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Nitrogen Isotopes in Mammalian Herbivores: Hair δ^{15}N Values from a Controlled Feeding Study

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ABSTRACT Nitrogen isotope analysis is a common technique for investigating dietary behaviour in modern and archaeological populations. One of its primary uses is to provide trophic level information. This application is possible because of a ~3‰ enrichment in ^{15}N along each step in the food chain, resulting in carnivores having higher δ^{15}N values than herbivores, which in turn have higher δ^{15}N values than plants. Much variation has also been observed within a trophic level, although the reasons for this are poorly understood. Here we present the results of a controlled feeding study designed to test the effects of gut anatomy and dietary protein levels on hair δ^{15}N values within a trophic level. The data reveal that mammalian herbivores eating identical diets can have hair δ^{15}N values that differ by as much as 3.6‰. This is particularly striking as it suggests that interspecific physiological differences can lead to larger shifts in δ^{15}N values than a shift in trophic level. We also found that diet-hair fractionation was 2.3‰ greater when herbivores were fed high-protein (19%) diets than when they were fed low-protein (9%) diets. The primary nitrogen losses in mammalian herbivores are ^{15}N-depleted urine and ^{15}N-enriched faeces. We reason that an increase in the ratio of urinary to faecal nitrogen efflux leads to greater diet-hair fractionation on the high-protein diet. Copyright © 2003 John Wiley & Sons, Ltd.

Key words: nitrogen isotopes; nitrogen excretion; hair; dietary reconstruction

Introduction

Nitrogen isotope analysis is a common tool for neoecological and archaeological studies. Ecologists have used nitrogen isotopes (in tandem with carbon isotopes) to determine the trophic behaviour of such diverse fauna as gulls (Thompson *et al.*, 1999), raptors (Harding & Stevens, 2001), bears (Hobson *et al.*, 2000), voles (Harding & Stevens, 2001), whales (Abend & Smith, 1997), fish (Fry *et al.*, 1999), and spiders (Oelbermann & Scheu, 2002). The technique has proven equally fruitful for archaeologists who have used it to distinguish between the consumption of terrestrial and marine foods (Schoeninger *et al.*, 1983; Schoeninger & DeNiro, 1984; Walker & DeNiro, 1986; Sealy *et al.*, 1987), and to investigate the prevalence of carnivory and piscivory in hominid populations (Bocherens *et al.*, 1999; Macko *et al.*, 1999; Richards *et al.*, 2000, 2001). Some of the more notable studies used nitrogen isotope analysis to show that Neanderthals subsisted largely on animal foods (Bocherens *et al.*, 1999; Richards *et al.*, 2000), and conversely, that the Tyrolean Iceman was largely vegetarian (Macko *et al.*, 1999).

Despite these successes, it is generally agreed that the mechanisms controlling nitrogen isotope

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compositions in mammalian foodwebs are poorly understood (Sealy *et al.*, 1987; Gannes *et al.*, 1997; Ambrose, 2000). It is known that $\delta^{15}N$ values increase by ~3‰ up every step in the food chain, although trophic level enrichments of as little as 1‰ and as high as 6‰ have been reported (DeNiro & Epstein, 1981; Yoneyama *et al.*, 1983; Minigawa & Wada, 1984; Schoeninger & DeNiro, 1984; Nakagawa *et al.*, 1985; Ambrose & DeNiro, 1986; Sutoh *et al.*, 1987; Fogel *et al.*, 1989; Koch *et al.*, 1991; Abend & Smith, 1997; Sealy *et al.*, 1987; O'Connell & Hedges, 1999a,b; Ambrose, 2000). Considerable variation has also been reported within a trophic level. This is not surprising as plant $\delta^{15}N$ values can vary greatly due to a number of physiological and abiotic factors. Physiological factors include differing nitrogen uptake mechanisms, differing pathways of assimilation and nitrogen recycling within the plant (see reviews by Evans, 2001 and Adams & Grierson, 2001). Abiotic factors include changes in aridity and salinity (Heaton, 1987), fire regime (Grogan *et al.*, 2000), and nitrogen availability (Hobbie *et al.*, 2000). As a result of these factors, even within a small area there can be variation of 10‰ or more in plant $\delta^{15}N$ values (Hobbie *et al.*, 2000; Sponheimer, unpublished data).

