Abstract

The application of bone collagen stable carbon and nitrogen isotope analysis to human palaeodietary reconstruction in tropical or arid regions is limited by two factors: (i) the overlap in C_4 and high marine protein (HMP) consumer bulk collagen δ^{13}C values, and (ii) the unpredictability of bulk collagen δ^{15}N values in regions of extreme aridity (<400 mm of rain per annum). Hence, the identification of HMP consumption among archaeological human populations can be problematic. In an endeavour to identify a more precise marine palaeodietary indicator, a range of collagen samples from archaeological faunal and human bone (n = 14 and 26, respectively), representing a spectrum of C_3, C_4 and HMP diets, were selected from coastal and near-coastal sites in the Western Cape, South Africa. Samples were subjected to compound-specific stable carbon isotope analysis of their constituent amino acids as trifluoroacetyl-isopropyl (TFA-IP) esters via gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). While human C_4 and HMP consumers were indistinguishable with respect to bulk collagen δ^{13}C values (−10.9±3.7‰ and −11.7±1.5‰, respectively) they were shown to be readily distinguished based on Δ^{13}C Glycine-Phenylalanine values (+4.0±1.6‰ and +12.0±1.9‰, respectively). The relationship between HMP consumption and elevated Δ^{13}C Glycine-Phenylalanine values was verified by: (i) the similarly elevated values exhibited by marine species when compared to terrestrial faunal species (+12.5±0.9‰ and +3.2±4.2‰, respectively), and (ii) the strong correlation observed between human Δ^{13}C Glycine-Phenylalanine and bulk collagen δ^{15}N values ($R^2 = 0.83, p < 0.001; n = 26$), the latter being a well-documented marine dietary indicator. It was concluded that Δ^{13}C Glycine-Phenylalanine values offer considerable potential as indicators of HMP consumption and a valuable substitute for bone collagen δ^{15}N values in arid regions where bulk δ^{15}N values are unpredictable.

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1. Introduction

The use of stable isotopes for palaeodietary reconstruction in certain environments can be problematic, and this paper focuses on those limitations specific to arid and tropical regions. Firstly, the utilisation of bulk collagen δ^{15}N values as indicators of marine protein consumption has been shown to be problematic in extremely arid environments [11,12,25,26]. For example, in particularly dry regions of South Africa (<400 mm of rain per annum), extremely enriched herbivore bone collagen δ^{15}N values, which overlap with the isotopic range for marine species, have been reported [12,30]. In an investigation of an arid South African environment, Heaton and co-workers observed a relationship between aridity and enriched plant δ^{15}N values (−0.3‰/100 mm
rain year$^{-1}$), however this relationship was shown to be less pronounced than that observed between aridity and animal bone collagen $\delta^{15}$N values ($-1.1$ to $-1.3\%_{\text{oo}}$) per 100 mm rain year$^{-1}$ [12]. A similar pattern was observed in the Cape region of South Africa, where the extensive range in animal bone collagen $\delta^{15}$N values was shown to correlate with rainfall; a pattern which was shown not to be reproduced in plant $\delta^{15}$N values [30]. Conversely, in an investigation of the archaeological site of Dakhleh, Egypt, where rainfall is essentially zero, highly enriched $\delta^{15}$N values were observed in both soils and plants, in conjunction with similarly $\delta^{15}$N-enriched herbivore and human bone collagen [25]. The authors hypothesised that the highly enriched $\delta^{15}$N values observed in these desert soils resulted from volatilization of isotopically light ammonia formed during bacterial activity. Hence, it may be that high bone collagen $\delta^{15}$N values exhibited in animals inhabiting arid regions result from a combination of: (i) the consumption of $\delta^{15}$N-enriched plants, and (ii) their physiological adaptation to water-stress, where drought-tolerant animals in particular, are able to conserve water by concentrating their urine with urea [3,12,23,30]. Since urea is $\delta^{15}$N-depleted with respect to diet [32,33], theoretically a relatively $\delta^{15}$N-enriched nitrogen pool remains upon excessive urea excretion. Elsewhere it has been hypothesised that the recycling of nitrogen (urea re-cycling) in low-protein consuming animals in arid environments increases the scope for isotopic fractionation, thereby resulting in higher $\Delta^{15}$N$^\text{Diet-Tissue}$ values [30].

