A study of stable carbon, nitrogen and oxygen isotopes in modern Australian marsupial herbivores, and their relationships with environmental conditions

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By

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Dedicated to Barbara, William and Charles Fraser

Declaration

Except where otherwise acknowledged in the text, this thesis represents original research by the author.

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Abstract

This research has examined the stable isotope ecology of three species of Australian marsupial herbivores; koalas, kangaroos and wombats, from a range of different geographic and climatic regions. The broad objective of this thesis research was to examine stable isotopes in modern marsupial species to provide baseline data that can be used to inform palaeoecological studies.

In the European, African and north American continents stable isotope analyses of terrestrial mammal remains are widely used to investigate the diet and ecology of modern and fossil species. By comparison, similar investigations using Australian native mammals are not as prevalent. Australian landscapes and the physiologies of some native marsupials are in many respects almost unique; therefore, an isotopic study of this continent is warranted. The investigation of modern specimens provided the experimental conditions to observe some of the baseline systematics of stable isotopes in marsupial tissues.

Firstly, the study explored the relationships between stable isotopes in the animals' diet, bone collagen and dental enamel, and the isotopic differences between these two tissues. Secondly, it examined isotopic variability on different levels: intra-population variability in $\delta^{13}C$ and $\delta^{15}N$ values in bone collagen and both inter and intra-tooth variability in $\delta^{13}C$ and $\delta^{18}O$ values in enamel. Thirdly, it examined the relationships between marsupial stable isotope ratios and environmental signals such as moisture availability, rainfall and habitat.

The koalas provided an opportunity to examine an obligate C_3 browser with an extremely consistent diet and conservative lifestyle. The constant nature of their diet enabled the effects of climate on stable isotope ratios to be examined with the knowledge that there is no C_4 dietary input. The stable isotopes in the kangaroos and the wombats were examined from a broader environmental perspective than the koalas because their geographic coverage is far more extensive- from the arid to the alpine. Because the kangaroos and wombats are both grazers, their $\delta^{13}C$ values reflected the availability of the C_3 and the C_4 plants in their respective habitats. Most significantly, microsampling of the continuously growing incisors of wombats revealed that variation in $\delta^{13}C$ and $\delta^{18}O$ values along the tooth can be used as a proxy for seasonality.

The results from this research provide empirical data for future research in isotopic biochemistry and significant baseline data for use in palaeodietary modelling and palaeoenvironmental interpretation of fossil marsupials. These findings are specific to the Australian palaeoecological context.

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Table of Contents

List of Figures

List of Tables

CHAPTER 1

1 INTRODUCTION

1.1 Dietary studies in palaeoecology and the potential use of stable isotopes

Animals respond to fluctuations in food resources and climate on a local scale. Biological adaptations to these fluctuations are seen as dominant driving forces that determine the broader temporal and spatial distributions of species. Therefore, from a palaeobiological viewpoint, knowledge of the palaeodiets and the palaeoenvironmental settings in which animals lived is crucial to understanding what has determined the biogeographical distributions and evolutionary success of species (Eisehberg 1981; Archer et al. 1999).

The incomplete nature of the fossil record means that palaeoecological reconstructions rely on, and integrate, multiple lines of evidence. In past research on terrestrial settings, evidence has been gained from many sources. Indirect evidence about animal diets has been obtained from studying cranial-dental morphologies and dental micro-wear patterns. Palaeoenvironmental and climatic evidence has been obtained from botanical remains (such as pollen and tree rings), the relative abundances and types of species present in fossil deposits, and geological information, such as sediment types and chemical signatures. These records provide information on a range of time and geographic scales. For example, evidence from cranial-dental morphology is largely based on similarities with modern functional analogues, it provides information related to broad long-term dietary adaptations (Janis 1990; 1997). Furthermore, evidence from species composition in faunal assemblages and the associated stratigraphic features can be subject to preservation and taphonomic biases (Behrensmeyer and Kidwell 1985). Palaeobiologists therefore, are particularly interested in an investigative technique that can determine what an extinct animal was eating in the period of its lifetime (Ambrose and DeNiro 1986, reviews in Koch 1998).

Stable isotope analysis of the chemical structure of animal tissues preserved in the fossil record, such as bones and teeth, is an additional tool that can provide unique and direct insights into animal diets and ecology. In the first-order, stable isotope analytical techniques are grounded in biochemistry, yet their broader application is interdisciplinary. Their use in palaeontological and archaeological lines of enquiry has largely benefited from research undertaken in the studies of plant physiology, animal nutrition, ecology, geology and climate. Much of this research has empirically observed that there are predictable flows in the chemical signatures of carbon, nitrogen and oxygen stable isotope ratios in air, water, soils, plants and animals, and subsequently, within wider ecosystems (Hoefs 1997). The theoretical premise behind the use of stable isotopes in palaeodietary studies is that the isotopic composition of an animal's diet is recorded in the isotopic structure of its body's tissues (DeNiro and Epstein 1978). In relation to mammalian herbivore diets in particular, the plant types and forage quantities available for consumption are closely linked to habitat and climate. Therefore, the stable isotope ratios of fossil herbivore tissues also provide proxy information about past environmental conditions (Tieszen 1991; van Klinken et al. 1994).

An accurate assessment of past diets and environments, however, requires detailed knowledge of how the biochemical structure of the tissues analysed reflect the long and short-term diets and local environmental conditions. Testing the nature and strength of these assumed associations can be undertaken in modern ecosystems. This has been viewed as an essential 'first step', which should be taken before analysing and interpreting stable isotopes in fossil animals (Gannes et al. 1997). Stable isotope research in many modern ecosystems has uncovered both limitations that affect the interpretation of data and new prospects for what can be interpreted from bone chemistry (Gannes et al. 1998; Kelly 2000). Laboratory experiments, on more detailed scales, have helped identify various metabolic and physiological mechanisms responsible for the transfer of the diet's isotopic composition into different body tissues (Ambrose and Norr 1993; Sponheimer et al. 2003a; 2003b). Both approaches have demonstrated that the relationships between stable isotopes in diet and mammalian body tissues can be complex. They can be, for example, species-specific and depend on the nutritional quality of the diet. Furthermore, environmental conditions affect both plants at the base of the food chain and animal physiologies.

1.2 Overview of the thesis objectives

The overarching aim of this thesis research is to examine the carbon, nitrogen and oxygen stable isotopes in the bones and teeth of modern Australian marsupial herbivores. The study focuses on three herbivore groups: 1) the koala (*Phascolarctos cinereus*), 2) three species of the grey,

red and wallaroo kangaroos (*Macropus giganteus/fuliginosus*, *M. rufus* and *M. robustus*), and 3) the common and hairy-nosed wombats (genera *Vombatus* and *Lasiorhinus*). These species are common in many Quaternary fossil deposits and have great palaeoecological significance. An important outcome of this research will be the provision of baseline information regarding the systematics of stable isotopes in these marsupials, which can be used as comparative data to aid in the future palaeoecological interpretations of fossil marsupial fauna assemblages.

The approaches used in modern stable isotope ecological studies can be viewed as 'natural experiments' (Gannes et al. 1998). This is because they can sample multiple numbers of individuals in populations, directly analyse animal diets (eg. plants or via faeces), undertake and/or utilise previous field research regarding ecology, physiology and behaviour, and also directly measure climatic conditions. These approaches were used in this research. The nature of these data sources allow hypotheses about the relationships between diet, tissues and environmental conditions to be rigorously tested. The three main lines of enquiry addressed in this study are: 1) the relationships between diet and the bone and tooth enamel tissues, 2) the levels of intra-population and inter-population variability in stable isotope values that exist in modern populations, and 3) how stable isotope ratios in diets and marsupial tissues reflect local climatic and habitat conditions. The results from this study may have implications for stable isotope analysis in palaeodietary research that is specific to the Australian context; per se, it will help to shape future research questions and appropriate sampling protocols. Furthermore, it may have far-reaching and positive suggestions for more accurate interpretations of stable isotope data.

1.3 Structure of the thesis

This research thesis has the following structure:

Chapter 2 begins with an overview of the science of stable isotope analysis using carbon, nitrogen and oxygen isotopes. The underlying premise of this work is based on principles of biochemistry, plant physiology, nutrition and animal metabolism. Therefore, the interrelationships between these disciplines that relate to using stable isotope analysis of faunal remains to provide palaeoecological information are discussed. Examples from archaeological and palaeontological studies of stable isotopes in prehistoric human and fossil animal remains are presented.

Chapter 3 provides an overview of how stable isotope analysis has been used to study Australian marsupials in modern ecological and palaeontological settings. This section describes previous research foci and serves to emphasize potential areas for future work. This is followed

by a detailed outline of the broad and specific research objectives of this thesis. The latter part of this chapter outlines the fieldwork and the experimental methods and procedures used in this study.

The separate stable isotope study of each species; koalas, kangaroos and wombats, are presented in Chapters 4 to 6. The stable isotope ratios of the different species are compared at sites where these species co-existed, and are summarised in Chapter 7.

In the final chapter of this thesis (Chapter 8), the findings from each of these studies are synthesised. In conclusion, the implications of these research results address the feasibility and implications for future stable isotope research on fossil marsupials.

CHAPTER 2

2 BACKGROUND: NATURAL ABUNDANCE OF STABLE ISOTOPES IN THE ENVIRONMENT

2.1 Chemical theory and mechanisms of fractionation

Although the isotopes of an element have the same chemical properties, their mass differences cause them to act differently in physical reactions. The heavier isotopic forms react slower and form stronger chemical bonds relative to the lighter isotopic forms. The differences in the reaction rates cause differences in the isotopic abundances between substrate and product, which is referred to as discrimination or 'isotopic fractionation'. Two types of fractionation dominate the Earths' natural systems. Kinetic fractionation occurs in reactions involving absorption, evaporation and diffusion, such as the steps involved in plant photosynthesis. Reactions governed by kinetics are irreversible and the lighter isotopes are concentrated in the products. Equilibrium isotope fractionation processes concern the partitioning of isotopes between phases, such as CO_2 in the air and dissolved bicarbonate in aquatic systems. These reactions are reversible and temperature dependent. Further detailed accounts of fractionation may be obtained from Hoefs (1997).

