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

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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 ($\varepsilon$). 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 $^{13}$C*$^{\varepsilon}_{\text{hair-diet}}$ and $^{15}$N*$^{\varepsilon}_{\text{hair-diet}}$ 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 $^{13}$C*$^{\varepsilon}_{\text{hair(infant-mother)}}$ and $^{15}$N*$^{\varepsilon}_{\text{hair(infant-mother)}}$ 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 (Schwert 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 sabaueus) 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 sabaueus) 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:

$$\delta x = 1000 \times \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) (\%e)$$ (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. $\delta x$ 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 $\leq 0.2$‰ for standards and $0.1$‰ for samples under replicate analyses.

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

$$\varepsilon_{\text{hair-5038}} = \left( \frac{R_{\text{hair}}}{R_{\text{diet}}} - 1 \right) \times 1000$$ (2)

where the asterisk (*) indicates that these are not reversible equilibrium reactions (Cerling & Harris 1999). $^{13}$C$_{\text{hair-5038}}$ and $^{15}$N$_{\text{hair-5038}}$ 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: $^{13}$C$_{\text{hair(infant-mother)}}$ and $^{14}$N$_{\text{hair(infant-mother)}}$ 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 $\varepsilon^{13}C_{\text{hair-5038}}$ and $\varepsilon^{15}N_{\text{hair-5038}}$ 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**

$\varepsilon^{15}N_{\text{hair-5038}}$ 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, $\varepsilon^{15}N_{\text{hair-5038}}$ was 4.0‰ ± 0.5‰ and $\varepsilon^{15}N_{\text{hair(infant-mother)}}$ was 1.8‰ ± 0.7‰.

$\varepsilon^{13}C_{\text{hair-5038}}$ for adult males and adult lactating females were 2.8 ± 0.4 and 2.4 ± 0.4, respectively. For infants, $\varepsilon^{13}C_{\text{hair-5038}}$ was 3.1‰ ± 0.5‰ and $\varepsilon^{13}C_{\text{hair(infant-mother)}}$ 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 $\varepsilon^{15}N_{\text{hair-5038}}$ 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 $\varepsilon^{15}N_{\text{hair-5038}}$ 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 $\varepsilon^{13}C_{\text{hair-5038}}$ values were only significant between infants and nursing adult females ($p < 0.05$, df = 1).

<table>
<thead>
<tr>
<th>Chow 5038</th>
<th>Protein (%)</th>
<th>Crude fat (%)</th>
<th>Crude fiber (%)</th>
<th>Ash (%)</th>
<th>$\delta^{13}C_W$</th>
<th>$\delta^{15}N_W$</th>
<th>$\delta^{13}C_O$</th>
<th>$\delta^{15}N_O$</th>
<th>%C$_W$ (mean ± SE)</th>
<th>%C$_O$ (mean ± SE)</th>
<th>%N$_W$</th>
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<tr>
<td></td>
<td>15.7</td>
<td>5.0</td>
<td>4.5</td>
<td>5.3</td>
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<td>-18.5</td>
<td>2.7</td>
<td>41.4 ± 0.3</td>
<td>42.9 ± 0.7</td>
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</table>
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 $\delta^{13}$C$_{\text{infant-mother}}$ and $\delta^{15}$N$_{\text{infant-mother}}$ values for mother to infant are estimated as differences in enrichments mothers and infants.

<table>
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<tr>
<th>Sample</th>
<th>Age (years)</th>
<th>$\delta^{13}$C$_{\text{hair}}$</th>
<th>$\delta^{15}$N$_{\text{hair}}$</th>
<th>$\delta^{13}$C$_{\text{hair-5038}}$</th>
<th>$\delta^{15}$N$_{\text{hair-5038}}$</th>
<th>$\delta^{13}$C$_{\text{infant-mother}}$</th>
<th>$\delta^{15}$N$_{\text{infant-mother}}$</th>
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<td>Infant</td>
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<td>1.3</td>
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<td>-15.4</td>
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<td>3.1*</td>
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<td>2.6</td>
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<td>L02-B_2008091/1565</td>
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<td>-15.9</td>
<td>6.7</td>
<td>2.5*</td>
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<td>1.4</td>
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<td>-14.5</td>
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<td>2.5</td>
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<td>-15.3</td>
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<td>1.3</td>
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<td>Mean ± SD</td>
<td></td>
<td>$-15.3 ± 0.5$</td>
<td>$6.8 ± 0.5$</td>
<td>$3.1* ± 0.5$</td>
<td>$4.0 ± 0.5*$</td>
<td>$0.7 ± 0.3$</td>
<td>$1.8 ± 0.7$</td>
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<td>Female, nursing</td>
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<td>Mean ± SD</td>
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<td>Male</td>
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<tr>
<td>Mean ± SD</td>
<td></td>
<td>$-15.6 ± 0.4$</td>
<td>$5.7 ± 0.2$</td>
<td>$2.8 ± 0.4$</td>
<td>$2.9 ± 0.2$</td>
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</table>

* 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. $^{13}$C for adult males were ~0.4 and ~0.6, respectively, greater than those for nursing females. Older females had greater $^{13}$C 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 $^{13}$C 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 $^{13}$C 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 (Balter 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 $^{13}$C and $^{15}$N-enriched metabolic amino acids would result in a difference in $\delta^{15}$N 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 ($\varepsilon^*$) 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 ($\varepsilon^*$) 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 $\varepsilon^*$ 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
Therefore, differences in amino acid composition of diets and tissues, and δ¹³C and δ¹⁵N 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 conditions 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 CO₂ within the small intestine affects diet-to-carbonate ε values because generation of methane is accompanied by a large ¹³C fractionation that results in CO₂ that, as compared with CH₄, is significantly enriched in ¹³C (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.

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References


