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Seasonal variation in kangaroo tooth enamel oxygen and carbon isotopes in southern Australia

Tom H. Brookman [a,*], Stanley H. Ambrose [b]

[a] School of Geographical and Environmental Studies, University of Adelaide, South Australia 5005, Australia
[b] Department of Anthropology, University of Illinois, 109 Davenport Hall, 607 South Mathews Avenue, Urbana, IL 61801, USA

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ABSTRACT

Serial sampling of tooth enamel growth increments for carbon and oxygen isotopic analyses of *Macropus* (kangaroo) teeth was performed to assess the potential for reconstructing paleoseasonality. The carbon isotope composition of tooth enamel apatite carbonate reflects the proportional intake of C_3 and C_4 vegetation. The oxygen isotopic composition of enamel reflects that of ingested and metabolic water. Tooth enamel forms sequentially from the tip of the crown to the base, so dietary and environmental changes during the tooth's formation can be detected. $\delta^{13}C$ and $\delta^{18}O$ values were determined for a series of enamel samples drilled from the 3rd and 4th molars of kangaroos that were collected along a 900 km north–south transect in southern Australia. The serial sampling method did not yield pronounced seasonal isotopic variation patterns in *Macropus* enamel. The full extent of dietary isotopic variation may be obscured by attenuation of the isotopic signal during enamel mineralisation. Brachydont (low-crowned) *Macropus* teeth may be less sensitive to seasonal variation in isotopic composition due to time-averaging during mineralisation. However, geographic variations observed suggest that there may be potential for tracking latitudinal shifts in vegetation zones and seasonal environmental patterns in response to climate change.

Introduction

Stable isotopic analysis of archeological and fossil bones and teeth has been a useful tool for paleoenvironmental reconstruction. Isotopic analysis of serial samples from hypsodont (high-crowned) herbivore teeth has aided in the study of prehistoric peoples (Balasse et al., 2002) and environments (Nelson, 2005) by providing seasonally resolved climate data. Environmental isotopic studies of Australian mammal bone collagen are well established (Pate et al., 1998; Roberts et al., 1999; Pate and Anson, 2008). However, the few systematic studies of intra-tooth isotopic variation in Australian ecosystems have been conducted only on hypsodont herbivores (Fraser et al., 2008). We are unaware of similar research for brachydont (low-crowned) herbivores. In Australia, where the dominant herbivores are brachydont kangaroos (*Macropus* spp.), the potential for obtaining seasonally resolved data from intra- and inter-tooth isotopic variation remains unknown.

Evaluating temporal and geographic variation in modern kangaroo teeth from known environments is a vital prerequisite for advancing the study of past environments. Intra-annual climate variation and seasonal events such as the summer monsoon documented in late Quaternary ratite shells by Johnson et al. (1999), and patterns of seasonality accompanying megafaunal extinction, changes in fire frequencies, decline in C_4 grass biomass and human colonization (Johnson et al., 2005; Prideaux et al., 2009; Field and Wroe, 2012; Murphy et al., 2012; Rule et al., 2012) could be evaluated. The impact of intra- and inter-annual climatic variation on prehistoric human populations could also be investigated because remains of *Macropus* spp. have been found in many late Quaternary archeological sites. The results presented here reveal both the potential and the limitations of isotopic studies of seasonal variation in the diet and climate of kangaroos, with global implications for the analysis of other brachydont species.

Tooth enamel apatite is extremely resistant to diagenesis (Wang and Cerling, 1994; Cerling et al., 1997; Lee-Thorp, 2000) and provides a reliable stable isotopic paleodietary and paleoenvironmental proxy. Isotopic analysis of apatite carbonate can reveal the sources of dietary carbon (Krueger and Sullivan, 1984) and ingested and metabolically formed water oxygen (Bryant and Froelich, 1995), and can provide information about paleoclimatic conditions.

Enamel forms gradually from the apex of the crown to the base of a tooth as the ameloblast cell layer secretes a largely organic apatite matrix on daily and weekly cycles of activity. As the tooth grows toward the cervix (neck) where the crown meets the root, the enamel progressively mineralises (Balasse, 2002; Hoppe et al., 2004), erasing short-term variation but preserving a permanent record of the isotopic composition of diet and ingested water during enamel mineralisation. Sampling at regular intervals parallel to the growth axis can provide an isotopic record of seasonal changes over the period during which the

* Corresponding author at: Department of Geological Sciences, University of Canterbury, Private Bag 4800, Christchurch 8140, New Zealand. Fax: +64 33642769.
E-mail address: tom.brookman@pg.canterbury.ac.nz (T.H. Brookman).

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tooth formed. However, even the highest resolution microsampling cannot recover short term isotopic variation during ameloblast activity.

Enamel mineralisation is not a constant process. Multi-stage enamel maturation results in time-averaging of the isotopic signal. In many hypsodont species there are two major phases, each with numerous components, so the isotopic signal of mature enamel is composed of multiple, temporally overlapping signals spanning up to six months from initial enamel organic matrix secretion to complete maturation (Balasse, 2002; Passey and Cerling, 2002; Hoppe et al., 2004). Apatite is likely most susceptible to isotopic exchange during the earliest stages of mineralization, so the isotopic composition may be biased toward the diet and water isotopic composition of the later stages of enamel maturation. Attenuation of isotopic variation has been overcome in studies of hypsodont teeth where seasonal changes in environment is pronounced (Balasse, 2002; Balasse et al., 2002; Higgins and MacFadden, 2004; Nelson, 2005). Enamel mineralisation patterns in brachydont teeth remain relatively unstudied. This study aims to determine whether seasonal isotopic variation within *Macropus* spp. can be detected with the hand-drilling sampling method used on hypsodont teeth (Balasse et al., 2002, 2003, 2005, 2006).

Bone apatite analyses were also conducted for comparison with enamel. Enamel accretion is generally a finite process because enamel becomes metabolically inert after mineralization. Conversely, bone is continually remodelled: old bone is re-absorbed and new bone deposited throughout life (Hedges et al., 2005). The isotopic composition of bone apatite integrates environmental variation over several years before the animal's death (Longinelli, 1984). Compared with the intra-annual resolution from enamel, bone apatite is a proxy for multi-year average environmental conditions.

Carbon and oxygen isotope analysis of seasonality in tooth enamel

Stable isotope ratios are expressed using the δ (delta) notation in parts per thousand (‰, per mil) difference in relative abundance of the heavier isotope from that of a standard, calculated as:

$$\delta‰ = \left(\left(R_{sample}/R_{standard}\right)-1\right) \times 1000,$$

where R is $^{13}C/^{12}C$ or $^{18}O/^{16}O$. Delta values presented here are expressed relative to the Pee Dee Formation *Belemnitella* (PDB) fossiliferous carbonate standard for carbon and Vienna Standard Mean Ocean Water (V-SMOW) (Coplen et al., 1983) for oxygen.

