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# Compound-specific amino acid isotopic proxies for detecting freshwater resource consumption



Emily C. Webb<sup>a</sup>, Noah V. Honch<sup>a</sup>, Philip J.H. Dunn<sup>a</sup>, Gunilla Eriksson<sup>b</sup>, Kerstin Lidén<sup>b</sup>, Richard P. Evershed<sup>a,\*</sup>

<sup>a</sup> Organic Geochemistry Unit, School of Chemistry, University of Bristol, Cantock's Close, Bristol BS8 1TS, United Kingdom

<sup>b</sup> Archaeological Research Laboratory, Stockholm University, SE-106 91 Stockholm, Sweden

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## ABSTRACT

Of central importance to palaeodietary reconstruction is a clear understanding of relative contributions of different terrestrial (i.e., C<sub>3</sub> vs. C<sub>4</sub> plants) and aquatic (i.e., freshwater vs. marine) resources to human diet. There are, however, significant limitations associated with the ability to reconstruct palaeodiet using bulk collagen stable isotope compositions in regions where diverse dietary resources are available. Recent research has determined that carbon-isotope analysis of individual amino acids has considerable potential to elucidate dietary protein source where bulk isotopic compositions cannot. Using  $\delta^{13}\text{C}_{\text{AA}}$  values for human and faunal remains from Zvejnieki, Latvia (8th – 3rd millennia BCE), we test several isotopic proxies focused on distinguishing freshwater protein consumption from both plant-derived and marine protein consumption. We determined that the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxies can effectively discriminate between terrestrial and aquatic resource consumption, and the relationship between essential  $\delta^{13}\text{C}_{\text{AA}}$  values and the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxies can differentiate among the four protein consumption groups tested here. Compound-specific amino acid carbon-isotope dietary proxies thus enable an enhanced understanding of diet and resource exploitation in the past, and can elucidate complex dietary behaviour.

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## 1. Introduction

Stable carbon and nitrogen isotopic analyses of archaeological human and faunal remains have been used for over three decades to reconstruct palaeodiet and to investigate palaeoecology, and these studies make valuable contributions to our understanding of subsistence practices in diverse environments, and of changing patterns of resource use temporally and spatially (Katzenberg and Pfeiffer, 2000). In recent years, however, it has become increasingly evident that there are significant limitations in the assessment of palaeodiet using bulk protein stable isotope compositions in ecologically-complex regions where diverse dietary resources are available (e.g., Hedges, 2004; Lidén et al., 2004; Milner et al., 2004). Differential resource access and exploitation by individuals or societies can have important implications for socioeconomic behaviour, or reveal the social and ecological impact of

environmental perturbations over time. Thus, of central importance in many archaeological contexts is the ability to determine the relative contributions of different protein sources to diet, such as terrestrial C<sub>3</sub> plant-derived protein, freshwater protein, terrestrial C<sub>4</sub> plant-derived protein, or marine protein resources. Bulk carbon-isotope compositions, however, may mask dietary variability if the contributions from specific resources (e.g., marine protein) are small (Hedges, 2004), or if the natural variability in carbon-isotope compositions between two classes of resources is low, as is often the case when comparing the isotopic compositions of terrestrial plants and freshwater fauna from adjacent lakes and rivers.

Nitrogen-isotope compositions reflect the trophic position of consumed protein and are known to increase several per mil per trophic level between producers and consumers, and often help in disentangling complex diets (DeNiro and Epstein, 1981; Schoeninger, 1985). There is considerable variability in the degree of <sup>15</sup>N-enrichment between diet and consumer tissues; among humans from European archaeological sites, for example, the tissue – diet nitrogen-isotope offsets ranged from ~+2 to +5‰ (reviewed

\* Corresponding author.

E-mail address: [r.p.evershed@bristol.ac.uk](mailto:r.p.evershed@bristol.ac.uk) (R.P. Evershed).

in Hedges and Reynard, 2007), and a recent controlled dietary study estimated trophic offsets as high as +6‰ (O'Connell et al., 2012). Trophic relationships inferred from nitrogen-isotope compositions can, however, be obscured or altered, for example, if manure is applied to terrestrial crops (Bogaard et al., 2007; Fraser et al., 2011), in particularly arid environments (Ambrose and DeNiro, 1986; Craine et al., 2009; Grocke et al., 1997; Heaton et al., 1986), or if nitrogen metabolism is affected by physiological stress (Reitsema, 2013; Williams et al., 2011). The possible influence of these factors on  $\delta^{15}\text{N}$  values can, however, be difficult to assess. Focusing on compound-specific carbon-isotope compositions, particularly of essential amino acids, eliminates the potentially confounding effect of tissue-diet fractionation and routing vs. biosynthesis of non-essential amino acids on bulk carbon-isotope compositions, and of the environmental and physiological effects discussed above on nitrogen-isotope compositions.

Controlled feeding studies and the relatively few published papers that have analyzed archaeological material have demonstrated that carbon-isotope analysis of individual amino acids has considerable potential to elucidate palaeodiet where traditional bulk isotopic data fail (Choy et al., 2010; Corr et al., 2005; Fogel and Tuross, 2003; Hare et al., 1991; Honch et al., 2012; Howland et al., 2003; Jim et al., 2006). For example, intra-individual offsets between the amino acids phenylalanine and glycine ( $\Delta^{13}\text{C}_{\text{Gly-Phe}}$ ; Corr et al., 2005) reliably distinguish among individuals consuming a high marine protein diet and those consuming a terrestrial  $\text{C}_4$  plant diet, and the feasibility of using the relationships between other paired amino acids to identify dietary protein source has also been investigated (e.g.,  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  for assessing freshwater resource consumption; Honch et al., 2012). Nonetheless, identifying instances of freshwater protein consumption isotopically remains challenging. In temperate environments, high freshwater protein consumption is typically inferred based on high nitrogen-isotope compositions,  $\text{C}_3$  plant-like carbon-isotope compositions, and the presence of fish bones or artefacts used to exploit aquatic resources in the archaeological record (Lillie and Richards, 2000; Minagawa and Wada, 1984; Richards et al., 2001). In the absence of corroborative archaeological evidence, however, it is very difficult to assess the significance of freshwater resource contribution to human diet. The development of an array of isotopic proxies which are sensitive to changes in dietary protein source and are highly discriminatory among different protein resources is thus essential to increasingly refined palaeodiet reconstruction.

Here, the objective is to test new and previously proposed isotopic proxies and relationships focused on distinguishing freshwater resource consumption from both plant-derived ( $\text{C}_3$  and  $\text{C}_4$ ) and marine protein consumption using compound-specific carbon-isotope compositions of collagen. We present new amino acid carbon-isotope data for archaeological humans and fauna from Zvejnieki, Latvia (c. 8th – 3rd millennia BCE). Archaeological and zooarchaeological data, as well as previously published bulk carbon- and nitrogen-isotope compositions, strongly indicate that the inhabitants consumed considerable quantities of freshwater resources. Hence, these hunter-gatherer-fisher settlements offer an ideal opportunity to evaluate and refine compound-specific isotopic proxies for disentangling palaeodiet, which can then be used in other, less well-understood archaeological contexts where multiple resources from marine, terrestrial and freshwater environments were available for consumption.

