Kangaroo metabolism does not cause the relationship between bone collagen $\delta^{15}N$ and water availability

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Summary

1. A negative relationship between water availability and the abundance of $^{15}N$ relative to $^{14}N$ (expressed as $\delta^{15}N$) in the bone collagen of herbivores has been widely reported. However, the relative importance of dietary $\delta^{15}N$ and animal metabolism in producing this effect remains unclear.

2. To evaluate the relative importance of these two factors, we examined variation in $\delta^{15}N$ of both grass foliage and kangaroo (Macropus spp.) bone collagen. We assessed whether the offset between grass and bone collagen $\delta^{15}N$ was constant with respect to water availability.

3. An index of water availability (annual actual evapotranspiration/annual potential evapotranspiration) explained a considerable proportion of the variation in both grass $\delta^{15}N$ ($R^2 = 0.40$) and bone collagen $\delta^{15}N$ ($R^2 = 0.57$), and the slopes of these negative relationships were similar, with a near-constant $\delta^{15}N$ offset between grass foliage and bone collagen.

4. This finding suggests that dietary $\delta^{15}N$ is the main cause of the negative relationship between kangaroo bone collagen $\delta^{15}N$ and water availability, with metabolic factors having little discernible effect.

Key-words: herbivore, Macropus, nitrogen, rainfall, stable isotope

Introduction

The abundance of $^{15}N$ relative to $^{14}N$ (expressed as $\delta^{15}N$) in animal tissues is commonly used to determine trophic structure and environmental factors influencing animal populations. Animal $\delta^{15}N$ increases by about 1–5‰ with each increasing trophic level and can be used to determine trophic position (Hobson & Montecvecchi 1991; Kwak & Zedler 1997). Animal $\delta^{15}N$ is also strongly affected by environmental factors, particularly water availability. A negative relationship between bone collagen $\delta^{15}N$ and water availability has been demonstrated in African (Heaton et al. 1986; Sealy et al. 1987), Australian (Gröcke, Bocherens & Mariotti 1997), and to a lesser extent, North American (Cormie & Schwarcz 1996) herbivores. As the $\delta^{15}N$ signal is often faithfully preserved in fossil material, this approach is particularly useful for reconstructing the trophic ecology of extinct animals (Bocherens et al. 1995; Hilderbrand et al. 1996), and past climate change (Ambrose & DeNiro 1989; Stevens & Hedges 2004).

The negative relationship between herbivore $\delta^{15}N$ and water availability is thought to arise because of the negative relationship between plant $\delta^{15}N$ and water availability, the direct effects of water availability on the nitrogen metabolism of the animal, or both. Plant $\delta^{15}N$ typically shows a negative relationship with water availability, a pattern that is now well documented at both regional (Heaton 1987; Austin & Vitousek 1998; Austin & Sala 1999; Schuur & Matson 2001; Aranibar et al. 2004; Swap et al. 2004) and global scales (Handley et al. 1999; Amundson et al. 2003). Austin & Vitousek (1998) proposed that the ‘openness’ of the nitrogen cycle may be an underlying cause of this relationship. They suggest that losses of nitrogen from an ecosystem via leaching and nitrogen transformation lead to $^{15}N$-enrichment of the nitrogen remaining in the system. While the magnitude of nitrogen losses tend to increase with water availability, the ratio of loss to intrasystem turnover tends to decrease, such that wetter ecosystems have relatively closed nitrogen cycles and are less $^{15}N$ enriched (see also Austin & Sala 1999; Handley et al. 1999; Schuur & Matson 2001).

A number of authors have suggested that the increase in plant $\delta^{15}N$ with decreasing water availability is insufficient to account for the increase in herbivore $\delta^{15}N$, and suggest that the metabolism of the animal is directly responsible for strong isotopic fractionation in arid areas (Heaton et al. 1986; Sealy et al. 1987).