The stable carbon isotope ratios of diet were calculated using the known diet-to-enamel enrichment (ε) of 11.7‰ for kangaroos (Murphy et al., 2007b) and the 'apparent fractionation factor' (α) equation required to accurately reconstruct dietary $\delta^{13}C$ from measured enamel values spanning a large range (Cerling and Harris, 1999). The notation '*' denotes a fractionation not associated with chemical equilibrium (Cerling and Harris, 1999).

$$\alpha*_{Enamel-Diet} = \left(1000 + \delta^{13}C_E\right)/\left(1000 + \delta^{13}C_D\right). \quad (1)$$

$$\varepsilon*_{Enamel-Diet} = (\alpha*_{E-D}-1) \times 1000. \quad (2)$$

The photosynthetic pathways of the plants consumed by herbivores are reflected in the carbon isotopic composition of mineralized tissues (DeNiro and Epstein, 1978; Ambrose, 1993; Ambrose and Norr, 1993). The most important distinction for herbivore diets is between plants with the C_3 photosynthetic pathway, including winter rainfall grasses and most trees and shrubs, and C_4 plants, which are primarily grasses adapted to summer rainfall, strong sunlight and low atmospheric CO_2 concentrations. C_3 plants are relatively ^{13}C depleted, with an average $\delta^{13}C$ of ~−27‰. C_4 plants discriminate less against isotopically "heavy" $^{13}CO_2$ and have an average $\delta^{13}C$ of ~13‰ (O'Leary, 1988).

Discrimination against air $^{13}CO_2$ in C_3 plants varies substantially, mainly in response to stomatal conductance, which is strongly influenced by humidity and water stress. C_3 leaf $\delta^{13}C$ values can vary by 3–6‰ within species in response to water stress, and are highest in hot, dry environments (Tieszen, 1991). This has been well-documented in C_3 pasture grasses in the South Australian context (Pate and Krull, 2007). In closed canopy forests ^{13}C-depleted CO_2 from C_3 plant respiration and decomposition can reduce forest understory food-web $\delta^{13}C$ values. Consequently $\delta^{13}C$ in C_3-based food-webs can be used to deduce detailed environmental attributes such as height and density of tree canopy cover (van der Merwe and Medina, 1989), light intensity (Farquhar et al., 1989; Buchmann et al., 1997), humidity (Francey and Farquhar, 1982; Ramesh et al., 1986), genetics (Farquhar, 1983; Tieszen, 1991) and/or water stress (Cerling et al., 2003).

C_4 plants do not grow in deep shaded habitats so their $\delta^{13}C$ values are not influenced by the canopy effect. Moreover, discrimination against $^{13}CO_2$ by C_4 plants is relatively insensitive to other environmental factors noted above (Marino and McElroy, 1991; Tieszen, 1991). Therefore, enrichment in $\delta^{13}C$ of herbivore tissues higher than that found in pure C_3 food-webs reflects the relative abundance of C_4 plants.

Diet to apatite ^{13}C enrichment varies from 9‰ to 14.3‰ between species due to differences in digestive strategies (DeNiro and Epstein, 1978; Ambrose and Norr, 1993; Tieszen and Fagre, 1993; Cerling and Harris, 1999). In kangaroos the average enrichment (mean ± 1 standard deviation) is estimated to be +11.7 ± 0.6‰ (Murphy et al., 2007b). Assuming C_3 and C_4 plant average end-member $\delta^{13}C$ values of −27‰ and −12‰, respectively, corresponding kangaroo apatite end-member values should be −15.6‰ and −0.4‰, ±0.6‰. Recent literature suggests that kangaroos exhibit an unusually negative range of $\delta^{13}C$ values compared to eutherian ruminant herbivores (Fraser, 2005; Murphy et al., 2007b), potentially due to low $\delta^{13}C$ of the vegetation they consume and the low abundance of C_4 plants (Johnson et al., 2005).

Oxygen isotope analysis of tooth enamel can provide complementary information about paleoenvironments (Levin et al., 2006). Among herbivores, $\delta^{18}O$ is largely determined by the varying proportions of ^{18}O-enriched leaf water and food-derived metabolic water, and ^{18}O-depleted drinking water (Connin et al., 1998). $\delta^{18}O$ variation has been applied as a proxy for changes in regional precipitation and humidity (Ayliffe and Chivas, 1990; Fricke et al., 1998; Balasse et al., 2002; Nelson, 2005). Plant leaf water and organic matter are enriched in ^{18}O to varying degrees due to stomatal water loss. Enrichment varies systematically with daily and seasonal variation in humidity and temperature, and is greatest with lowest relative humidity (Yakir, 1992; Helliker and Ehleringer, 2002). Consequently, care must be taken when using $\delta^{18}O$ as a proxy for factors associated with rainfall $\delta^{18}O$, such as latitude, temperature, evaporation, humidity, continentality, amount of rainfall and altitude (Craig, 1961; Dansgaard, 1964; Rozanski et al., 1993; Fricke et al., 1998; Darling et al., 2005; Wang et al., 2008). Kangaroos have particularly low drinking-water requirements and obtain a substantial amount of water from plants, so their apatite $\delta^{18}O$ is most closely correlated with relative humidity (Ayliffe and Chivas, 1990) and temperature (Murphy et al., 2007a). Seasonal changes in $\delta^{18}O$ in response to these environmental factors have been observed in tooth enamel in a wide range of hypsodont herbivores on other continents (Balasse et al., 2002; Higgins and MacFadden, 2004; Nelson, 2005; Balasse et al., 2006; Passey and Cerling, 2006).

Kangaroo physiology and ecology

Macropus spp. were chosen for this study because they are common in most modern Australian environments (Dawson, 1995) and

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in the fossil record. Australian paleontology has focussed heavily on megafaunal extinctions that accelerated around 46,000 yr ago (Roberts et al., 2000; Murphy et al., 2012); more recent environmental changes have not been studied as intensively despite the occurrence of significant climatic events and most of Australia's archeological record (Lourandos and David, 2002). Isotopic analysis of modern and fossil kangaroo remains has the potential to link with existing paleontological research and adds new dimensions to the Quaternary paleoclimatic record. To successfully extend this record to a seasonal resolution using kangaroo teeth would unlock extensive, continent-wide archives of palaoclimatic information.

The feeding ecology of the larger kangaroos, *Macropus rufus*, *M. giganteus*, *M. fuliginosus* and *M. robustus*, makes them a valuable source of isotopic information. These larger species are considered preferential grazers (Hume, 1982; Dawson, 1995; McCullough and McCullough, 2000) but can revert to browsing, particularly in arid and semi-arid areas where chenopods (a C_4 plants), are evidently more palatable than some grass species (Bailey et al., 1971). Chenopods may have been a significant component of the diet of one extinct kangaroo species (Prideaux et al., 2009). Consequently, the carbon isotope component of diet can provide information on dietary and therefore vegetative changes. In areas of seasonally variable rainfall with sufficient moisture to support C_4 grasses in summer and C_3 in winter, change in their relative abundance may be evident through $\delta^{13}C$ analysis (Balasse et al., 2002; Nelson, 2005; Balasse et al., 2006; Passey and Cerling, 2006).