2.1.1 Notation of stable isotope values

Fractionation during physical, chemical and biological processes in the Earth's systems produce substances with different isotopic compositions. In nature, the abundance of the heavier isotope is always much less than the lighter form, for example the percentages of the stable isotope abundances of carbon, nitrogen and oxygen are:

- Carbon ^{12}C abundance is 98.89% and ^{13}C abundance is 1.11%.
- Nitrogen ^{14}N abundance is 99.63% and ^{15}N abundance is 0.36%.
- Oxygen ^{16}O abundance is 99.76% and ^{18}O abundance is 0.204%

The stable isotope ratios based on actual percentages of abundance of the heavier isotope are not used because they occur as hundredths of a percent. Instead, measurement of stable isotopes involves the calculation of the ratio of the heavier isotope to the lighter isotope relative to the ratio in a laboratory standard of known isotopic composition. The carbon, nitrogen and oxygen isotope ratios used in palaeodiet studies are written in a delta (δ) notation which express fractions in per mil (‰) according to the following:

$$\delta‰ = [(R_{sample}/R_{standard}) -1] \times 1000$$

where R is the ratio of the heavier to the light isotope. A negative δ value for a sample indicates that it contains less of the heavier isotope than the standard, whereas a positive δ value indicates enrichment in the heavier isotope. The global standard for nitrogen is atmospheric N_2 (AIR). The initial global standard for carbon and oxygen was the fossil marine carbonate Pee Dee Belemnite (PDB). However, because the sources of PDB are now exhausted, the International Atomic Agency, Vienna (IAEA), National Bureau of Standards (NBS) 19, 18 and 20 standards, calibrated to the original PDB, are used. The $\delta^{13}C$ and $\delta^{18}O$ values are now given relative to Vienna-PDB' (VPDB). The $\delta^{18}O$ of non-carbonate materials is commonly measured relative to the Standard Mean Ocean Water (SMOW). For comparison, the $\delta^{18}O$ values measured relative to VPDB can be converted to $\delta^{18}O$ SMOW using the following equation (Coplen et al. 1983):

$$\delta^{18}O \text{ VSMOW} = \delta^{18}O \text{ VPDB} \times 1.03091+30.91$$

2.2 Stable isotopes in different body tissues

Stable isotopes can be measured on nearly all animal tissues. For dietary studies, tissues such as blood, flesh, muscle, hair, feather, bone and tooth enamel, as well as metabolic outputs such as urine, faeces and breath have been analysed. The timing of formation and turnover of tissues influences which dietary signals are recorded. The turnover times of blood and internal organ tissues are on the scale of a few days to weeks. For muscle and fat tissues, the turnover can take weeks to months. These tissues therefore record the diets from relatively short periods. Hard tissues such as bone take many years to turnover and therefore record the diets from much longer periods (Tieszen et al. 1983). Other tissues such as tooth enamel, hair and feathers form once and preserve the diets from discrete time periods.

Tissues that form once or turn over quickly are useful for the investigation of short term changes in diet. In modern studies, the isotopes in feathers grown at different times have been

used to examine the changing diets of migrating birds (Hobson, 1999) and isotopes in hair to track seasonal diets in wolves (Darimont and Reimchen 2002) and elephants (Cerling et al. 2004b). The time span represented in a specific tissue is an important factor because a methodological bias may be introduced when stable isotope values from non-contemporaneous parts of tissues are compared.

In palaeontological and archaeological studies, one typically analyses stable isotopes in bones and teeth because these tissues are often the only ones preserved in the fossil record. Typically, $\delta^{13}C$ and $\delta^{15}N$ values of bone and dentine collagen and $\delta^{13}C$ and $\delta^{18}O$ values in enamel carbonate are measured. These two body tissues and isotopes were analysed in this thesis, and will therefore, be described in more detail. Particular attention is given to tooth enamel, because collagen is more susceptible to post mortem alteration (Grupe et al. 1989; Grupe 1995), and is often not preserved in the Australian environment (Pate 1998).

2.2.1 Bone collagen

Collagen is a fibrous protein that makes up to 90% of the organic component of fresh bone and approximately 40% of its dry weight. The remaining 60% of the dry weight of bone comprises mainly inorganic calcium hydroxyapatite (bone apatite), this gives bone its rigidity and compressive strength. Bone collagen proteins have a unique amino acid structure, consisting predominantly of glycine (~30 %), hydroxyproline (10%) and proline (12.3%), and smaller amounts of glutamate (7.2 %) and aspartate (4.7%) (Herring 1972 in Koch et al. 1994; (O'Connell and Hedges 2001; Fogel and Tuross 2003).

Bone collagen is constantly reabsorbed and remodelled throughout life. Complete turnover in humans is estimated to take around 10 years (Libby et al. 1964). Consequently, the stable isotopes in bone collagen is regarded a representing an individual's 'average lifetime diet' (Sealy et al. 1995).

2.2.1.1 Collagen purity tests

Chemical and physical processes in the burial and surface environments may alter the biological information preserved in organic tissues, which may result in collagen loss and changes in the original $\delta^{13}C$ and $\delta^{15}N$ values (Grupe 1995). An examination of weathered unburied modern bones in East Africa by Koch et al. (2001) concluded that although bone proteins degraded there was little impact on isotope chemistry. Post-mortem collagen loss and chemical degradation can be detected using elementary laboratory procedures. One method is to reject samples where the

final collagen yield is less than 1% of the original dry bone weight (see discussions in Ambrose (1990) and Schoeninger et al.(1989). A second method, established by DeNiro (1985), specifies that well preserved collagen has an atomic carbon and nitrogen ratio (C:N ratio) of between 2.9-3.6. This is the range found in modern unaltered bone collagen based on laboratory studies of animals with known dietary preferences.

2.2.2 Tooth enamel

Enamel is the outer layer of the tooth crown. It is comprised almost entirely of calcium hydroxyapatite $Ca_5(OH)(PO_4)_3$ (Hillson 1986). Within this composition, carbon occurs as structurally bound carbonate that commonly substitutes in the phosphate and hydroxyl sites in quantities of less than 3% of total weight (Hillson 1986). A smaller portion of absorbed carbon occurs along hydration layers on hydroxyapatite crystal surfaces (Koch et al. 1997). Oxygen isotopes can be measured from the phosphate and carbonate portions.

Enamel is preferred over bone and dentine apatite for stable isotope analysis of fossil samples (Schoeninger and DeNiro 1982; Lee-Thorp and Sponheimer 2003), because the hydroxyapatite crystals in enamel are larger and more densely packed, with fewer defects and substitutions compared to those in bone and dentine (LeGeros 1981, in Koch 1997). Enamel is less porous, hence there is less diffusion of solutions (Pike et al. 2002) and less chemical and structural change (Lee-Thorp and van de Merwe 1991; Lee-Thorp 2000).

2.2.2.1 Tooth enamel formation

Tooth formation begins at the crown and proceeds to the base in two main stages. First, a lightly mineralized protein matrix (amelogenin) is secreted by ameloblast cells at the developing enamel-dentine boundary. During the second stage, amelogenin is removed and replaced by the inorganic crystalline apatite (Hillson 1986; Finchmam et al. 1999). Enamel deposition proceeds by gradual accruement and after maturation it is not remodelled. Serial bands, called Retzuis lines, are visible under light magnification and represent successive temporal phases of mineral production (Hillson 1986). Enamel layers are not always deposited perpendicular to the growth axis, and the angle of deposition varies between species and teeth types within a species (Hillson 1986; Kohn et al. 1998; Fox 2000; Passey and Cerling 2001).

Mammals typically produce two sets of teeth. The first teeth ('milk teeth') develop before or slightly after birth, and then some or all of these are replaced (usually after weaning) by the permanent adult teeth. The time taken for the adult dentition to be in place is highly variable and

species dependent. Enamel growth rates vary between taxa and between different teeth of the same species, and is partially related to tooth size and morphology (Sharman et al. 1964; Sanson 1980; Hillson 1986; Finchmam et al. 1999; Passey and Cerling 2001).

2.2.3 Time resolution and characteristics of isotopic signals recorded in teeth

Tooth enamel forms once and therefore records the isotopic signals of the diet and climate at the time of its formation. In order to understand the isotopic signals in teeth, it is important to know the eruption sequence of teeth in the jaw and the enamel formation time. The juvenile milk teeth record the dietary signals of early life stages, such as the diets received *in utero* and during subsequent suckling and weaning periods (Wright and Schwarcz 1999; Balasse and Tresset 2002). The teeth formed after this record the adult diets.

Teeth of small animals typically form quicker than larger teeth and record the isotopic signal on a time scale of a few weeks or months. Large, high-crowned (hypsodont) teeth, such as those of equids and bovids, or continuously growing teeth, such as the incisors of rodents and wombats, as well as highly modified teeth like proboscidean tusks, take longer to form and preserve long temporal records of isotopic signals from months to many years. In light of this, recent studies have used intra-tooth microsampling of these teeth types to investigate seasonal variations in climate and diet (Koch et al. 1995; Fricke et al. 1998; Kohn et al. 1998; Balasse et al. 1999; Gadbury et al. 2000; Fox and Fisher 2001).

2.2.3.1 The effect of sampling technique

The dietary signals obtained from enamel can be influenced by sampling techniques. A homogenized bulk sample, which includes enamel layers from crown to base, will provide an isotopic value that represents a time-averaged diet value. Sequential micro-samples taken along a tooth's growth axis will provide a time-series of isotopic values. It is often not possible to sample each growth layer separately, especially when layers are small and cutting precision can be difficult to control. Consequently, a sample may include enamel from layers formed at different times and with varying isotopic compositions. If the change in the seasonal signal is short compared to the interval represented by the consecutive samples, a dampening of the estimated seasonality may occur. The potential isotopic biases introduced by enamel sampling methods have been have discussed in more detail by various authors (Wiedermann et al. 1999; Fox 2000; Wiedermann 2000; Passey and Cerling 2001; Balasse 2003).