## 2. Theoretical considerations for stable carbon-isotope analysis

The reconstruction of palaeodiet using stable isotope analysis is based on the well-tested assumption that tissue isotopic

composition reflects the isotopic composition of consumed food (Ambrose, 1993). Systematic differences exist in isotopic composition between tissue and diet (Ambrose, 1993; DeNiro and Epstein, 1978), which are a result of isotopic discrimination, the differential partitioning of isotopes between phases in a reaction (e.g., ingested food  $\rightarrow$  consumer tissue) caused by the slight mass differences among isotopes of the same element (e.g.,  $^{13}\text{C}$ ,  $^{12}\text{C}$ ). When diet is protein-sufficient, the  $\delta^{13}\text{C}$  values of proteinaceous consumer tissues (e.g., collagen and dentin) largely reflect the carbon-isotope composition of dietary protein as it is derived from the base of the food web (Ambrose, 1993; Kellner and Schoeninger, 2007). Tissue carbon-isotope composition is a weighted average of the isotopic compositions of all component essential and non-essential amino acids ( $\delta^{13}\text{C}_{\text{AA}}$ ). Essential amino acids (e.g., threonine, valine, methionine, isoleucine, leucine, histidine, lysine, and phenylalanine) cannot be generated by the body and therefore must be ingested in sufficient quantities. In contrast, non-essential amino acids (e.g., asparagine/aspartic acid, hydroxyproline, glutamic acid/glutamate, serine, glycine, alanine, proline and arginine) can be assimilated with minimal modification from a dietary source, or may be synthesised *de novo* using components drawn from the body's biochemical pools; the latter will result in isotopic fractionation (Ambrose and Norr, 1993; Newsome et al., 2014). Tissue essential amino acid  $\delta^{13}\text{C}$  values are thus expected to closely approximate dietary essential amino acid  $\delta^{13}\text{C}$  values due to direct routing, i.e.,  $\Delta^{13}\text{C}_{\text{tissue AA} - \text{diet AA}} \approx 0\text{‰}$ . Non-essential amino acid  $\delta^{13}\text{C}$  values, however, may show evidence of both direct routing and biosynthesis, depending on the quality, digestibility and amino acid composition of consumed food.

Differences in the way that  $\text{C}_3$  and  $\text{C}_4$  plants incorporate  $^{13}\text{C}/^{12}\text{C}$  during photosynthesis allow assessment of relative dietary contributions of these different classes of foods, consumed as both plants and plant-consuming fauna.  $\text{C}_3$  plants (e.g., grains, rice, tubers, fruits and vegetables) have lower (i.e., more negative)  $\delta^{13}\text{C}$  values (global average  $\approx -26.5\text{‰}$ ), whereas  $\text{C}_4$  plants (e.g., tropical grasses, maize, millet and sorghum) have higher  $\delta^{13}\text{C}$  values, with an average of around  $-12.5\text{‰}$  (Ambrose, 1993). Terrestrial plants obtain carbon from atmospheric  $\text{CO}_2$  (average  $-8\text{‰}$ ; Marino and McElroy, 1991). In contrast, marine plants obtain carbon from several inorganic sources (e.g., dissolved bicarbonate, carbonate-containing minerals), which are generally  $^{13}\text{C}$ -enriched relative to the terrestrial carbon source. Dissolved inorganic carbon enters the marine food web via photosynthetic uptake by algae and phytoplankton (Corbisier et al., 2006; Mook and Tan, 1991). The carbon-isotope compositions of marine organisms are therefore dependent on several factors, including the  $\delta^{13}\text{C}$  values of the dissolved inorganic carbon, isotopic fractionation associated with photosynthetic uptake, and the rate of diffusion of  $\text{CO}_2$  across photosynthetic cells in plants, which is slow for marine plants compared to terrestrial plants (Mook and Tan, 1991; Rau et al., 1996). As a result,  $\text{C}_3$  marine plant carbon-isotope compositions are often indistinguishable from the isotopic compositions of terrestrial  $\text{C}_4$  plants. This comparatively small difference in carbon-isotope compositions makes disentangling consumption of marine protein ( $-5$ – $-17\text{‰}$ ) from  $\text{C}_4$  plant-derived protein ( $\approx -12.5\text{‰}$ ) challenging. The isotopic composition of freshwater carbon is contingent on a similarly complex array of environmental sources, including, for example,  $\text{CO}_2$  and dissolved organic carbon evolved from bacterial decay of terrestrial organic matter, dissolution of inorganic carbonates from rocks and soils in the catchment area, photosynthesis of aquatic flora, and carbonate precipitation (Mook and Tan, 1991). In the predominately  $\text{C}_3$  plant-based ecosystems of northern Europe, freshwater resource  $\delta^{13}\text{C}$  values are  $\approx -27\text{‰}$  (Dufour et al., 1999; Katzenberg and Weber, 1999).

### 3. Archaeological background and sampling

The Zvejnieki archaeological complex encompasses a large Neolithic and Mesolithic hunter-fisher-gatherer cemetery, and two adjacent settlement sites: the extensively excavated Mesolithic Zvejnieki II and the Neolithic Zvejnieki I, which has been partially excavated (Eriksson et al., 2003; Larsson and Zagorska, 2006; Zagorskis, 2004). The cemetery is located on the eastern side of Lake Burtnieks, approximately 50 km inland from the Gulf of Riga (Fig. 1). During the Mesolithic and Neolithic, water levels in Lake Burtnieks were high, and the Zvejnieki site would have been on a small island rather than part of the mainland during this time. The cemetery, consisting of in excess of 300 human burials, is one of the largest hunter-fisher-gatherer cemeteries in Europe. Radiocarbon evidence indicates that the cemetery was in use from the Middle Mesolithic (c. 8th millennium BCE) to the Late Neolithic (c. 5th–3rd millennium BCE). Most human graves are single interments, although some graves contain multiple individuals.

Zooarchaeological and stable isotope analyses suggest that freshwater fish were a vital dietary resource for the individuals buried in the Zvejnieki cemeteries. Numerous fish bones (e.g., pike, perch and various species from the carp family), as well as eel and salmon, have been recovered from the habitation layers of Zvejnieki I and II, together with a wide variety of fishing implements. A diverse range of mammalian species, the most prevalent of which are moose, wild boar and beaver, were also found at the settlements. In the burials, in contrast, tooth pendants made of teeth from hunted game, such as moose, wild boar and red deer, predominate, and badger, marten, wild horse, brown bear, fox, wolf, aurochs, seal, otter, beaver and dog also occur (Eriksson et al., 2003). Bulk stable carbon- and nitrogen-isotope analysis has determined that freshwater fish were consumed throughout the period of use of the cemetery, although dependency on this resource appears to have declined by the Late Neolithic (Eriksson, 2006; Eriksson et al., 2003). Terrestrial protein resources also contributed to diet. The  $\delta^{13}\text{C}$  values of many of the humans and fish are low ( $\sim -24$ – $-21\text{‰}$ ) relative to local terrestrial herbivores, but are similar to isotopic compositions from freshwater fish in shallow lakes elsewhere in central Europe (Dufour et al., 1999; Eriksson et al., 2003).

Here, a subset of human skeletal remains from Zvejnieki were analyzed for their compound-specific amino acid carbon-isotope compositions ( $n = 8$ ; Fig. 2 and Table 1). Individuals with low  $\delta^{13}\text{C}$  and high  $\delta^{15}\text{N}$  values, which are characteristic of high freshwater protein consumption, were selected for further compound-

specific isotopic analysis. Several archaeological faunal specimens were also selected for amino acid carbon-isotope analysis, representing a variety of dietary ecologies ( $n = 16$ ; Fig. 2 and Table 1). The archaeological material spans the Middle Mesolithic to Middle Neolithic, after which time freshwater resources were not as widely consumed.

### 4. Laboratory procedures

Collagen was extracted from bone or tooth dentin powder at the Archaeological Research Laboratory, Stockholm University, using the method described in Brown et al. (1988). Collagen samples of  $\sim 0.5$  mg were weighed into tin capsules and isotopic analysis was performed using a Carlo Erba NC2500 elemental analyzer coupled to a Finnigan MAT Delta<sup>Plus</sup> mass spectrometer via a split interface. Bulk collagen isotopic analyses were performed at the Stable Isotope Laboratory (SIL), Department of Geological Sciences, Stockholm University. The  $\delta^{13}\text{C}$  values were calibrated to VPDB using NBS-18, NBS-19, IAEA-CO-1 and IAEA-CO-8, and the  $\delta^{15}\text{N}$  values were calibrated to AIR using IAEA-N1, IAEA-N2, IAEA-NO-3 and USGS25. Precision was monitored throughout using in-house standards (acetanilide and pepton), and was  $\pm 0.1\text{‰}$  for both carbon- and nitrogen-isotope analyses (Eriksson, 2006).

