Ecological niche of Neanderthals from Spy Cave revealed by nitrogen isotopes of individual amino acids in collagen

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Article info
Article history:
Received 6 November 2014
Accepted 31 January 2016
Available online xxx

Keywords:
Neanderthal
Nitrogen isotope
Amino acid
Ecological niche
Subsistence strategy
Plant diet

Abstract
This study provides a refined view on the diet and ecological niche of Neanderthals. The traditional view is that Neanderthals obtained most of their dietary protein from terrestrial animals, especially from large herbivores that roamed the open landscapes. Evidence based on the conventional carbon and nitrogen isotopic composition of bulk collagen has supported this view, although recent findings based on plant remains in the tooth calculus, microwear analyses, and small game and marine animal remains from archaeological sites have raised some questions regarding this assumption. However, the lack of a protein source other than meat in the Neanderthal diet may be due to methodological difficulties in defining the isotopic composition of plants. Based on the nitrogen isotopic composition of glutamic acid and phenylalanine in collagen for Neanderthals from Spy Cave (Belgium), we show that i) there was an inter-individual dietary heterogeneity even within one archaeological site that has not been evident in bulk collagen isotopic compositions, ii) they occupied an ecological niche different from those of hyenas, and iii) they could rely on plants for up to ~20% of their protein source. These results are consistent with the evidence found of plant consumption by the Spy Neanderthals, suggesting a broader subsistence strategy than previously considered.

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1. Introduction
Dietary subsistence is a major component of the biology of a human population. Neanderthals became extinct around 40,000 years ago (Higham et al., 2014), and their subsistence strategies have attracted a lot of scientific attention since this information is crucial to evaluating their cognitive abilities and the possible reasons for their extinction. Several hypotheses regarding the demise of the Neanderthals at the time when early anatomically modern humans entered Europe involve dietary differences between the two types of hominins (Bocherens and Drucker, 2006; Froehle and Churchill, 2009; Hoffecker, 2009). Numerous studies, based on different approaches such as zooarchaeology and tooth wear patterns, have provided convincing evidence for a Neanderthal diet comprised largely of meat from large terrestrial herbivores (Lalueza Fox and Pérez-Pérez, 1993; Gardeisen, 1999; Boyle, 2000; Ready, 2010; Gaudzinski, 2014; Fiorenza et al., 2015). In this context, the first isotopic study of Neanderthal bone collagen published over two decades ago showing an isotopic composition close to those of animal predators such as wolves and hyenas was not surprising (Bocherens et al., 1991). Since then, many Neanderthal specimens have been analysed, and in all cases where collagen was well-preserved, a similar pattern was found (Fizet et al., 1995; Bocherens et al., 1999, 2005, 2013; Richards et al., 2008; Richards and Trinkaus, 2009). On the other hand, recent findings of plant remains trapped in a tooth calculus testify to the consumption of plants by Neanderthals (Henry et al., 2011, 2014; Hardy et al., 2012). Studies of dental microwear support a more diverse diet in some Neanderthal groups, which has been linked with increasing tree-cover (El Zaatari et al., 2011). However, as none of the palaeodietary approaches is able to accurately quantify the proportions of meat or...
plants eaten, the question remains as to how much plant material the Neanderthals consumed.

Recently an isotopic approach that provides accurate determination of trophic positions was developed for use in ecological and palaeoecological studies: nitrogen isotope analysis of individual amino acids. This approach represents a powerful tool for estimating animal trophic positions (TP: 1 = primary producer, 2 = primary consumer, 3 = secondary consumer, and so on) in aquatic (McClelland and Montoya, 2002; Chikaraishi et al., 2007, 2009; Popp et al., 2007), terrestrial (Chikaraishi et al., 2010, 2011), and complex ecosystems (Ishikawa et al., 2014). This method is based on the fact that the $\delta^{15}N$ of phenylalanine ($\delta^{15}N_{\text{Phe}}$) in organisms shows a small shift of 0.4 ± 0.4‰ from prey to consumer, and the $\delta^{15}N$ of glutamic acid ($\delta^{15}N_{\text{Glu}}$) shows a large prey–predator shift of 8.0 ± 1.1‰, reflecting the trophic position of the consumers in their respective ecosystems (McClelland and Montoya, 2002; Chikaraishi et al., 2009, 2014). An important point here is that $\delta^{15}N$ of phenylalanine reflects that of the nitrogen source of the ecosystems, therefore the primary producers (i.e., plants in most cases for terrestrial ecosystems; McClelland et al., 2003; Chikaraishi et al., 2011). Using the equation below based on $\delta^{15}N$ of glutamic acid and phenylalanine, it is possible to estimate the TP of animals in terrestrial ecosystems (Chikaraishi et al., 2011):