2.3 Natural abundance variations in carbon stable isotopes

Carbon exchanges between the atmosphere, oceans and the terrestrial and freshwater systems. Thermodynamic equilibrium fractionation of the ^{13}C and ^{12}C isotopes in CO_2 occurs as it interchanges between the ocean and the atmosphere, and results in the relative depletion of ^{13}C in the atmosphere. The modern-day atmosphere has a relatively constant $\delta^{13}C$ value of approximately -7.5‰ (Hoefs 1997). The entry point for carbon into living tissues is photosynthesis in plants, after which it is passed on to higher levels in the biological food chain from herbivores to carnivores.

2.3.1 Photosynthesis and carbon fractionation in plants

During photosynthesis plants take in CO_2 through leaf stomata and convert it to sugars and carbohydrates via a series of enzymatic pathways (Raven et al. 1992). Plants discriminate against ^{13}C during photosynthesis due to kinetic fractionations that occur during the initial diffusion of CO_2 into the leaf, and then as CO_2 is incorporated through photosynthetic enzymatic pathways (O'Leary 1981; Farquhar 1989).

Terrestrial plants can be divided into three main groups based on their photosynthetic pathways: Calvin-Benson (C_3); Hatch-Slack (C_4) and Crassulacean-Acid-Metabolism (CAM) (O'Leary 1988). The C_3 group includes trees, shrubs and cool temperate grasses as well as crops such as wheat, rice, oats, legumes and most fruits. C_3 plants dominate the higher latitudes and prefer cooler and moister temperate climates with winter rainfall regimes (Hattersley 1983; Winslow et al. 2003; Swap et al. 2004). C_3 plants discriminate heavily against ^{13}C and have $\delta^{13}C$ values ranging from -22‰ to -33‰, with a mean value of -27.5‰ (Smith and Epstein 1971; O'Leary 1981; Teiszen and Boutton 1989). The C_4 group includes warm climate and tropical grasses, as well as crops such as maize, sugarcane and millet. C_4 plants dominate the lower latitudes and are well adapted to high light intensities, high temperatures, dry growing conditions and summer rainfall regimes (Hattersley 1983; Tieszen 1991; Winslow et al. 2003; Swap et al. 2004). C_4 plants discriminate less against ^{13}C than C_3 plants and have $\delta^{13}C$ values ranging from -21‰ to -9‰, with a mean value -13‰ (Smith and Epstein 1971; O'Leary 1981; Farquhar 1983; Tieszen 1991). The CAM group includes many succulents, such as cacti and pineapple. They can use either the C_3 or the C4 photosynthetic pathway depending on environmental factors. In cooler temperate conditions, CAM plants function as C_3 plants, but in hot arid conditions they can use parts of the C_4 pathway. Consequently, the range of $\delta^{13}C$ values of CAM plants is more variable but their mean value is reported as -19‰ (Smith and Epstein

1971; O'Leary 1988; Tieszen 1991). CAM plants are rarely consumed by herbivores and are not discussed further.

2.3.2 Effects of climate and environment on the carbon isotopes in plants

Carbon isotope values of plants within these groups are affected by environmental factors such as climate and microenvironment (Farquhar and Richards 1984; Ehleringer and Cooper 1988; O'Leary 1988; Farquhar 1989; Tieszen 1991; van de Merwe and Medina 1991; van Klinken et al. 1994; Ehleringer et al. 1997; Dawson et al. 2002). For example, early studies on the $\delta^{13}C$ values of tree rings showed that temperature and moisture influences plant isotope values. Francey and Farquhar (1982) observed that wider rings, which grew when conditions were cooler, wetter and more humid, had more negative $\delta^{13}C$ values. In contrast, smaller rings grew during hot and dry conditions and had more positive $\delta^{13}C$ values. Studies by O'Leary (1981), Farquhar et al.(1982) and Farquhar (1989) have subsequently established that climate and environmental factors affect isotope discrimination by influencing the degree of stomatal closure and the rates at which photosynthetic enzymes catalyse carboxyl groups from CO_2 (carboxylation rates). For example, hot dry conditions cause water stress in plants that leads to increased stomatal closure and carboxylation rates, which, reduces CO_2 uptake and results in is less discrimination against ^{13}C (Farquhar et al. 1982).

The climate and environmental factors observed to decrease plant $\delta^{13}C$ values are: low temperatures and light intensities, low soil nutrients, near-ground position beneath a closed canopy (canopy effect) and high levels of CO_2. Factors that increase plant $\delta^{13}C$ values are: high temperatures and light intensities, soil salinity, water stress and low CO_2 levels. These observations and the fractionation mechanisms responsible are discussed further in the reviews of (Ehleringer and Cooper 1988; Tieszen 1991; van de Merwe and Medina 1991; Ehleringer et al. 1997; Heaton 1999; Dawson et al. 2002) and the references therein).

Although climate and environmental conditions can affect the individual $\delta^{13}C$ values of C_3 and C_4 plants, the range of values of these two photosynthetic groups do not overlap; C_3 plants have values ranging from -22‰ to -33‰, and C_4 plants have values ranging from -21‰ to -9‰. Thus, it is possible to determine the dietary source of carbon using $\delta^{13}C$ values in animal tissues based on this C_3 /C_4 dichotomy (Tieszen 1991).

2.3.3 Carbon isotopes in animal tissues

The patterns of carbon isotopes in plants are passed on through the food. Studies have established there is little fractionation of carbon within herbivores that can be attributed to age

and sex (DeNiro and Schoeninger 1983; Lovell et al. 1986), therefore, variations in $\delta^{13}C$ values are due to dietary intake. In controlled feeding experiments, DeNiro and Epstein (1978, 1981) found that $\delta^{13}C$ values of 'whole bodies' were slightly more positive than those of their diets. Specifically, that body lipids were always depleted in $\delta^{13}C$ relative to diet and that both bone collagen and carbonate were enriched, but had different $\delta^{13}C$ values to each other. Bone collagen was enriched by approximately 3‰ and carbonate by 9‰. This pattern of lipids being depleted, while bone collagen and carbonate being progressively more enriched was observed in subsequent laboratory studies by Ambrose and Norr (1993) and Tieszen and Fagre (1993). van de Merwe (1977) and Vogel and van der Merwe (1987) noticed an enrichment in $\delta^{13}C$ values in bone collagen of ancient humans from woodland North America and estimated the magnitude of this effect to be 5.1‰.

The average diet to bone collagen fractionation for larger mammals ranges between +3‰ to +5‰; a summary of the fractionation values of various mammal species can be found in Ambrose and Norr (1993). The fractionation between diet and bone and enamel carbonates also varies between species; quoted values range from +9‰ and +14‰ (DeNiro and Epstein 1978; Krueger and Sullivan 1984; Ambrose and Norr 1993; Tieszen and Fagre 1993; Cerling et al. 1997a; 1997b; 1999). Stable isotope analysis has determined that terrestrial mammalian herbivores eating a C_3 diet will therefore have an average bone collagen $\delta^{13}C$ value of -23‰ and enamel carbonate $\delta^{13}C$ value of -13‰. Those eating C_4 diets will have an average bone collagen $\delta^{13}C$ value of -9‰ and enamel carbonate value of +1‰.

2.3.4 Laboratory experiments on the carbon isotopes in diet and animal tissues

Controlled feeding experiments have demonstrated that carbon isotope diet to tissue fractionations may be influenced by the diet's nutritional composition. DeNiro and Epstein (1978; 1981) initially suggested that the proportions of different "biochemical fractions" in the diet could be responsible for the observed differences in isotopic variability seen between tissues. Several authors suggested that the main dietary macronutrients (lipids, carbohydrates and proteins) were perhaps routed into specific tissues in the body (Chisholm et al. 1982; Krueger and Sullivan 1984).

The two experiments conducted by Ambrose and Norr (1993) and Tieszen and Fagre (1993) investigated the effect of dietary composition on the isotopic relationships between diet and bone collagen and carbonate. These studies placed rats on diets where the protein and carbohydrate components had different isotopic compositions (distinctly C_3 and C_4), as well as varying protein levels. They found that the $\delta^{13}C$ values of dietary proteins correlated most

highly with the $\delta^{13}C$ values of bone collagen, moreover, that this relationship was stronger on high protein diets (~70%) than low protein diets (~5%). The $\delta^{13}C$ values of bone carbonate correlated most highly with blood bicarbonate, lipids and 'bulk diet'. In summary, these studies demonstrated that carbon in dietary proteins is preferentially routed to bone collagen and that carbon in protein, carbohydrates and lipids are combined and used to synthesize bone carbonate.

From these two studies is it has been inferred that the carbon in bio-apatites represent the $\delta^{13}C$ value of overall bulk diet, whereas bone collagen may under represent bulk diet $\delta^{13}C$ values, but specifically tracks the $\delta^{13}C$ value of dietary proteins. Furthermore, they have shown that different levels of dietary stress can change the routing of nutrients. In this case, it appears that animals on low protein diets may have to synthesise nonessential amino acids from the carbon supplied by carbohydrates, but when there is ample protein then protein can contribute to the body's energy sources along with carbohydrates and lipids (Ambrose and Norr 1993; Tieszen and Fagre 1993; Schwarcz 2000). It follows, that the effects of preferential routing on the diet to tissue fractionations will be more pronounced in species that consume nutritionally complex diets and where the protein and carbohydrate components have different isotopic compositions, such as those of humans and omnivores (Ambrose and Norr 1993; Schwarcz 2000). Ambrose (1993) noted that animals on low protein diets might have larger diet to bone collagen fractionation values, because they rely on a greater synthesis of non-essential amino acids from the diet, which leads to further isotopic enrichment.

