

RESEARCH ARTICLE

Reconstructing Temporal Variation in Great Ape and Other Primate Diets: A Methodological Framework for Isotope Analyses in Hair

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Stable isotope analysis of carbon and nitrogen in hair provides a versatile tool for reconstructing feeding behavior in elusive primate species. Particularly in great apes, researchers can sample long hair completely non-invasively from nests, allowing the investigation of inter- and intra-individual dietary variation. Given its incremental growth pattern, hair records temporal shifts in diet over long periods and allows one to reconstruct seasonal dietary patterns in species that cannot be directly observed. However, as for other sample materials, there are potential drawbacks related to the properties of hair keratin. Here I review some important facts on the nature of primate hair and also introduce new isotopic data from infant bonobo hair to provide methodological recommendations for future sample collection in the field and sample preparation in the laboratory. While these methodological guidelines focus on great apes which can be sampled strictly non-invasively, I also consider applications to other free-ranging primates. The biochemical composition, growth cycle, isotope turnover rate and isotopic fractionation in hair keratin are particularly relevant for data analysis and interpretation. Also, one can microscopically identify infant hairs and analyze them separately to study nursing and weaning behavior in primates. The goal of this article is to encourage primatologists to analyze the stable isotope ratios of hair to assess primate feeding ecology. *Am. J. Primatol.* © 2015 Wiley Periodicals, Inc.

Key words: carbon; nitrogen; seasonality; hair keratin; nursing

INTRODUCTION

In the past decade, primatological research has increasingly used light stable isotope analytics to assess primate feeding behavior in the wild. Stable isotope ecology is a well-established analytical tool in many other zoological disciplines [Gannes et al., 1997; Wolf et al., 2009], as well as archaeology and physical anthropology [e.g., Schwarcz & Schoeninger, 1991]. One of the earliest isotope studies of non-human primates by Schoeninger et al. [1997] highlighted some key advantages of using stable isotopes to study primate ecology: (i) the diet of elusive species can be assessed despite the lack of long-term direct observations, and (ii) the method can help researchers to reconstruct the diet of recently extinct primate populations (i.e., specimens curated in museum collections).

Hair is an ideal sampling material for the most commonly applied combination of stable isotope ratios, (δ) carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$), as analyses require only minute amounts (0.5 mg). Researchers can sample hair either non-invasively from animal resting spots, minimally-invasively during animal captures, or minimally-destructively from pelts stored in museum collections. In addition to isotope analysis, fresh hair samples are also

potentially useful for genetic [Vigilant, 1999] and endocrinological analyses [Carlitz et al., 2014; Yamashita et al., 2013].

Hair keratin is a useful sample matrix for investigating temporal and inter- and intra-individual dietary variation by means of stable isotopes [Cerling et al., 2004; Oelze et al., 2011; Schwertl et al., 2003], including differences between sympatric primate species [Oelze et al., 2014]. Most forest habitats show temporo-spatial shifts in young leaf flushing and fruit ripening, both of which are determined by abiotic factors including the annual

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variation in precipitation, temperature and insolation, but also by biotic factors such as the interaction with consumers [Van Schaik et al., 1993]. Seasonal changes in food resources have large effects on many aspects of primate ecology, such as the timing of reproduction, mobility, and party size [e.g., Brockman & van Schaik, 2005]. The key advantage of hair is that it records the dietary signature of a single individual over extended periods and one can directly compare isotopic data obtained from different individuals [Oelze et al., 2011, 2014]. Particularly in taxa with long hair (e.g., *Gorilla* and *Pongo*), it is possible to gain dietary information over several seasons. However, each hair is a complex tissue and it is important to consider both the life cycle of hair and its biochemical and physiological properties in carrying out analyses.

The present study comprises a) a review of the nature of primate hair, b) methodological guidelines for the field and the laboratory to aid future stable isotope studies using primate hair, and c) novel isotope data from the hair of nursing infant bonobos.

THE NATURE OF PRIMATE HAIR KERATIN

Hair Keratin as a Sample Tissue for Isotope Analysis

Hair is a protein-rich hard keratin. The keratin fiber develops in multiple steps in the hair follicle, a tubular structure of the epidermis that extends into the dermis, which connects the developing tissue directly to the blood supply. While the hair structure emerges from the dermis, it is bathed in various lipids produced by sebaceous and apocrine glands [Harkey, 1993]. The keratin fiber itself consists of polypeptides that form intermediate filaments embedded by a matrix of cysteine and glycine-tyrosine-rich proteins [Marshall et al., 1991]. The amino acid composition of mammalian keratin varies only slightly, but there are larger differences between eukeratins of different animal classes [Gillespie & Frenkel, 1974]. As the amino acid composition differs between keratin and other proteinaceous tissues such as bone collagen (Table I), there are differences between stable isotope data obtained from hair and bone collagen [Crowley et al., 2010].

Cysteine, a molecule containing sulfur (S), is the most abundant amino acid in keratin, and distinguishes it from any other protein. The keratin of primates seems to be particularly enriched in cysteine relative to other mammals [Block et al., 1939]. Therefore, primate keratin is high not only in nitrogen and carbon, but also in sulfur [Nehlich, 2015], allowing for the analysis of multiple isotope systems in the same matrix (see Table I). While this potentially also includes the stable isotopes of oxygen and hydrogen,

there are analytical limitations, as bulk organic materials with high N and S content can cause unintended byproduct gases during analysis that may interfere with the isotopic measurements [Hunsinger et al., 2013]. Once these technical obstacles are overcome, oxygen stable isotopes in hair will be useful to assess the past and present climatic conditions in primate habitats [Chenery et al., 2011; O'Grady et al., 2012; Sharp et al., 2003].