There have been a number of attempts to explain mammalian $\delta^{15}N$ diversity within a trophic level. Ambrose & DeNiro (1986) argued that drought tolerant herbivores are enriched in ^{15}N compared to other herbivores because they lose more ^{15}N-depleted urea in urine, the inevitable consequence of their marked ability to increase urine osmolality and conserve water. Since maximum urine concentrating ability is only manifest during heat stress or dehydration, this model holds that the $\delta^{15}N$ values of herbivores should be most similar when the weather is mild and water is plentiful. In contrast, Sealy *et al.* (1987) suggested that differences in the recycling of urea might lead to differences in tissue $\delta^{15}N$ values. For example, grazers eat diets with less crude protein than sympatric browsers. As a result, they might be forced to recycle ^{15}N-depleted urea more extensively, possibly resulting in grazers having higher $\delta^{15}N$ values than browsers. In short, this model argues that tissue $\delta^{15}N$ values should increase as dietary protein decreases and urea recycling increases. It has also been suggested that digestive anatomy plays a role in determining herbivore $\delta^{15}N$ values (Sealy *et al.*, 1987; van Klinken *et al.*, 2000). Foregut fermenters can digest most of their microflora because of their anterior placement in the gastrointestinal tract, while the posterior or distal location of microflora in hindgut fermenters necessitates that much of the microfloral component escapes digestion and is lost in faeces. Consequently, foregut fermenters should be functionally at a higher trophic level than hindgut fermenters, causing them to have relatively higher $\delta^{15}N$ values.

Given the multitude of physiological, anatomical, and dietary factors that potentially govern herbivore $\delta^{15}N$ values, it is little wonder that our understanding of nitrogen isotopes in mammals remains nascent. Clearly, experimental studies that control one or several of these variables are needed if we are to better our understanding of the determinants of mammalian $\delta^{15}N$ values. Ambrose (2000) conducted one such study to test the influence of heat and protein stress on the nitrogen isotope composition of rat tissues. Surprisingly, he found little evidence that either of these factors significantly influenced rat $\delta^{15}N$ values. He cautioned, however, that these results might not be applicable to humans or mammalian herbivores. Here, we present results from two controlled feeding studies that comprise part of the Stable Isotope Biology (SIB) project, run under the auspices of Brigham Young University and the University of Utah. The first study was designed to test the influence of digestive anatomy on herbivore $\delta^{15}N$ values (Sealy *et al.*, 1987; van Klinken *et al.*, 2000), and the second examined the importance of dietary protein levels in determining herbivore nitrogen isotope compositions (Sealy *et al.*, 1987).

Methods

Experiment 1 was designed to determine whether or not foregut fermenters have elevated $\delta^{15}N$ values compared to hindgut fermenters. To this end, we fed 17 foregut fermenters (four llamas (*Lama glama*), two alpacas (*Lama pacos*), four goats (*Capra hircus*), and seven cattle (*Bos taurus*)) and nine

hindgut fermenters (five horses (*Equus caballus*) and four rabbits (*Oryctolagus cuniculus*)) high-protein (19% crude protein 'CP') alfalfa (*Medicago sativa*) diets. Experiment 2 was designed to test whether or not dietary protein levels affect herbivore $\delta^{15}N$ values. This was accomplished by feeding two alpacas, four goats, six cattle, and two horses low-protein (9% CP) coastal bermudagrass (*Cynodon dactylon*) diets and comparing their diet-hair fractionation values with those obtained from the same species in Experiment 1.

In both experiments, each species was housed in an individual pen and provided with alfalfa or coastal bermudagrass hay *ad libitum*. Each pen also contained automatic waterers so that water was available *ad libitum*. Both the alfalfa and coastal bermudagrass hays were obtained from single cuttings of one farm to minimize isotopic heterogeneity. To further constrain variability in feed $\delta^{15}N$ values, hay was sampled and analysed from feed troughs over 30 times during the course of the experiment; means for the $\delta^{15}N$ of alfalfa and coastal bermudagrass hays were 0.1 ± 0.4‰ and 7.8 ± 0.9‰ respectively. Each animal was on its respective grass or alfalfa diet for over 24 wk prior to isotope analysis of its hair. Biweekly hair sampling established this period to be sufficient for diet-hair nitrogen isotope equilibration in herbivores (Sponheimer *et al.*, 2001; Figure 1). After hair was equilibrated, it was clipped back and the most recently grown hair (~1 mm) was removed for stable isotope analysis. Approximately 0.5 to 0.7 mg was combusted in an automated Carlo-Erba device and stable isotopes were analysed using a flow-through inlet system on a continuous flow isotope ratio mass spectrometer. The standard deviation for replicate measurements of a yeast standard was <0.2‰ for nitrogen isotope ratios. The means of at least two measurements for each individual animal were used in this study.