The second limitation to palaeodietary reconstruction in arid environments results from the observed overlap in bulk collagen $\delta^{13}$C values of human HMP and C$_4$ consumers, which results from the insubstantial difference between marine foods (mean $\delta^{13}$C $-17\%_{\text{oo}}$) and C$_4$ terrestrial plants (mean $\delta^{13}$C $-12.5\%_{\text{oo}}$ [2,6]).

A combination of these two problems was identified in a recent investigation of marine protein consumption in Later Stone Age hunter-gatherers inhabiting the southern and western Capes of South Africa [26]. The southern Cape is a temperate well-watered area which receives 600–1200 mm rainfall per year. While sections of the region are today covered with dense forest or thick scrub and bush, grass species include several C$_4$ varieties [26,36]. There are challenges in predicting the extent to which prehistoric vegetation types can be extrapolated from those found there today, primarily owing to land clearance for agriculture. However, the mean bone collagen $\delta^{13}$C value for all animals in the region for the last 11,000 years ($-11 \pm 1.9\%_{\text{oo}}$) verifies the high abundance of C$_4$ grasses present in the region during the Holocene period [26]. Herbivore bone collagen $\delta^{15}$N values typically exhibit low $\delta^{15}$N values ($+5.0 \pm 1.2\%_{\text{oo}}$), as characteristic of well-watered environments. However, because the southern Cape is characterised by a mixed C$_3$/C$_4$ terrestrial environment, bone collagen $\delta^{13}$C values were considered inappropriate in establishing the extent of marine food consumption because of the overlap observed in human bone collagen $\delta^{13}$C values of C$_4$ and marine food consumers [26].

The western Cape is dominated by C$_3$ terrestrial flora and although a minor region of C$_4$ grasses has also been identified, ultimately bone collagen $\delta^{13}$C values can be successfully utilised to distinguish between C$_3$ terrestrial and marine food consumption [26]. However, the nitrogen isotope ecology of the western cape is complicated in the area north of Saldanha Bay, which receives below the threshold 400 mm of rain per annum and terrestrial herbivore $\delta^{15}$N values are in excess of $10\%_{\text{oo}}$, and commonly plot in the range observed for marine species. Bone collagen $\delta^{15}$N determinations of 12 terrestrial animals in this region produced a highly enriched mean value of $+13.6 \pm 1.8\%_{\text{oo}}$ [26]. Thus, in this region of the Cape the utilisation of bone collagen $\delta^{15}$N determinations for palaeodietary reconstruction has been shown to be problematic. In summary, bone collagen $\delta^{15}$N analysis may be applied in the southern Cape and $\delta^{13}$C analysis in the western Cape, however, it is difficult to accurately compare the palaeodietary habits of humans between the two regions when different dietary techniques are utilised. These problems constitute a major limitation in palaeodietary investigations in the Cape region because there is a wealth of evidence for major marine food exploitation in the Later Stone Age [26–29] and bone collagen $\delta^{13}$C and $\delta^{15}$N values normally represent valuable marine dietary indicators [19,21].

We describe here the compound-specific measurement of human bone collagen amino acids $\delta^{13}$C values, via GC-C-IRMS, as a means of circumventing these problems. Our assertion is based on the results obtained in our laboratory from studies of isotopically-controlled rat [16] and pig feeding experiments [13], which have demonstrated that essential amino acids are routed from dietary sources to bone collagen with minor isotopic fractionation ($\Delta^{13}$C$_{\text{Bone collagen amino acid-Dietary amino acid}} \approx 2–3\%_{\text{oo}}$). In addition, the considerable influence of dietary energy components (predominantly lipids and carbohydrates) on the isotopic composition of bone collagen non-essential amino acids was demonstrated for the first time. However, differences in bone collagen non-essential amino acid $\delta^{13}$C values between 20% and 70% marine protein (tuna) consuming rats indicated that the relative balance between non-essential amino acid absorption and de novo biosynthesis was governed by dietary protein intake [16]. Hence, in high protein consumers, bulk collagen and non-essential amino acid $\delta^{13}$C values would necessarily reflect that of dietary protein to a higher degree than in normal protein consumers.