Isotopic Turnover in Hair

Given its incremental nature, hair has the potential to retain a dietary signal over time until it ceases growth and is shed. As in other body tissues, dietary isotopic signatures are incorporated in hair during its formation. The metabolic formation rate of keratin is an essential factor to be considered in dietary reconstructions using stable isotope analysis. Compared to hard tissue formation times, keratinization has a much quicker turnover rate and, unlike other incremental tissues such as bone and teeth, it is constantly formed and fully replaced throughout life history. The isotopic signatures of the amino acids forming keratin are the same ones present in the blood cycle during hair formation in the follicle. As the body recycles large amounts of internal amino acids, several days or weeks may pass until the body's amino acid reserve has changed its isotopic signature, even after intense and rapid shifts in dietary isotopic inputs. An immediate response in horse (*Equus ferus caballus*) tail hair $\delta^{13}\text{C}$ ratios following a dietary shift from C_3 to C_4 foods suggests ~41% of the body's amino acid pool alters after one day [Ayliffe et al., 2004]. This corresponds well with the reported turnover of ~8 hr for isotopic markers injected into the circulatory system detected in the hair follicle [Ryder, 1958]. In the horse hair study, however, up to ~140 days passed until the last 44% of the horse's amino acid pool had reached equilibrium with the new dietary signature [Ayliffe et al., 2004]. This study showed that hair $\delta^{13}\text{C}$ ratios can indeed be used to trace rapid dietary change, but the isotopic response will be considerably dampened in hair stable isotope ratios as only roughly half of the amino acid pool undergoes rapid turnover whereas the other half does not change for an extended period.

Much less is known about hair's response to shifts in nitrogen, presumably because it is difficult to provide experimental diets with distinct $\delta^{15}\text{N}$ values, in contrast to the 13 ‰ difference in $\delta^{13}\text{C}$ between two horse fodders [Ayliffe et al., 2004]. For a single domestic cow (*Bos primigenius taurus*), a 50% shift in hair $\delta^{15}\text{N}$ occurred ~20 days after the introduction of a new fodder, and only after another 20 days had passed did hair reach isotopic equilibrium with the new diet [Schwertl et al., 2003]. After 30 and 40 days, shaved hair samples of rats (*Rattus rattus*) reached isotopic

TABLE I. Amino Acid (AA) Composition of Hair Keratin Compared to Bone Collagen

Amino acid	Nitrogen atoms per AA	Carbon atoms per AA	Sulfur atoms per AA	Human hair keratin (%)	Human bone collagen (%)
Half-Cystine ^a	6	2	1	17.8	0.0
Serine	1	3	0	11.7	3.6
Glutamic acid	1	5	0	11.4	7.4
Proline	1	5	0	8.4	8.9
Threonine	1	4	0	6.8	1.7
Glycine	1	2	0	6.4	33.4
Leucine	1	6	0	5.8	2.3
Valine	1	5	0	5.8	2.5
Arginine	4	6	0	5.8	5.0
Asparagine	1	4	0	4.9	4.4
Alanine	1	3	0	4.6	11.2
Lysine	2	6	0	2.7	2.7
Isoleucine	1	6	0	2.6	0.9
Tyrosine	1	9	0	2.0	0.3
Phenylalanine	1	9	0	1.6	1.2
Histidine	3	6	0	0.9	0.5
Methionine	1	5	1	0.6	0.5
Hydroxylysine	2	5	0	0.0	0.5

^aHere levels of cysteine are expressed in terms of half-cystine, data from [Ambrose, 1993; Gillespie & Frenkel, 1974].

equilibrium with the experimental feed for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ [Caut et al., 2008]. However, feeding studies have found contradictory patterns regarding the turnover of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in different species and body tissues [e.g., Fisk et al., 2009; MacAvoy et al., 2005, summarized by Dalerum & Angerbjörn, 2005]. In fact, there is good reason to assume that the two isotope systems differ not only in their discrimination factors (see below), but also in their turnover rate as (a) they are metabolized differently, (b) they are represented in amino acids in different proportions (see Table I), and (c) they are excreted via different pathways [Schoeller, 1999]. Isotopic research on modern or archaeological human hair suggests there may be a temporal delay in the $\delta^{13}\text{C}$ response compared to $\delta^{15}\text{N}$, but the sample size is small [i.e., one individual as in O'Connell & Hedges, 1999] or the underlying human diet resulting in this pattern is unknown [Williams et al., 2011]. No comprehensive data on non-human primates are currently available.

Hair Growth Rate and Cycle

In addition to the time lags for isotopic turnover in hair, it is important to consider hair growth rates when examining the isotope values of hair. For obvious reasons, there are no data on great ape hair growth rates in the wild. In other primates, where capture-recapture surveys are feasible, it would be desirable to measure growth rates using small scale hair marking (with bleach or paint) or shaving techniques. So far, researchers have consulted human hair growth rates when assessing temporal

isotopic variation in hair of apes [Oelze et al., 2011, 2014]. The growth rate of human hair differs slightly between the various surfaces of the body, with scalp hair averaging 0.3–0.4 mm/day resulting in 0.9–1.2 cm/month [Bost, 1993]. It also varies among individuals, which makes it difficult to obtain precise hair growth rates [Auerswald et al., 2010; Yamanashi et al., 2013], even in humans [Harkey, 1993]. A recent study of endocrinological stress markers in hair reported growth rates for three captive adult orangutans and found a remarkably consistent rate of 0.98 cm/30 days, which matches human hair growth rates [Carlitz et al., 2014]. Subjective observations suggest that shaving may affect hair growth rates in primates, but there is no supporting empirical evidence [e.g., Lynfield & Macwilliams, 1970]. The rough estimate of 1 cm per month may be reasonable for humans and great apes, but there is a need for further experiments, particularly in other primate taxa. For instance, Loudon et al. [2007] shaved the tails of wild ring-tailed lemurs (*Lemur catta*) and visually monitored hair growth to assess the timing of isotope ratios in hair. This may be a useful approach in many other taxa.