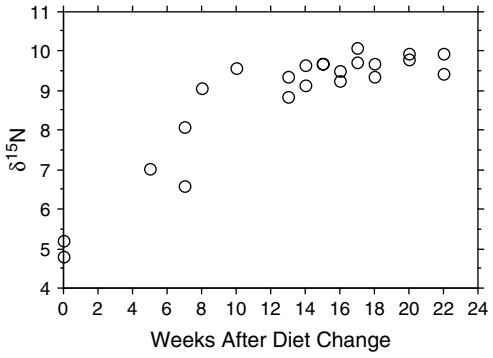

Figure 1. Scatterplot showing diet-hair nitrogen isotope equilibration for two horses. Dietary $\delta^{15}N$ values were changed from 2.5‰ to 7.8‰ at wk 1 and were equilibrated with hair $\delta^{15}N$ in less than 24 wks. Note that from wk 8 to wk 24 $\delta^{15}N$ changed less than 1.5‰.

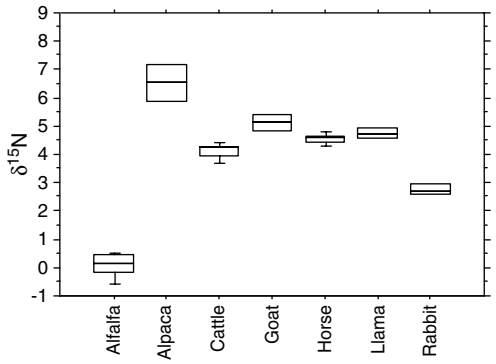

Figure 2. Box plot showing $\delta^{15}N$ values for the alfalfa diet and six mammalian herbivore species that had been eating this diet. The range in trophic enrichment is between 2.8‰ and 6.4‰. Note the difference between the alpacas and rabbits is 3.6‰, more than enough to be attributed to a shift in trophic level. The hindgut fermenting rabbits have lower $\delta^{15}N$ values than all of the foregut fermenters, possibly because they digest a lower percentage of their microflora.

Results and Discussion

Experiment 1

The most conspicuous result was the marked $\delta^{15}N$ variation found between herbivores eating the same diet, even when they experienced no heat or water stress (Figure 2). Indeed, the difference between the rabbits and alpacas was 3.6‰, more than large enough to be ascribed to a trophic level difference in most neoecological and archaeological studies. This would seem to strengthen arguments that foregut fermenters are enriched in ^{15}N due to the digestion of symbiotic microflora, as does the fact that analysis of variance post hoc tests show that the mean $\delta^{15}N$ value for each foregut fermenting species (cattle, goats, llamas, and alpacas) is enriched compared to rabbits (Scheffe; $P < 0.01$).

However, this interpretation is confounded by the relatively enriched $\delta^{15}N$ values of the hindgut fermenting horses, which are not significantly different from the foregut fermenters, save the alpacas. That horses and rabbits are rather dissimilar despite both being hindgut fermenters is perhaps unsurprising as they usually have different digestive strategies. In particular, rabbits are noted for producing hard nitrogen-poor faeces as well as caecotrophs which are moist and nitrogen-rich, the latter of which they are known to consume (Carabano & Piquer, 1998). Horses, in contrast, have only been observed to consume their own faeces on very low-protein diets (Schurg *et al.*, 1977). Thus, one might expect rabbits to digest a larger fraction of their microflora through caecotrophy, leading to relatively elevated $\delta^{15}N$ values compared to horses—just the opposite of what was observed in this study. Nontheless, van Klinken *et al.* (2000) also noted that European rabbits are depleted in ^{15}N compared to sympatric goats, cattle, and horses. We proffer no explanation for this pattern at present.