For each sample, approximately 2 mg of collagen were hydrolyzed by heating at  $110^\circ\text{C}$  in 6 M HCl for 24 h. Collagen hydrolysates of free amino acids were dried under  $\text{N}_2$  at  $60^\circ\text{C}$  and re-dissolved in 1 ml HPLC-grade water for LC/IRMS analysis. Amino acid  $\delta^{13}\text{C}$  values were obtained using a Surveyor HPLC connected to a Delta V Plus mass spectrometer via an LC Isolink interface (Thermo Scientific). Amino acids were resolved on a Primesep A column ( $250\text{ mm} \times 3.2\text{ mm}$ ,  $5\ \mu\text{m}$  particle size,  $100\ \text{\AA}$  pore size, SiELC Technologies Ltd.) using a linear two-phase mobile phase gradient progressing from 100% water to 100%  $0.024\text{ M H}_2\text{SO}_4$  (Dunn et al., 2011; Honch et al., 2012; McCullagh et al., 2006, 2010). Reference  $\text{CO}_2$  gas was calibrated to VPDB against IAEA L-Glutamic acid (USGS40,  $\delta^{13}\text{C}$ :  $-26.2\text{‰}$ ; Qi et al., 2003). Accuracy and reproducibility were monitored throughout using a standard amino acid mix incorporating a wide range of  $\delta^{13}\text{C}$  values ( $\delta^{13}\text{C}_{\text{Asp}}$ :  $-22.4\text{‰}$ ;  $\delta^{13}\text{C}_{\text{Glu}}$ :  $-26.2\text{‰}$ ;  $\delta^{13}\text{C}_{\text{Gly}}$ :  $-32.4\text{‰}$ ;  $\delta^{13}\text{C}_{\text{Ala}}$ :  $-26.1\text{‰}$ ;  $\delta^{13}\text{C}_{\text{Pro}}$ :  $-10.6\text{‰}$ ; Sigma Aldrich). The standard deviations of the  $\delta^{13}\text{C}$  values for the known amino acids ranged from  $\pm 0.4$  to  $\pm 0.6\text{‰}$ , and compared well with the certified or EA-IRMS determined accepted values ( $\pm 0.02$ – $\pm 0.5\text{‰}$ ). All archaeological samples were analyzed at least twice and the average  $\delta^{13}\text{C}_{\text{AA}}$  value is reported herein. All compound-specific carbon-isotope analyses were performed in



Fig. 1. Location of the Zvejnieki archaeological complex in the interior of Latvia.

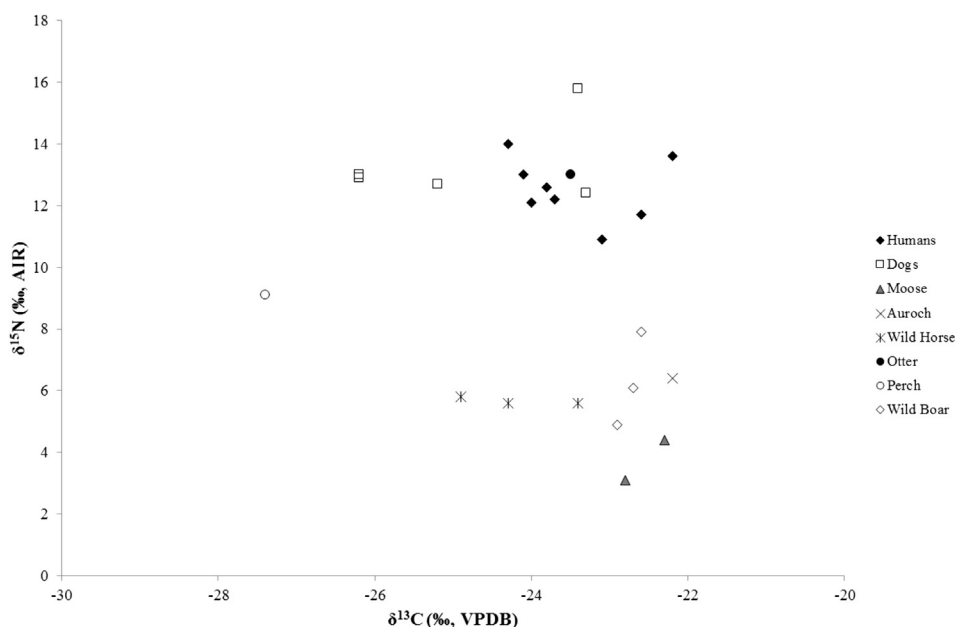


Fig. 2. Bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for humans and faunal specimens analysed in this paper from Zvejnieki.

Table 1

Descriptions of archaeological human and faunal specimens.

Sample ID	Species	Context	Element	Notes	Date <sup>a</sup>
ZVE15	Human	Burial 158	Incisor	Adult (M)	Early Neolithic
ZVE27	Human	Burial 228	Scapula	Adult (M)	Middle Neolithic; 5090 ± 55
ZVE29	Human	Burial 277	Femoral bone	Young Adult (M)	Middle Neolithic; 5545 ± 65
ZVE32	Human	Burial 62	1st incisor	Tooth Pendant	Late Mesolithic
ZVE45	Human	Burial 208	Cranial bone	Juvenile	Middle Neolithic; 5345 ± 60
ZVE56	Human	Burial 121	Cranial bone	Juvenile	Early Neolithic; 6145 ± 80
ZVE57	Human	Burial 201	1st molar	Child, 9–11 yrs	Middle Neolithic; 4865 ± 75
ZVE59	Human	Burial 197	Scapula	Adult (M)	Early Neolithic; 6410 ± 95
AZV70	Dog	Burial 114	Canine	Tooth Pendant	Early Neolithic
AZV72	Dog	Burial 132	Canine	Tooth Pendant	Early Neolithic
AZV73	Dog	Burial 146	Canine	Tooth Pendant	Early Neolithic
AZV77	Dog	Burial 153	Canine	Tooth Pendant	Early Neolithic
AZV82	Dog	Burial 167/8	Canine	Tooth Pendant	No date
AZV56	Moose	Zvejnieki II Settlement	Bone		Middle Mesolithic
AZV96	Moose	Burial 221	Antler	Modified Antler	Middle Neolithic; 5290 ± 105
AZV18	Aurochs	Burial 164	Incisor	Tooth Pendant	Middle Neolithic
AZV11	Wild Horse	Burial 42	Incisor	Tooth Pendant	Late Mesolithic
AZV12	Wild Horse	Burial 86	Incisor	Tooth Pendant	No date
AZV14	Wild Horse	Burial 122	Incisor	Tooth Pendant	Early Neolithic
AZV88	Otter	Burial 190	Canine	Tooth Pendant	Late Mesolithic
AZV64	Perch	Zvejnieki II Settlement	Bone	Tooth Pendant	Middle Mesolithic
AZV26	Wild Boar	Burial 146	Incisor	Tooth Pendant	Early Neolithic
AZV35	Wild Boar	Burial 168	Canine	Tooth Pendant	No date
AZV37	Wild Boar	Burial 206	Canine	Tooth Pendant	Middle Neolithic

<sup>a</sup> AMS  $^{14}\text{C}$  dates (uncalibrated BP) reported when available.

facilities housed in the Organic Geochemistry Unit, School of Chemistry at the University of Bristol.