Another physiological factor that leads to a delayed or extenuated response in hair is the hair growth cycle. The life cycle of each hair undergoes several stages characterized by growth (anagen phase), regression (catagen phase) and rest (telogen phase) until shedding [Harkey, 1993]. Magnification allows one to distinguish these phases morphologically [Petraço & Kubic, 2004, Appendix A, p. 225]. In the anagen phase, hair is metabolically active and

grows. Typically the root is pigmented and the hair shaft is embedded several millimeters deep into the subcutaneous tissue. When pulled from the skin, the root has a frazzled and long root sheath consisting of follicular tissue. In the catagen phase, cell division ceases and the root reduces pigmentation and turns club shaped as the hair ceases growth. Finally, in the telogen phase, hair is metabolically inactive and rests until it is shed [Harkey, 1993]. The root bulb enlarges, loses pigmentation and gains a light-yellowish color [Petraco & Kubic, 2004; Fig. 1A]. When illuminated the enlarged root bulb is easily visibly with the naked eye.

The telogen growth stage in human scalp hair lasts about 10 weeks [Harkey, 1993; Williams et al., 2011]. In humans, 10–15% of all scalp hairs are in the telogen phase and will not absorb the recent dietary isotopic signal incorporated in physiologically active hair. To avoid this potential source of temporal asynchrony, Williams et al. [2011] suggested that one select only hairs in the active growth phase. While selecting active hair may be an appropriate approach in archaeology and forensic sciences when one focusses on dietary change over time, this strategy is not practical in non-invasive sampling. In nests of apes, for example, hairs containing intact roots fall almost exclusively in the telogen phase (Oelze, per. observation in all African ape species). During captures of wild primates however, one can obtain hair when in the anagen phase if one pulls each hair individually and forcefully. When possible, it is best to select hairs in the anagen phase only as the isotopic signature will be the most recent and the hair sample will benefit from a high degree of growth synchrony.

In captive Japanese macaques (*Macaca fuscata*), monthly hair shavings promptly captured the timing of isotopic shifts induced by new fodder, whereas isotope ratios in whole (unshaved) body hair changed with some delay [Nakashita et al., 2013]. This study indicated that short term dietary shifts are detectable in naturally growing hair comprising different growth stages, but a time lag is likely. Trimmings and resampling of hair from shaved body parts allows one to assess much more

short-term responses in hair as the newly emerging hairs are formed simultaneously. The shaving approach may be particularly practical for nutritional studies in captive settings [Carlitz et al., 2014; Yamanashi et al., 2013], but may also be useful for studies in which animals are repetitively darted or trapped. For each primate species and population, one would need to determine hair growth rates by estimating precisely the length of the newly emerging hair within a given time frame.

In summary, it is desirable to consider growth rate phases when hair can be pulled from the animals' fur, for example in captive settings, in studies employing capture-recapture, but also in museum collections. Isotopic research on wild apes however is limited to naturally shed hair in the telogen phase. The telogen phase implies an uncertain asynchronicity of isotopic signatures in hair that needs to be accounted for when interpreting hair isotope data. It is especially important to know the duration of the hair's resting phase, which likely differs between species but also between individual hairs of the same animal. There are no data documenting these variations but they are likely to differ by days to weeks.

Isotopic Fractionation in Hair $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$

Isotopic fractionation (Δ) is the enrichment of isotope ratios within the consumer's body relative to its diet. The heavy isotope appears to be more inert in many physiological processes within and between cells, whereas the lighter isotope is more mobile and preferentially excreted from the body [Ambrose, 1993; DeNiro & Epstein, 1981]. While the general statement "you are what you eat plus a few per mill" [DeNiro & Epstein, 1976; Kohn, 1999] holds true, there are differences between body tissues, such as keratin, bone collagen, bone apatite and tooth enamel. These differences can vary between species and are more closely related to body size, diet and diet quality than to phylogenetic relationships [Crowley et al., 2010]. The following studies on primates and their known food items suggest the

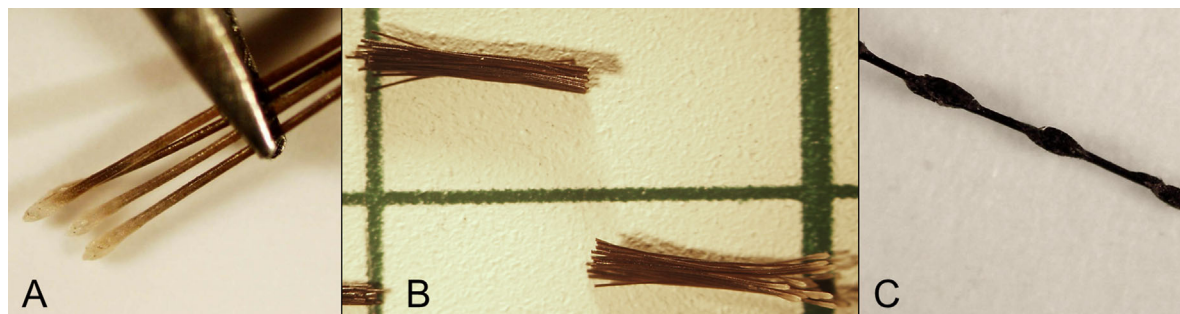


Fig. 1. Adult bonobo hair samples under 15-fold magnification. (A) Enlarged white root bulbs of hair in the telogen phase. (B) Sectioning of hair into 5 mm sections on a 1cm scale cutting mat. (C) Hair with modifications of the hair shaft structure, presumably caused by ectoparasites.