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Herbivores from arid areas may have low drinking water requirements and conserve water by excreting urine with a high concentration of $^{15}$N-depleted urea, leading to increased animal $\delta^{15}$N (Ambrose & DeNiro 1986; Ambrose 1991). Using controlled experiments, Sponheimer et al. (2003b) confirmed a close relationship between urea excretion and the $\delta^{15}$N of large herbivores, while Ambrose (2000) was unable to show that water or heat stress affected the $\delta^{15}$N of rats. The low protein content of forage in arid areas may also promote urea recycling in ruminant herbivores, which could also increase animal $\delta^{15}$N (Sealy et al. 1987). Despite such theories, there is remarkably little evidence that the relationship between herbivore $\delta^{15}$N and water availability is not simply a reflection of the relationship between plant $\delta^{15}$N and water availability. While a handful of studies have compared the two relationships, none have done so in a statistically rigorous way (Heaton 1987; Sealy et al. 1987; Vogel et al. 1990).

Materials and methods

**Specimen collection and analysis**

Between March 2003 and November 2004, 408 grass specimens were collected from relatively undisturbed vegetation at 173 locations throughout Australia (Fig. 1). At each location, only the most abundant grasses were collected, usually numbering between three and four different taxa. Owing to the paucity of reproductive material available to be collected, grasses could not be identified to species level in most cases. Samples of live foliage were collected, pressed in newspaper and air-dried. When no live foliage was available, senescent foliage was collected. Both young and mature leaves were selected from each sample, rinsed with deionized water, dried and ground to a fine powder.

The analysis of kangaroo bone collagen was restricted to larger members of the kangaroo genus *Macropus*, a group that are predominantly grazers, consuming relatively small amounts of dicotyledonous plant material (Dawson 1989; Sanson 1989). Specimens of bone from 779 road-killed individuals were collected throughout Australia between March 2003 and November 2004 (Fig. 1). Where possible, the age of each animal at death was determined (Kirkpatrick 1985). Approximately 0.4 g of bone was removed from the horizontal ramus of the mandible, and collagen extracted using the modified Longin method of Brown et al. (1988).

Approximately 4 mg of each grass foliage sample and 2 mg of each bone collagen sample were placed in tin capsules, and analysed with an EA 1110 elemental analyser (CE Instruments, Rodano, Italy) coupled with an IsoChrom mass spectrometer (Micromass, Manchester, UK). For the grass foliage samples, $\delta^{15}$N was measured, and for the bone collagen samples, both $\delta^{13}$C and $\delta^{15}$N were measured. $\delta^{13}$C and $\delta^{15}$N were expressed in per mil (‰) units, relative to the atmospheric N$_2$ and Vienna Pee Dee Belemnite standards, respectively. Bone collagen $\delta^{13}$C was measured because it provided a convenient
Bone collagen has a very slow rate of turnover, such that bone collagen $\delta^{13}N$ represents the kangaroo’s isotopic signal averaged over much of its lifetime (Thompson & Ballou 1956; Libby et al. 1964). While some individuals of certain kangaroo species may disperse over large distances (> 200 km) during their lifetime, these individuals are uncommon (Priddel 1987). Most individuals of even the larger, less sedentary kangaroo species have home ranges of less than 10 km$^2$, and rarely disperse more than 20 km (Priddel 1987; Priddel, Shepherd & Wellard 1988; Croft 1991). The potential error due to kangaroo movements may decrease the strength of the relationship between $\delta^{15}N$ and environmental variables, but is unlikely to bias the results in any particular direction.

ENVIRONMENTAL DATA

Digital maps of mean annual areal actual evapotranspiration (AET) and areal potential evapotranspiration (PET), covering Australia at a resolution of 0.05° (Bureau of Meteorology, Canberra, Australia), were used to estimate a soil water availability index (WAI). This was calculated as the ratio of AET to PET (Walker & Landridge 1997). Other environmental variables used in the analysis (Table 1) were estimated from digital maps of mean slope (CSIRO Land and Water 2001), vegetation (National Land and Water Resources Audit 2001), and soils (Bureau of Rural Sciences after Commonwealth Scientific and Industrial Research Organisation 1991).