The aims of the present study were to use bone collagen amino acid $\delta^{13}$C values to address the
fundamental limitations of bulk collagen $\delta^{13}C$ and $\delta^{15}N$ values in the southern and western Cape of South Africa, specifically to: (i) assess whether individuals consuming C₄ and high marine protein (HMP) diets with consequently overlapping bulk collagen $\delta^{13}C$ values may have distinguishable amino acid $\delta^{13}C$ values, and (ii) develop an alternative marine dietary indicator to substitute for bulk collagen $\delta^{15}N$ values in arid regions.

2. Materials and methods

2.1. Archaeological samples

Human skeletons ($n = 26$) from the southern (Plettenberg Bay, East of Wilderness, Matjes River, Drury’s Cave, Whitchers Cave, van der Walt’s Cave and Robberg Peninsula) and western Cape (Melkbosch, Hout Bay, Gordons Bay, Ysterfontein, Bokbaai, Klipfonteinrand, Watervalrivier, Eland Cave, Noordhoek, Cape Point and Kommetjie) with bulk collagen $\delta^{13}C$ and $\delta^{15}N$ values reflecting a range of C₃ and C₄ terrestrial and HMP consumption were selected for compound-specific amino acid $\delta^{13}C$ determinations. A range of archaeological terrestrial (2×steenbok, rock hyrax and buffalo, and 4×tortoise) and marine species (2×seal and 2×whale) common to South African Holocene sites were also investigated to provide a range of amino acid $\delta^{13}C$ values for dietary sources available for human consumption.

2.2. Sample preparation

The acid-insoluble bone protein (collagen) was obtained by surface-cleaning of 1–2 g fragments of bone followed by demineralisation in dilute HCl (2%). The bone fragments were then rinsed in distilled water and soaked overnight in 0.1 M NaOH, and rinsed repeatedly in distilled water until neutral. Prepared collagens were then freeze-dried prior to determinations of bulk $\delta^{13}C$ and $\delta^{15}N$ values [26] or hydrolysis for the determination of compound-specific amino acid $\delta^{13}C$ values. Approximately 2 mg of collagen was hydrolysed under vacuum in Young’s tubes (6 M HCl, 500:1 v/w; 100 °C, 24 h). On cooling the samples were transferred to screw-capped vials with double distilled water (3×0.5 ml) and methanol (3×0.5 ml). The solutions were evaporated under a gentle stream of N₂, re-dissolved in 2 ml methanol and stored at –18 °C until required for analysis.

Fractions (0.5 ml) of the protein hydrolysates obtained from the hydrolysis of bone collagen were transferred to screw-capped test tubes and dried under N₂. γ-Amino- $n$-butyric acid (40 µl of 0.2 mg ml⁻¹ solution in 0.1 M HCl) was added to each tube as an internal standard. Acidified isopropanol (0.5 ml, 2.8 M with acetyl chloride) was added to each culture tube, which were sealed with Teflon-lined caps and heated (100 °C, 1 h). The reaction was terminated by placing the tubes in a freezer and the residual isopropanol was removed under a gentle stream of N₂ at 40 °C. Excess H₂O and isopropanol were removed by addition of dichloromethane (DCM, 2×0.25 ml) which was also evaporated under N₂. Trifluoroacetic anhydride (TFAA, 0.5 ml) and DCM were added to the culture tubes, which were sealed with Teflon-lined caps and heated (100 °C, 10 min). The tubes were then placed in an ice bath where the excess TFAA and DCM were removed under a gentle stream of N₂. The derivatized amino acid trifluoroacetyl-isopropyl (TFA-IP) esters were dissolved in 0.25 ml DCM and stored at –18 °C until required for GC-C-IRMS analysis.

2.3. Instrumental analyses

Bone collagen bulk $\delta^{13}C$ and $\delta^{15}N$ values were measured on a Finnigan-MAT 252 mass spectrometer coupled to a Carlo-Erba NA1500 elemental analyser as described in Sealy [26].