fractionation between diet and hair is $\sim 3\%$ in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$. A controlled feeding study on captive Japanese macaques found mean $\Delta\delta^{13}\text{C}_{\text{diet-hair}}$ and $\Delta\delta^{15}\text{N}_{\text{diet-hair}}$ values of 2.8% and 3.4% respectively [Nakashita et al., 2013]. Oelze et al. [2011] reported mean $\Delta\delta^{13}\text{C}_{\text{diet-hair}}$ and $\Delta\delta^{15}\text{N}_{\text{diet-hair}}$ values of 3.2% and 2.8% , respectively, for wild bonobos [Oelze et al., 2011]. A controlled feeding experiment in various herbivorous mammals also showed an average $\Delta\delta^{13}\text{C}$ value of $\sim 3\%$ [Sponheimer et al., 2003a]. However, fractionation of $\delta^{15}\text{N}$ appears to be complex and varies between species with different digestive systems. As the isotope ratios of proteinaceous tissues relate directly to the dietary protein fraction, the quantities of dietary protein had an effect on $\Delta\delta^{15}\text{N}_{\text{diet-hair}}$ [Sponheimer et al., 2003b]. There is still a need to improve our understanding of the factors influencing $\Delta\delta^{13}\text{C}_{\text{diet-hair}}$ and $\Delta\delta^{15}\text{N}_{\text{diet-hair}}$ values among primates and this may be largely accomplished with controlled feeding studies for different species.

Crowley et al. [2014] assumed the $\Delta\delta^{13}\text{C}_{\text{diet-hair}}$ and $\Delta\delta^{15}\text{N}_{\text{diet-hair}}$ values for reddish-gray mouse lemurs (*Microcebus griseorufus*) were 2.4% and 2.1% , respectively, based on food web data from other tropical ecosystems. These authors interpreted deviations from a predicted $\Delta_{\text{diet-hair}}$ scenario based on the average value of site specific C_3 non-leguminous plants as indicating consumption of arthropods, legumes or CAM plants. Indeed, in the different lemur groups there was variation in the mean $\Delta\delta^{13}\text{C}_{\text{diet-hair}}$ and $\Delta\delta^{15}\text{N}_{\text{diet-hair}}$ values, which ranged from 3.6% to 5.6% and from 4.2% to 5.9% , respectively, suggesting differing diets between groups, sexes and seasons. This approach highlighted the relevance of a standardized plant food selection to establish a representative isotopic plant baseline across habitats, sites and species (see also Crowley et al., this volume). Clearly, the necessity of local plant baseline values also depends on the specific research question addressed using stable isotopes, but such a baseline will always facilitate data interpretation and meta-analyses of published isotope data. Some isotopic research aims can, however, largely avoid vegetation baseline study. One example is the study of infant nursing and weaning behavior (see below). Here, the mother provides the isotopic baselines to its infant, as I show below with data from infant hairs taken from the nests of adult female bonobos.

TECHNICAL CONSIDERATIONS FOR ISOTOPE ANALYSIS IN HAIR

Non-Invasive Hair Sample Collection From Free-Ranging Great Apes

All great apes construct individual nests for night sleeping or day resting. Individuals learn to

construct nests early in ontogeny. The first playful attempts occur around 1 year of age or even earlier, commonly during the day while other group members are resting or feeding [Fruth & Hohmann, 1994]. These clumsily built infant nests are easily distinguished from night nests built by adults. During the weaning process females refuse to allow their offspring to join them in the same nest [Fruth & Hohmann, 1996]. While weaning and independent nesting in bonobos (*Pan paniscus*) and chimpanzees (*Pan troglodytes*) generally occurs at the age of 4–5 years, orangutan (*Pongo sp.*) and gorilla (*Gorilla sp.*) infants may construct their own nests slightly earlier, at 3–4 years of age [Fruth & Hohmann, 1996; Prasetyo et al., 2009].

When sampling hair from great ape nests, it is important to consider nest construction and nest re-use frequencies to avoid unintentional resampling of the same individual, which can result in considerable statistical errors if unrecognized [Mundry and Oelze, this volume]. Nest reuse is generally low in great apes. It appears to be more common in orangutans and occurs rarely in mountain gorillas (*Gorilla beringei beringei*) [Fruth & Hohmann, 1996; Rothman et al., 2006]. In western and eastern chimpanzees, the average daily nest production rate is 1.1 nests per weaned individual, including the production of day nests [Plumptre & Cox, 2006; Plumptre & Reynolds, 1997], whereas in bonobos it is 1.4 nests per individual [Mohneke & Fruth, 2008]. In orangutans, nest production rate is higher (1.7 nest/individual), particularly among females with offspring [Van Schaik et al., 1995]. Gorillas show the most variation, as they construct nests during the night and during the day, on the ground and up in the trees, and use a range of vegetative materials [Tutin et al., 1995]. This diversity is particularly useful to differentiate nests built by sympatric gorillas and chimpanzees. Besides other signs such as feces and hair, the nesting location and form can aid the identification of the constructing species [Sanz et al., 2007]. Finally, the positioning of nests within nest groups can provide information on the sociodemographic structure of a foraging group. In chimpanzees and bonobos, females tend to nest higher in the canopy than males, and gorilla females nest in trees more often than males do [Fruth & Hohmann, 1996; Plumptre & Reynolds, 1997].

Hair sample collection from nests is a non-invasive strategy to sample body tissues of wild great apes. As nests are often in high-crowned trees, the use of climbing gear and rope-based access methods is advisable to assure safety [Anderson et al., 2015; Houle et al., 2004; Van Casteren et al., 2012]. In habituated communities, nest sampling can be combined with routine dawn-to-dusk follows, with the identification of the nest building individual in the evening and sampling on the following day [Oelze et al., 2011]. In unhabituated ape populations, nests are commonly the most abundant indirect sign of

their presence. Hair sampling should be limited to fresh nests if seasonal isotopic variation is the focus of the study. A fresh nest contains fresh green vegetation and feces and/or the smell of urine is present [Tutin & Fernandez, 1984]. For fresh nests, the times of nest construction and nest sampling will differ negligibly. Nests with apparent reuse (e.g., new on old vegetation) should be avoided for hair sampling.