STATISTICAL ANALYSIS

General approach

To analyse the data from this study, we used an information-theoretic approach, sensu Burnham & Anderson (2002). Rather than focusing on a single null hypothesis, this approach utilizes a set of multiple hypotheses, or models, that are ranked for suitability using Akaike’s Information Criterion (AIC). AIC favours both model fit and model simplicity, with lower values indicating greater support for a model, relative to other models in the same candidate set. Burnham & Anderson (2002) suggest that a model with an AIC value within 2 of the lowest AIC value in the candidate set can be considered well supported, and a model with an AIC value within 4–10 of the lowest can be considered weakly supported. For each model in the candidate set, AIC can be used to calculate an Akaike weight ($w_i$), which represents the probability of that model being the best in the candidate set (Burnham & Anderson 2002). In cases where AIC does not clearly distinguish the most highly ranked model in the candidate set, multimodel averaging can be used as the basis for inference (Burnham & Anderson 2002). The predictions of all models with an AIC, value within 10 of the best model are weighted according to $w_i$ and combined.

Visual inspection of grass and bone collagen $\delta^{15}N$ plotted against WAI suggested a nonlinear relationship, so we applied an inverse transformation to WAI prior to any analysis, i.e. WAI$^{-1}$.

Offset between grass $\delta^{15}N$ and bone collagen $\delta^{13}N$

Our primary aim was to determine whether the offset between grass $\delta^{13}N$ and bone collagen $\delta^{15}N$ was constant with respect to water availability. To do this, we made $\delta^{15}N$ the response variable, and ‘sample type’ (either grass or bone collagen) a predictor variable. We constructed a set of three candidate models using the variables WAI and ‘sample type’ (Table 2). One model contained an interaction between WAI and ‘sample type’, i.e. the offset varied with respect to water availability; one model contained WAI and ‘sample type’ with no interaction, i.e. the offset was constant with respect to water availability; and the third model included WAI only, i.e. there was no offset with respect to water availability. The models were fitted using generalized least-squares (GLS) linear regression in the computer program R (Ihaka & Gentleman 1996). Inspection of

Table 1. Variables examined in the analysis of kangaroo bone collagen $\delta^{13}N$, and key references that suggest a relationship between each variable and plant or animal $\delta^{15}N$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAI</td>
<td>Water availability index</td>
<td>Handley et al. (1999)</td>
</tr>
<tr>
<td>slope</td>
<td>Mean slope (%) within 20 km radius</td>
<td>Amundson et al. (2003)</td>
</tr>
<tr>
<td>coastal</td>
<td>Coastal (&lt; 20 km); or inland (&gt; 20 km)</td>
<td>Heaton (1987)</td>
</tr>
<tr>
<td>chenopod</td>
<td>Proportion of the surrounding area (20 km radius) dominated by chenopod shrubland, as a proxy for soil salinity</td>
<td>Heaton (1987)</td>
</tr>
<tr>
<td>soil</td>
<td>Predominant soil type within 20 km radius</td>
<td>Rodière et al. (1996)</td>
</tr>
<tr>
<td>$\delta^{13}C$</td>
<td>Bone collagen $\delta^{13}C$ as a proxy for C$_4$ contribution to diet</td>
<td>Kelly (2000)</td>
</tr>
<tr>
<td>group</td>
<td>Subgenus Notamacropus; or subgenera Macropus and Osphranter</td>
<td></td>
</tr>
<tr>
<td>age</td>
<td>Age of the animal at death</td>
<td>Trueman, McGil &amp; Guyard (2005)</td>
</tr>
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empirical variograms of the model residuals suggested that the residuals were spatially autocorrelated. We accounted for this by incorporating an exponential spatial autocorrelation function into the GLS models (Crawley 2002). The candidate models were compared using AICc, the second order form of AIC, appropriate for small sample sizes (Burnham & Anderson 2002).