2.3.4.1 C_3 and C_4 linear mixing models

Linear mixing models have been used to quantify the proportions of C_3 and C_4 plants in herbivore diets (Chisholm et al. 1982; Schwarcz et al. 1985; Schoeninger 1989; White and Schwarcz 1989; Little and Schoeninger 1995). Many have relied on the assumption that the isotopic composition of an animal's tissues equals the weighted average of the isotopic composition of its dietary constituents (Gannes et al. 1997). Moreover, all dietary components are 'scrambled' and different tissues are synthesized from this homogenised pool (Schoeninger 1989; Schwarcz 1991). Experimental evidence has shown that dietary components are preferentially routed to certain tissues (as described in section 2.3.4). Therefore, linear mixing models may not be considered appropriate for reconstructing isotopically diverse and nutritionally complex diets (Ambrose and Norr 1993; Phillips 2001; Phillips and Gregg 2001; Phillips and Koch 2002). They can only provide a general indication of the proportions of C_3 and C_4 plants consumed.

Schwarcz et al. (1985) calculated the dietary proportions of C_3 and C_4 plants from bone collagen $\delta^{13}C$ values of prehistoric humans in Ontario using the following simple linear mixing model:

Proportion of C_4 plants in diet (PC_4) = $((\delta c - \delta 3 + \Delta dc)/\delta 4 - \delta 3) \times 100$

where, $\delta 3$ and $\delta 4$ were the assumed values for C_3 and C_4 plant components; Δdc the diet to collagen fractionation amount (here 5‰) and δc was the measured bone collagen $\delta^{13}C$ value. Using the global averages of C_3 and C_4 plants of approximately -27.5‰ and 13‰, respectively (as discussed in section 2.3.1), this model implies that a bone collagen $\delta^{13}C$ value of -19‰ represents a diet of 24.5% C_4 plants. Whilst such precise measurements are attractive to dietary interpretations, they are somewhat unrealistic. The deviation of the $\delta 3$ and $\delta 4$ end member values from expected global $\delta^{13}C$ plant averages and incorrect diet to tissue fractionations will lead to errors in diet reconstruction (Ambrose 1987; Ambrose and Norr 1993). Schwarcz et al. (1985) noted, that a 1‰ shift in $\delta 4$ results in an 8‰ variation in the predicted percentage of C_4 plants in the diet. Ambrose (1993) discussed that for these models to be useful, the correct diet to tissue fractionation and the isotopic composition of the likely plant dietary sources must be known. However, for fossil species these are often unknown. Therefore, dietary reconstruction should use the diet to tissue fractionation of closely related modern-day species and the isotopic analysis of associated fossil plant remains or nearby regional plant $\delta^{13}C$ values.

2.3.5 The use of carbon isotopes in archaeological and palaeoecological studies

The first palaeodiet studies used $\delta^{13}C$ values to characterize human diets based on either C_3 or C_4 plants. The first focus was the timing of the introduction of maize, a C_4 cultigen, into different regions in North America. Vogel and van de Merwe (1977) and (van der Merwe and Vogel 1978) investigated bone collagen from archaeological sites in New York State and observed pre-horticultural hunter-gathers had bone collagen $\delta^{13}C$ values between -21.3‰ to -19.8‰, showing they relied heavily on C_3 plants. In contrast, horticulturists dated from younger periods had more enriched $\delta^{13}C$ values between -13.5‰ and -16.6‰, showing that significant proportions of C_4 plants had been included in their diet. Similar studies using $\delta^{13}C$ continued to explore evidence of changes in prehistoric human diets in North America (Schwarcz et al. 1985; White and Schwarcz 1989; Katzenberg et al. 1995; Little and Schoeninger 1995), Africa (Lee-Thorp and Sillen; Parkington 1991; Iacumin et al. 1998) and Europe (Lubell et al. 1994; Richards et al. 1998; Katzenberg and Weber 1999; Schulting and Richards 2002). Carbon stable isotopes have been used to address other archaeological topics, such as: the estimation of the proportions of marine and terrestrial foods in the diet, based on the ~7‰ difference between marine and terrestrial carbon sources(Chisholm et al. 1982; Schoeninger and DeNiro 1984;

Sealy and van der Merwe 1988; Pate 1997; Cannon et al. 1999; Richards and Hedges 1999), and determining dietary status within populations (Schutowski et al. 1999; White et al. 1999; Privat and O'Connell 2002; White et al. 2004b).

Due to the different climatic conditions that favour C_3 or C_4 plants, the $\delta^{13}C$ signals preserved in fossil animal bones and teeth are a proxy record of climate and environmental conditions. Stable isotope analysis of fossil herbivores from well dated sites has provided evidence for vegetation and climate change through time. One of the initial applications of carbon stable isotope analysis in palaeoecology focused on evidence for climatic change through the late Tertiary Period. Quade et al. (1992) tracked the changes in C_3 and C_4 plant dominance in Siwalik, northern Pakistan, over 16 million years using $\delta^{13}C$ values in herbivore teeth. They found that prior to 7.3 Ma, the $\delta^{13}C$ values of herbivores had averages of -11‰ indicating a pure C_3 diet, whereas after 6 Ma and into the Plio-Pleistocene, the average value increased to 1.9‰, indicating a diet that included predominantly C_4 plants. This isotopic change was used to infer that near the end of the Miocene, a major ecological shift occurred at Siwalik where a C_3 environment dominated by shrubs and trees was replaced by more open C_4 grassland. Other isotopic studies have continued to examine the global timing of this particular ecological change using fossil deposits in the Americas (MacFadden and Cerling 1994, 1996; MacFadden et al. 1994; Cerling et al. 1997b; MacFadden 2000; Passey et al. 2002). Using similar methods, MacFadden et al. (1999) studied the $\delta^{13}C$ teeth values of North and South American grazing equids over the last 1.8 million years, and concluded that the present-day pattern of increasing C_4 grass dominance at lower latitudes and higher temperatures was in place by beginning of the Pleistocene. Lee-Thorp and Beaumont (1995), Sealy (1996) and Smith et al. (2002) used bone collagen $\delta^{13}C$ values of herbivores to determine shifts in the geographic distribution of seasonal rainfall in South Africa during the late Quaternary.

Carbon stable isotope analysis has been used to show that the feeding niches of some animal lineages have changed between C_3 browse and C_4 graze over time. This evidence has also been used in conjunction with cranial-dental morphologies to examine the evolutionary adaptations and biogeographical distributions of animal groups (MacFadden and Cerling 1994; Feranec 2003, also the reviews in MacFadden 2000a). Cerling et al. (1999) observed that African and Asian elephants and other ancient proboscideans ate predominantly C_4 grass (based on enamel $\delta^{13}C$ values greater than -1.5‰) through the late Miocene to early Pleistocene. However, present day elephants have C_3 or mixed C_3/C_4 diets. The development of specialized high crowned teeth by elephants in the late Miocene is considered an adaptation to abrasive grass diets. This agrees with the isotopic evidence for widespread C_4 grassland savannah ecosystems at that time. Cerling et al. (1999) noted that the elephants moving to C_3 browse is an interesting

dietary change that could be due to recent changes in feeding competition with other grazers in their dynamic woodland and grass habitats.

It must be noted that carbon stable isotopes can be used to distinguish between browsers and grazers in sub-tropical, tropical and warm climates because the grasses in these environments are predominantly C_4 and isotopically distinct to C_3 browse. These two feeding strategies can not be isotopically distinguished in pure C_3 plant environments.

2.4 Natural abundance variations in nitrogen stable isotopes

The primary reservoir of nitrogen is atmospheric N_2, which has a relatively constant $\delta^{15}N$ value of 0‰ (as the Standard for nitrogen measurements is air). Plants and animals can not fix nitrogen directly from the atmosphere to synthesise tissues. Specialised soil bacteria fix atmospheric N_2 into inorganic ammonium (NH_4^+) and during the decomposition of organic matter different bacteria convert soil nitrites (NO_2^-) to nitrates (NO_3^-) (nitrification). Nitrogen in these two forms is can be taken up by plants and incorporated into the food chain (Raven et al. 1992).

The $\delta^{15}N$ values of plants do not simply reflect the $\delta^{15}N$ values of its N_2 sources; it involves complex interactions between sources and metabolic $^{14}N/^{15}N$ fractionations (Robinson et al. 1998). Despite the empirical collection of many plant $\delta^{15}N$ data from different vegetation communities, the direct casual determinants of plant $\delta^{15}N$ values are not well understood (see reviews in Hogburg 1997 and Dawson et al. 2002).

Isotopic differences have been observed in terrestrial plant $\delta^{15}N$ values depending on how they obtained N_2. Plants can be divided into two groups: N_2-fixers or non-N_2 fixers. N_2-fixers (eg. leguminous plants such as acacia, beans, peas and some trees and shrubs) have symbiotic nitrogen fixing bacteria or mycorrhiza on their roots and obtain most of their N_2 from NH_4^+ compounds fixed directly from the air. There is little fractionation in the process so that N_2-fixers have lower $\delta^{15}N$ values similar to air, between 0 to +1‰ (Robinson 2001; Schmidt and Stewart 2002). Non-N_2fixing plants take up N_2 from the soil from NO_3^-. Typically, their $\delta^{15}N$ values are enriched over air; however, $\delta^{15}N$ values can be highly variable due to site specific soil properties and fractionation by microbial activities during nitrification. Variations of up to 10‰ have been observed in plant $\delta^{15}N$ values within some ecosystems (Handley and Scrimgeour 1997). It should be noted that N_2-fixers may also obtain nitrogen from soil NO_3^-, which contributes to the isotopic variability in this group.

Many environmental and land use factors influence plant $\delta^{15}N$, such as: soil moisture, age, salinity, pH and disturbance; rainfall and evaporation, fire frequency, fertilizers and grazing (Yoneyama et al. 1997; Schulze et al. 1998; Penuelas et al. 1999; Cook 2001; Schmidt and Stewart 2002 and the reviews in Hoburg 1997). Although plant $\delta^{15}N$ values are influenced by many factors, some broad trends in $\delta^{15}N$ values exist in terrestrial ecosystems and across landscapes. High foliar $\delta^{15}N$ values have been correlated with low moisture availability globally by Handley et al. (1999) and similar patterns have been observed across northern Australia by Schulze et al. (1999), and Schmidt and Stewart (2002). High soil salinity levels have also been correlated with enriched plant $\delta^{15}N$ values by Virginia and Delwiche (1982) and Heaton (1987). Depleted $\delta^{15}N$ values have been associated with low soil nitrogen levels, mycorrhiza associations and $\delta^{15}N$ depleted soils in moist closed forest and cold tundra ecosystems (Schultze et al. 1994; Michelsen et al. 1998; Falkengren-Grerup et al. 2004).