Kangaroo water use varies significantly depending on environmental conditions. Typically, between 25 and 40% of water is ingested in plant matter (Ayliffe and Chivas, 1990), and often much more for *M. robustus* (Ealey et al., 1965; Ayliffe and Chivas, 1990). Consequently, the fractionation of plant water $\delta^{18}O$ by evapotranspiration may play a significant role in determining kangaroo $\delta^{18}O$. Dry grass cannot contribute significantly to the herbivore water budget, so grazers without access to green grass must obtain water from surface sources and/or leaves of C_3 plants during the dry season. Dietary shifts to C_3 plants due to water stress, often coupled with low abundance of grasses, may be reflected in the results obtained by Murphy et al. (2007b), whose isotopic data suggest that *M. fuliginosus* browses significantly more than expected. Changing to a C_3 diet during the dry season would cause a decrease in enamel $\delta^{13}C$ and an increase in $\delta^{18}O$. Intra-tooth variation in wombats from some study sites show this kind of pattern (Fraser et al., 2008).

It is important to select teeth formed after weaning for study, because stable isotope ratios of tissues formed before weaning can be influenced by those of mother's milk (Balasse, 2002). This may be particularly important in kangaroos, as the chemical composition of milk alters with the needs of developing joeys (Dawson, 1995). Both isotopic records (Fraser, 2005; Murphy et al., 2007b) and studies of molar development in kangaroos (Kirkpatrick, 1978) indicate that the 3rd and 4th molars of the larger kangaroos post-date weaning and thus reflect an adult diet.

Methods and materials

Sample collection and geographical context

Heads from four of the largest and most common species of kangaroos, the red kangaroo (*Macropus rufus*), the euro (or wallaroo) (*M. robustus*), the western grey (*M. fuliginosus*) and the eastern grey (*M. giganteus*) were opportunistically collected from road-killed or culled animals along a transect stretching south from The Flinders Ranges, and south-southeast from Woomera, South Australia, covering 7.3° in latitude, which is equivalent to ~900 km (Fig. 1). The transect moves from hot, arid (Stuart Highway) and semi-arid (Flinders Ranges) environments in the north, to cool, temperate areas in the south. There is very little elevation change (~10–600 m asl), minimising the potential for elevation-based $\delta^{18}O$ variation.

The southernmost specimens, from Portland, Victoria, were collected to provide a control sample for the C_3 carbon isotope end member. Bone collagen isotope analyses (Pate and Noble, 2000) indicate that southern herbivores subsist entirely on C_3 vegetation, a finding consistent with Hattersley's (1983) floral survey and Murphy and Bowman's (2006) isotopic vegetative survey, which suggests that the mean $\delta^{13}C$ of C_3 grasses near the southern of the transect is −29.4‰.

The northern end members of the transect, in contrast, support C_4 grasses: 51% of grass species in the Flinders Ranges and 82% in the Gairdner–Torrens region (Hattersley, 1983) (referred to in this study as the Stuart Highway). Murphy and Bowman (2006) measured $\delta^{13}C$ in grasses from a similar environment close to the Stuart Highway and their data suggest that the arid environment causes elevated $\delta^{13}C$ in C_3 plants (~−25.8‰). In contrast, average $\delta^{13}C$ in C_4 grasses is lower (−14.9‰) than the expected average of –13‰ (O'Leary, 1988).

Winter rainfall in these arid areas generally originates from frontal systems and summer rainfall from monsoonal systems and thunderstorm events (Schwerdtfeger and Curran, 1996; B.O.M. [Bureau of Meteorology], 2008). This may provide the basis for seasonal $\delta^{18}O$ shifts but the scarcity of rainfall isotope monitoring stations around Australia makes prediction difficult. The Stuart Highway and Flinders Ranges lie between Adelaide and Alice Springs; Adelaide displays small seasonal $\delta^{18}O$ shifts in rainfall (<6‰) around an average $\delta^{18}O$ of −4.5‰ (Liu et al., 2010). Alice Springs, in the centre of the Australian continent, is much more seasonally variable (~12‰ range) around an average of −6.5‰ (Liu et al., 2010). Despite being removed from Adelaide it is likely that our samples are more similar to Adelaide's seasonal precipitation $\delta^{18}O$ regime due to their proximity to the Southern Ocean. Samples from the southern extreme of this transect are also likely to be similar to Adelaide as they are located between Adelaide and Melbourne, both coastal cities, which display very similar $\delta^{18}O$ precipitation seasonality.

Animals were assigned to species where possible but this was often difficult for decomposed specimens and in the overlapping range of *M. fuliginosus* and *M. giganteus* (Caughley et al., 1984) where the species display few morphological differences. Specimens from the overlap zone were treated as generic 'grey kangaroos', as in Wilson (1975).

Skulls were assigned an 'age at death' using an index of molar progression (Kirkpatrick, 1985; Jackson, 2003) and species-specific progression-to-age formulae (Kirkpatrick, 1965, 1970). Third and fourth molars were cut from jaws using diamond-coated cutting disks mounted in a high-speed drill. Teeth exhibiting advanced development and minimal wear were chosen. Mandibular teeth were also preferred as they were easily extracted and their posterior loph (the rear half of the tooth) was conducive to finer-resolution sampling. While sampling aimed to include a varied population, there were instances of multiple sampling from single animals to test the isotopic symmetry between left and right, and upper and lower molars. Some teeth were sampled along both posterior and anterior lophs to determine whether they produced similar results.

Isotopic analyses

Samples were prepared for analysis at the University of Illinois Department of Anthropology Environmental Isotope Palaeobiogeochemistry Laboratory. Remnant plaque, organic matter and cementum were removed from the teeth using a carbide drill-bit. A diamond-coated 0.9-mm diameter dental drill burr was used to drill out a horizontal groove of enamel at approximately 1-mm intervals from the apex of the crown to the 'neck' (enamel–root dentine junction) along the posterior loph (Fig. 2). Sample groove depth (~0.5–1 mm) was determined by the colour change evident when dentine was just below the enamel surface; every effort was made to prevent contamination with dentine beneath the enamel. The length of each groove was dependent on the size of the tooth/width of the loph. Samples generally weighed 5–10 mg. A total of