Bone collagen amino acid $\delta^{13}C$ values were measured in triplicate following derivatisation to TFA-IP esters using a HP 6890 gas chromatograph coupled to a Finnigan MAT DELTAplus XL isotope ratio monitor via a Finnigan MAT GC III combustion interface (electron ionisation 100 eV, three Faraday cup collectors m/z 44, 45 and 46, CuO/NiO/Pt combustion reactor set to 940 °C, Cu reduction reactor set to 600 °C) as described in Howland et al. [13]. The GC was fitted with a ZB-5ms column (60 m×0.32 mm, 0.25 µm film thickness, 5% phenyl, 95% dimethylpolysiloxane equivalent stationary phase). The split-splitless injector temperature was held at 200 °C. The following oven temperature programme was used: initial temperature was held isothermally at 40 °C (1 min), then increased to 70 °C at a rate of 10 °C min⁻¹, then to 170 °C (20 min) at a rate of 3 °C min⁻¹, then to 250 °C (10 min) at a rate of 15 °C min⁻¹.

Co-injected standards of alanine, phenylalanine and lysine TFA-methyl esters (Sigma-Aldrich products T3381, T5006 and T4631) of known isotopic composition were added immediately prior to GC-C-IRMS analysis. Amino acid $\delta^{13}C$ values were calculated from the $\delta^{13}C$ values of their TFA/IP derivatives using correction factors [5,14,31].

Carbon isotope ratios are reported relative to the Pee Dee Belemnite limestone standard in parts per thousand (‰). Results are expressed as:

$$\delta^{13}C = \left[ \frac{(R_{\text{sample}}/R_{\text{standard}}) - 1}{1000} \right]$$

where $R_{\text{sample}}$ and $R_{\text{standard}}$ are the ratios of $^{13}C/^{12}C$ for the sample and the standard, respectively.
3. Results and discussion

Of the 20 amino acids in bone collagen, baseline resolution (which is essential for the determination of reliable compound-specific δ^{13}C and δ^{15}N values) was obtained for five essential (threonine, valine, leucine, isoleucine and phenylalanine) and seven non-essential amino acids (alanine, glycine, serine, proline, hydroxyproline, aspartate and glutamate), which in total constitute 84.8% of the carbon in collagen. Bulk bone collagen δ^{13}C and δ^{15}N values, and individual amino acid δ^{13}C values for humans and faunal species are displayed in Tables 1 and 2, respectively. Within both the faunal (17.6±2.3‰, n = 17) and human bone collagens (22.3±5.9‰, n = 26) a relatively wide range in δ^{13}C values was observed between the 12 amino acids. A similarly wide isotopic range in bone collagen amino acid δ^{13}C values has been observed previously in rats (23.6‰) [16] and pigs (25.4‰) [10], and 25‰ [13] subjected to controlled-feeding experiments. This reflects the fact that the isotopic composition of tissue amino acids is governed by: (i) the isotopic composition of their biosynthetic precursors, and (ii) potential isotopic fractionation associated with amino acid de novo biosynthesis [1,8,10,15,18,34].

The wide range in bone collagen amino acid δ^{13}C values is illustrated in Fig. 1, which presents the amino acid δ^{13}C values from three human individuals that were defined via assessment of bulk collagen δ^{13}C and δ^{15}N values as: (i) a predominantly C₃ consumer (UCT 333, δ^{13}C = −19.0‰, δ^{15}N = +9.9‰), (ii) a consumer who ate significant amounts of C₄ plant-based foods (UCT 67, δ^{13}C = −11.6‰, δ^{15}N = +10.7‰), and (iii) a HMP consumer (SAM-AP 4304A, δ^{13}C = −12.3‰, δ^{15}N = +17.3‰). Predictably, the C₃ consumer exhibited the most depleted δ^{13}C values in all of its bone collagen amino acids. However, the isotopic composition of the C₄ and HMP consumers’ bone collagen amino acids was more instructive. Predictably, the C₄ (δ^{13}C = −11.6‰) and HMP consumer (δ^{13}C = −12.3‰) could not be distinguished based on bulk collagen δ^{13}C values. However, with the exception of glycine, the HMP consumer exhibited amino acid δ^{13}C values depleted relative to the C₄ consumer. Proline, hydroxyproline and glutamate which comprise 15.4, 12.9 and 8.4% of the carbon atoms in collagen were more depleted in the HMP consumer’s bone collagen by 5.9, 6.9 and 4.7‰, respectively (Fig. 2). In the HMP consumer, glycine was enriched by 6.2‰ relative to the C₄ terrestrial consumer and because glycine contributes 17.5% of the carbon atoms to bone collagen this enrichment compensates for the depletion observed in the remaining 11 amino acids and, hence, similar C₄ terrestrial and HMP consumer bone collagen δ^{13}C values were observed. The nature of this overlap in high C₄ and HMP consumer bone collagen δ^{13}C values has not been revealed at the molecular level previously. The pattern observed for these 3 individuals was similar in all South African individuals classified as high C₃ consumers (n = 10), high C₄ consumers (n = 3) and HMP consumers (n = 13).