## 5. Results

Bulk protein carbon- and nitrogen-isotope compositions are summarized in Fig. 2 and Table 2, and fully reported in Eriksson (2006). For humans, the average  $\delta^{15}\text{N}$  value was  $+12.5 \pm 1.0\text{‰}$  and, for  $\delta^{13}\text{C}$  values,  $-23.5 \pm 0.8\text{‰}$ . The average  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for categories of fauna are as follows:  $-24.9 \pm 1.4\text{‰}$  and  $+13.4 \pm 1.4\text{‰}$  for dogs,  $-22.6 \pm 0.4\text{‰}$  and  $+3.8 \pm 0.9\text{‰}$  for moose,  $-23.3 \pm 1.0\text{‰}$  and  $+6.0 \pm 0.9\text{‰}$  for terrestrial fauna,  $-25.5 \pm 2.8\text{‰}$  and  $+11.1 \pm 2.8\text{‰}$  for freshwater fauna. For amino

acid isotopic analyses, collagen preservation was assessed by comparing each sample amino acid distribution to the theoretical distribution for collagen, determined relative to glycine and expressed as a percentage. All amino acids were preserved within 10% of the theoretical value (Dunn, 2011). An example chromatogram of archaeological dentin collagen is presented in Fig. 3. The  $\delta^{13}\text{C}$  values are reported for the following amino acids: aspartic acid/asparagine, hydroxyproline, glutamic acid/glutamine, threonine, serine, methionine, histidine, glycine, alanine, proline, valine, isoleucine/leucine, lysine, phenylalanine and arginine. Isoleucine and leucine co-elute and are thus reported as a single value. Aspartic acid and asparagine and glutamic acid and glutamine are reported as single values, because asparagine and

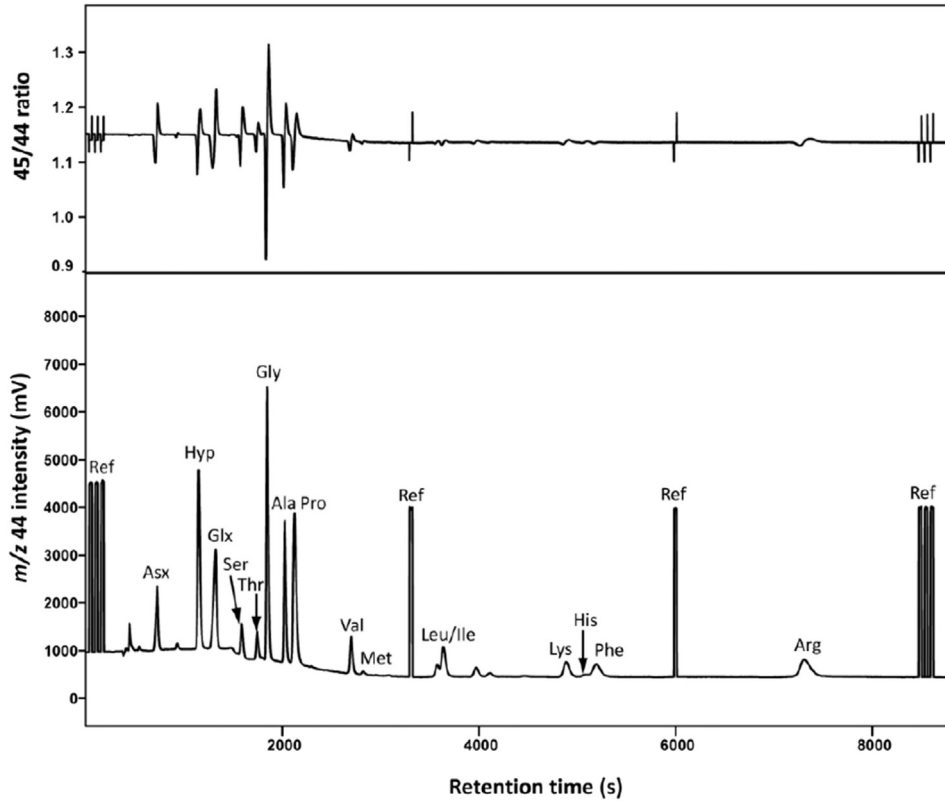
**Table 2**  
Zvejnieki human (ZVE prefix) and faunal (AZV prefix) stable isotopic compositions.

	ZVE15	ZVE27	ZVE29	ZVE32	ZVE45	ZVE56	ZVE57	ZVE59
$\delta^{13}\text{C}_{\text{bulk}}$ (‰, VPDB)	−24.3	−24.0	−22.6	−23.8	−22.2	−23.1	−23.7	−24.1
$\delta^{15}\text{N}_{\text{bulk}}$ (‰, AIR)	+14.0	+12.1	+11.7	+12.6	+13.6	+10.9	+12.2	+13.0
<i>Essential amino acids</i> (‰, VPDB)								
Threonine	−21.9	−18.2	−16.4	−20.6	−16.2	−17.1	−18.3	−18.2
Valine	−32.1	−29.0	−27.9	−31.4	−26.7	−28.5	−29.6	−31.1
Methionine	−36.3		−30.9	−32.8	−31.8	−29.4	−31.1	
Isoleucine/leucine	−32.4	−32.4	−30.1	−31.9	−29.6	−30.9	−32.0	−32.0
Histidine	−27.6	−24.8	−28.1	−26.7	−22.8	−22.2	−25.2	
Lysine	−27.6	−27.7	−26.3	−27.5	−25.5	−26.7	−27.0	−28.1
Phenylalanine	−32.2	−34.5	−30.7	−33.4	−31.3	−33.8	−31.8	−34.8
<i>Nonessential amino acids</i> (‰, VPDB)								
Aspartic acid/asparagine	−26.5	−25.6	−24.3	−25.7	−24.7	−24.2	−25.1	−25.5
Hydroxyproline	−25.6	−22.1	−22.4	−24.9	−27.7	−25.3	−25.6	−25.4
Glutamic acid/glutamate	−23.1	−25.6	−23.6	−21.7	−17.1	−19.8	−20.4	−23.4
Serine	−5.3	−20.8	−8.6		−19.9	−20.9	−22.5	
Glycine	−15.7	−17.0	−15.6	−15.6	−15.8	−15.3	−16.7	−17.1
Alanine	−27.9	−24.9	−24.9	−27.2	−24.4	−23.2	−26.3	−26.7
Proline	−26.1	−24.6	−24.0	−25.7	−23.3	−23.8	−24.6	−27.0
Arginine	−25.6	−27.0	−26.2	−28.7	−25.8	−26.4	−26.4	−26.0
$\Delta^{13}\text{C}_{\text{Gly-Phe}}$ (‰)	+16.5	+17.5	+15.1	+17.8	+15.5	+18.5	+15.1	+17.7
	AZV70	AZV72	AZV73	AZV77	AZV82	AZV56	AZV96	AZV18
$\delta^{13}\text{C}_{\text{bulk}}$ (‰, VPDB)	−23.3	−25.2	−26.2	−23.4	−26.2	−22.8	−22.3	−22.2
$\delta^{15}\text{N}_{\text{bulk}}$ (‰, AIR)	+12.4	+12.7	+13.0	+15.8	+12.9	+3.1	+4.4	+6.4
<i>Essential amino acids</i> (‰, VPDB)								
Threonine	−19.3	−22.0	−21.9	−20.8	−24.2	−13.8	−16.6	−15.5
Valine	−31.7	−33.4	−34.7	−31.4	−34.8	−30.2	−29.1	−29.6
Methionine	−44.6					−24.7	−26.3	
Isoleucine/leucine	−31.8	−33.7	−34.3	−32.1	−34.8	−31.6	−30.7	−31.6
Histidine	−26.7	−28.2	−30.6	−27.0	−30.0	−21.5	−21.3	−21.0
Lysine	−25.0	−27.0	−28.4	−27.0	−28.8	−22.5	−22.2	−22.4
Phenylalanine	−33.8	−35.7	−36.4	−34.9	−36.1	−28.6	−27	−27.1
<i>Nonessential amino acids</i> (‰, VPDB)								
Aspartic acid/asparagine	−23.9	−26.4	−26.2	−24.2	−27.4	−23.5	−22.2	−19.2
Hydroxyproline	−26.4	−27.2	−28.6	−25.1	−28.4	−24.8	−24.2	
Glutamic acid/glutamate	−19.4	−22.8	−23	−21.5	−23.5	−19.3	−17.8	
Serine	−9.9	−15.4	−13.3	−10.3	−11.6			−20.6
Glycine	−14.8	−16.3	−18.6	−14.3	−18.1	−18.1	−17.4	−17.0
Alanine	−28.2	−29.8	−29.8	−28.1	−31.4	−27.4	−26.4	−27.6
Proline	−24.0	−26.3	−27.7	−24.6	−27.8	−23.9	−23.4	−22.3
Arginine	−25.7	−28.2	−28.1	−27.8	−29.2	−23.4	−25.0	−24.1
$\Delta^{13}\text{C}_{\text{Gly-Phe}}$ (‰)	+19.0	+19.4	+17.8	+20.6	+18.0	+10.5	+9.6	+10.1
	AZV11	AZV12	AZV14	AZV88	AZV64	AZV26	AZV35	AZV37
$\delta^{13}\text{C}_{\text{bulk}}$ (‰, VPDB)	−24.9	−23.4	−24.3	−23.5	−27.4	−22.7	−22.6	−22.9
$\delta^{15}\text{N}_{\text{bulk}}$ (‰, AIR)	+5.8	+5.6	+5.6	+13.0	+9.1	+6.1	+7.9	+4.9
<i>Essential amino acids</i> (‰, VPDB)								
Threonine	−20.1	−16.0	−17.1	−17.4	−22	−16.6	−15.3	−12.2
Valine	−31.7	−30.1	−32.2	−28.3	−33.4	−28.0	−27.3	−27.1
Methionine		−28.5	−28.4				−25.1	−28.9
Isoleucine/leucine	−32.7	−30.8	−32.3	−30.9	−32.7	−29.9	−29.0	−30.5
Histidine	−29.5	−23.3	−25.2	−24.9		−22.6	−21.9	−30.6
Lysine	−26.2	−25.7	−25.0	−27.4	−28.3	−24.6	−24.5	−23.1
Phenylalanine	−29.2	−29.1	−27.1	−32.2	−35.8	−28.0	−28.5	−26.9
<i>Nonessential amino acids</i> (‰, VPDB)								
Aspartic acid/asparagine	−23.6	−22.1	−23.6	−23.8	−29.5	−23.4	−20.4	−22.1
Hydroxyproline	−26.3	−26.4	−27.4	−23.3	−26.1	−26.4	−25.7	−24.0
Glutamic acid/glutamate	−21.2	−18.7	−18.4	−23.5	−22.5	−18.5	−15.8	−18.6
Serine	−23.5			−19.1		−18.9	−20.6	
Glycine	−20.5	−18.0	−18.4	−16.1	−20.0	−19.2	−18.2	−18.6
Alanine	−28.1	−27.3	−27.5	−27.0	−30.1	−24.7	−23.6	−25.6
Proline	−24.9	−24.4	−25.1	−24.2	−30.9	−22.9	−22.3	−23.36
Arginine	−23.6	−25.2	−26.3	−25.2	−30.0	−25.7	−25.6	−24.7
$\Delta^{13}\text{C}_{\text{Gly-Phe}}$ (‰)	+8.7	+11.1	+8.7	+16.1	+15.8	+8.8	+10.3	+8.3