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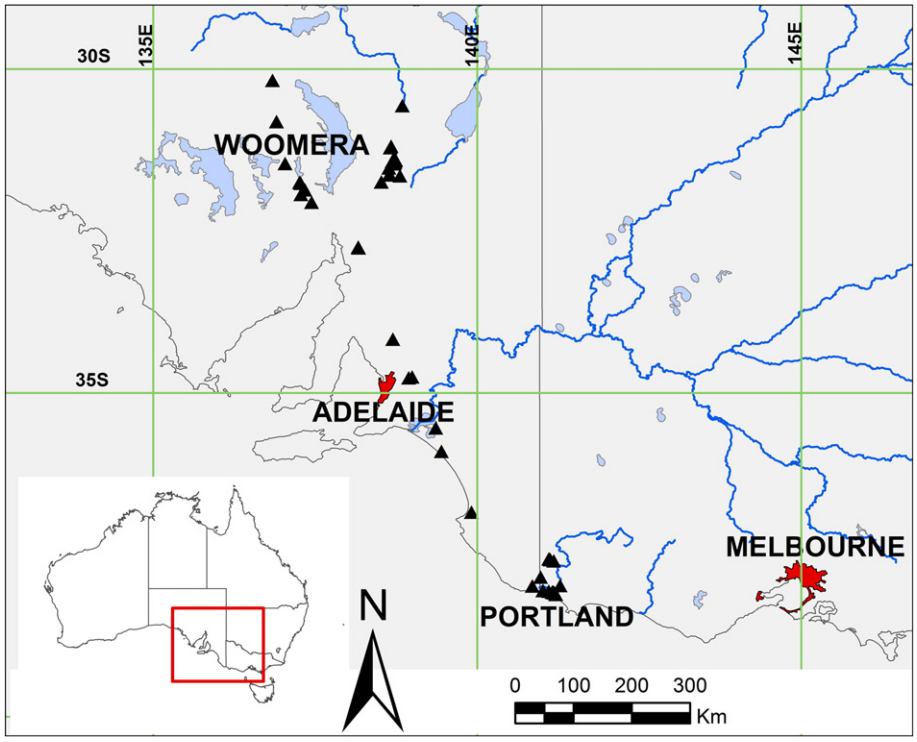

Figure 1. The study area. The study area in southeastern Australia with sample locations represented by black triangles (compiled using ArcMAP 9.2 module of ArcView 9.2, ESRI International). This figure incorporates material that is © Commonwealth of Australia (Geoscience Australia) 2008.

383 samples were drilled from 52 teeth of 30 animals. Samples were prepared for isotopic analysis following Balasse et al. (2002): powdered enamel was treated with 50% sodium hypochlorite (Clorox) for one day to remove organic matter and then rinsed 4× with distilled water.

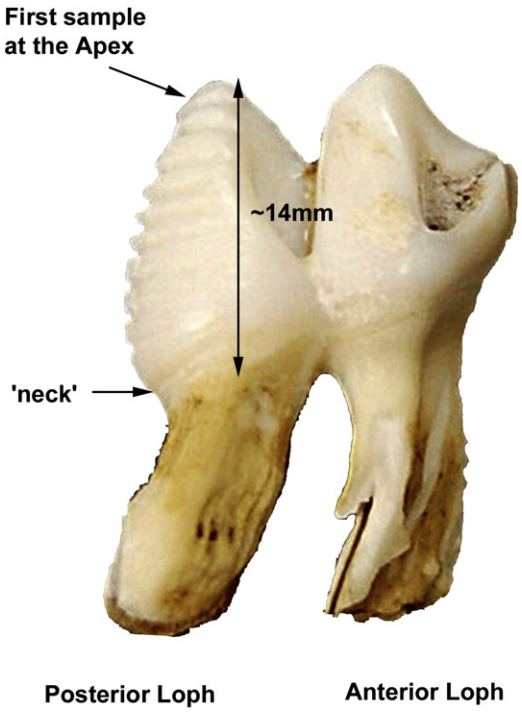

Figure 2. Sampling system. Right lower 3rd molar of a *M. rufus*, displaying 11 sample drilling grooves from apex to 'neck' of the posterior loph.

Treatment with weak (0.1 M) acetic acid (0.1 ml per 1.0 mg of enamel) removed diagenetic and adsorbed carbonate. Bone samples from the mandibles of 28 animals were prepared and analysed following the same method, but with Clorox treatment for two days because bone has more organic matter than enamel.

Approximately 700–800 μg of treated enamel or 600–700 μg of bone was placed in an individual vial in a Kiel III autocarbonate device interfaced with a Finnegan MAT 252 isotope ratio mass spectrometer at the Illinois State Geological Survey. Samples were reacted with 100% phosphoric acid under vacuum at 70°C, and CO_2 was cryogenically distilled for simultaneous carbon and oxygen isotope ratio measurements. The analytical precision, based on measurements of 46 NBS-18 and 44 NBS-19 standards, was better than 0.08‰ for $\delta^{13}C$ and 0.3‰ for $\delta^{18}O$. Results are reported relative to the PDB standard for carbon and the V-SMOW standard for oxygen.

Results

Isotope data are presented according to the animal from which they were obtained. The animal is represented by 'R' (Roo) followed by a sample number and the tooth is denoted as a left or right molar (LM/RM) followed by the molar number. Molars taken from the maxilla are denoted by the addition of a lower case 'm'.

Consistency of isotopes within and between teeth

Six teeth were sampled along the anterior loph in addition to the original posterior samples. The $\delta^{13}C$ and $\delta^{18}O$ values of opposing lophs are compared in Figures 3a and b respectively. The absolute mean difference between lophs ranged from 0.07 to 0.72‰ for $\delta^{13}C$ and 0.06 to 0.83‰ for $\delta^{18}O$ (Table 1).

In most cases different lophs recorded similar carbon isotopic information. Apart from one sample in R036, all $\delta^{13}C$ values are within

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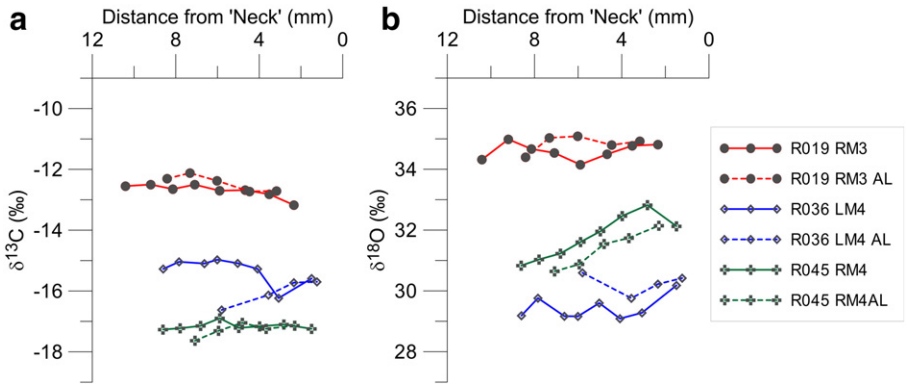

Figure 3. Inter-loph variability. a) $\delta^{13}C$ variation in anterior and posterior lophs of the same tooth. 'AL' denotes anterior loph. b) $\delta^{18}O$ variation between anterior and posterior lophs. 'AL' denotes anterior loph.