$$TP = (\delta^{15}N_{\text{Glu}} - \delta^{15}N_{\text{Phe}}) / \beta + 1$$

where TP, $\delta^{15}N_{\text{Glu}}$, $\delta^{15}N_{\text{Phe}}$, and $\beta$ indicate trophic position in an ecosystem, $\delta^{15}N$ of glutamic acid, $\delta^{15}N$ of phenylalanine, and isotopic difference between $\delta^{15}N$ of glutamic acid and $\delta^{15}N$ of phenylalanine, respectively. The $\beta$ value for terrestrial plants was estimated to be -8.4‰ (Chikaraishi et al., 2010, 2011). However, to date, only ancient humans from the Holocene have been studied using this approach, and most studies took place in temperate humid ecosystems. In these studies, marine protein consumptions (Styring et al., 2010; Naito et al., 2010a, b), the relative proportions of plant protein vs meat protein in diets (Naito et al., 2013a), and freshwater resource consumption (Naito et al., 2013b) were evaluated in South Africa, Japan, and France.

We applied this novel isotopic approach to Neanderthal specimens from the Spy Cave, Belgium and quantitatively evaluated this group’s consumption of plant and animal foods. Three Neanderthal specimens, attributed to two individuals, were analysed: Spy-94a from Spy I and Spy-92b and Spy-430a from Spy II (Supplementary Online Material (SOM) Table S1). These specimens were selected because consumption of plants was suggested by both microwear and tooth calculus micro-fossils found on Neanderthal remains at this site (El Zaatari et al., 2011; Henry et al., 2011).

2. A brief introduction to trophic position (TP) estimation

Trophic position estimates for the Neanderthals and animals in this study are based on well-founded empirical data: (i) the large $\delta^{15}N$-enrichment of glutamic acid from prey to consumer (+8.0 ± 1.1‰) and the limited $\delta^{15}N$-enrichment of phenylalanine (+0.4 ± 0.4‰) as mentioned above (McClelland and Montoya, 2002; Chikaraishi et al., 2009, 2011); and (ii) the characteristic $\beta$ value ($\delta^{15}N_{\text{Glu}} - \delta^{15}N_{\text{Phe}}$) in the primary producer among terrestrial plants are -8.4‰, while aquatic ones such as algae and cyanobacteria present a $\beta$ value of +3.4‰ (Chikaraishi et al., 2007, 2010; Fig. 1).

Chikaraishi et al. (2007) hypothesised that, during metabolism, glutamic acid rapidly undergoes transamination and the C–N bond is cleaved, leading to the large enrichment in $\delta^{15}N$, while, in contrast, the dominant metabolic step of phenylalanine adds a hydroxyl group to form tyrosine and does not cleave the C–N bond, leading to the small enrichment in $\delta^{15}N$ (Chikaraishi et al., 2007; SOM Fig. S1). Thus, the $\delta^{15}N$ of phenylalanine reflects that of the primary producers (e.g., plants and algae) at the base of food webs (McClelland et al., 2003; Chikaraishi et al., 2011). Though factors controlling $\beta$ values in plants are not fully understood, one promising hypothesis has been proposed: an isotope fractionation during the synthesis of lignin from phenylalanine through the phenylpropanoid pathway, used by terrestrial plants for structural reinforcement, may cause elevated $\delta^{15}N_{\text{Phe}}$ values relative to those of other amino acids (Ohkouchi and Takano, 2014; Ohkouchi et al., 2015; SOM Fig. S2). Because aquatic primary producers do not utilize this pathway, they may have less elevated $\delta^{15}N_{\text{Phe}}$ values than that of terrestrial plants. Based on these observations, the following equations that estimate the trophic position of animals were proposed (Chikaraishi et al., 2009, 2011; see Fig. 1):

