 0.5‰ and $^{13C}\varepsilon^*_{hair(infant\text{-}mother)}$ was 0.7‰ ± 0.3‰.

The independent samples Kruskal-Wallis test established that at least one of the groups (infants, adult nursing females, and adult males) had a significantly different $\varepsilon^{*15}N_{hair\text{-}5038}$ values for the diets ($\chi^2_2 = 14.08$, $n = 23$, $p < 0.01$). The post-hoc comparisons between groups revealed significant differences in the distribution of $\varepsilon^{*15}N_{hair\text{-}5038}$ values between adult nursing females and adult males ($p < 0.05$, df = 1), between adult nursing females and infants ($p < 0.05$, df = 1), and between adult males and infants ($p < 0.05$, df = 1). However, the differences in the distribution of $\varepsilon^{*13}C_{hair\text{-}5038}$ values were only significant between infants and nursing adult females ($p < 0.05$, df = 1).

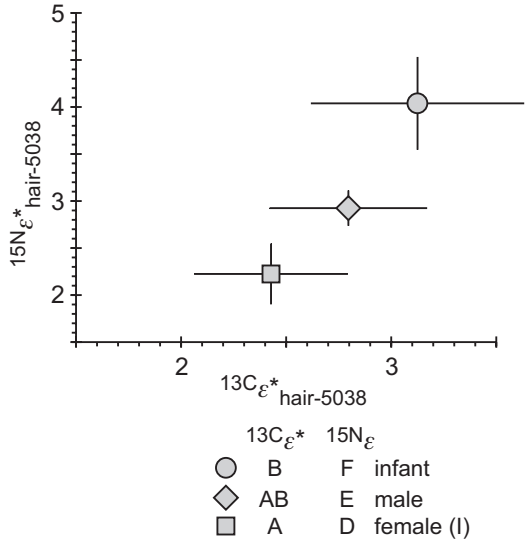

Fig. 1. Carbon and nitrogen isotope enrichment between hair and source diet for males, lactating (l) females, and for infants. The diet was Purina formulation LabDiet #5038 (*see* Table 1). The differences in ranges of $\delta^{13}C$ values, A and B corresponding to infants and adult females respectively, are statistically significant while the differences in ranges of $\delta^{15}N$ values, D, E, and F, are statistically significant for infants, males, and females respectively. The difference in ranges of $\delta^{13}C$ values in males AB, is not statistically significant from adult female and infants.

Table 1. Formulations, carbon and nitrogen fractions, and stable C- and N-isotope values for whole ($\%C_w$, $\delta^{13}C_W$ and $\delta^{15}N_W$) and delipified ($\%C_D$, $\%N_D$, $\delta^{13}C_D$ and $\delta^{15}N_D$) diets used in this study. %N, $\delta^{13}C$ and $\delta^{15}N$ uncertainties (standard error of the mean) are 0.1‰, while those of %C are included in the table.

	Chow 5038
Protein (%)	15.7
Crude fat (%)	5.0
Crude fiber (%)	4.5
Ash (%)	5.3
$\delta^{13}C_W$	–18.4
$\delta^{13}C_D$	–18.5
$\delta^{15}N_W$	2.7
$\delta^{15}N_D$	2.7
$\%C_W$ (mean ± SE)	41.4 ± 0.3
$\%C_D$ (mean ± SE)	42.9 ± 0.7
$\%N_w$	2.5
$\%N_D$	2.7

Table 2. Individual $\delta^{13}C$ and $\delta^{15}N$ values for subjects of this study; isotope enrichments between hair and diet are calculated from diet values in Table 1. Isotope enrichments between hair from paired infants and mothers is shown separately. The $^{13C}\varepsilon^{*}_{\text{infant-mother}}$ and $^{15N}\varepsilon^{*}_{\text{infant-mother}}$ values for mother to infant are estimated as differences in enrichments mothers and infants.