1‰ between lophs. Many of the larger discrepancies in both $\delta^{13}C$ and $\delta^{18}O$ appear to be an artefact of the sampling technique, whereby the apparent isotopic difference may actually reflect slightly different ages of samples between lophs rather than different isotopic compositions. Some differences may be due to differences between timing of mineralization of different lophs.

While there is more variation in $\delta^{18}O$ than in $\delta^{13}C$ values, the posterior and anterior lophs seem to preserve the same signal. This is also reflected in the signed and absolute mean differences of the data (Table 1). The mean difference is much less than the maximum observed in R036, suggesting that the teeth generally preserve similar information. The lack of any trend in the signed mean difference between teeth also suggests that the variation observed is random. It appears that both lophs record essentially the same signal but that there is significant 'noise' in the signal.

Six sets of paired left and right molars were analysed in order to determine whether the same signal occurred in teeth that are expected to mineralise contemporaneously. The absolute mean difference between left and right paired molars ranged from 0.04 to 1.04‰ for $\delta^{13}C$ and 0.03 to 1.67‰ for $\delta^{18}O$. Most paired molars appeared to record a similar $\delta^{13}C$ signal (e.g., R042, Fig. 4a), however two sets displayed matching patterns of variation that included systematic disparities of ~0.5–1‰ (e.g., R070, Fig. 4a). There were no systematic differences in the results from standards run with the teeth. Ultimately, the results are promising because in four of six pairs, carbon isotope ratios were close enough to overlap at various stages and the absolute mean difference between all paired left and right molars was only ~0.36‰ (Table 1).

$\delta^{18}O$ values from the paired molars (Fig. 4b) were less consistent. In sample R070, the left and right molars differ by 2‰. In sample R015, which has similar $\delta^{13}C$ values between molars, $\delta^{18}O$ values also diverge significantly. However, these differences are not systematic, suggesting that they cannot be explained by analytical inconsistencies or normal physiological differences between mineralisation of the left and right molars, and that discrepancies represent inherent isotopic variability, perhaps driven by asynchronous formation of enamel/molars. It is also possible that some of this variation is due to slight variations in the depth and interval of sampling between paired teeth.

Despite some discrepancies, the absolute mean difference is less than 1‰ for $\delta^{13}C$ and $\delta^{18}O$ (Table 1) and the variation in the signed mean differences suggests that no systematic fractionation existed between the left and right sides of the jaw. Furthermore, some paired molars (e.g., R042, Fig. 4b) displayed very similar $\delta^{18}O$ values. Given the consistency of $\delta^{13}C$ ratios between teeth it appears that the variation seen in $\delta^{18}O$ is essentially a function of greater inherent variability of oxygen isotopes in kangaroo enamel.

Similar tests were run on upper and lower molars and both $\delta^{13}C$ and $\delta^{18}O$ results show that they preserve similar isotopic signals. The absolute mean differences between upper and lower molars range from 0.05 to 0.67‰ for $\delta^{13}C$ and 0.01 to 0.48‰ for $\delta^{18}O$. R017 and R021 had paired right M4s that displayed marked $\delta^{13}C$ and $\delta^{18}O$ similarities. Similar levels of co-variation were evident in other pairs. The co-variation between teeth observed in Figures 5a and b is reflected by the low absolute mean difference between upper and lower molars (<0.33‰, Table 1).

Table 1
Mean isotopic differences (in ‰) between lophs and paired molars.

Signed difference (posterior–anterior)		Signed difference (left–right)		Signed difference (lower–upper)	
$\delta^{13}C$ (‰)	$\delta^{18}O$ (‰)	$\delta^{13}C$ (‰)	$\delta^{18}O$ (‰)	$\delta^{13}C$ (‰)	$\delta^{18}O$ (‰)
R008		R008		R004	
0.234	0.241	−0.039	−0.033	−0.666	0.007
R019		R013		R017	
−0.250	−0.236	0.582	0.069	0.247	0.155
R029		R015		R021	
0.293	−0.833	−0.061	1.377	0.051	−0.485
R036		R042		R084	
0.724	−0.821	0.042	−0.389	0.347	−0.283
R045		R045			
0.117	0.370	0.362	−1.049		
R084		R070			
−0.069	−0.058	1.044	−1.672		
Absolute mean difference		Absolute mean difference		Absolute mean difference	
0.281	0.427	0.355	0.765	0.328	0.233

Intra-tooth variation

Among all teeth, $\delta^{13}C$ ranged between −17.6‰ and −3.5‰, and $\delta^{18}O$ between 27.2‰ and 39.7‰. Significant intra-tooth variation was found in $\delta^{13}C$ in animals from the Flinders Ranges and in $\delta^{18}O$ of individuals collected along the entire latitudinal transect. The maximum range of variation was generally 2–3‰ for both $\delta^{13}C$ and $\delta^{18}O$. Teeth exhibited diverse patterns of isotopic variation, ranging from abrupt 'spikes' (Fig. 6a: R056 RM4) to sinusoidal variations (Fig. 6b: R099 RM3) and gradual, unidirectional change (Fig. 6a: R087 LM4). Variations of 2–3‰ in $\delta^{13}C$ could represent a ~15–22% or greater dietary shift in C_4 grass consumption. Variation of 2–3‰ in $\delta^{18}O$ could reflect a significant change in the proportions of plant leaf vs. drinking water and/or dry vs. wet-season leaf and surface–water isotopic composition.

Mean intra-tooth $\delta^{18}O$ variation was similar from all sampled regions (Table 2 and Fig. 6b. Mean intra-tooth $\delta^{13}C$ variation was greater by ~25%

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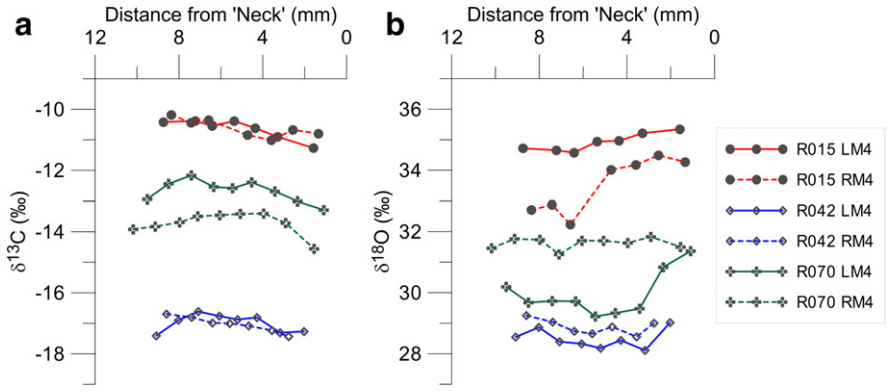

Figure 4. Left vs. right molar variability. a) $\delta^{13}C$ variation between paired left and right molars of the same individual. b) $\delta^{18}O$ variation between paired left and right molars.

in the Flinders Ranges (Table 2 and Fig. 6a) than elsewhere along this transect, reflecting the seasonal changes in the abundance of C_4 grass.