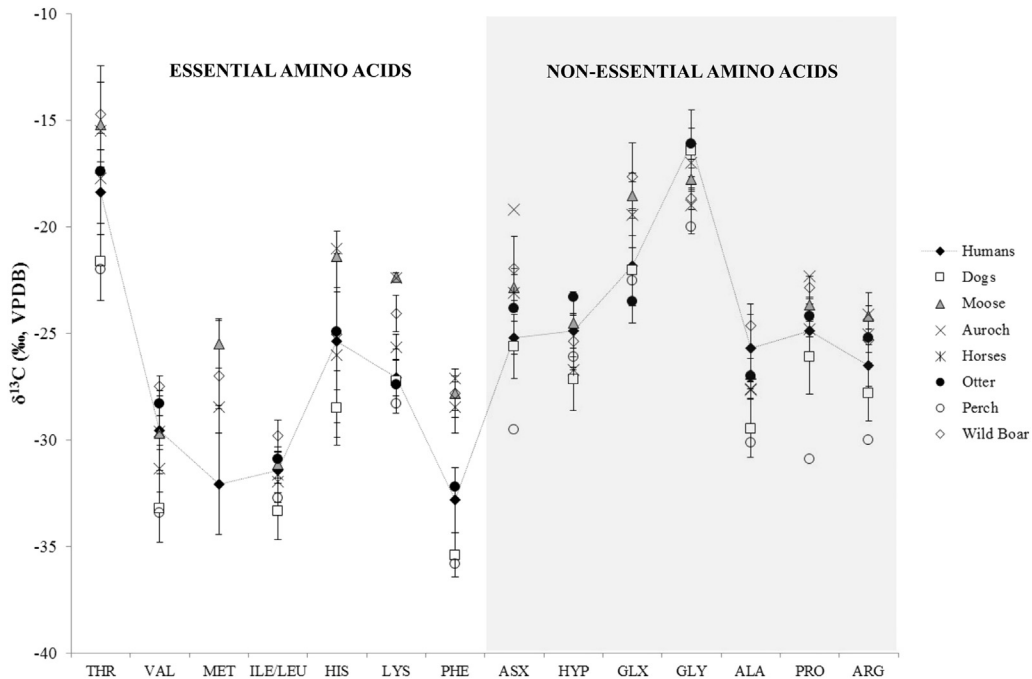
glutamine are deaminated during hydrolysis and are thus indistinguishable from aspartic acid and glutamic acid, respectively. Serine typically elutes in a region where the baseline drops with the introduction of the sulphuric acid component of the mobile phase, and the  $\delta^{13}\text{C}_{\text{Ser}}$  values are less accurate because of the difficulty of integrating peaks in such a dynamic region. Low abundance amino

acids (i.e., methionine and histidine) are likewise not considered interpretively useful because of poor reproducibility compared to the more abundant amino acids. All  $\delta^{13}\text{C}_{\text{AA}}$  values are reported in Table 2 and shown in Fig. 4.

For archaeological humans, the overall range of the  $\delta^{13}\text{C}$  values for non-essential amino acids is 13‰ (−29–−15‰), and the overall



**Fig. 3.** An example of an LC/IRMS chromatogram (human dentin collagen, ZVE15). Note that serine (Ser) elutes where the baseline drops due to the introduction of the mobile phase acid (see text). Ref: reference CO<sub>2</sub> gas pulses; Asx: asparatic acid/asparagine; Hyp: hydroxyproline; Glx: glutamic acid/glutamate; Ser: serine; Thr: threonine; Gly: glycine; Ala: alanine; Pro: proline; Val: valine; Met: methionine; Leu/Ile: leucine and isoleucine; Lys: lysine; His: histidine; Phe: phenylalanine; Arg: arginine.



**Fig. 4.** Average essential and non-essential amino acid carbon-isotope compositions for Zvejnieki humans and fauna. Error bars represent ±1σ where n > 1. The line connecting the human δ<sup>13</sup>C<sub>AA</sub> values is to serve as a visual aid only, and has no interpretive meaning. Asx: asparatic acid/asparagine; Hyp: hydroxyproline; Glx: glutamic acid/glutamate; Thr: threonine; Gly: glycine; Ala: alanine; Pro: proline; Val: valine; Met: methionine; Leu/Ile: leucine and isoleucine; Lys: lysine; His: histidine; Phe: phenylalanine; Arg: arginine.

range of  $\delta^{13}\text{C}$  values for non-essential amino acids for archaeological fauna is slightly larger 17‰ (–31––14‰). No meaningful trends exist in non-essential amino acid carbon-isotope compositions among the humans and various fauna, although perch have moderately lower  $\delta^{13}\text{C}_{\text{AA}}$  values for some non-essential amino acids. The narrow range in bulk collagen and dentin  $\delta^{13}\text{C}$  values thus appears to be reflected in the low variability of the  $\delta^{13}\text{C}$  values for non-essential amino acids relative to the essential amino acids (see below). The narrow range in non-essential amino acid  $\delta^{13}\text{C}$  values is likely the underlying cause of the similarly low variation determined for the bulk  $\delta^{13}\text{C}$  values of collagen and dentin, because nonessential amino acids constitute a large proportion of the carbon (~80%; Ambrose, 1993) present in type I collagen protein.