Hair Sampling From Other Primates

For free-ranging primates that do not build nests, hair samples can be retrieved only through invasive means, such as traps or darting [Crowley et al., 2014; Glander et al., 1991; Jolly et al., 2003]. As mentioned above, shaving strategies during capture-recapture surveys may open new avenues for primate isotope research focusing on dietary variation over time. Additionally, this approach could provide information on hair growth and isotope turnover rates in different species. Blunt darts equipped with sticky tape or glue to could also be tested in different settings and species to retrieve hair from individual animals. Most hair that sticks to these darts is likely to be in the telogen phase.

Hair Preservation and Contamination

Once keratin is formed, its robust structure is not prone to contamination or biochemical alterations related to UV radiation or abrasion [Auerswald et al., 2011]. As a result, one can recover hair even from archaeological contexts dating back several thousand years [Britton et al., 2013; Macko et al., 1999]. One can sample hair without protective gloves and store at ambient temperature in paper or pergamin envelopes that remain dry. These features are particularly convenient for field work in moist habitats as little equipment is required, and small quantities of silica are sufficient to store large numbers of hair samples, for example in plastic collection bags. One can also sample pelts stored over multiple decades in museum collections [Gibson, 2011; Macho & Lee-Thorp, 2014]. However, some preservatives, such as formalin, may complicate analysis of treated pelts, as they are likely to affect the isotopic signature of proteinaceous animal tissues [Sarakinis et al., 2002].

Sample Cleaning

Prior to analysis, it is beneficial to clean hair samples with detergent to remove undesirable lipids. Besides sweat, the skin leaves various oily and waxy lipids that surround the hair keratin matrix and actually protect it from extrinsic factors [Harkey, 1993]. Furthermore, dirt particles may attach to the hair, and handling with bare hands during sampling

may lead to contamination. For isotopic analysis it is desirable to extract these contaminants and lipids to measure the true isotope ratios of hair protein. At the same time, these extracts could potentially be useful for endocrinological studies on the same study subjects, for example to measure levels of physiological stress [Carlitz et al., 2014]. It is likely that the production of lipids by sebaceous glands varies between the various surfaces of the body, individuals, populations and species [Harkey, 1993; Mykytowycz, 1972]. The isotope signatures of sebum will probably be related to diet as several essential amino acids can be identified in primate epidermal lipids [Feingold, 1991]. At present there is no information on the turnover and production rate of these lipids, nor is it possible to assess how they would proportionally contribute to the isotope signal of hair. Hence, sebum can be considered a minor yet unpredictable bias for isotope analysis in hair keratin. To improve reproducibility and consistency in hair isotope analysis, it is desirable to remove these components or to separate them and, if quantities allow, measure them separately.

The classical lipid extraction method using a 1:2 solution of chloroform and methanol [after Bligh & Dyer, 1959] is suggested for hair keratin to remove any lipids [O'Connell & Hedges, 1999]. Acetone, ethyl alcohol [Loudon et al., 2007] or nonionic surfactants [Schilliacci et al., 2014] and even water [Macho & Lee-Thorp, 2014] have been used to clean hair. I propose that a standard exogenous contaminant and lipid extraction method could be applied to hair samples used for isotope analysis to aid future comparisons across studies. The protocol of O'Connell & Hedges [1999] for modern and archaeological human hair may be too laborious for wild primates that are rarely exposed to anthropogenic hair contaminants other than gland secretions, but may be applied to museum curated specimens where previous preservative treatments are difficult to assess [O'Regan et al., 2008]. Removal of preservatives is particularly relevant to facilitate comparisons with specimens not chemically treated, for example modern material from the wild or hair samples from other collections. The chloroform/methanol extraction procedure reliably dissolves non-polar neutral and polar lipids from the hair. The hair can be bathed overnight in this solution in 15 ml tubes using a rotator or, for a more rapid process, in an ultrasonic bath for at least 30 min. Once the solution is poured from the sample, three additional rinses with deionized water ensure the removal of solvents attached to the hair. Subsequently, samples can be dried at 40–100°C in an oven incubator or freeze dryer.

Under the Microscope

Sectioning of hair samples allows one to assess temporal variation in hair isotope values. For

sectioning, tubes with cleaned and dried hair samples are transferred to a clean microscope working space with a microbalance. From this point, I recommend to handle the hair sample with cleaned, precision tweezers to avoid contamination. Wearing rubber gloves is not advisable as they often cause an electrostatic charge in which hair may respond in an uncontrollable way. A simple stereo microscope with 10–15× magnification is sufficient to control visually the quality and orientation of individual hairs. Fractured hair or hairs without roots should be excluded from analysis. Also, any hairs altered by unidentified ectoparasites, which can influence hair formation (Fig. 1C), should be removed from the sample.

For sectioning into sequential segments, it is ideal to select hairs with roots in the same (e.g., telogen) hair growth phase, and hairs of similar length and thickness. Short and thin hair may derive from infants sleeping in their mother's nest and should be removed or analyzed separately (see Figs. 2A and 3).

Once one selects a homogenous hair sample, consisting of multiple hairs in the same growth phase and in consistent length and thickness, it is weighed on a microbalance to determine the exact number of recoverable sub-samples. Commonly, chimpanzee and bonobo hairs are 5–6 cm long and towards the tip a hair strand tends to thin out, thus the weight of the distal hair sections will decrease. 1 cm of a single adult great ape hair weighs on average 0.1 mg (personal observation and personal communication E. Carlitz). Hence, while single hairs cannot be segmented for

analysis, bulk analysis allows one to determine an average isotope value for a single individual for a longer period (at least 5–6 subsequent months). A sample of 20 *Pan* hairs is equivalent to 10 mg of hair keratin. Depending on the sensitivity of the mass spectrometer, the ideal sample weight for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis in keratin can be as low as 0.5 mg, but successful measurements are possible even from slightly lower weights. In the above example, the sample can be cut into 10 sections of 5 mm length each. The hair sample is aligned under the microscope at the root bulbs (see Fig. 1A and B) on an elastic cutting mat with preprinted scale and is cut with a clean scalpel. To aid the straightening and sectioning of hair on the cutting mat, one can add several drops of deionized water to prevent the small hair fragments from clinging to the scalpel or the tweezers. Once placed in separate 0.5 ml tubes, the sections are dried overnight once more and are weighed into tin capsules with adequate size (e.g., for 5 mm hair sections the respective 6 mm capsules). During weighing, one can again assess the precision of the cutting procedure (e.g., subsequent 5 mm sections should weigh the same).