Bone apatite results

$\delta^{13}C$ in bone apatite ranged between −18.7 and −9.4‰, while $\delta^{18}O$ varied between 28.0 and 35.8‰. Bone and enamel apatite $\delta^{13}C$ and $\delta^{18}O$ values of the same individuals were frequently significantly different: up to ~7‰ for $\delta^{13}C$ and ~4‰ for $\delta^{18}O$ (Fig. 7). If bone and enamel apatite formed at the same time can be expected to have the same isotopic composition, then these disparities confirm that enamel and bone were preserving isotopic signals from distinct developmental periods or at least, in younger animals, over different timescales.

Discussion

Consistency of results

Most studies of intra-tooth isotopic variation do not investigate similarity of isotopic values between paired molars, or different lophs of the same tooth. In order to obtain consistent and comparable results, establishing continuity within and between teeth is of fundamental importance. Limited published results of isotopic comparisons between paired upper and lower or left and right bovid molars have shown excellent concordance in patterns of isotopic composition (Balasse, 2003; Balasse et al., 2006). In our study of *Macropus* molars several pairs produced greater differences, although the majority were concordant.

These data showed that paired kangaroo molars generally display marked isotopic similarities. The absolute mean isotopic differences between upper and lower molars were small, as were the actual $\delta^{13}C$ differences between six pairs of left and right molars and upper and lower molars. $\delta^{18}O$ values from paired teeth were also generally similar, though larger disparities (~2‰) were observed in some teeth. These data suggest that the same isotopic information is retrievable from different areas of the tooth, albeit with limited accuracy due to isotopic 'noise', which may reflect differences in timing of mineralisation of different lophs. Ultimately, the absolute mean difference between molars was low and there was no consistent trend in the results to suggest systematic fractionation between lophs or molars.

Results from different lophs and paired molars also provide an indication of the inherent isotopic variability within kangaroo enamel. Based on the analytical precision in this study and the absolute mean differences between posterior and anterior lophs, left and right molars and upper and lower molars, it appears that only variations greater than 0.36 ± 0.08‰ for $\delta^{13}C$ and 0.77 ± 0.3‰ for $\delta^{18}O$ can be attributed to environmental influences. The use of absolute mean differences means that these figures are conservative; the maximum isotopic discrepancies between 'paired' samples were significantly larger. The level of variability observed suggests that further research aimed at refining the understanding of the isotopic variation between pairs of the same element is warranted.

Monitoring environmental variation using enamel and bone apatite

Bone apatite and enamel $\delta^{13}C$ and $\delta^{18}O$ values were compared from the same animals in order to contrast 'long-term' isotopic averages with the shorter duration signal from enamel (Fig. 7). Many teeth recorded isotopic signals significantly different from the average represented by bone. Discussion below is predicated on the assumptions that bone and enamel apatite formed in the same environment should

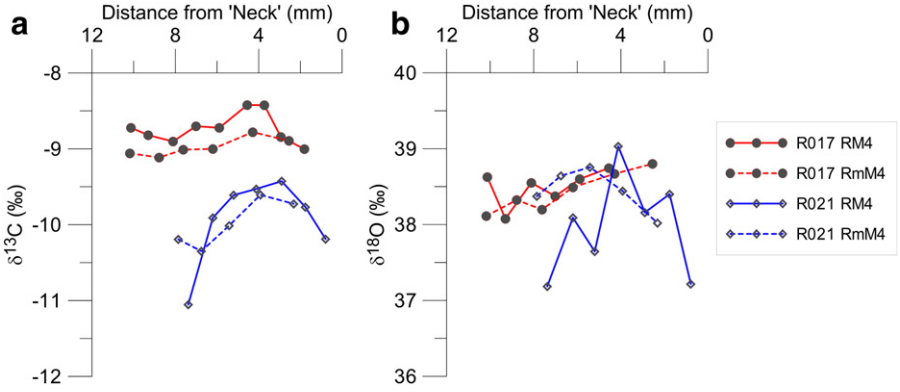

Figure 5. Upper vs. lower molar variability. a) $\delta^{13}C$ variation between paired upper and lower molars of the same individual. b) $\delta^{18}O$ variation between paired upper and lower molars.

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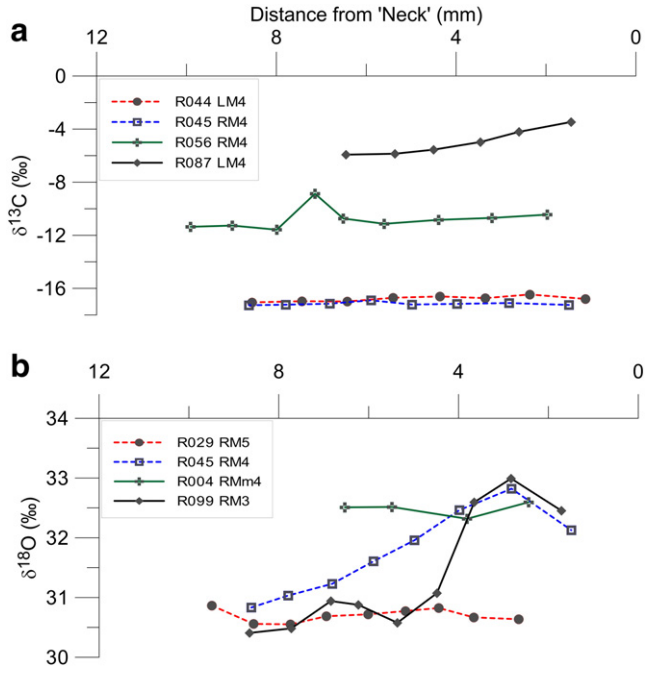

Figure 6. Contrasting amplitudes of isotopic variability. a) Contrasting amplitudes of $\delta^{13}C$ variation, reflecting the greater amplitude of variation typical of the Flinders Ranges. Flinders Ranges animals are shown with solid lines; animals from southeastern South Australia with dashed line: R087, *M. robustus* from Flinders Ranges; R056, *M. robustus* from Flinders Ranges; R045, *M. giganteus/fuliginosus* from SE South Australia; R044, *M. giganteus/fuliginosus* from SE South Australia. b) Contrasting amplitudes of $\delta^{18}O$ variation, showing regional similarities. Flinders Ranges animals shown with solid lines, animals from southeastern South Australia with dashed line: R045, *M. giganteus/fuliginosus* from SE South Australia; R029, *M. giganteus/fuliginosus* from SE South Australia; R004, *Unidentified species* from Flinders Ranges; R099, *M. robustus* from Flinders Ranges.

have the same isotopic composition, and pretreatment affects bone and enamel in the same way. However, the first assumption has not been adequately tested by isotopic analysis of enamel and bone of individuals raised on isotopically constant diets (Warriner and Tuross, 2009). Moreover, bone–enamel differences vary non-systematically among marine species (Clementz et al., 2007), so further research is needed to evaluate this assumption.