$$TP (\text{Terrestrial}) = (\delta^{15}N_{\text{Glu}} - \delta^{15}N_{\text{Phe}} + 8.4) / [8.0–0.4] + 1$$

for terrestrial ecosystems, and

$$TP (\text{Aquatic}) = (\delta^{15}N_{\text{Glu}} - \delta^{15}N_{\text{Phe}} - 3.4) / [8.0–0.4] + 1$$

for aquatic ecosystems.

It is supposed that these equations can offset the background fluctuation in $\delta^{15}N$ of ecosystems because they utilize the isotopic spacing between two amino acids from the same individual organism, providing a means that the traditional isotopic method based on bulk protein lacks to reconstruct trophic positions (Fig. 2; Naito et al., 2013b).

3. Materials and methods

3.1. Materials

The Spy Cave is located in the village of Spy in the municipality of Jemeppe-sur-Sambre (province of Namur, Belgium), about 18 m above the present level of the Orneau River, a tributary of the Sambre. It opens to the southwest in a carboniferous limestone massif below a vast plateau. In 1886, the discovery of two adult Neanderthal skeletons in the terrace sediments along with Ice Age fauna and Middle Palaeolithic artefacts was a major milestone in the history of palaeoanthropology. The important skeletal assemblage was presented in the first monograph dedicated to Neanderthal fossils (Fraipont and Lohest, 1887). A multidisciplinary research project (2004–2007, BELPO [J36/0112]) enabled the recent discovery of new (unvarnished) Neanderthal remains amongst the faunal collections. These specimens have recently been re-evaluated, and have undergone dating (Semal et al., 2009, 2013) and new isotopic analysis (Bocherens et al., 2013). This new anthropological study produced a new inventory of the human remains and re-attributed the fragments to the Spy I and Spy II individuals (Rougier et al., in press). Based on this work, the three specimens analysed in the present study could be attributed to either Spy I or Spy II (SOM Table S1).

In addition to human and animal bone material from Spy Cave, we also analysed faunal remains from the nearby cave of Schladina (Sclayn, Belgium), where a contemporary rich and diverse mammal fauna has been found and already analysed for bulk collagen isotopic values (Bocherens et al., 1997, 2011, 2013; SOM Tables S2 and S3). We analysed bone and tooth collagen samples that were already extracted from those skeletal remains in previous studies (Bocherens et al., 1997, 1999, 2011, 2013). All samples have atomic C/N ratios within established criteria (2.9–3.6; DeNiro, 1985).
3.2. Sample preparation and instrumental analyses

All amino acid samples were prepared following established protocols (Chikaraishi et al., 2007). The bone and tooth collagen samples were subjected to hydrolysis by 12 N HCl at 100 °C for 12 h, followed by derivatisation with thionyl chloride/2-propanol (1:4, v/v) at 110 °C for 2 h and pivaloyl chloride/dichloromethane (1:4, v/v) at 110 °C for 2 h. The nitrogen isotopic compositions of the individual amino acid derivatives were measured by gas chromatography/combustion/IRMS (GC/C/IRMS) using an Agilent Technology instrument.

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**Figure 1.** Schematic illustration of the relationship between animal trophic position and nitrogen isotopic composition of glutamic acid and phenylalanine in: a) a terrestrial ecosystem and b) an aquatic ecosystem (modified after Fig. 1 in Chikaraishi et al., 2011). There are characteristic β (δ15N_Chu – δ15N_Thm) values in primary producers among terrestrial plants (−8.4‰) and aquatic plants such as algae and cyanobacteria (+3.4‰; Chikaraishi et al., 2007, 2010).