NURSING SIGNALS IN WILD PRIMATE INFANT HAIR

In great apes, infants sleeping in the nests of adult females create a potential bias in analyses of the stable isotope ratios of hair. The diet of juveniles may differ considerably from mature individuals, particularly if the majority of dietary protein

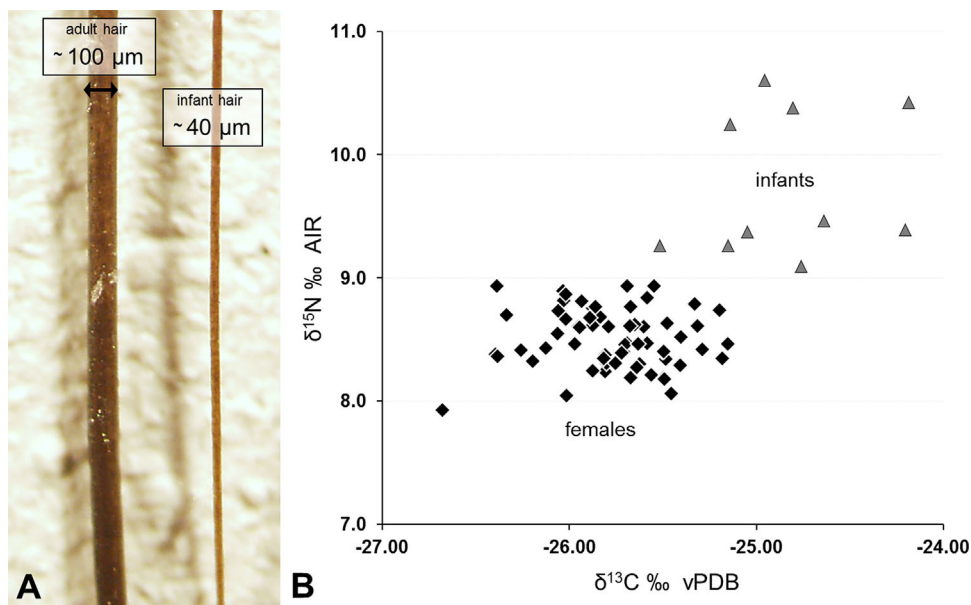


Fig. 2. (A) Microscopic comparison of a thick adult female bonobo hair compared to a thin potential infant hair from the same nest. (B) Separate analysis of potential infant hairs reveals nursing isotopic signatures in samples from infants younger than 2 years [see Tables 3 and 4, female bonobo data from Oelze et al., 2011].

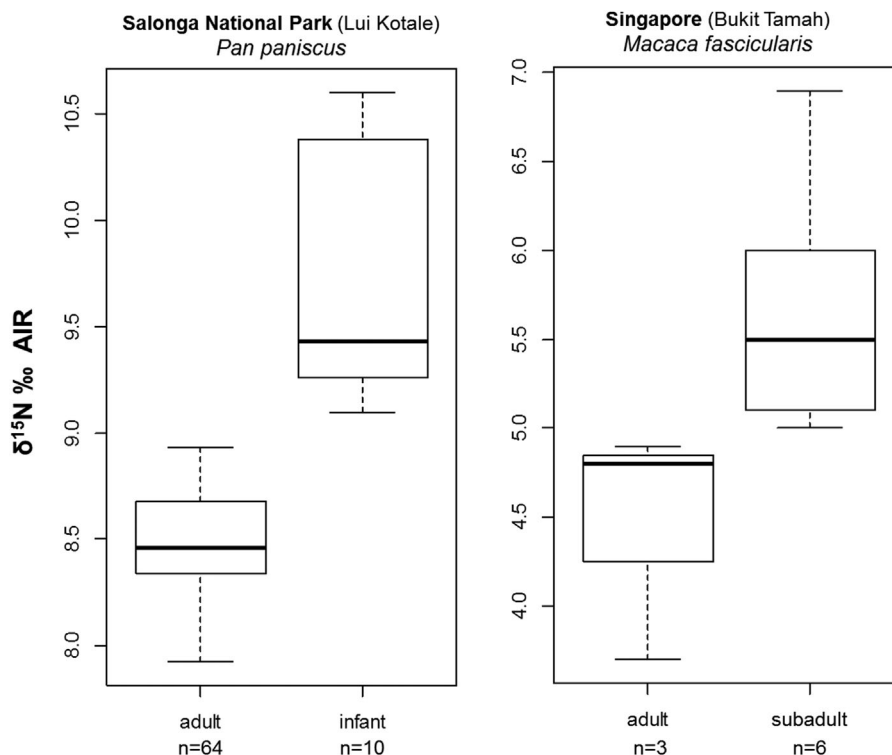


Fig. 3. Whisker plot with the $\delta^{15}\text{N}$ values of wild bonobos and macaques illustrating the dietary and isotope differences between adults and infant/premature of the same population [macaque isotope data are taken from Schillaci et al., 2014 who differentiate only between adult and subadult age classes].