Despite the generally low amplitude of kangaroo intra-tooth variability in seasonal environments in southeast Australia (see Magnitude and pattern of intra-tooth variation section), paired bone and enamel analyses provided an alternative means of assessing isotopic variability within an environment. Isotopic differences between enamel and bone reached ~7‰ for $\delta^{13}C$ and differences of 3–4‰ were common in $\delta^{13}C$ and $\delta^{18}O$. For instance, R027 (Fig. 7a) exhibits a large discrepancy between enamel and bone apatite $\delta^{13}C$, suggesting teeth formed during seasonal periods of low C_4 availability compared to the environmental 'average'. Based on molar progression the third and fourth molars mineralised ~5.5 and ~4.5 yr before the 'current' bone began forming, meaning the bone and enamel apatite record distinct time periods. Similarly, R021's upper and lower fourth molars $\delta^{18}O$ values are similar but differ significantly (2–4‰) from the bone apatite $\delta^{18}O$. Based on molar progression calculations, teeth preserved a signal likely to have originated 8–9 yr before that from bone apatite. Similar discrepancies between bone and enamel apatite isotope ratios were observed in animals in which enamel likely mineralised contemporaneously with bone (e.g. R023 and R024; Supplementary Table 1). These discrepancies clearly demonstrate significant changes in the animals' dietary and water intake, meaning that if isotope ratios fractionate consistently between bone and enamel apatite then comparisons between the two can reveal environmental changes.

Table 2
Mean range of intra-tooth isotope values for sample regions.

	Flinders Ranges	Stuart Highway	SE'n South Australia/W'n Victoria
$\delta^{13}C$ (‰)	1.012	0.845	0.863
$\delta^{18}O$ (‰)	1.015	1.113	1.078

The discrepancies observed in enamel and bone comparisons in animals such as R027 and R021 suggest that the maximum intra-tooth variations should be greater than the 2–3‰ observed. This is especially so considering that the discrepancies between bone and enamel apatite are based on the 'long-term' data from bone apatite, which dampens the amplitude of isotopic variation from extreme environmental conditions. The actual isotopic variation in animal diets during extreme seasonal changes is likely to be even larger than the 7‰ maximum observed. This indication of isotopic variability within the environment, from enamel–bone comparisons, confirms that the attenuation of environmental signals within kangaroo teeth is significant.

The comparison between bone and tooth apatite in this study provided an indication of seasonal variability despite the small variations in intra-tooth measurements. A compelling case exists for the paired analysis of bone and tooth enamel apatite wherever possible, as data from one can provide context for data from the other.

Magnitude and pattern of intra-tooth variation

Compared with the isotopic variation within hypsodont teeth of some larger herbivores, the 2–3‰ maximum variations in $\delta^{13}C$ and $\delta^{18}O$ measured within kangaroo teeth seem insignificant. Balasse has shown up to 6‰ changes in $\delta^{13}C$ and 5‰ in $\delta^{18}O$ in sheep molars (Balasse et al., 2003; Balasse et al., 2005) and up to 12‰ $\delta^{13}C$ in cattle molars (Balasse, 2002). In the Australian context, seasonal changes of up to 9‰ in $\delta^{13}C$ and 7‰ in $\delta^{18}O$ have been documented in wombat incisors (Fraser et al., 2008). Isotopic comparisons with bone apatite in this study reveal that this is probably not due to a lack of environmental variation, but more likely to time-averaging of the isotopic signal. It is likely that the small changes observed along kangaroo teeth may reflect significant dietary and/or environmental change (Balasse, 2002; Passey and Cerling, 2002; Kohn, 2004; Codron et al., 2008).

Mineralisation is a gradual process with distinct but overlapping phases that can dampen the magnitude of isotopic signals (Balasse, 2002; Passey and Cerling, 2002; Hoppe et al., 2004). A significant part of the isotopic signal from a sample taken across the breadth of the enamel derives from food and water ingested over several months after the initial matrix secretion during the prolonged period of gradual enamel maturation (Balasse, 2002). Within a single sample, a number of environmental influences may be represented by enamel from separate mineralization events (Zazzo et al., 2005).

Serial sampling of hypsodont teeth can provide meaningful data despite attenuation (Balasse et al., 2002). However, it appears that attenuation is a larger problem in kangaroo teeth, most likely due to their brachydont form. Brachydont teeth of baboons appear to have similar attenuation of isotopic variation, which reduces the amplitude of seasonal variation documented by fecal carbon isotope analysis (Codron et al., 2008). Micro-milling (Zazzo et al., 2005) or laser ablation (Passey and Cerling, 2006; Sponheimer et al., 2006) may provide a higher resolution record of intra-tooth isotopic variation. Both methods isolate smaller developmental increments of enamel for sampling, minimising the time averaging of multiple mineralisation events. However, the scale of time averaging of hypsodont teeth seems to be at the millimetre scale along the enamel growth and mineralization axis. High-resolution analyses of teeth from controlled diet experiments such as those conducted by Passey and Cerling (2006), and Balasse (2002) should be performed on kangaroos. The combination of these

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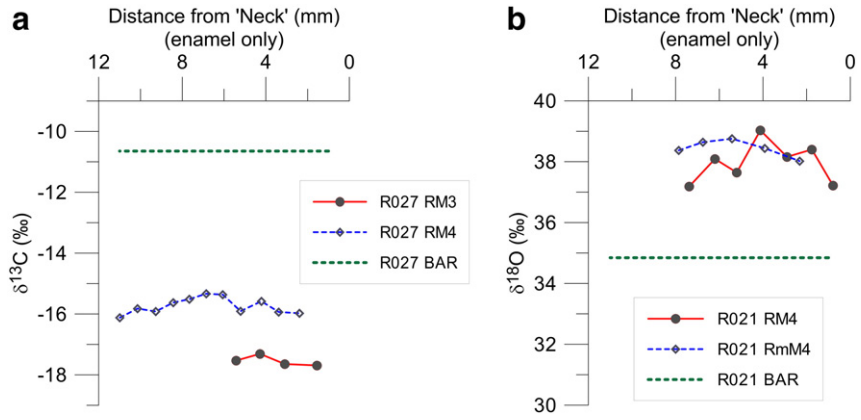

Figure 7. Comparing 'long-term' bone data to 'short term' enamel data. a) Contrasting $\delta^{13}C$ records from bone (BAR) and enamel apatite. The dashed line represents the long-term average provided by a single bone sample. b) Contrasting $\delta^{18}O$ records from bone and enamel apatite. The dashed line represents the long-term average provided by a single bone sample.

techniques with mathematical modelling (Passey and Cerling, 2002; Passey and Cerling, 2004; Zazzo et al., 2005) could provide a more accurate picture of the original magnitude of environmental events recorded within enamel.