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**Figure 2.** A comparison of the TP (trophic position) estimations based on: a) carbon and nitrogen isotopic composition of bulk collagen (TP_Bulk), or b) nitrogen isotopic composition of glutamic acid and phenylalanine (TP_AA). In the TP_Bulk approach, δ15N of consumers with the same trophic position can vary depending on the δ15N value of a baseline (e.g., plants) of a food chain and it is often difficult to estimate the baseline δ15N. On the other hand, in the TP_AA approach, estimations of a baseline δ15N are not required because the approach is based on the two amino acids in the same individual consumer. In addition, organisms with same TP should show values on the same trophic line (dashed lines in Fig. 2b) in δ15N_Chu – δ15N_Phe plots, because characteristic β (δ15N_Chu – δ15N_Phe) values in terrestrial plants have been suggested to be consistent through a variety of environments (Naito et al., 2013b), thus enabling a clearer TP evaluation of consumers.
6890 GC (Agilent Technologies, Palo Alto, CA) coupled to a Thermo Finnigan DeltaPlus XP IRMS (Thermo Fisher Scientific, Bremen, Germany) via combustion and reduction furnaces. Instrumental analysis was performed according to previous methods, with a few modifications of the equipment settings (Chikarashii et al., 2007). The amino acid derivatives were injected into the GC column using the Gerstel programmable temperature vaporising (PTV) injector (Gerstel, Mülheim an der Ruhr, Germany) in solvent vent mode. The PTV temperature program was as follows: 50 °C (initial temperature) for 0.2 min, heating from 50 °C to 250 °C at the rate of 600 °C min⁻¹; isothermal hold at 250 °C for 10 min, heating from 250 °C to 350 °C at the rate of 600 °C min⁻¹; and isothermal hold at 350 °C for 10 min. Combustion and reduction furnaces were set at 950 °C and 550 °C, respectively. The GC was equipped with an Ultra-2 capillary column (50 m × 0.32 mm i.d. 0.52-µm film thickness; Agilent Technology). The GC oven temperature was programmed as follows: isothermal hold at 40 °C for 3 min; temperature ramp to 110 °C at the rate of 15 °C min⁻¹; ramp to 150 °C at the rate of 3 °C min⁻¹; ramp to 220 °C at the rate of 6 °C min⁻¹; and subsequent holding isothermally at 220 °C for 13 min. Carrier gas (He) flow rate through the GC column was 1.4 ml min⁻¹. CO₂ generated in the combustion furnace was eliminated by a liquid nitrogen trap. Standard mixtures of nine amino acids with known δ¹⁵N values were injected into the GC/C/IRMS every five runs to confirm the reproducibility of the isotope measurements. The mean accuracy and precision of the reference mixtures were 0.0% and 0.4–0.7% (mean of 1%), respectively. Nitrogen isotope composition of the following 10 amino acids were determined: alanine (Ala), glycine (Gly), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), serine (Ser), glutamic acid (Glu), phenylalanine (Phe), and hydroxyproline (Hyp). All reported δ¹⁵N values for glutamic acid included a contribution from the δ¹³C values and atmospheric nitrogen (AIR) values for δ¹⁵N values (Coplen, 2011).