Other factors influencing bone collagen $\delta^{15}N$

Our secondary aim was to determine what factors, other than water availability, influenced bone collagen $\delta^{15}N$. We chose seven variables other than WAI that were considered likely to influence bone collagen $\delta^{15}N$ (Table 1). These variables were combined with WAI to construct an a priori set of eight biologically plausible candidate models to explain variation in bone collagen $\delta^{15}N$ (Table 3a). Again the models were constructed as GLS regression models incorporating an exponential spatial autocorrelation structure and ranked using AICc.

The variable ‘age’ could not be included in the initial a priori candidate set, because age at death was not known for all samples. Therefore, to examine the effect of ‘age’ on bone collagen $\delta^{15}N$, we removed the samples for which age at death was not known (109 of 779 samples), and fit two models: the first incorporating WAI; and the second incorporating WAI and the variable ‘age’. The importance of ‘age’ was determined by comparing the AICc values of the two models (Table 3b).

The entire data set used in the analysis is provided in the Supplemental material.

Results

OFFSET BETWEEN GRASS $^{15}N$ AND BONE COLLAGEN $^{15}N$

Both grass and bone collagen $\delta^{15}N$ showed a strong negative correlation with water availability ($R^2 = 0.40$ and 0.57, respectively), and the slopes of these relationships were very similar, with a near-constant offset between grass and bone collagen $\delta^{15}N$ (Fig. 2). The model suggesting a constant $\delta^{15}N$ offset was 8.4 times more likely to be the best in the candidate set than the model suggesting a $\delta^{15}N$ offset that varied with respect to water availability (Table 2).

Despite there being strong support for the model suggesting a constant $\delta^{15}N$ offset, we used multimodel averaging of the entire candidate model set as the basis for inference (Burnham & Anderson 2002). Multimodel averaging suggested that the offset between grass and bone collagen $\delta^{15}N$ varied by only 0.05‰ over the entire range of the data, from 4.74‰ at the highest water availability, to 4.79‰ at the lowest (Fig. 2).

When kangaroo bone collagen $\delta^{15}N$ is plotted against mean annual rainfall, the relationship is relatively strong ($R^2 = 0.61$), but clearly nonlinear (Fig. 3).
The relationship between the δ15N of herbivore bone collagen and mean annual rainfall. The upper panel shows kangaroo bone collagen δ15N from the present study. The middle panel shows the kangaroo data of Gröcke et al. (1997); the data for north-western and south-eastern Kangaroo Island have been averaged and appear as two data points only. The lower panel shows data for southern African (filled circles, Heaton et al. 1986; Sealy et al. 1987) and North American herbivores (empty circles, Cormie & Schwarz 1996); the southern African data are site averages. The lines represent a GLS regression using the kangaroo data from the present study: δ15N = 1830 × WAI−1 + 2.39. R² for the regression is 0.61.

**OTHER FACTORS INFLUENCING BONE COLLAGEN 15N**

Other than water availability, the only variable that was correlated with bone collagen δ15N was bone collagen δ13C, which we used as a proxy for the contribution of C₄ grasses to the diet. The model incorporating both WAI and δ13C was clearly better than the model incorporating WAI alone, with a probability of 1.00 of being the best model in the candidate set (Table 3a).

While the evidence for a relationship between bone collagen δ15N and δ13C was very strong, the relationship itself appeared to be very weak, with δ13C only explaining a small proportion of the variation in δ15N. Adding the variable δ13C to the model that incorporated WAI alone made very little difference to R² (Table 3a). The relationship between δ15N and δ13C was negative, with δ15N predicted to differ by 2-6‰ between the most 13C depleted (δ13C = −26.1‰) and enriched individuals (δ13C = −8.4‰; Fig. 4).