Despite the limitations noted above, the results of this study suggest strong potential for intra-tooth isotope studies in kangaroos. A number of teeth yielded 2–3‰ isotopic variations that indicate changes in their environment. The $\delta^{13}C$ values of teeth such as R087 LM4 (*M. robustus*) suggest significant change resembling a steady change in diet, in this case from a mixed C_4 grass/browse or C_4/C_3 grass feeding pattern to an almost 100% C_4 grass diet (Fig. 8a). Others, like R070 LM3 (*M. rufus*) however, show little variation at all, or incorporate more abrupt changes most likely associated with periods of relatively rapid dietary shifts (e.g., R056 RM4) brought on by rapid growth of C_4 grasses after summer rains. The range and absolute values of our data compare well with the limited available vegetative $\delta^{13}C$ data. Murphy and Bowman's (2006) grass $\delta^{13}C$ data from slightly west of the Stuart Highway, at similar latitude to our samples, averaged −14.9‰ for C_4 to −25.8‰ for C_3. Flinders Ranges animals range from −14.5‰ to −3.5‰, approximately matching 100% C_3 to 100% C_4 consumption while Stuart Highway animals display less variability (−8‰ to −13‰) probably reflecting a lower C_4 grass biomass (Murphy, personal communication), despite relatively high species diversity, and increased moisture stress on C_3 vegetation. The Portland area in the south, which supports C_3 grasses with $\delta^{13}C$ of ~−29.4‰, provided kangaroo teeth with $\delta^{13}C$ ranging from −15.0 to −17.7‰ which fit well with a 100% local C_3 diet with perhaps some small influence from water-stressed vegetation.

$\delta^{18}O$ values (Fig. 8b) are similarly varied in their patterns. R008 RM3 (unknown species) is representative of numerous teeth that display a pattern characteristic of seasonal $\delta^{18}O$ changes observed in other studies (Balasse et al., 2003). Some teeth like R070 RM4 (*M. rufus*) present little change, suggesting substantial periods with a stable water source, whereas other teeth (e.g., R056 RM4 — *M. robustus*) present brief, abrupt changes that may represent the influence of short-term seasonal climate changes interrupting the often inconsistent seasons in the Flinders Ranges.

Unfortunately, the interpretation of all isotopic results is complicated by a lack of concrete knowledge about the duration of development of kangaroo molars, so intra-tooth isotope variation cannot be examined in the context of a firm time scale. Similarly, the paucity of isotopic data on modern precipitation along this sampling transect makes it difficult to determine the expected range of variation in tooth enamel $\delta^{18}O$. Apart from Darwin, all of Australia's current monitoring stations are located on the coast and document mean annual $\delta^{18}O$ ranges of <6‰, all essentially within the −8 to −2‰ range (Liu et al., 2010).

In contrast, our data indicate that kangaroos living in environments with likely very similar mean precipitation $\delta^{18}O$ (−4.5‰ using the Adelaide value) display a 12.5‰ range. Mean $\delta^{18}O$ from Stuart Highway kangaroos was ~40.7‰ enriched compared to Adelaide precipitation's mean $\delta^{18}O$, compared with Flinders Ranges animals (36.6‰) and significantly more than those from around Portland (34.5‰). This suggests that at least on an inter-regional scale 'drinking' behaviour (i.e., plant water vs. standing water) and/or $\delta^{18}O$ enrichment, through evaporation of standing water and/or leaf water evapotranspiration,

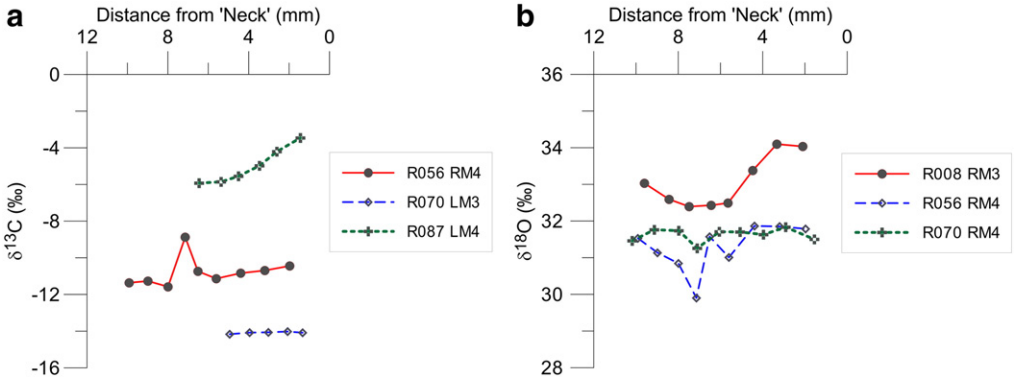

Figure 8. Patterns of variability. a) Contrasting patterns of $\delta^{13}C$ variation in Flinders Ranges kangaroo teeth. b) Contrasting patterns of $\delta^{18}O$ variation in Flinders Ranges kangaroo teeth.

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control kangaroo $\delta^{18}O$ rather than any systematic seasonal changes. Within regions, $\delta^{18}O$ may be more directly affected by factors related to water availability (e.g., relative humidity and temperature) than to seasonal shifts in precipitation $\delta^{18}O$ due to source region. Samples from the Alice Springs region may shed more light on this issue.

Variations of 2–3‰ are evident within teeth, despite the presumably significant attenuation due to time-averaging during mineralization and the coarse sampling method. Evidence of much greater environmental change may thus be retrievable from kangaroos with forward modelling (Passey and Cerling, 2002), opening a window on a continent-wide record of Pleistocene seasonality. However, this method should be cautiously applied in the Australian context given the uncertainty and variability observed here.

Due to that variability, a program following the controlled dietary change model of Balasse (2002, 2003) or Passey and Cerling (2006) may help establish the difference between observed and actual isotopic variations. Important information about the length of tooth growth and mineralisation could also be gained from such experiments, adding detail to the pioneering work of Kirkpatrick and others in the 1970s and 1980s. With more information about the growth patterns of kangaroo teeth and a more precise sampling technique, accurate records of seasonal variation could likely be retrieved from kangaroo teeth.

Conclusions

The results of this study suggest that a largely unexplored, high-resolution continent-wide paleoclimate record exists in intra-tooth isotopic variation in kangaroos in Australia. The data presented here suggest that it may be possible to retrieve stable isotopic records of seasonal variability from prehistoric kangaroos, providing a missing link between the megafauna and modern climate studies.

However, the brachydont nature of kangaroo molars means that the attenuation of isotopic signals is likely significant and the amplitude of environmental variation is not captured by sampling methods used in this study. The error margins associated with isotopic 'noise' mean that significant isotopic changes, dampened during enamel mineralisation, are not evident. Controlled feeding studies and the application of more precise sampling methods such as laser ablation or micro-milling are likely to provide better estimates of the duration of mineralisation and attenuation of isotopic variation within teeth.

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