Enriched tissue glycine δ^{13}C values have been reported previously [1,8,9], and result in the relative enrichment in bulk collagen δ^{13}C values because glycine comprises 17.5% of the carbon in collagen. The α-carboxyl carbon in amino acids is substantially enriched in δ^{13}C relative to the other carbons by up to 10–20‰; this accounts for the enrichment of two carbon-containing glycine relative to other amino acids [1,17]. In addition, the association between highly enriched glycine δ^{13}C values and marine sediments and organisms has been reported previously [7,17]. Significantly, a glycine δ^{13}C value of approximately −2.0‰ for fossil whale bone collagen has also been reported [10]. Elsewhere, in a food web analysis of the blue crab, δ^{13}C-enrichment of glycine was shown to correlate positively with increasing trophic level [7]. Furthermore, in an investigation of sediments along the Washington coast of North America extremely depleted glycine δ^{13}C values were observed in sediments exhibiting the highest concentrations of terrigenous organic matter, and, conversely, those with the highest concentrations of marine-derived organic matter exhibited the most enriched glycine δ^{13}C values [17]. The latter was proposed to reflect the fact that plankton are amino acid-rich relative to terrigenous plants which are carbohydrate-dominated; hence, the higher glycine δ^{13}C values observed in marine sediments reflected a higher degree of microbial re-working of planktonic amino acids [17].

In order to rationalize the δ^{13}C-enrichment observed in bone collagen glycine in humans consuming HMP diets, glycine and phenylalanine δ^{13}C values were compared. Phenylalanine is an essential amino acid which has been shown to be directly incorporated from the diet into rat [16] and pig bone collagen [13] with little fractionation (1–2‰). Hence, it can be hypothesized that the δ^{13}C value of collagen phenylalanine should faithfully reflect the isotopic composition of terrestrial/marine plants at the bottom of the foodweb. Conversely, it was hypothesized that dietary sources of the non-essential amino acid glycine would not be enough to accommodate the enormous demand for glycine in the body [22], thus the majority of bone collagen glycine is thought to be biosynthesised de novo via glycolytic precursors, and as a result, should largely reflect the isotopic composition of dietary carbohydrates. Since marine ecosystems contain more trophic levels than their terrestrial counterparts a larger scope for isotopic fractionation of glycine is expected within marine biomes. As such, it was considered that higher Δ^{13}C Glycine-Phenylalanine values may be exhibited in humans consuming marine rather than terrestrial diets.
Table 1
Bulk bone collagen δ¹³C and δ¹⁵N values and δ¹³C values for the 8 most abundant amino acids identified in the human bone collagens (n = 26) from the Cape region of South Africa

<table>
<thead>
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<th>Accession number</th>
<th>Site</th>
<th>Alanine</th>
<th>Glycine</th>
<th>Aspartate</th>
<th>Glutamate</th>
<th>Proline</th>
<th>Hydroxyproline</th>
<th>Leucine</th>
<th>Phenylalanine</th>
<th>δ¹³C_Bulk</th>
<th>δ¹⁵N_Bulk</th>
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<td>East of Wilderness</td>
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<td>UCT 333</td>
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<td>−18.3</td>
<td>−14.4</td>
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<td>−15.4</td>
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<td>−11.5</td>
<td>−10.9</td>
<td>−12.5</td>
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<td>−3.2</td>
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<td>−6.3</td>
<td>−9.2</td>
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<tr>
<td>SAM-AP 4203A</td>
<td>Kommetjie</td>
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<td>−4.1</td>
<td>−8.4</td>
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<td>−10.5</td>
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<td>SAM-AP 4308</td>
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<td>−10.4</td>
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<td>−11.2</td>
<td>−21.1</td>
<td>−16.9</td>
<td>11.8</td>
<td>16.4</td>
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</table>

All purified bone collagens exhibited C/N ratios within the range of 2.9–3.6.

Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Site</th>
<th>C/N Ratio</th>
<th>13C (‰)</th>
<th>13C (‰)</th>
<th>17O (‰)</th>
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</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>Nelson Bay Cave</td>
<td>2.6 (0.7)</td>
<td>-23.7 (0.8)</td>
<td>-12.5 (3.9)</td>
<td></td>
</tr>
<tr>
<td>Rock hyrax</td>
<td>Elands Bay Cave</td>
<td>2.6 (1.4)</td>
<td>-23.0 (1.0)</td>
<td>-14.9 (1.3)</td>
<td></td>
</tr>
<tr>
<td>Tortoise</td>
<td>Elands Bay Cave</td>
<td>2.5 (1.4)</td>
<td>-22.0 (0.8)</td>
<td>-16.5 (1.3)</td>
<td></td>
</tr>
</tbody>
</table>

All purified bone collagens exhibited C/N ratios within the range of 2.9–3.6.

and bone collagen, B, values (|0.3|0.5|), explaining the

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well-documented $^{15}$N enrichment observed in marine environments [4,20]. Indeed, a very significant correlation was shown between $\Delta^{13}$C Glycine-Phenylalanine and bone collagen $\delta^{15}$N values ($R^2 = 0.84$, $p < 0.001$; $n = 26$), and an unambiguous distinction was observed between HMP (black triangles) and both C3 and C4 terrestrial consumers (grey squares and white diamonds, respectively).

The same pattern in $\Delta^{13}$C Glycine-Phenylalanine values was also observed among South African archaeological faunal species, where seals (+12.2%$^{\text{oo}}$, $n = 2$) and whales (+12.9%$^{\text{oo}}$, $n = 2$) exhibited significantly higher values than the terrestrial species (+3.2±4.2%$^{\text{oo}}$, $n = 13$). Interestingly, in the case of faunal species, a weaker degree of correlation between $\Delta^{13}$C Glycine-Phenylalanine and bone collagen $\delta^{15}$N values was observed than in humans ($R^2 = 0.16$, $p > 0.05$, $n = 12$). This is a consequence of the $^{15}$N-enrichment exhibited in the drought-tolerant tortoises and rock hyraxes originating from the arid western Cape, which exhibited mean bone collagen $\delta^{15}$N values of +9.6±1.1%$^{\text{oo}}$ and +17.7±2.8%$^{\text{oo}}$, respectively. Importantly, this uncharacteristic enrichment observed in the bone collagen $\delta^{15}$N values of the drought-tolerant rock hyraxes and tortoises was not correlated with an increase in $\Delta^{13}$C Glycine-Phenylalanine values, as observed in the marine faunal species. Hence, $\Delta^{13}$C Glycine-Phenylalanine values provide a means of determining whether highly enriched bulk $\delta^{15}$N values correspond to the consumption of high trophic level/marine foods or result from a response to severe aridity. Indeed, if the rock hyrax and tortoise originating from the arid western Cape are omitted from the dataset the correspondence between faunal $\Delta^{13}$C Glycine-Phenylalanine and $\delta^{15}$N values is greatly improved ($R^2 = 0.56$, $p < 0.001$; $n = 17$).