There was little evidence that any of the other variables examined were correlated with δ15N. Inclusion of the variables ‘slope’ and ‘chenopod’ in models with WAI increased the support for those models only very slightly (Table 3a). The variables ‘group’, ‘coastal’, ‘soil’ and ‘age’ decreased support for any model in which they were included (Table 3).

**Discussion**

A strong negative relationship between herbivore bone collagen δ15N and water availability has been demonstrated extensively (Heaton et al. 1986; Sealy et al. 1987; Gröcke et al. 1997), yet there is considerable debate about the relative roles of plant δ15N values and animal metabolism in causing the relationship (Ambrose & DeNiro 1986; Heaton 1987; Vogel et al. 1990; Ambrose 1991). Our findings show that the strong relationship between kangaroo bone collagen δ15N and water availability is very similar to that between grass foliage δ15N and water availability, with a near-constant δ15N offset between grass foliage and bone collagen. This finding strongly suggests that dietary δ15N is the main cause of the relationship between kangaroo bone collagen δ15N and water availability and that metabolic factors have little discernible effect.

Despite belonging to a phylogenetically distinct group of mammals, there is little in the biology of kangaroos to suggest that the pattern we have identified is exceptional. The two main mechanisms that have been proposed to explain the metabolic enrichment of herbivores in arid areas are high urine osmolality in response to low drinking water intake and urea recycling in response to low protein intake. It has been shown that arid zone kangaroos have urine osmolalities that are comparable to large arid-zone herbivores of other continents (Ealey, Bentley & Main 1965). Furthermore, kangaroos are foregut fermenters that recycle urea in response to low protein intake (Kinnear & Main 1975; Taylor 1985). Certainly, the bone collagen δ15N data for kangaroos are consistent with published data for eutherian herbivores in southern Africa and North America (Fig. 3).

Gröcke et al. (1997) found a very strong negative linear relationship between kangaroo bone collagen...
Kangaroo bone collagen $\delta^{15}N$ and water availability

$\delta^{15}N$ and annual rainfall ($R^2 = 0.92$), yet the relationship we found was much weaker ($R^2 = 0.61$), and clearly nonlinear (Fig. 3). Given that our sample size was over 21 times that of Gröcke et al. (1997: 779 vs. 36), and came from a much wider distribution throughout Australia, it appears that the smaller sample of Gröcke et al. did not show the true variability of $\delta^{15}N$ nor the true shape of the relationship between rainfall and $\delta^{15}N$. Such a difference underscores the need for a large sample of geographically widespread samples before meaningful inferences can be drawn.

We found strong evidence of a negative relationship between bone collagen $\delta^{15}N$ and $\delta^{13}C$. Bone collagen $\delta^{13}C$ closely reflects the abundance of C$_4$ plants relative to C$_3$ plants in a herbivore's diet (DeNiro & Epstein 1978), and in the case of kangaroos, which are predominantly grazers, bone collagen $\delta^{13}C$ would largely reflect the relative abundance of C$_4$ vs. C$_3$ grasses in the diet. Hence, the relationship between $\delta^{15}N$ and $\delta^{13}C$ may arise because of differences in protein content between C$_3$ and C$_4$ plants, as well as between C$_3$ woody plants and grasses. Sponheimer et al. (2003a,b) have shown that animals that consume low protein diets tend to have low tissue $\delta^{15}N$, relative to dietary $\delta^{15}N$. The foliage of C$_4$ plants contains considerably less nitrogen, and hence protein, per unit area than C$_3$ plants, due to their higher nitrogen use efficiency (Ehleringer & Monson 1993). Woody plants (mostly C$_3$) could also be expected to have a higher protein content than grasses (C$_3$ and C$_4$). For these reasons, we would expect a negative relationship between tissue $\delta^{15}N$ and $\delta^{13}C$, even if dietary $\delta^{15}N$ was constant.