The overall range of the  $\delta^{13}\text{C}$  values for essential amino acids for humans is 20‰ (–36––16‰) and 32‰ (–45––12‰) for fauna. These ranges are, as expected, larger than those observed for non-essential amino acids, and the faunal range is larger than that determined for humans, which is likely a reflection of diverse dietary strategies. Species that consume terrestrial (i.e., aurochs, wild horse and wild boar) or mixed aquatic-terrestrial (i.e., moose) plant resources tend to have higher essential  $\delta^{13}\text{C}_{\text{AA}}$  values than species which consume freshwater resources (i.e., otter and perch), and this is most clearly discernable in the  $\delta^{13}\text{C}_{\text{Phe}}$  values (Fig. 4). The similarity of the essential  $\delta^{13}\text{C}_{\text{AA}}$  values between humans and freshwater fauna (e.g., otter and perch) indicates significant consumption of freshwater fish by the humans included in this study. Further, it is likely that dogs were consuming freshwater fish as well, either by scavenging or by direct feeding.

Kruskal–Wallis (KW) non-parametric one-way analysis of variance tests were used to compare the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values, bulk  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, and individual essential and non-essential amino acid isotopic compositions of humans, dogs, terrestrial fauna, moose and freshwater fauna. Both proxies, the  $\delta^{15}\text{N}$  values, and the  $\delta^{13}\text{C}$  values of lysine, phenylalanine, glycine, alanine, glutamate/glutamic acid, aspartate/aspartic acid, and arginine were statistically significantly different (KW,  $p < 0.005$ ), whereas the bulk  $\delta^{13}\text{C}$  values and  $\delta^{13}\text{C}_{\text{Pro}}$  and  $\delta^{13}\text{C}_{\text{Val}}$  values were not statistically distinguishable. Note, however, that the sample sizes for each group of specimens was small ( $n = 2$ – $n = 8$ ), adding uncertainty to these statistical determinations. Further, the non-normal distribution of the data and small sample sizes preclude additional post-hoc testing (i.e., to assess the extent to which pairs of groups are different from each other). For the two isotopic proxies ( $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$ ) a visual assessment of Fig. 5a and b indicates that humans, dogs and freshwater fauna have similar  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  values, and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values, although there is some overlap between the lowest human proxy values and the highest terrestrial fauna proxy values for the latter.

## 6. Discussion

Comparative  $\delta^{13}\text{C}_{\text{AA}}$  values from a variety of ecological and archaeological settings are used to contextualize the Zvejnieki human isotopic results. All data were generated using a similar laboratory methodology and are fully reported in Honch et al. (2012). Briefly, archaeological human isotopic compositions

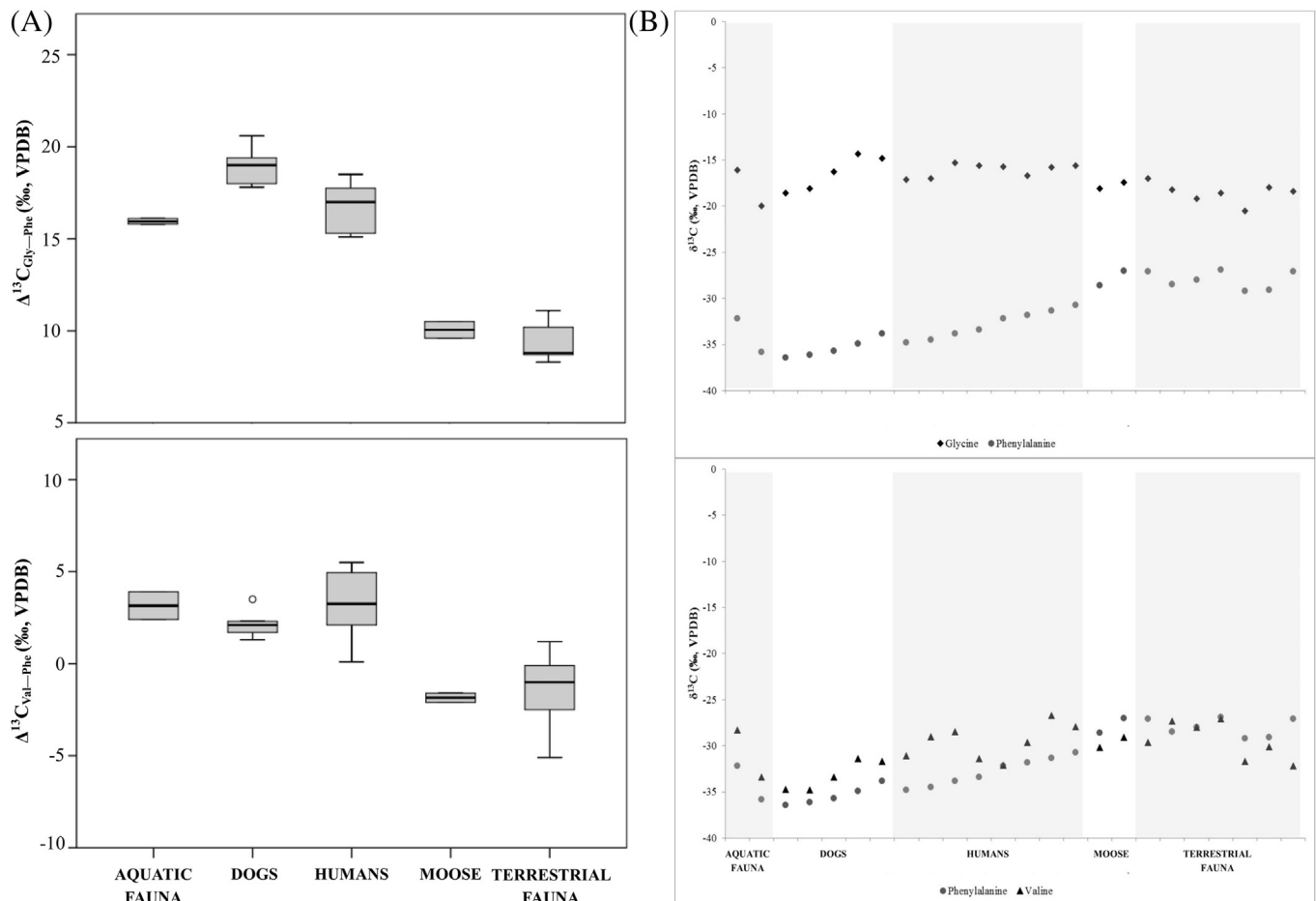


Fig. 5. The (A)  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  proxy and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxy and (B) the underlying  $\delta^{13}\text{C}_{\text{Gly}}$ ,  $\delta^{13}\text{C}_{\text{Val}}$  and  $\delta^{13}\text{C}_{\text{Phe}}$  values for Zvejnieki humans and fauna.

representing high marine protein consumption (Japan and Greenland), terrestrial C<sub>4</sub>-derived protein consumption (Mesoamerica), high freshwater protein consumption (Iron Gates Region, Europe) and high C<sub>3</sub>-derived protein consumption (Eastern Europe) are used to assess the efficacy of the proxies tested here in distinguishing among different dietary regimes. Although there is necessarily some degree of uncertainty in all palaeodietary reconstructions, the interpretation of the dietary isotopic compositions for these four groups is well-supported by archaeological and zooarchaeological evidence (Honch et al., 2012). Statistical analysis has determined that there are significant differences among C<sub>3</sub> terrestrial protein consumers, C<sub>4</sub> terrestrial protein consumers, freshwater protein consumers (including Zvejnieki human samples) and marine protein consumers for both  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  (Corr et al., 2005) and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  (Honch et al., 2012) proxies (KW,  $p < 0.005$ ; Fig. 6). Further, for those individual amino acids for which sufficient data were obtained (see Honch et al., 2012), there are significant differences among dietary protein groups (valine, lysine, phenylalanine, glycine, alanine, proline, glutamate/glutamic acid, aspartate/aspartic acid, and arginine; KW,  $p < 0.005$ ).