In order to elucidate whether high $\Delta^{13}$C Glycine-Phenylalanine values are indicative of trophic level position or are confined to marine species and human HMP consumers, human $\Delta^{13}$C Glycine-Phenylalanine and $\delta^{15}$N values were plotted alongside values for marine faunal species (Fig. 4). The relationship between high $\Delta^{13}$C Glycine-Phenylalanine and $\delta^{15}$N values and HMP consumption was again verified, since human HMP consumers and marine fauna can be seen at the top of the scatterplot while human C3 and C4 terrestrial consumers plotted at the base. With the exception of one seal bone, all HMP consumers exhibited more enriched bone collagen $\delta^{15}$N values than marine fauna, indicating the utility of $\delta^{15}$N values as trophic level indicators. However, no pattern was observed in $\Delta^{13}$C Glycine-Phenylalanine values, where human HMP consumers and marine species formed a tight but mixed

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Table 3: $\delta^{13}$C Glycine, $\delta^{13}$C Phenylalanine and $\Delta^{13}$C Glycine-Phenylalanine values (%$^{\text{oo}}$) for C3, C4 and HMP consumers

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>C3 consumers</th>
<th>C4 consumers</th>
<th>HMP consumers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>$\delta^{13}$C Depleted (DeB): $-3.2\pm1.8%^{\text{oo}}$</td>
<td>$\delta^{13}$C Enriched (DeB): $-10.6\pm1.2%^{\text{oo}}$</td>
<td>$\delta^{13}$C Enriched (DI): $-16.7\pm1.3%^{\text{oo}}$</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>$\delta^{13}$C Depleted (DI): $-18.5\pm1.8%^{\text{oo}}$</td>
<td>$\delta^{13}$C Enriched (DI): $-13.9\pm2.4%^{\text{oo}}$</td>
<td>$\delta^{13}$C Depleted (DI): $-16.7\pm1.3%^{\text{oo}}$</td>
</tr>
<tr>
<td>$\Delta^{13}$C Glycine-Phenylalanine</td>
<td>+5.1±1.8%$^{\text{oo}}$</td>
<td>+4.0±1.6%$^{\text{oo}}$</td>
<td>+12.0±1.9%$^{\text{oo}}$</td>
</tr>
</tbody>
</table>

DeB and DI denote de novo biosynthesis and direct incorporation, respectively.

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Fig. 3: Relationship between human bone collagen $\Delta^{13}$C Glycine-Phenylalanine and bulk collagen $\delta^{15}$N values of predominantly C3 consumers, C4 consumers and HMP consumers.

Fig. 4: Relationship between bone collagen $\Delta^{13}$C Glycine-Phenylalanine and $\delta^{15}$N values of predominantly human C3 consumers, C4 consumers and HMP consumers and marine faunal species.
group. Hence, it is likely that $\Delta^{13}C_{\text{Glycine-Phenylalanine}}$ values are diagnostic of HMP consumption but not trophic level positioning.

The distinguishably high $\Delta^{13}C_{\text{Glycine-Phenylalanine}}$ values identified in HMP consumers requires explanation. Since glycine is a non-essential amino acid bone collagen glycine may derive from either: (i) de novo biosynthesis, or (ii) direct incorporation from dietary sources. Although glycine can be biosynthesised via two major pathways, the majority of the body’s supply of biosynthesised glycine derives from serine and most of the carbons in serine derive from 3-phosphoglycerate, which, as a glycolytic intermediate, derives from glucose. Thus it follows that if the majority of glycine in bone collagen derives from de novo synthesis its $\delta^{13}C$ values should largely reflect that of the carbohydrate component of the diet. Since marine mammal and fish flesh contain negligible carbohydrates (a very minor glycogen component), glycine, if biosynthesised de novo, should reflect the isotopic composition of the minor terrestrial plant food input into the HMP consumers’ diets. Thus, in the HMP consumers of the southern Cape of South Africa the highly enriched glycine $\delta^{13}C$ values could be explained by the availability of C$_4$ terrestrial foods, however, this explanation cannot satisfy the situation on the exclusively C$_3$ western Cape. Hence, the origins of the majority of glycine in the bone collagen of these individuals may not be via de novo synthesis, rather the majority of glycine probably derives directly from dietary sources. This is further supported by the almost indistinguishable glycine $\delta^{13}C$ values observed between the marine faunal species ($-4.8 \pm 1.5^{\circ}/{_o}$, $n = 4$) and the HMP consumers ($-4.5 \pm 1.6^{\circ}/{_o}$, $n = 13$), however, the slightly more enriched glycine $\delta^{13}C$ values observed for humans agrees with the small $^{13}C$-enrichment ($1–2^{\circ}/{_o}$) observed in rat and pig bone collagen essential amino acids over the dietary amino acid $^{13}C_{\text{phenylalanine}}$ [13,16].