comprises mothers' milk. Suckling infants are typically significantly enriched in the isotope ratios as compared to their mothers [Fuller et al., 2006]. This difference is based on the trophic level effect, which leads to isotope fractionation with each step in the food chain, particularly in $\delta^{15}\text{N}$, but also in $\delta^{13}\text{C}$ [DeNiro & Epstein, 1976, 1981]. Dependent offspring consuming mother's milk live on the body tissue of their mothers and as a consequence reveal a "carnivorous" isotope signature in relationship to adult females of the same population. During the process of weaning, the isotopic enrichment in juveniles relative to females gradually declines [Fuller et al., 2006; Schurr, 1998]. Conclusions on feeding behavior can be considerably flawed if isotopic data of juveniles and adults are combined [e.g., Schillaci et al., 2014]. In hair samples from great apes, it is essential to remove potential infant hair from each sample to exclude biased isotope signatures for adult females. Infant hair should differ from adult hair in size [Bakuneeta et al., 1993]. I have proposed the removal of hair with reduced diameter and length from the female nest [Oelze et al., 2011], or in the case of unhabituated groups, in all nests sampled [Oelze et al., 2014].

I tested this sampling strategy in hair samples collected from nests of adult females with infants younger than 4 years of age. These infants still

slept in their mothers' nest and were still suckling. I measured hair length, hair shaft diameter and stable isotope ratios to show that hair from suckling infants can indeed be identified and separated from an adult female ape hair sample.

METHODS

Hair samples of free-ranging bonobos were collected at Salonga National Park, Democratic Republic of Congo (DRC) in 2010 [see Oelze et al., 2011 for details on the site and the procedure]. Collection and export procedures adhered to the legal requirements of DRC and the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES permit N/Ref. 00651/ICCN/DG/ADG/KBY/2011) and to the American Society of Primatologists principles for the ethical treatment of non-human primates.

For this study, I selected hair samples from the nests of five females with offspring younger than 4 years of age and cleaned them as outlined above. I removed short (>4 cm) and apparently thin hairs ($n = 41$) from the remaining sample under a stereo microscope with $10\times$ magnification and measured them separately from the adult female hair (Fig. 2A). I calculated the hair diameter at two different locations perpendicular to the shaft using a stereo

microscope (Olympus SZX9) at 30× magnification with the Stream Motion (Olympus, Version 1.6.1) software package. Subsequently, I weighed >0.2 mg of hair identified as potential infant hair into tin capsules for measurement of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in a Flash EA 2112 (Thermo-Finnigan[®], Bremen, Germany) coupled to a DeltaXP mass spectrometer (Thermo-Finnigan[®], Bremen, Germany). I compared the resulting data to isotopic data from adult female bonobos from the previous year [Oelze et al., 2011]. I expected “potential infant hair” to yield $\delta^{15}\text{N}$ values higher than female $\delta^{15}\text{N}$ ratios (<9.0‰).

RESULTS

I obtained isotope data from 15 “potential infant hair” samples (Table II) all of which yielded acceptable atomic C:N ratios between 2.6 and 3.8 [O’Connell et al., 2001]. In three samples of infants aged 0–1 years, the $\delta^{15}\text{N}$ values were equivalent to the female mean value, which suggested that the thin hair may derive from the female rather than the offspring. In ten hair samples from nests with infants aged 1–2 years, both isotope ratios were enriched. Based on the isotopic signal, these samples can be classified as hair samples of sucking bonobo infants (Fig. 2B). The $\delta^{15}\text{N}$ ratios of two further samples of older infants were ^{15}N -enriched compared to adult females, but a less clear nursing signal may relate to the

increased consumption of solid foods by the age of 3 and 4 years [Fuller et al., 2006].

The mean diameter of the infant hair samples identified by their isotopic nursing signature is clearly lower than the mean diameter of adult females (one way ANOVA, $F=137.6$, $DF=1$, $P<0.001$). There is some degree of overlap in hair thickness between infants and adults, but the combination of hair thickness and length seems to be a good indicator of infant hair presence (Table III). According to these data, false negatives may occur in cases where fine short female hair is misclassified as potential infant hair. Repeated sampling and isotope analysis should help to identify and exclude such false negatives. The majority of the “potential infant hair” samples will provide true positives with information about the infants nursing behavior and the process of weaning.

DISCUSSION

Hair keratin is a robust incremental proteinous tissue that can be readily collected in the wild and analyzed for multiple stable isotopes. Repeated sampling of hair from the same individual allows studying temporal or even seasonal dietary variation in elusive free-ranging primates. The main limitation for investigating temporal aspects in hair isotope signatures is related to the response delay. Time lags in isotope turnover and hair growth need

TABLE II. Stable Isotope Ratios Measured in Hair Samples Microscopically Classified as “Potential Infant Hair”

Sample ID	Female	Infant birth	Length (cm)	mg	$\delta^{13}\text{C}\text{‰}$	$\delta^{15}\text{N}\text{‰}$	%C	%N	C:N
21722 inf	Zoe	2010	2	0.23	-25.5	8.4	42.6	14.5	3.4
		Newborn (<1 month)			-25.5	8.4			
21692 inf	Iris	2009	2	0.15	-25.9	8.0	46.5	17.8	3.0
21696 inf	Susi	2009	3	0.25	-25.4	8.9	42.2	14.3	3.5
		Infants aged 1 year		mean	-25.7	8.4			
		no nursing signal		1σ	0.3	0.6			
21643 inf	Olga	2009	3	0.36	-24.8	10.4	40.4	13.5	3.5
21614 inf	Olga	2009	2	0.21	-25.0	10.6	45.3	17.4	3.0
21628 inf	Olga	2009	2	0.20	-25.0	9.4	41.0	13.8	3.5
21643 inf	Olga	2009	3	0.39	-25.5	9.3	45.3	15.3	3.5
21654 inf	Susi	2009	3.5	0.50	-25.1	10.2	42.0	13.8	3.5
21637 inf	Uma	2009	2.5	0.51	-24.2	10.4	39.7	13.2	3.5
21683 inf	Uma	2008	2.5	0.33	-24.2	9.4	39.7	13.4	3.4
21702 inf	Uma	2008	2.5	0.46	-25.2	9.3	41.3	13.7	3.5
21717 inf	Uma	2008	2.5	0.45	-24.6	9.5	40.6	13.4	3.5
21627 inf	Uma	2008	2	0.31	-24.8	9.1	40.1	13.6	3.4
		Infants aged 1–2 years		mean	-24.8	9.7			
		with nursing signal		1σ	0.4	0.6			
21708 inf	Gwen	2007	2.2	0.51	-26.5	8.9	45.7	14.0	3.8
21569 inf	Hanna	2006	3.5	0.45	-25.8	8.6	46.9	14.7	3.7
		Infants aged 3–4 years		mean	-26.1	8.8			
		no clear nursing signal		1σ	0.4	0.1			
		adult females (n = 64)		mean	-25.8	8.5			
				1σ	0.3	0.2			