The application of the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  proxy to detect high marine protein consumption is well-established, and has been tested on archaeological material (Choy et al., 2010; Corr et al., 2005; Honch et al., 2012). It is expected that relatively high  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  values will be observed at high levels of marine protein consumption, and lower  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  values (i.e., less than +10‰) will be observed when the dietary protein source is predominately terrestrial. The precise relationship between % contribution of marine protein to

diet and the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  value for humans is currently unknown, although we hypothesize that more marine protein will lead to increasingly large  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  values. For the Zvejnieki human and faunal data, the human, dog, otter and perch  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  values range from +15 to +21‰, whereas the moose, horse, wild boar and aurochs values are generally  $\leq +10\text{‰}$  (Table 2). On the basis of ecological, archaeological and zooarchaeological evidence, this division is consistent with the presence and absence of substantial freshwater resources in the diet. Corr et al. (2005) determined that, for high marine protein consumers, the high  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  values relative to terrestrial protein consumers were primarily caused by high  $\delta^{13}\text{C}_{\text{Gly}}$  values, as is also the case for the high marine protein consumer values included here. The high freshwater protein consumers from the Iron Gates Region have low  $\delta^{13}\text{C}_{\text{Phe}}$  values ( $-30.4 \pm 1.1\text{‰}$ ) and, although the absolute  $\delta^{13}\text{C}_{\text{Phe}}$  and  $\delta^{13}\text{C}_{\text{Gly}}$  values differ from those determined for the Zvejnieki humans, the spacing is similar compared to other dietary groups ( $+16.7 \pm 1.3\text{‰}$  for Zvejnieki and  $+19.7 \pm 1.5\text{‰}$  for Iron Gates; Fig. 6). The high  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  values for freshwater protein consumers are driven primarily by the very low  $\delta^{13}\text{C}_{\text{Phe}}$  values. We hypothesize that the low  $\delta^{13}\text{C}_{\text{Phe}}$  values observed here and in other studies for freshwater resources may be influenced by contributions of terrestrial carbon to total carbon present in freshwater lakes and rivers, which is likely to be <sup>13</sup>C-depleted relative to marine carbon (see Section 2).

Honch et al. (2012) proposed instead that the relationship between  $\delta^{13}\text{C}_{\text{Val}}$  and  $\delta^{13}\text{C}_{\text{Phe}}$  values may be useful in distinguishing among marine, freshwater, C<sub>4</sub> plant-derived, and C<sub>3</sub> plant-derived protein consumers. Controlled feeding studies have confirmed

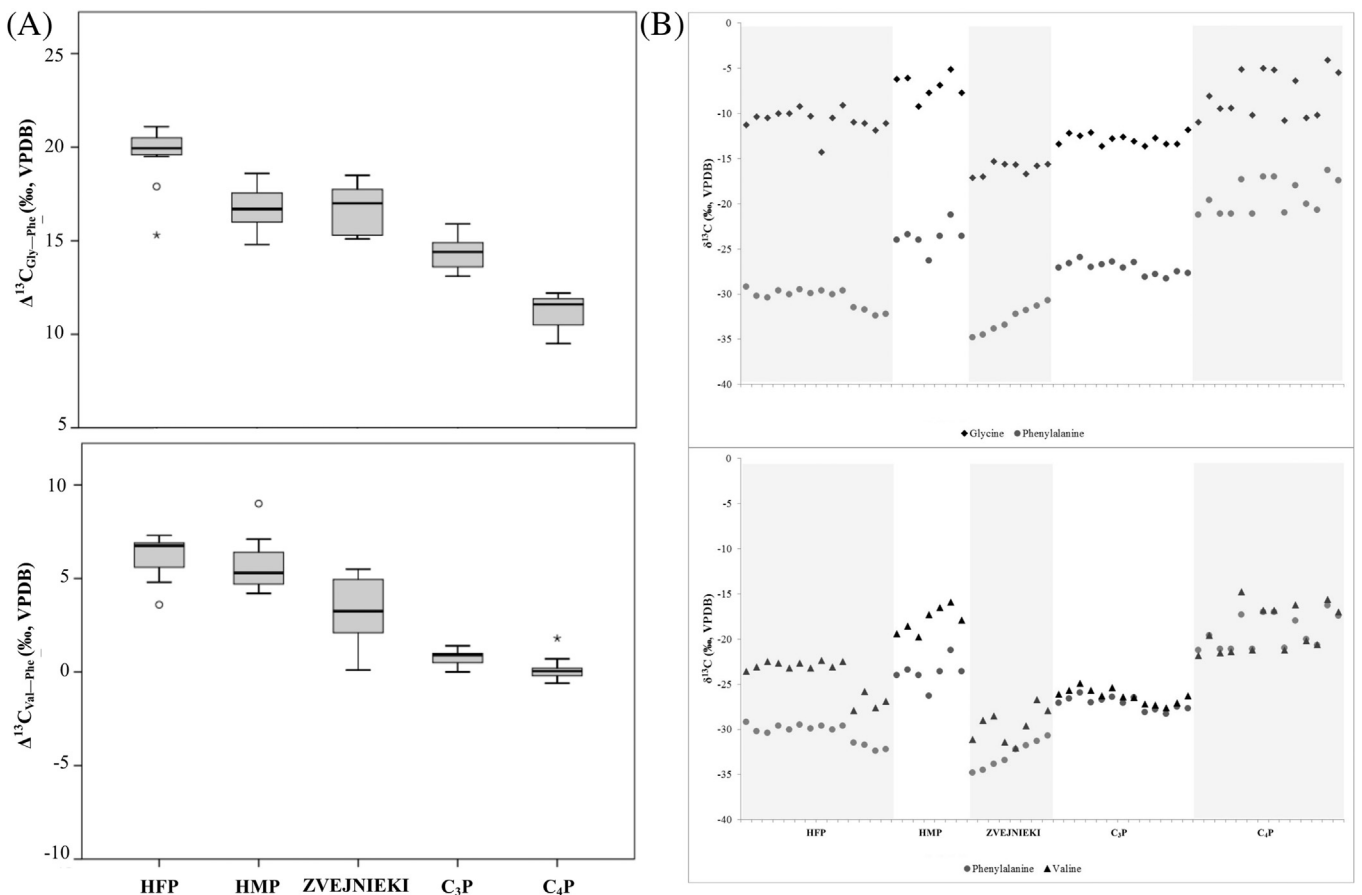


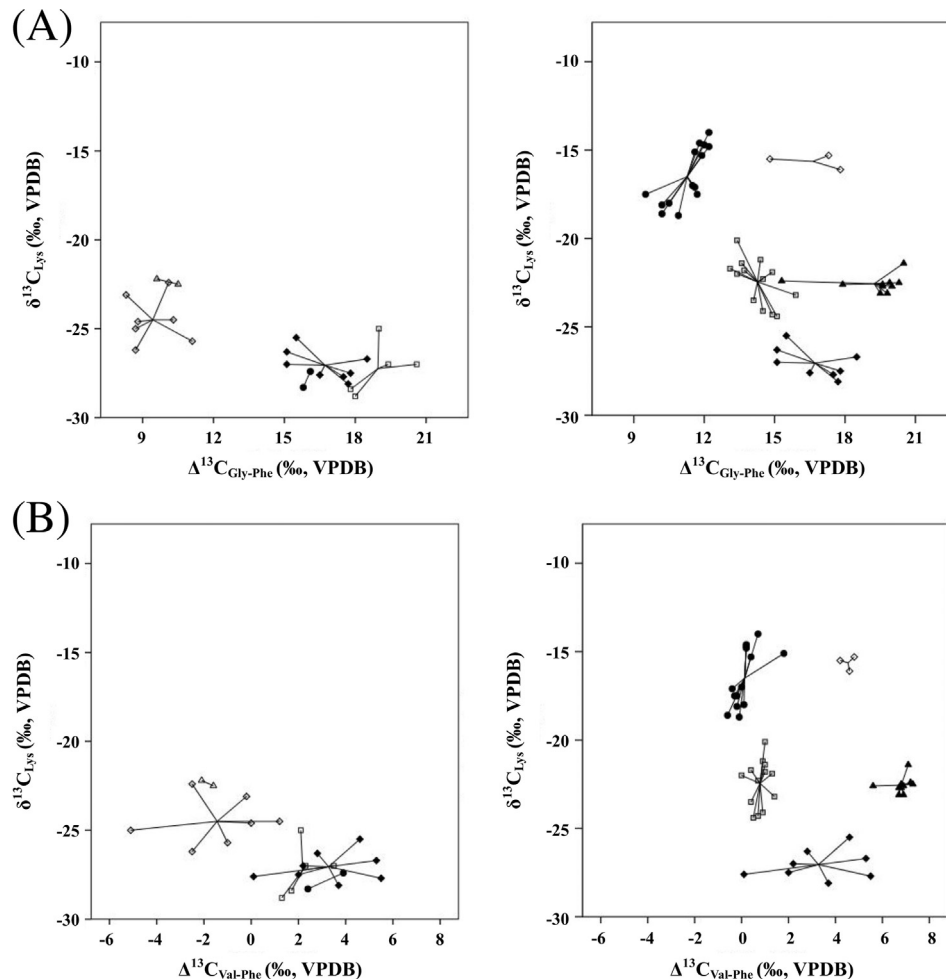
Fig. 6. The (A)  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  proxy and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxy and (B) the underlying  $\delta^{13}\text{C}_{\text{Gly}}$ ,  $\delta^{13}\text{C}_{\text{Val}}$  and  $\delta^{13}\text{C}_{\text{Phe}}$  values for Zvejnieki humans and additional human specimens with well-understood diets from Honch et al. (2012).