2.4.1 Nitrogen isotopes in animal tissues

Herbivores must source their nitrogen from plant proteins, therefore their $\delta^{15}N$ values are related to the $\delta^{15}N$ of the plants they consume. There is an observed fractionation between plant diet and bone collagen in mammals of approximately +3 to +5‰ (DeNiro and Epstein 1981; Minagawa and Wada 1984). Research also suggests that the diet to tissue fractionation in nitrogen isotopes can be affected by the diet's nutritional composition. The early studies by DeNiro and Epstein (1981) observed that mice fed different diets had varying diet to bone collagen fractionations, ranging from +1.4 to +3.4‰. A study by Sponheimer et al. (2003a) examined the influence of dietary protein levels on the hair $\delta^{15}N$ values of a range of herbivores and found that diet to hair fractionation was 2.3‰ greater on high protein diets than low protein diets.

An approximate +3‰ enrichment repeats with each consecutive step up the food chain and is termed the 'trophic level effect' (DeNiro and Epstein 1981; Minagawa and Wada 1984; Ambrose 1991). An interesting aspect of this effect is the apparent enrichment of the $\delta^{15}N$ values of nursing infants and over the values of their mothers (Fogel et al. 1989). After weaning, their $\delta^{15}N$ values change to reflect the adult diet. The significance of the trophic level effect is most evident in the marine environment because the aquatic food chains are longer than those in the terrestrial environments are. Comparatively, top trophic level consumers in aquatic environments can have more positive $\delta^{15}N$ values than those in terrestrial systems (DeNiro and Epstein 1981; Schoeninger and DeNiro 1984; Sealy et al. 1987; Kelly 2000; McCarthy and Waldron 2000).

2.4.1.1 The effects of climate on the nitrogen isotope values in bone collagen

There is considerable empirical evidence suggesting mammal $\delta^{15}N$ values are influenced by environmental conditions. Studies by Heaton et al. (1986) and Sealy (1987) found strong correlations between annual precipitation and $\delta^{15}N$ values in the bone collagen of South African animals. In these cases, animals from drier habitats had more positive $\delta^{15}N$ values than those from wetter habitats. This relationship has been observed in *Macropus* bone collagen $\delta^{15}N$ values in Australia (Gröcke et al. 1997; Pate and Noble 2004). A study on white-tailed deer by Cormie and Schwarcz (1996) found that high $\delta^{15}N$ values correlated with low precipitation only when their diets contained more than 10% C_4 plants. In the study of Sealy et al. (1987) herbivores with $\delta^{15}N$ values of greater than +10‰ came from areas where annual rainfall was less then 400 mm. Heaton et al. (1986) suggested a causal link between higher $\delta^{15}N$ values and nitrogen metabolism within the animal. Further studies by Ambrose and DeNiro (1986a; 1989) and Ambrose (1991) noted that variations in $\delta^{15}N$ values within species from the same trophic level and between drought tolerant and water dependent animals, which could not be explained by dietary preferences alone. Two main models have been put forward to explain elevated $\delta^{15}N$ levels in more arid adapted mammals.

The first proposed model by Ambrose and DeNiro (1986, 1987) and Ambrose (1991) suggested that variation in urea output affects $\delta^{15}N$ values. Animals that concentrate their urine to conserve water excrete more urinary nitrogen, which is considered to be depleted ^{14}N; as a result, the remaining body nitrogen pool is expected to become more enriched in ^{15}N. Subsequently, the tissues synthesised from this pool become more enriched in ^{15}N. Ambrose (1991) added that this effect should be more apparent in drought tolerant species (usually browsers) than in obligate drinkers (usually grazers).

A different model was proposed by Sealy et al. (1987), who suggested that in addition to urea output, differences related to urea recycling also affect animal $\delta^{15}N$ values. This arose from their observations that in contrast to the expected model of Ambrose (1991), the grazers in their arid study areas had higher $\delta^{15}N$ values than browsers. They postulated that animals on lower protein diets, such as grazers (compared to sympatric browsers), are forced to recycle nitrogen from urea and thereby also leading to an enrichment in ^{15}N. Ambrose (1991) disagreed with this, from two perspectives; firstly that the synthesis of tissues from urea, which is usually ^{14}N depleted, would not lead to a decrease in ^{14}N in these body tissues, and secondly, because mass balance must be maintained, no tissue enrichment can occur until ^{14}N depleted urea is excreted.

Recent experimental studies have begun to investigate the effects of water stress and dietary protein levels on $\delta^{15}N$ values of body tissues. Ambrose (2000) studied the effects of water and

heat stress, as well as high and low diet protein levels, on rat bone collagen. He found little evidence that these factors significantly influenced the rat $\delta^{15}N$ values. However, he suggested that rats may be an inappropriate model for such experiments and cautioned against using these results to explain $\delta^{15}N$ variation in other animals. Sponheimer et al. (2003b) conducted a study on llamas fed low and high protein diets to test if the well held assumption that ^{14}N is preferentially excreted, and to explain the hypothesis that diet to collagen fractionation increases under nutritional stress. The study measured total diet nitrogen influx and efflux (including urine and faeces) and found that only urinary nitrogen was depleted in ^{14}N on low protein diets (yet counterbalanced with higher ^{15}N faeces nitrogen), but total nitrogen efflux on both diets was not depleted in ^{14}N. These data challenge the assumption that ^{14}N is preferentially excreted in urine leading to the enrichment of body tissues. This work could be considered as lending support to the model suggested by Ambrose (1991), however, it did not discount the role that ^{14}N-depleted urea plays in animals that are under nutritional stress or not in a steady state of growth. Clearly more research into nitrogen fractionation in body tissues is required to understand what determines $\delta^{15}N$ tissue values.

The relationship between low precipitation and high $\delta^{15}N$ values in mammals has been primarily attributed to physiological responses in the animal (Ambrose and DeNiro 1986b; Ambrose 1987; Sealy et al. 1987; Ambrose 1991; Sponheimer et al. 2003b). Schwarcz et al. (1999) speculated that the mammalian $\delta^{15}N$ values could be primarily influenced by the plants at the base of the food chain, where a similar relationship between low precipitation and high plant $\delta^{15}N$ values has been observed (see section 2.4).

2.4.2 The use of nitrogen isotopes in archaeological and palaeoecological studies

Nitrogen isotopes have been used most extensively to explore trophic interactions in animal food webs and the diets of human populations. Ambrose and DeNiro (1986b) studied prehistoric humans in Africa and found that high average $\delta^{15}N$ bone collagen values were consistent with the higher trophic level sources of dietary protein. Subsequently, studies have examined the relative levels of dietary protein in other populations, such as those from ancient Nubia (Iacumin et al. 1998; White et al. 2004a), Siberia (Katzenberg and Weber 1999; Weber et al. 2002) and Europe (Richards et al. 2000; Polet and Katzenberg 2003). Aspects of class structure and status have been explored using $\delta^{15}N$ values because it has been assumed that wealthy people had preferential access to larger quantities of protein rich foods (Schutowski et al. 1999; Cox et al. 2001; Privat and O'Connell 2002; Ambrose et al. 2003). Nitrogen isotopes have also been used to distinguish between marine and terrestrial diet sources (Schoeninger and DeNiro 1984; Sealy et al. 1987; Fogel et al. 1998; Richards and Hedges 1999; Clementz and Koch

2001), and $\delta^{15}N$ values in juvenile teeth have been used to determine the weaning ages of human infants (Schurr 1997; Wright and Schwarcz 1999; Richards et al. 2002) and non-human animals (Wright et al. 1999; Balasse et al. 2001; Polischuk et al. 2001; Balasse and Tresset 2002).

Isotopic studies have used $\delta^{15}N$ values to examine animal trophic levels within marine environments (McCarthy and Waldron 2000; Clementz and Koch 2001; Kurle and Worthy 2001) and forests (Drucker et al. 2003; Krigbaum 2003; Cerling et al. 2004a). Further studies have examined trophic level relationships between extinct animals in mixed fossil assemblages (Bocherens et al. 1995; Bocherens and Billiou 1997; Iacumin et al. 2000; Palmqvist et al. 2003), and examined specific topics, such as the physiologies of fossil bears (Bocherens et al. 1994; Nelson et al. 1998; Liden and Angerbjorn 1999; Keeling and Nelson 2001).

Despite the uncertainty of what exactly determines the $\delta^{15}N$ values of herbivores (plant $\delta^{15}N$ values and/or metabolic processes when water stressed), nitrogen isotopes in bone collagen have provided additional lines of evidence about environmental conditions (Ambrose 1987, 1991; Sealy et al. 1987; Iacumin et al. 1996, 1997, 2000; Hedges et al. 2003; Cerling et al. 2004a). Because animal $\delta^{15}N$ bone collagen values have been strongly correlated with rainfall, Johnson et al. (1997) used the variability in the $\delta^{15}N$ values of ostrich eggshells, from Equus Cave in South Africa, to infer changes in mean annual rainfall through the Holocene. Similarly, Gröcke and Bocherens (1996) and Gröcke et al. (1997) used $\delta^{15}N$ values of late Pleistocene mammals to infer past rainfall levels in South Australia.

2.5 Natural abundance variations in oxygen stable isotopes

2.5.1 Oxygen isotopes in environmental waters

Within the hydrological system, oxygen isotopes exchange between oceans, the atmosphere and terrestrial surface reservoirs. The average isotopic composition of the ocean is the accepted International Standard of $\delta^{18}O$, so that SMOW = 0‰. Compared to the oceans, meteoric waters (precipitation, atmospheric moisture and ground and surface waters) are more depleted in the heavier oxygen isotope, ^{18}O (Hoefs 1997). Variability in meteoric water $\delta^{18}O$ values is due to fractionations during Rayleigh distillation and evaporation. Fractionation of oxygen isotopes occurs as it moves states between water and vapour, and this is fundamentally controlled by temperature; at thermodynamic equilibrium between vapour and water, the water has the higher ^{18}O content. With increasing temperature, precipitation becomes more enriched in the heavier isotope ^{18}O in a linear relationship. Under the Rayleigh process, precipitation becomes more

depleted in ^{18}O as it moves further away from the source ('rain-out' effects). Evaporation causes a standing water source to become enriched in the heavier isotope because ^{16}O evaporates quicker than ^{18}O. (Rozanski et al. 1993; Bowen and Wilkinson 2002).