Sample	Age (years)	$\delta^{13}C_{hair}$	$\delta^{15}N_{hair}$	$^{13C}\varepsilon^{*}_{\text{hair-5038}}$	$^{15N}\varepsilon^{*}_{\text{hair-5038}}$	$^{13C}\varepsilon^{*}_{\text{infant-mother}}$	$^{15N}\varepsilon^{*}_{\text{infant-mother}}$	Mother ID
Infant								
R07-X_2008100/1574	0.3	–15.4	6.1	3.0#	3.3#	0.6	1.3	2001057
L02-_2008043/1517	0.3	–15.4	7.3	3.1#	4.5#	1.0	2.6	2001039
L02-_2008091/1565	0.3	–15.9	6.7	2.5#	3.9#	0.4	1.4	2000037
R07-B_2008022/1496	0.4	–14.5	7.2	3.9#	4.5#	1.1	2.5	1996040
L02-Y_2008066/1540	0.3	–15.3	6.7	3.2#	3.9#	0.4	1.3	1995115
Mean ± SD		–15.3 ± 0.5	6.8 ± 0.5	3.1# ± 0.5	4.0 ± 0.5#	0.7 ± 0.3	1.8 ± 0.7	
Female, nursing								
R07-Z_2001057/1198	7.4	–16.0	4.7	2.4	2.0			
L02-B_2001039/1366	7.5	–16.3	4.7	2.1	2.0			
L02-E_2000037/1369	8.4	–16.4	5.3	2.0	2.5			
R07-H_1996040/1187	12.4	–15.6	4.7	2.8	2.0			
L02-Y_1995115/1388	13.5	–15.7	5.3	2.8	2.6			
Mean ± SD		–16.0 ± 0.4	5.0 ± 0.3	2.4 ± 0.4	2.2 ± 0.3			
Male								
R07-Q_2004084/1193	4.3	–15.6	5.8	2.8	3.1			
R07-D_2002106/1179	6.1	–15.4	5.7	3.0	3.0			
R07-Y_2002056/1180	6.4	–15.6	5.9	2.8	3.1			
L02-L_2000012/1376	8.6	–15.2	5.5	3.2	2.7			
L02-V_2000001/1385	8.8	–16.2	5.5	2.2	2.7			
Mean ± SD		–15.6 ± 0.4	5.7 ± 0.2	2.8 ± 0.4	2.9 ± 0.2			

# indicates total isotope enrichment from diet (-5038) to the infant hair; i.e., dietary contribution from milk is unaccounted for.

Discussion

This study demonstrates the ranges of ^{13}C and ^{15}N isotope enrichments in primate populations having identical diets. In this study, an average difference of 0.7‰ in $\delta^{15}N$ in hair between adult males and lactating females was found; differences of this order need to be accounted for in wild population studies when hair or collagen is used to infer dietary differences or trophic status. The enrichment in infant hair as compared with that in mother's is, in effect, a trophic enrichment (Fogel *et al*. 1989, Stegall *et al*. 2008). Thus, the range of $\delta^{13}C$ and $\delta^{15}N$ values within a population is even greater if hair from infants are included in a study; this should be considered in studies in which hairs are collected from nests or from otherwise unidentified individuals (Schoeninger *et al*. 1997, 1998, 1999).

Although the sample sizes in this study were small, there were notable differences in measured values between sexes among adults (adult males *versus* nursing females) as well as nursing females of varying ages. $^{13C}\varepsilon^*_{\text{hair-5038}}$ and $^{15N}\varepsilon^*_{\text{hair-5038}}$ for adult males were ~0.4 and ~0.6, respectively, greater than those for nursing females. Older females had greater $^{13C}\varepsilon^*_{\text{hair-5038}}$ than the younger ones by ca. 0.5. The effect of protein (Balter *et al*. 2006, Florin *et al*. 2011, Poupin *et al*. 2011) and energy balance (energy intake minus its expenditure) (Tsuji *et al*. 2008) on $^{13C}\varepsilon^*_{\text{hair-5038}}$, $^{15N}\varepsilon^*_{\text{hair-5038}}$ variations was not considered in this study. However, emerging evidence suggest that omnivores such as primates require greater amounts of dietary protein to synthesize tissues than herbivores (Sare *et al*. 2005), and that $\delta^{13}C$ of dietary protein is the main determinant of hair $\delta^{13}C$, rather than the composition of the whole diet (Tieszen & Fagre 1993). While carbohydrates and lipids are metabolized for energy, certain essential amino acids (AAs) in the diet maybe preferentially routed to the tissues for protein synthesis. For instance, Miller *et al*. (2011) found that juveniles of a breeding small mammal (*Peromyscus maniculatus*) were more enriched in ^{13}C relative to adults when feeding on milk, and that tissue $\delta^{13}C$ decreased slightly after the animal was weaned to the laboratory diet, notwithstanding the latter's slight enrichment in ^{13}C relative to milk. Consequently, variations in $^{13C}\varepsilon^*_{\text{hair-5038}}$ with age and sex may be influenced by differences in the rates of tissue carbon incorporation during protein synthesis and isotopic routing. In contrast, tissue $\delta^{15}N$ are significantly affected by tissue turnover (Lee *et al*. 2012).