Although non-essential amino acids can be biosynthesised by the body, they need not be. On high-protein diets the incorporation of non-essential amino acids is more biologically plausible than their de novo biosynthesis in terms of energy efficiency. Indeed, glycine (and proline and hydroxyproline) requires four enzymes for its biosynthesis. Non-essential amino acids can enter the bloodstream directly from the liver and subsequently be transported to cell-sites for protein biosynthesis. Furthermore, the comparison of $\Delta^{13}C_{\text{Amino acid-Amino acid}}$ values in the bone collagen of rats subjected to an isotopically controlled feeding experiment demonstrated that an increase in dietary protein (from 20 to 70%) resulted in a higher degree of absorption of dietary non-essential amino acids into collagen [16]. The direct incorporation of dietary glycine into bone collagen has been previously reported from a pig-feeding experiment, where minor $\Delta^{13}C_{\text{Collagen amino acid-Diet amino acid}}$ values (1.4 and 0.9$^{\circ}/{_o}$, for a C$_3$ and C$_4$ diet, respectively) were interpreted as resulting from the limited fractionation associated with its incorporation into collagen [10].

High protein diets are also associated with an inhibition of amino acid biosynthesis. There are two well-established methods of inhibition associated with high protein diets [24]: (i) the enzyme that controls the committed step of amino acid synthesis is inhibited by the presence of substantial intracellular concentrations of the amino acid [35], and (ii) the product amino acid can inhibit the synthesis of the enzyme utilised in the committed step of amino acid synthesis [24]. In addition to the fact that these HMP consumers obtained a diet which has been interpreted as containing >70% marine sources [29], the protein of marine species contains more glycine than any other foods. Glycine contributes a higher number of residues per thousand to fish (77) than casein (18), beef (51), pork (47), bean (42), seed (58) or maize (38) [37]. Thus, approximately twice the amount of glycine present in plants is obtainable from fish and if the protein bioavailability difference between fish (high) and plant protein (lower) is considered, the available glycine in fish compared to plants is further increased. This demonstration of the direct incorporation of dietary glycine in HMP consumers provides evidence that the dietary isotope signals of non-essential amino acids are also governed by their dietary availability, in addition to more widely established factors, i.e. age, nitrogen balance, health, physiology and species.

4. Conclusions

In this paper we have demonstrated the utility of compound-specific stable isotope analysis of individual bone collagen amino acids in human palaeodietary reconstruction. We have shown that: (i) the overlap in C$_4$ and HMP consumer bone collagen $\delta^{13}C$ values can be attributed to the relative $^{13}C$-enrichment observed in the glycine of HMP consumers when compared to C$_4$ terrestrial consumers, and (ii) due to their contrasting metabolic pathways the $\delta^{13}C$ values of the essential amino acid phenylalanine and the non-essential amino acid glycine in bone collagen preserve very different palaeodietary signals and this difference ($\Delta^{13}C_{\text{Glycine-Phenylalanine}}$) may be exploited to distinguish between HMP and terrestrial consumers. The application of this compound-specific approach has been shown to be particularly useful in the assessment of palaeodiet in South Africa where a possible resolution of the problems associated with bulk collagen $\delta^{13}C$ (overlap of C$_4$ and marine consumer $\delta^{13}C$ values) and $\delta^{15}N$ analysis ($^{15}N$ enrichment in arid environments) has been presented. The finding of high $\Delta^{13}C_{\text{Glycine-Phenylalanine}}$ values in marine species and HMP consumers verifies
that this novel indicator offers considerable potential as a marine dietary indicator and a valuable substitute for bone collagen $\delta^{15}$N values in arid regions where $\delta^{15}$N values are unpredictable.

Acknowledgements

This work was completed whilst LTC was in receipt of a University of Bristol studentship. NERC is thanked for mass spectrometry facilities (GR3/2951, GR3/3758 and FG6/36101). We would like to thank Drs Jim Carter and Ian Bull (University of Bristol) for assistance with GC-C-IRMS analysis.

References