Thus, this study provides only marginal support for the hypothesis that foregut and hindgut fermenters have different $\delta^{15}N$ values. At the very least, however, it is clear that rabbits are distinct from the four foregut fermenters, and the mean $\delta^{15}N$ value for horses is at least marginally lower than the mean value for goats, alpacas, and llamas, but not cattle. Some research has shown that tissue $\delta^{15}N$ values can increase with age (Ambrose, 2000; Overman & Parrish, 2001), so it is possible that the cattle (12 months old) used in this study might have been more enriched in ^{15}N had they been fully mature; however, most studies have not demonstrated an increase in tissue $\delta^{15}N$ with age (Minigawa & Wada, 1984; Fogel *et al.*, 1989; Katzenberg *et al.*, 1993), so we doubt that this would prove to be the case.

Experiment 2

The data provide strong support for the hypothesis that dietary protein levels influence $\delta^{15}N$ values (Figure 3). In all four species for which we have data for high-protein alfalfa and low-protein coastal bermudagrass, diet-hair fractionation is

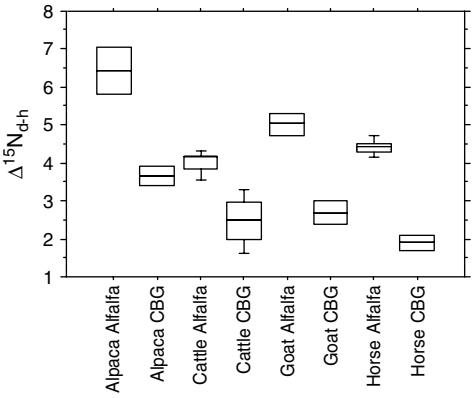

Figure 3. Box plot showing diet-hair fractionation (Δ_{d-h}) for herbivores eating high-protein alfalfa diets and low-protein coastal bermudagrass (CBG) diets. Note the consistently higher diet-hair fractionation for animals eating the high-protein alfalfa.

1.5‰ to 2.8‰ greater for the high-protein diets (Figure 2). Intriguingly, this change moves in the opposite direction of that predicted by Sealy *et al.* (1987), who reasoned that animals on low-protein diets would be more likely to recycle urea and consequently have higher $\delta^{15}N$ values. Admittedly, the low-protein diet used here (9% CP) may still have too much protein to initiate the intensive urea recycling envisioned by Sealy *et al.* (1987). Nonetheless, the increased diet-hair fractionation on high-protein diets requires explanation. Clearly another mechanism must be found to explicate the strong impact of dietary protein levels on $\delta^{15}N$ values.

One possible mechanism may be the changing urinary/faecal nitrogen efflux ratios that are the concomitants of changing dietary protein levels. While nitrogen is lost in hair and skin, this represents a very small fraction of nitrogen efflux. Faeces and urine are the primary nitrogen effluxes, but only urinary nitrogen losses have been considered important determinants of mammalian $\delta^{15}N$ values (Steele & Daniel, 1978; Ambrose & DeNiro, 1986; Ambrose, 1991; Sealy *et al.*, 1987; Gannes *et al.*, 1997). This tendency is problematic, however, as faecal nitrogen losses (which include undigested nitrogen from food, sloughed tissues, microflora, etc.) are often a very large percentage of nitrogen efflux, especially when herbivores consume low-protein diets. More specifically, faecal nitrogen losses frequently represent more than 40% of all nitrogen losses of herbivores on

low-protein diets, while urinary nitrogen losses usually constitute the bulk of total nitrogen efflux for herbivores on high-protein diets (e.g., Robbins *et al.*, 1975; Milton *et al.*, 1980; Mousa *et al.*, 1983; Warmington *et al.*, 1989; Carmean *et al.*, 1992; Sutoh *et al.*, 1993; Zervas & Zijlstra, 2001).