Climate and geography influence global and local meteoric water $\delta^{18}O$ values and the observed patterns are best understood in terms of temperature. At mid to high latitudes, $\delta^{18}O$ values follow more of a seasonal pattern where summer precipitation is more enriched in ^{18}O than winter precipitation. At lower latitudes seasonal isotopic patterns are not as pronounced and site specific $\delta^{18}O$ values can be influenced by the 'amount effect' (i.e. the greater the amount of rainfall the lower the $\delta^{18}O$). More negative $\delta^{18}O$ values have been correlated with colder environments and higher latitudes. Due to the combined effects of temperature and rain-out effects, the $\delta^{18}O$ values of precipitation becomes more negative with altitude, latitude and increasing distance from the coast (Rozanski et al. 1993). The isotopic composition of surface meteoric waters such as rivers and lakes can be highly variable because they depend on the $\delta^{18}O$ of the input sources and levels of evaporation.

2.5.2 Oxygen isotopes in plants

Variation in plant $\delta^{18}O$ values are the result of variations in the $\delta^{18}O$ of source waters (meteoric waters) taken up by the plant and the enrichment of leaf water during transpiration (Roden and Ehleringer 1999; Barbour et al. 2004). The dominance of these effects varies due to climatic conditions and differences in plant physiology (Roden and Ehleringer 1999). Meteoric water $\delta^{18}O$ values can be highly variable and plant root structures can obtain water from many of sources; smaller plants like grasses source water from the upper soil layers and large trees may tap into groundwater sources. The plant $\delta^{18}O$ values of some species may track the seasonal variation in meteoric water values; hence, the $\delta^{18}O$ values of cellulose in tree rings have been used in palaeoclimatic studies (Macfarlane et al. 1999; McCarroll and Loader 2004).

Evaporation of water from the leaf during transpiration results in the enrichment of ^{18}O in leaf water because ^{16}O evaporates faster than ^{18}O (Farquar and Lloyd 1993). Accordingly, leaf $\delta^{18}O$ values are often enriched over source water $\delta^{18}O$ values (Roden and Ehleringer 1999; Cernusak et al. 2003). A strong negative correlation between $\delta^{18}O$ values of leaves and levels of stomatal conductance was found in studies by Farquhar and Lloyd (1993) and Barbour and Farquhar (2000). Barbour et al. (2004) observed that plants grown under low humidity were enriched in ^{18}O more than those grown under higher humidity because of reduced stomatal conductance. Hence, the $\delta^{18}O$ values of leaves can reflect leaf evaporative conditions related to humidity and transpiration (Barbour and Farquhar 2000; Barbour et al. 2000). The enriched leaf $\delta^{18}O$ values

are subsequently passed on to plant organic material when tissues are synthesized (Barbour and Farquhar 2000).

2.5.3 Oxygen isotopes in mammalian teeth and bone

The oxygen isotope composition of mammalian tooth enamel and bone apatite is directly related to the $\delta^{18}O$ of its body water reservoir (Longinelli and Nuti 1973; Longinelli 1984). This reservoir is a combination of three main oxygen inputs: ingested water (drinking water and water in food), solid food and atmospheric O_2 (Luz et al. 1984; Luz and Kolodny 1985; Kohn 1996). Drinking water is the most dominant input and its isotopic composition varies according to the meteoric source, which (as discussed above) is highly dependent on environmental temperature and evaporative enrichment (Luz and Kolodny 1985, 1989; D'Angela and Longinelli 1990; Bryant and Froelich 1995; Kohn 1996). Food and food water isotopic ratios are related to meteoric water, but are also modified by changes in relative humidity through the evapotranspirative enrichment of leaves (Cormie et al. 1994). The atmospheric O_2 input has a near constant $\delta^{18}O$ value of 23‰ and may be fractionated when it enters the blood to form oxyhaemoglobin (Luz et al. 1984). The $\delta^{18}O$ value of the body water reservoir is further complicated by each animal's thermoregulatory strategy (eg. sweating and panting), water dependence (obligate and non-obligate drinkers) body size and behaviours (seeking shelter during heat, nocturnal feeding) that influence water utilization (Bryant and Froelich 1995; Kohn 1996; Longinelli et al. 2003).

2.5.3.1 Variability in bio-apatite $\delta^{18}O$ values; implications for palaeoclimatic reconstruction

Early on, Longinelli and Nuti (1984), Longinelli 1984 and Luz et al. 1984 recognised the potential of biogenic carbonates and phosphates as indicators of terrestrial palaeoenvironmental conditions and to quantify palaeotemperatures. This is based on the fact that oxygen precipitates in equilibrium with body water at a constant 37°C, and is independent of external temperatures (Longinelli and Nuti 1973). Early studies by Longinelli (1984), Luz et al. (1984), Luz and Kolodny (1985) and D'Angela and Longinelli (1990) showed that the $\delta^{18}O$ of modern animal phosphate values were related to the mean meteoric $\delta^{18}O$ values, and that different species had a specific linear relationship. Specific equations have been formulated to quantify these relationships for various mammal species (Luz et al. 1990; Cormie et al. 1994; Iacumin and Longinelli 2002; Longinelli et al. 2003). Under different climatic conditions, and depending on the species, a linear trend is not always evident (D'Angela and Longinelli 1990; Cormie et al. 1994; Huertas et al. 1995). Huertas et al. (1995) studied the relationship between bone

phosphate $\delta^{18}O$ values and meteoric waters of ten species of mammals. Overall, the correlations between mammalian $\delta^{18}O$ values and local meteoric waters were very strong, yet they found unreliable results for rabbits. They suggested that because rabbits rarely drink and rely on water in their food their body water $\delta^{18}O$ was influenced by enriched leaf $\delta^{18}O$ water rather than meteoric water $\delta^{18}O$ values. Similarly, Ayliffe and Chivas (1990) studied modern *Macropus* bone phosphate $\delta^{18}O$ values and found correlations with mean annual relative humidity that were independent of Australian meteoric water $\delta^{18}O$ values. It has become apparent that the factors influencing body water $\delta^{18}O$ values are more complex than had previously been considered; and clearly, some mammal species are not suitable for quantitatively reconstructing palaeotemperatures (D'Angela and Longinelli 1990; Iacumin and Longinelli 2002).

Detailed models have been developed to explain the $\delta^{18}O$ variations caused by different diets, drinking behaviours and physiologies that influence water utilisation (Bryant and Froelich 1995; Kohn 1996; Kohn et al. 1996, 1998; Longinelli et al. 2003). Kohn et al. (1996), for example, predicted that humidity should be a moderate influence on drought-tolerant animals in dry areas, and browsers, which are often not obligate drinkers and obtain more water from leaves, should be more enriched in $\delta^{18}O$ than grazers, which are mostly obligate drinkers. This pattern has been observed in modern African fauna (Kohn et al. 1998; Sponheimer and Lee-Thorp 2000) and in fossil faunal assemblages from Equus Cave and Swartkran's Cave in South Africa (Sponheimer and Lee-Thorpe 1999). In contrast, Bocherens et al. (1996) found that browsing black rhinoceros and mixed-feeding elephants from Northern and East African fossil deposits were depleted in $\delta^{18}O$ compared to grazers. This shows that mammalian $\delta^{18}O$ values are not due to feeding niche *per se*, but depend on how water is incorporated and fractionated in the specific plants they consume. In this case, for example, grazers may have eaten grasses heavily enriched in ^{18}O due to evaporation, and drank from highly evaporated surface waters, whereas large trees able to tap deeper into soil and groundwater may have maintained high leaf water levels, so that browsers relied less on surface waters (Sponheimer and Lee-Thorpe 1999).

2.5.4 The use of oxygen isotopes in archaeological and palaeoecological studies

Despite the complexity of deciphering isotopic inputs that affect body water $\delta^{18}O$ values, fossil teeth and bone $\delta^{18}O$ values have provided qualitative data relating to animal ecology, physiology and changing environmental conditions. For example, bone and enamel $\delta^{18}O$ values of fossil equids (Chillon et al. 1994), reindeers and mammoths (Genoni et al. 1998; Longinelli et al. 2003) have been used to infer relative palaeotemperature differences during glacial and post glacial phases in Europe and Eurasia. The $\delta^{18}O$ values in juvenile teeth have been used to examine breastfeeding practices in prehistoric humans (Wright and Schwarcz 1998) and to

establish the season of birth of animals (Bryant et al. 1996; Zazzo et al. 2002; Balasse et al. 2003).

Recent studies have used serial intra-tooth microsampling to investigate long-term and seasonal variation in $\delta^{18}O$ values of many fossil mammals such as: equids (Bryant et al. 1996; Sharp and Cerling 1998; Hoppe et al. 2004a, 2004b), bison (Fricke and O'Neil 1996; Gadbury et al. 2000), beavers (Stuart-Williams and Schwarcz 1997) and proboscideans (Fox and Fisher 2001). Many of these studies found repetitive and/or sinusoidal isotopic patterns along the teeth; often the more depleted $\delta^{18}O$ values have been correlated with winter precipitation and the more enriched values with summer precipitation. The patterns and range of inter-tooth $\delta^{18}O$ values have been used to infer the nature and magnitude of seasonal variations in precipitation, temperature and humidity (Cerling and Sharp 1996; Fricke and O'Neil 1996; Sharp and Cerling 1998; Fricke and O'Neil 1999; Hoppe et al. 2004a). The interpretation of intra-tooth $\delta^{18}O$ variability in terms of precipitation and temperature 'winter lows' and 'summer highs' is only one simple model. Other factors that affect body water $\delta^{18}O$ values (as discussed in section 2.5.3) will also contribute to seasonal patterns in $\delta^{18}O$ values. Consequently, mammalian $\delta^{18}O$ intra-tooth values may not always track seasonal meteoric water $\delta^{18}O$ patterns precisely (Kohn 1996; Sharp and Cerling 1998; Higgins and MacFadden 2004).