Generally, protein catabolism and anabolism are thought to be in balance at steady states in feeding animals but may change with protein availability (Batler *et al*. 2006). During nutritional stress, catabolism of body proteins (splanchnic tissues and skeletal muscles) into metabolic amino acids would decrease lean body mass and is accompanied by ^{15}N enrichment. The ^{15}N enrichment occurs because hydrolysis of amino acids in the Krebs–Henseleit cycle results in production of ^{15}N-depleted urea that is excreted and causes an increase in $\delta^{15}N$ in the residual metabolic amino acid pools in the body (Hobson *et al*. 1993, Balter *et al*. 2006, Lee *et al*. 2012). Tissue synthesis from these ^{15}N-enriched metabolic amino acids would result in an increase in tissue $\delta^{15}N$. The effect of energy and protein balance on $^{13C}\varepsilon^*_{\text{hair-5038}}$, $^{15N}\varepsilon^*_{\text{hair-5038}}$, $^{13C}\varepsilon^*_{\text{hair(infant-mother)}}$, and $^{15N}\varepsilon^*_{\text{hair(infant-mother)}}$ among nursing females remains a matter of further investigations.

There are considerable potential physiological and dietary differences resulting from age, sex, and trophic factors; therefore, experiments designed to evaluate enrichment factors (ε^*) for hair and other proteinaceous tissues (e.g., bone collagen, and muscle) in different species of the primate order are necessary. Dietary macromolecular composition (i.e., protein, lipid, and carbohydrate), differences in isotopic composition of ingested amino acids (AAs), and differences in isotopic enrichments (ε^*) among non-essential AAs and essential AAs, may provide insights into the biosynthetic pathways by which amino acids are incorporated into proteinaceous tissues under different states of protein and energy balance. Further, differences in amino acid composition among tissues are expected to influence diet-to-tissue ε^* values. For instance, glycine makes up about one third of amino acid residues in collagen, a tissue that is generally more enriched in ^{13}C relative to other tissues, while cysteine and serine comprise about one third of all amino acids in keratins (O'Connell *et*

al. 2001). Therefore, differences in amino acid composition of diets and tissues, and $\delta^{13}C$ and $\delta^{15}N$ of diets need to be considered when designing experiments to study diet-to-tissue ε* values (Crowley *et al*. 2010). Such studies would also help reveal the isotopic effects of deamination and transamination of amino acids, processes that are poorly understood (O'Connell *et al*. 2001, Sponheimer *et al*. 2003a, 2003b, Balter *et al*. 2006).

Evaluation of diet-to-apatite carbonate ε* values should be carried out in controlled conditons because as omnivores, different species of primates differ in the amount of plant and animal matter consumed, and how plant and animal matter is digested (Crowley *et al*. 2010). During digestion, methanogenic reduction on CO_2 within the small intestine affects diet-to-carbonate ε* values because generation of methane is accompanied by a large ^{13}C fractionation that results in CO_2 that, as compared with CH_4, is significantly enriched in ^{13}C (Hedges 2003). Consequently, the diet-to-apatite carbonate, which is assumed to form in isotopic equilibrium with blood bicarbonate, is likely to differ depending on the amount methane produced.

Acknowledgements

We thank Tara Chavanne, Hilaree Sorenson, and Jill Byrnit for assistance with sample collection, and Ellen Miller for discussions. Funding for the isotope analyses was provided by National Science Foundation Grant BCS0621543. The monkeys from which samples were obtained were supported in part by National Institutes of Health grant P40 RR019963/OD010965 and all procedures used here followed Wake Forest IACUC guidelines (protocol A07-115).

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