This was further evidenced by Robinson *et al.* (2001) who showed that on a grass hay diet (11% CP) alpacas lost 5.3 g/day of faecal nitrogen (41% of nitrogen efflux) and 7.5 g/day of urinary nitrogen (59% of nitrogen efflux). On an alfalfa diet (20% CP), however, faecal and urinary nitrogen losses were 4.6 g/d (28% of nitrogen efflux) and 11.6 g/d (72% of nitrogen efflux) respectively. Robinson *et al.* (1995) also demonstrated that Holstein steers on grass hay (11% CP) excreted 34.8 g/d of urinary nitrogen (56% of nitrogen efflux), while faecal nitrogen excretion was 27.2 g/d (44% of nitrogen efflux). But when 120 g/d of supplemental casein was infused into their abomasums, relatively little of it was utilized, leading to dramatically increased urinary nitrogen excretion of 142.4 g/d (81% of nitrogen efflux), while faecal nitrogen excretion held reasonably steady at 34.0 g/d (19% of nitrogen efflux). Thus, once an herbivore's protein requirements are exceeded, urinary nitrogen losses increase rapidly, while faecal nitrogen losses do not. But when dietary protein does not significantly exceed maintenance requirements, which is at least seasonally the case for most wild herbivores, faecal nitrogen losses will constitute an important part of total nitrogen efflux.

Furthermore, the nitrogen isotope composition of herbivore faeces is consistently enriched in ^{15}N compared to diet by 0.5‰ to 3‰ (Steele & Daniel, 1978; Sutoh *et al.*, 1987, 1993). Urinary nitrogen, in contrast, is usually depleted in ^{15}N by 0.5‰ to 5.0‰ compared to diet (Steele & Daniel, 1978; Sutoh *et al.*, 1987, 1993). Thus, in the case of an herbivore on a low-protein diet, it is possible that more than half of all nitrogen losses would be in the form of ^{15}N-enriched faeces. For herbivores consuming high-protein diets, however, ^{15}N-depleted urinary nitrogen would comprise the bulk of total nitrogen efflux. As a result, herbivores on high-protein diets should have higher diet-tissue fractionations than the same animals on low-protein diets, which is exactly what was observed in this study.

Differences in the proportions of faecal and urinary nitrogen losses may also account for the 3.6‰ disparity observed in hair $\delta^{15}N$ values of herbivore species eating the alfalfa diet. This would be consistent with the observation that alpacas, which had the highest diet-hair fractionations, lose relatively more urinary nitrogen than most herbivores, even when on very low-protein diets (Robinson *et al.*, 2001). Alternatively, processes such as nitrogen recycling might lead to different urinary or faecal $\delta^{15}N$ values between species that, in turn, affect hair $\delta^{15}N$ values.

Conclusion

This study makes two important points. The first is that mammalian herbivores on identical diets without water, heat, or protein stress can have widely varied diet-hair fractionations. Significantly, the diet-hair fractionation was between 4‰ and 6.5‰ for all taxa save the rabbits when eating alfalfa. Such large fractionations have usually been associated with aridity, but clearly non-climatically induced mechanisms can also lead to highly-enriched $\delta^{15}N$ values. More important, however, is the fact that interspecific physiological differences led to 3.6‰ differences in hair $\delta^{15}N$ values, even when animals consumed the same diet—a shift that is as great or greater than many changes in trophic level. This intimates that caution is needed when using nitrogen isotope compositions as trophic-level indicators.

This study has also shown that a change in dietary protein levels can induce large changes in diet-hair fractionation in herbivores (2.3‰ on average). Ambrose's (2000) data reveal a much smaller increase in diet-hair fractionation (0.6‰) in rats fed high-protein diets (70%). While we cannot explain why dietary protein levels have a much greater effect on large herbivores than on rats, the disparity underscores the need for experimentation on a wide variety of taxa. Otherwise, we will be forced to interpret all mammalian $\delta^{15}N$ values using taxon-specific models that may or may not be appropriate. Lastly, while we have confined ourselves to the discussion of hair $\delta^{15}N$ values, it is likely that similar interspecific and diet-related differences

will be found in bone collagen, as an individual's hair and collagen $\delta^{15}N$ values are known to be correlated (O'Connell & Hedges, 1999b; O'Connell *et al.*, 2001). This suggests that the discussion here will prove equally relevant for bone collagen.

Nonetheless, this equivalence between bone and hair can only be established through controlled feeding studies in which physiologically and trophically distinct mammals are fed isotopically homogenous diets for their entire lives. Hopefully, through such experiments and detailed studies of plant $\delta^{15}N$ values in varied ecosystems, we will better our understanding of the interspecific variability observed in neoecological and archaeological populations.

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