TABLE III. Metrics of Bonobo Infant Hair (Isotopic Nursing Signal) and of Hair Samples of Adult Female Bonobos

	Nursing infants	Adult females
N individuals	3	5
N hairs	41	58
length	2–3.5 cm	5–7 cm
mean ϕ	55 μm	77 μm
1 σ	$\pm 6 \mu\text{m}$	$\pm 11 \mu\text{m}$
max ϕ	63 μm	111 μm
min ϕ	39 μm	60 μm

to be taken into account. Also, the isotopic response after dietary shifts will be dampened compared to the isotopic signatures of the new food sources due to the recycling of the body's own amino acids and the fact that not all hairs are forming simultaneously. The most critical points for isotope analysis in hair include:

- A reduced isotopic response ($\sim 40\%$) immediately after hair emergence is likely due to the recycling of the body's own amino acids. Complete isotopic reflection of an isotopically distinct recently consumed food source in hair may require several weeks or even months.
- The $\Delta\delta^{13}\text{C}_{\text{diet-hair}}$ and $\Delta\delta^{15}\text{N}_{\text{diet-hair}}$ values for primates are likely to be $\sim 3\%$, but more data from controlled feeding experiments are needed to assess processes influencing Δ , such as reproduction, growth rate or the nutritional quality of consumed foods. In the wild, $\Delta_{\text{diet-hair}}$ values need to be established for new study species and populations. The use of $\Delta_{\text{diet-hair}}$ values has great potential for cross-site comparisons and requires a standardized isotopic plant baseline.
- Naturally shed hair of primates consists of hair in the resting (telogen) phase, which likely induces asynchrony and delay in the isotopic response.

Above, I have proposed methodological guidelines for the field and the laboratory to sample, purify, handle and select hair for studies using isotope analysis, particularly for studies focusing on temporal variation in feeding behavior. The most critical points include:

- In great apes, optimal hair samples come from fresh nests, which have not been used previously, and information on nest position should be recorded.
- Lipid extraction from hair ensures the removal of contaminants and lipids and is recommended prior to isotope analyses.

- Infant and adult hair should be identified and separated with care not to mix subadult and adult specimens when reconstructing the feeding ecology of primates.
- Hair sampled invasively can be limited to hair in the anagen phase. In captive subjects, shaving can facilitate hair synchronization.
- Hair growth rates of approximately 1 cm per month seem most appropriate for hominoids, but differential growth rate studies of non-human primates would be valuable.

Dietary shifts in free ranging primates are not rapid but rather linked to seasonally induced shifts in food availability, which commonly last several weeks or months [Brockman & van Schaik, 2005]. For example, specific fruit species may be more abundant in certain months of the year, whereas in other periods fall back foods dominate the diet [Marshall & Wrangham, 2007]. The study of primates adapted to highly seasonal environments such as woodland/savanna or dry deciduous forests is particularly promising using isotopes in hair [Codron et al., 2008; Crowley et al., 2014]. Also, faunivory can be seasonal [Boesch & Boesch-Achermann, 2000; Crowley et al., 2014; Stanford et al., 1994]. In Tai chimpanzees for example, the hunting season lasts approximately 2 months [Boesch & Boesch-Achermann, 2000]. Vertebrate meat consumption [Fahy et al., 2013], as well as the consumption of invertebrates [Crowley et al., 2014], is detectable in hair isotope ratios.

A temporal perspective also has the potential to trace anthropogenic impacts on primate feeding behavior [Engel et al., 2010; Schurr et al., 2012]. Pienkowski et al. [1998] demonstrated that crop raiding by different primate species is often linked to seasonal climatic shifts or shortages in fruit availability. Many tropical domestic crops are C_4 plants and their consumption can be traced in hair $\delta^{13}\text{C}$ ratios [Loudon et al., 2014]. Stable isotope analysis in hair samples of wild primates provides a novel tool to investigate these human-primate conflicts and may aid the implementation of primate conservation policies.

Finally, the sampling approach discussed in this study can be applied to reconstruct nursing and weaning behavior in great apes and other elusive primates, as illustrated for free-ranging bonobos (Fig. 3). While I show here how infant hair can be obtained non-invasively from great apes nests, hair sampling may require more invasive strategies in other primate taxa. Stable isotope analysis in infant tissues such as hair is a valuable tool to assess the relative proportions of mother's milk ingested by infant and juvenile primates, particularly when direct observations on sucking behavior are impossible [Reitsema, 2012]. In habituated primate communities

this method may be combined with isotope analysis from feces [Reitsema, 2012] to improve accuracy in timing when assessing suckling frequencies during the process of weaning.

Future studies on captive and wild primates will improve our understanding of the time lags to expect for isotopic turnover in the body and on hair growth rates in wild primates living in different habitats and climates. Climatic and phenological information can be matched to changes in hair isotope signatures over time, and information on seasonal feeding behavior and habitat use can be inferred in many elusive species and even in extinct populations existing only in museum collections.

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