that the  $\delta^{13}\text{C}$  values of essential amino acids do not change substantially from diet to primary and secondary consumer tissues (Hare et al., 1991; Howland et al., 2003; Jim et al., 2006). Thus, the  $\delta^{13}\text{C}$  values of phenylalanine and valine are expected to primarily reflect isotopic variability at the base of the food web with minimal isotopic modification. Although there are differences in the absolute  $\delta^{13}\text{C}_{\text{Val}}$  and  $\delta^{13}\text{C}_{\text{Phe}}$  values of different freshwater ecosystems, the  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values of the Zvejnieki humans compared to local terrestrial fauna and the comparative human samples are distinct from terrestrial protein consumers. Terrestrial fauna had  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values of less than +1.5‰, whereas freshwater fauna had values greater than +2.0‰. Humans and dogs had a range of  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values, from as low as +0.1‰ to +5.5‰. Interestingly, some of the Zvejnieki individuals appear to have consumed a mixed C<sub>3</sub> terrestrial and freshwater diet, which was not apparent at the bulk isotopic level and not clearly resolved by the sole use of the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  proxy. The high marine protein consumers and high freshwater protein consumers had average  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values of  $+5.8 \pm 1.7\text{‰}$  and  $+6.3 \pm 1.1\text{‰}$ , respectively, whereas C<sub>3</sub> and C<sub>4</sub> terrestrial protein consumers had considerably lower values ( $+0.8 \pm 0.4\text{‰}$  and  $+0.1 \pm 0.6\text{‰}$ , respectively; Figs. 5 and 6).

Although the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxies clearly distinguish between aquatic protein consumers and terrestrial C<sub>3</sub> and C<sub>4</sub> plant-derived protein consumption, they cannot as yet be reliably used to differentiate between marine and freshwater resource

consumption. Thus, although the proxies are valuable in regions where only one kind of aquatic resource is likely to have been available (marine or freshwater), they will be of limited use in more complex socioeconomic or ecological contexts. Instead, bivariate plots of these indices vs. other essential  $\delta^{13}\text{C}_{\text{AA}}$  values can be used to distinguish among all four dietary protein groups, and may potentially be able to characterise the protein sources consumed in mixed diets (Fig. 7). For example, the relationship between  $\delta^{13}\text{C}_{\text{Lys}}$  and the  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxy visually discriminates among all four diet groups, clearly separating terrestrial from aquatic, C<sub>3</sub> from C<sub>4</sub> and freshwater from marine protein consumers (Fig. 7b). Lysine, an essential amino acid, is a limiting amino acid in cereals and most plants, and is found in somewhat larger quantities in legumes. Meat and other animal products, however, are the most abundant dietary source of lysine, especially aquatic fauna (Young and Pellett, 1994). Here, freshwater consumers tended to have lower  $\delta^{13}\text{C}_{\text{Lys}}$  values and higher  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values, whereas marine protein consumers had higher  $\delta^{13}\text{C}_{\text{Lys}}$  and higher  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values. Because there is some variation globally in  $\delta^{13}\text{C}$  values of terrestrial and freshwater resources, the measured  $\delta^{13}\text{C}_{\text{Lys}}$  values are not expected to be the same across regions; however, the Zvejnieki human  $\delta^{13}\text{C}_{\text{Lys}}$  values agree well with those for freshwater fauna from the same archaeological site, and the  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxy values are, as expected, higher than those of C<sub>3</sub>-plant consuming terrestrial fauna. Further analyses from well-constrained archaeological human and faunal



**Fig. 7.** Bivariate comparisons of (A)  $\delta^{13}\text{C}_{\text{Lys}}$  vs.  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and (B)  $\delta^{13}\text{C}_{\text{Lys}}$  vs.  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  values for Zvejnieki humans and fauna (left) and all archaeological human data (right). For the Zvejnieki plots:  $\blacklozenge$  Humans;  $\square$  Dogs;  $\bullet$  Freshwater Fauna;  $\blacktriangle$  Moose;  $\diamond$  Terrestrial Fauna. For archaeological human comparative data plots:  $\blacksquare$  C<sub>3</sub> Terrestrial Protein Consumers;  $\bullet$  C<sub>4</sub> Terrestrial Protein Consumers;  $\blacktriangle$  Freshwater Protein Consumers;  $\blacklozenge$  Zvejnieki Humans;  $\diamond$  Marine Protein Consumers.

contexts, as well as modern controlled feeding studies, are needed to improve the certainty with which such relationships can be applied, but this outcome shows considerable promise for ultimately refining palaeodietary reconstruction in complex ecosystems around the world.

## 7. Conclusion

Compound-specific carbon-isotope analysis of amino acids shows considerable potential as a method for resolving complex palaeodietary reconstructions. As the body of published  $\delta^{13}\text{C}_{\text{AA}}$  values grows, the isotopic proxies discussed herein and elsewhere (Corr et al., 2005; Honch et al., 2012) will become increasingly valuable to archaeologists in distinguishing among terrestrial ( $\text{C}_3$  vs.  $\text{C}_4$ ), freshwater and marine resource consumption. Here, our goal was to explore how freshwater resource consumption by humans is expressed at the amino acid level compared to other dietary protein sources ( $\text{C}_3$  terrestrial,  $\text{C}_4$  terrestrial, and marine protein), in order to develop broadly applicable isotopic proxies and relationships for use in less well-understood archaeological contexts. We determined that (i) the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxies can effectively discriminate between terrestrial and aquatic resource consumption, and (ii) the relationship between essential  $\delta^{13}\text{C}_{\text{AA}}$  values and the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  and  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxies can differentiate among the four protein consumption groups tested here. Further, individuals with 'hidden' mixed diets, such as the few Zvejnieki individuals for whom a mixed  $\text{C}_3$  and freshwater protein-based diet may be hypothesised, can be distinguished from those with a diet dominated by one type of dietary resources. Crucially, however, the compound-specific amino acid isotopic proxies discussed here must be applied in a context-sensitive manner. For example, the  $\Delta^{13}\text{C}_{\text{Gly-Phe}}$  or  $\Delta^{13}\text{C}_{\text{Val-Phe}}$  proxies could be used to identify freshwater protein consumption for inland sites, but not in an estuarine environment, where both freshwater and marine aquatic resources would be readily available for human consumption. Instead, either proxy coupled with an essential amino acid (e.g., lysine) would be more useful. This investigation has demonstrated that amino acid stable isotopic compositions and compound-specific isotopic proxies enhance our understanding of diet and resource exploitation in the past, and can elucidate complex dietary behaviours.

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