CHAPTER 3

3 THE AUSTRALIAN CONTEXT

3.1 An overview of stable isotope studies of Australian marsupial fauna

In the European, African and North American continents stable isotope analyses of terrestrial mammal remains are widely used to investigate the diet and ecology of modern and fossil species. By comparison, similar investigations using Australian native mammals are not as prevalent, and to date, around ten Australian studies have been published. The majority of these studies have concentrated on modern specimens of *Macropus*. The topics most commonly explored related to using stable isotopes in bone to indicate levels of moisture availability and the relative distributions of C_3 and C_4 grasses within environments.

The work of Ayliffe and Chivas (1990) was the first isotopic study of modern *Macropus* bone. They found a strong relationship between bone phosphate $\delta^{18}O$ values and relative humidity, and emphasized it as a potential source of palaeoclimatic information. Also using modern samples, Gröcke (1997) reported a strong negative linear relationship between *Macropus* bone collagen $\delta^{15}N$ values and annual precipitation. This relationship was considered robust, and was used to calculate annual palaeo-precipitation levels from the $\delta^{15}N$ values of fossil *Macropus* from sites in southern Australia. Pate et al. (1998) reported a similar negative correlation between $\delta^{15}N$ and annual precipitation from modern *Macropus* bone collagen that were sampled along a coastal to inland transect through South Australia. Recently, Pate and Noble (2004) combined the two datasets of Pate et al. (1998) and Gröcke (1997) to further convey these findings. Due to these studies, the $\delta^{15}N$ values in *Macropus* bone collagen have been actively promoted as a measure of environmental moisture availability.

The different ranges and mean $\delta^{13}C$ values of C_3 and C_4 plants has led to the $\delta^{13}C$ values of herbivore tissues being used to estimate the percentage of these plant types in the diet; and thus infer their frequency distribution in the environment. Pate and Noble (2000) and Pate et al. (1998) observed that modern *Macropus* bone collagen $\delta^{13}C$ values became more enriched from

the coast to the continental interior along the South Australian transect (mentioned above). These bone collagen $\delta^{13}C$ values followed a predictable geographic trend given the modern-day distribution of C_3 and C_4 grasses estimated by Hattersley (1983). Carbon isotopes in *Macropus* were examined from a different perspective by Witt and Ayliffe (2001). Their study analysed the $\delta^{13}C$ values in bone collagen and hair of 13 individuals, as well as the $\delta^{13}C$ values of their diet (via faeces) over a 15 month period. They used a single population in south-west Queensland. This was the first Australian study to investigate intra-population isotopic variability and seasonal changes in diet $\delta^{13}C$ values. Although bone collagen $\delta^{13}C$ variability was positively correlated with age in this study, this has rarely been observed and supported elsewhere (Lovell et al. 1986). In addition, isotopic studies on small marsupials have been undertaken. Mcilwee and Johnson (1998) used carbon isotopes to investigate seasonal diet selection of bandicoots. Klaaussen et al. (2004) examined variation in the discrimination between diet and blood cell and blood plasma $\delta^{15}N$ values of bandicoots fed low and high protein diets.

3.1.1 Stable isotope studies on Australian fossil marsupial and non-marsupial fauna

Only three studies have published stable isotope ratios from fossil marsupial remains. Gröcke (1997, 1998) analysed bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of *Macropus*, *Sthenurus* and *Diprotodon* from fossil sites in South Australia. The $\delta^{13}C$ values were used to interpret the percentage of C_3 and C_4 plants in the extinct animal's diet. Interestingly, these sites lay on a similar geographic transect as the modern specimens measured by Pate et al. (1998). Yet there has been little comparison of the fossil and modern *Macropus* stable isotope values from this region. This is understandable, however, given that few of the fossil sites have detailed chronologies. This limits the temporal comparisons and subsequent interpretations of palaeoecological change.

The combined work of Miller et al. (1999, 2005) and Johnson et al. (1999) have examined the $\delta^{13}C$ ratios of eggshells of *Dromaius* (emu) and *Genyornis* (extinct mihirung) from within the central Lake Eyre and Murray-Darling basins. Although not focused on marsupials, these projects represent by far the most extensive stable isotope studies of fossil material in Australia and are worthy of mention. Miller et al. (1999) used the eggshell $\delta^{13}C$ values to characterise the feeding ecologies of these two birds over the last approximately the last 140,000 years to the present. In contrast to the emu, the *Genyornis* eggshell $\delta^{13}C$ values were generally more depleted and confined in range, and the species went extinct at around 50 ka, whereas the emu persists today. The authors suggested the isotopic evidence indicates *Genyornis* had more selective dietary requirements, especially for C_3 plants, than the emu. Furthermore, the more opportunistic and flexible diet choices of the emu perhaps made it less susceptible to extinction. Within the latter

part of their studies, the $\delta^{13}C$ values of fossil marsupial wombat tooth enamel were also examined (Miller et al. 2005). Over the time period from approximately >50 ka to < 45 ka, fossil wombat $\delta^{13}C$ values became more depleted and confined in range, similar to the temporal pattern of $\delta^{13}C$ values observed in the emu eggshells. The wombat stable isotope data, therefore, provided an additional line of evidence for ecological and vegetation change. Within a backdrop of little substantial evidence for detrimental climatic change within the region, the authors proposed that the change in eggshell and wombat tooth $\delta^{13}C$ values indicated substantial vegetation change during this time period. One of the proposed causes of this change was the land use, primarily increased fire regimes, of early Australians (Flannery 1994).

Figure 3-1. Location of specimens used in previous stable isotope studies in Australia

3.1.2 Summary

In summary, the previous work within Australia has concentrated on the genus *Macropus*. Predominantly, areas along the north-western coast and southern regions of Australia were sampled. The locations of specimens in the abovementioned studies are shown in Figure 3-1. Modern bone has been the most common tissue analysed, with the exception being the two fossil *Macropus* teeth by Gröcke (1998) and bulk tooth enamel from wombats by Miller et al. (2005). Collectively, these studies have made valuable contributions to stable isotope research, yet clearly, there are many avenues for further investigation.

3.2 A modern baseline study of stable isotopes in Australian marsupial herbivores

The majority of observations and theories that link the relationships between stable isotopes in diets, animals and their environments are based on studies in the northern hemisphere and Africa. Australia has a unique set of native fauna, vegetation and environments. They warrant their own investigation. The majority of Australia's fauna are marsupials. They develop in a pouch, many are characterised by low metabolic rates and they are largely nocturnal. On mainland Australia, native marsupial carnivores are now extinct and the largest herbivore is the red kangaroo, which reach a maximum weight of approximately 80 kg (Strahan 2000). Australia's fauna have evolved unique behavioural and physiological adaptations to cope with factors such as: high temperatures, high climate variability, lack of surface water to drink, and vegetation that is often fibrous and low in nutrients (Hobbs 1998; Hume 1999).

This thesis presents a study of modern Australian stable isotope ecology. It builds on the previous Australian studies of marsupials (described in section 3.1) by analysing additional species and extending the geographic coverage of sample locations. This will include the eastern-half of the continent. A large component of this research involves the analysis of $\delta^{13}C$ and $\delta^{18}O$ in tooth enamel carbonate. Bones is rarely preserved in the hot arid and humid Australian environments (Schoeninger et al. 1989; Reed 2001). Therefore, understanding the nature of stable isotopes in the enamel is crucial because this tissue is more often preserved in the fossil record (Lee-Thorp 2002).

3.2.1 Thesis research objective and specific areas of investigation

The broad objective of this thesis research is to examine stable isotopes in modern marsupial species to provide baseline data that can be used to inform palaeoecological studies.

This work centres on three marsupial herbivore groups: the koala, kangaroos and wombats. The study of each group is presented separately. These animals eat different diets and occupy different ecological niches. In addition, each has a different pattern of enamel formation and tooth eruption sequence. These differences not only shaped the necessary lines of enquiry about each species, but also provided unique opportunities for investigation. For example, one of the distinctive and novel lines of enquiry in this thesis is the preliminary investigation of seasonality using $\delta^{13}C$ and $\delta^{18}O$ values in the continuously-growing teeth of wombats.

Within the scope of available and relevant sample materials, the studies of the three marsupial species will each address these three specific research areas:

1) The isotopic nature of the animal's diet and its relationship to bone collagen and enamel carbonate tissues.

Estimations of diet $\delta^{13}C$ and $\delta^{15}N$ values will be obtained from analysing plants or via the animal's faeces (for koalas and wombats). Previous studies have shown that the enrichment, or fractionation, between diet and body tissues can vary between species (DeNiro and Schoeninger 1983; Cerling and Harris 1999; Sponheimer et al. 2003). Although fractionation values may only differ by a few per-mil, this still has consequences for palaeodietary interpretations that compare the diets of different co-existing species. This modern isotopic study provides the opportunity for these fractionation values to be more accurately estimated for koalas and wombats. In addition, the analysis of bone collagen and enamel carbonate in the same individual will enable the spacings between $\delta^{13}C$ in these tissues to be calculated.

2) The levels of isotopic variability.

The study will examine the intra-population variability in the bone collagen $\delta^{13}C$ and $\delta^{15}N$ and enamel carbonate $\delta^{13}C$ and $\delta^{18}O$ values. This is to gain an insight into the range of isotope values that can exist in natural populations of animals living in same location at the same time. This is particularly useful for palaeo studies where the availability of samples is often limited. It is appropriate to ask, in what circumstances will the temporal and spatial interpretations of stable isotope values from only two or three fossil samples actually be biologically meaningful or merely descriptive? These modern studies can help to better identify the numbers of samples are required to obtain a meaningful estimation of a population's mean isotopic value. Within the populations themselves, isotopic heterogeneity might be due to factors such as age or sub-species. Inter-tooth variability, such as systematic patterns in isotope values between teeth types, may provide information about the isotopic composition of diets during development or life. From a sampling perspective, identifying these factors can help avoid inappropriate comparisons between data.