Another factor that may contribute to the relationship between bone collagen $\delta^{15}N$ and $\delta^{13}C$ is differences in $\delta^{15}N$ between C$_3$ and C$_4$ grasses. We have previously found that $\delta^{15}N$ is about 1-1‰ higher in C$_4$ grasses than C$_3$ grasses collected throughout Australia (Murphy & Bowman unpublished; Fig. 5). Because $\delta^{13}C$ is lower in C$_3$ than C$_4$ grasses (Bender 1971), we would expect a negative relationship between $\delta^{13}C$ and $\delta^{15}N$. The relative abundance of C$_3$ and C$_4$ plants in the diet has not previously been reported to affect $\delta^{15}N$ in herbivores, despite several authors briefly addressing the issue (Heaton et al. 1986; Heaton 1987). Reasons for a relative $\delta^{15}N$ enrichment of C$_3$ grasses is unclear, but may relate to ecological differences between C$_3$ and C$_4$ grasses, particularly differences in the timing of their respective growing seasons (Winslow, Hunt & Piper 2003). It should be noted, however, that this hypothesized difference in $\delta^{15}N$ between C$_3$ and C$_4$ grasses has yet to be rigorously tested.

It is unlikely that the relationship between bone collagen $\delta^{15}N$ and $\delta^{13}C$ arises because of a relationship between C$_3$ grass abundance (and hence bone collagen $\delta^{13}C$) and water availability. In Australia, the abundance of C$_4$ grasses is not thought to be closely related to water availability; C$_4$ grasses are most closely associated with high summer temperatures and a high ratio of summer to winter rainfall (Hattersley 1983). In fact, there was only a very weak positive relationship between bone collagen $\delta^{13}C$ and the water availability index ($R^2 = 0.03$). We would actually expect this relationship to be negative if it was responsible for the negative relationship between bone collagen $\delta^{15}N$ and $\delta^{13}C$.

**Implications**

While the $\delta^{15}N$ of ancient herbivore bones is commonly used to reconstruct trophic positions of ancient animals and past changes in water availability, little attention has been given to the effects of changes in atmospheric CO$_2$ concentration on plant and animal $\delta^{15}N$. CO$_2$ concentration is thought to strongly influence ecosystem $\delta^{15}N$ via its strong effect on the water use efficiency (WUE) of plants (Morison 1993; Peñuelas & Estiarte 1997). Decreased CO$_2$ concentrations, such as that at the last glacial maximum, would lower plant WUE and hence net primary productivity (NPP), for a given level of water availability. A reduction in NPP would reduce the biological demand for nitrogen, increasing the openness of the nitrogen cycle and increasing plant $\delta^{15}N$. Given that our results suggest a close relationship between plant and herbivore $\delta^{15}N$, we could expect herbivore $\delta^{15}N$ to be strongly affected by changes in CO$_2$ concentration. Herbivore $\delta^{15}N$ can only be an accurate palaeoecological tool if the effects of CO$_2$ concentration on ecosystem $\delta^{15}N$ are taken into account.

Researchers have recently turned to isotopic analysis of plant specimens stored in herbaria to provide evidence of historical changes in nitrogen cycling (Peñuelas & Estiarte 1997; Peñuelas & Filella 2001). Given the close relationship we have demonstrated between grass and kangaroo $\delta^{15}N$, isotopic analysis of the remains of kangaroos, and possibly other herbivores, stored in museums has the potential to yield similar information about changes in nitrogen cycling. As a proxy for the openness of the nitrogen cycle, an advantage of kangaroo
bone collagen δ¹⁵N over plant δ¹⁵N is that it appears to give a much less noisy signal, probably because the isotopic signature of bone collagen represents a bulk sample of the kangaroo’s diet, integrated over much of the individual’s lifetime.

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