3) The relationships between marsupial stable isotope values and the environment.

This study will examine how effectively the stable isotope composition of bone and teeth reflect the environmental signals such as moisture availability, ambient temperature and habitat type at a variety of spatial scales; local and continent wide. One critical test will be to see if the trends that have been observed in previous studies in Australia and on other continents hold true for the sites examined in this study. Palaeobiological studies examine multiple species to obtain a broader palaeoecological picture. Observing how the stable isotope values of co-existing species, which occupy different ecological niches, reflect the climatic conditions in the same geographic area will be insightful. It is hypothesised that the stable isotope analysis of each of the three species will provide different types of palaeoecological information. For example, stable isotope analysis of one species could be a better indicator of a particular set of environmental variables than another is.

3.2.2 Additional background information

Additional background information about the distribution of C_3 and C_4 grasses and climate zones in Australia are provided below. This information is important for understanding the environmental context from which the specimens were sampled.

The work of Hattersley (1983) remains a comprehensive resource regarding the distribution of C_3 and C_4 grasses in Australian. Hattersley's map of the estimated distribution of C_4 grasses is shown in Figure 3-1, and will be referred to during the subsequent chapters. Hattersley (1983) plainly states that this map represents the proportions of species type found in each environment. The important distinction between species abundance and species diversity needs to be considered when using this map definitively. For example, a location may have twice as many individual C_4 grass species than C_3 species, yet the C_3 grasses may comprise a larger proportion of the total grass biomass. Nonetheless, this map provides a good indication of the overall distribution of these grass types. Hattersley (1983) found that C_4 species are more abundant in areas that have hot wet summers, and the C_3 species in areas that have a cool wet spring. Overall, C_4 species declined with decreasing temperature and lower levels of summer rainfall. These geographic trends corroborate with the global trends regarding the distribution of these grass types in relation to climate (discussed in Section 2.3.1). Three climate maps published by the Australian Bureau of Meteorology (www.bom.gov.au) are shown in Figure 3-3. These maps provide an overall picture of Australia's climatic zones and annual precipitation.

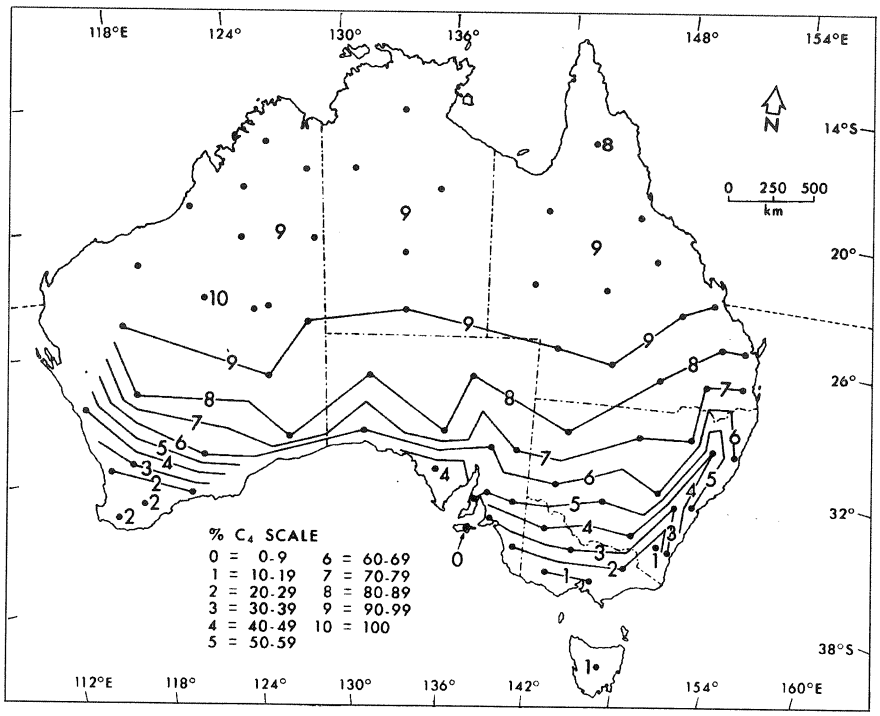

Figure 3-2. The distribution of C_4 grasses in Australia. Source: Hattersley (1983, p.114)

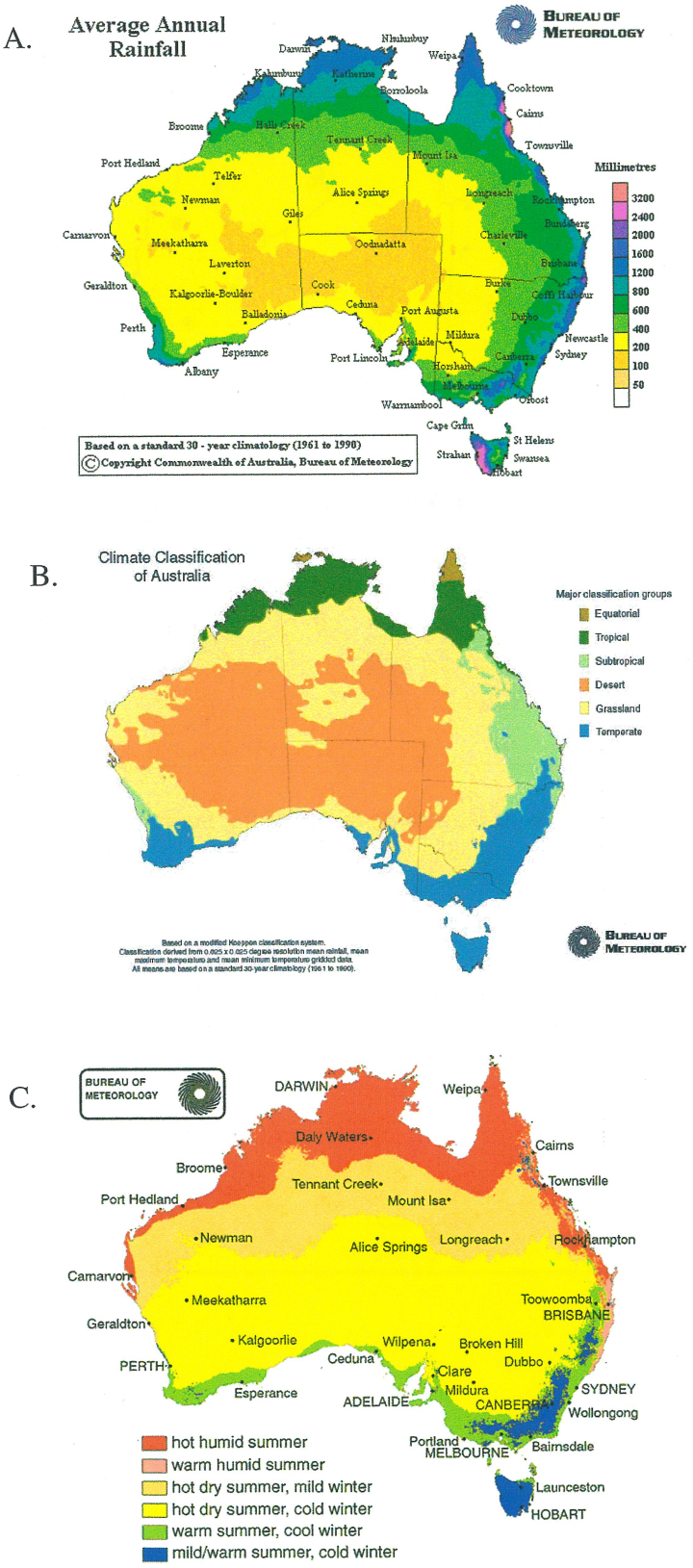

Figure 3-3. Climate in Australia. **A**. Annual precipitation. **B** and **C**. Climatic zones. Maps sourced from the Australian Bureau of Meteorology (www.bom.gov.au).

3.3 Experimental methods

3.3.1 Field work components and specimen collection

The aim of fieldwork was to collect samples of modern bone, tooth, faeces and plant-diet items from a wide range of different climatic regions in Australia. Attempts were made to collect multiple samples from populations at geographically distinct sites in a region, rather than single samples widely spaced over a region. Between December 2001 and October 2003, 1306 bone and tooth samples were collected.

The following protocol was followed at each site: skulls were collected to obtain both bone and teeth from each individual and to enable taxonomic identification. Postcranial elements were collected if cranial material was unavailable, and if disarticulated skeletons were mixed then single skeletal elements from one side of the body were taken to avoid sampling an individual twice (eg. 2 left femurs). Exact specimen locations were obtained with a GARMIN GPS and general descriptions of the surrounding environment and land uses were recorded (eg., open woodland, alpine grassland, cropping and forestry etc).

Koala skulls were obtained from: Dr A Melzer at the University of Queensland; the Brisbane Moggill Koala Veterinary Hospital, Mr R Schalgloth of the Koala Foundation (Ballarat) and Dr D Higgins at the University of Sydney. Northern hairy-nosed wombat bones, lower incisors and faeces samples were provided by Dr A Horsup of the Queensland Parks and Wildlife Service, Rockhampton.

Faeces were collected from animal carcasses (if available) and the surrounding habitats. Descriptions by Briggs (1999) helped to relate faeces to species. *Eucalyptus* leaves were collected from koala habitats. At the field site, plant and faeces samples were stored in paper bags and kept cold in an icebox to minimise decay and microbial growth.

3.3.2 Sample preparation methods and isotopic measurement

3.3.2.1 Bone and dentine collagen preparation and extraction

The best solution for standardising bone sampling is to choose the same part of the skeleton from each individual. Subject to availability, bone was preferentially cut, first from the horizontal ramus of the dentary, second from the zygomatic arch of the skull, and finally from the mid-shaft of postcranial elements.

Bones were scraped clean of flesh, and the outer enamel layer on teeth was removed using a drill and diamond edged blade. Samples were then washed in water and air-dried at ambient temperature. Modern bone and dentine contain lipids that need to be removed, and to do this,