3.3. Statistical analysis

In the present study, all statistical analyses were performed using the statistical program R, version 3.0.2 (R Core Team, 2014). To explore groupings among prey animals according to isotopic similarities rather than to biological species, we conducted a cluster analysis using the Ward’s hierarchical clustering based on their δ¹³C and δ¹⁵N values of collagen or δ¹³Cn and δ¹⁵Nphe values. This procedure clarifies comparable ecological niches in terms of diet of the prey animals. For the quantification of each food source to predators including the Neanderthals, we applied the package software “SIAR” (Stable Isotope Analysis in R; Parnell et al., 2010) running under R. SIAR is a Bayesian mixing model based on multiple isotope values for multiple sources. This model can incorporate uncertainties in consumer body tissues (i.e., predators in this study), sources (i.e., prey animals in this study), and trophic enrichment factors, though we incorporated no uncertainty for consumer isotope values because we performed each simulation for each individual predator. It is also important to note that SIAR produces true probability densities for the proportion estimates of each prey species to the predators’ diet. We performed mixing models based on δ¹³Cn and δ¹⁵Nphe data (AA model; but see also SOM for models based on bulk δ¹³C and δ¹⁵N data [Bulk model]). Isotopic compositions of each predator mean isotopic compositions with one standard deviation of prey animals and trophic enrichment factors (prey-predator isotopic enrichments) of 8.0 ± 1.1% and 0.4 ± 0.4% for δ¹³Cn and δ¹⁵Nphe, respectively, were used. The simulations were implemented under 500,000 iterations with the first 50,000 results to be discarded. For the estimation of mean isotopic composition of seven herbivorous prey species, we aggregated data for bone collagen and deciduous tooth collagen (a DP² for wooly rhinoceros and an M2 for mammoth; Germonpré et al., 2014). The δ¹³Cn and δ¹⁵Nphe values of plant foods for the Neanderthals were estimated by subtracting the enrichment factors from those of cave bears, because their diets could best represent a pure vegetarian end-point in that: i) palaeontological and isotopic data demonstrate their purely vegetarian diet; ii) hibernation has no significant impact on the isotopic compositions of bone collagen in this species; iii) the plant food typically consumed by bears covers a very similar spectrum as the plant food edible to human beings; and iv) the bears have a simple digestive tract lacking the special adaptations of most ungulates, as humans do (Bocherens, 2009 and references cited therein).

4. Results

4.1. Nitrogen isotopic composition of glutamic acid and phenylalanine

The results show large inter-individual variation in the Neanderthal δ¹⁵Nphe, with values of 6.8–11.0%, and relatively consistent δ¹³C values of 10.8–11.4% for bulk collagen (Fig. 3). Similar δ¹³Cn and δ¹⁵Nphe values between SPY-92b and SPY-430a could support their attribution to the same individual, Spy II (Rougier et al., in press). Terrestrial herbivores also showed a wide range of δ¹⁵Nphe values (4.2–16.0%), with mammoths (12.3–16.0%) representing the highest value, indicating that their high δ¹³C value of bulk collagen was due to the high baseline value of the ecosystem they inhabited. Mammoth, horse, and bovine showed a value (9.8‰) that fell within the Neanderthal range (see SOM Figs. S3–S5 for δ¹³C data of the other amino acids). The cluster analysis indicates groupings among the prey animals according to the isotopic similarity: animals are divided into three clusters (Cluster A1, A2, and A3) with low, high, and medium δ¹³Cn and δ¹⁵Nphe values, respectively (Fig. 4). The Cluster A2 includes only mammoths, while the other clusters include several prey species (see SOM for clusters based on δ¹³C and δ¹⁵N of collagen).

4.2. Trophic position estimates for the animals

Based on Eq. (1), the TP of the herbivores and wolf were estimated to be around 2 (±1.8–2.1) and 2.9, respectively, therefore being reasonable for pure herbivores and carnivores (SOM Table S4). These estimates confirm that this method is probably yielding reasonable results for the terrestrial ecosystem in which the Spy Neanderthals lived. In addition, very high TPs (3.4–3.8) were shown for hyenas. Dentine collagen in teeth (deciduous teeth and even adult teeth for carnivores) generally shows a higher δ¹³C value than that of bone collagen, probably because it records dietary signals during infancy that could be affected by breastfeeding (i.e., suckling effect; Fogel et al., 1989; Fuller et al., 2006), and those
signals do not change during growth due to a lack of the protein remodelling once it is formed (Bocherens et al., 1994). In this study, the collagen in the deciduous teeth of mammoth and woolly rhinoceros showed not only $\delta^{15}N_{\text{Phe}}$ values, but also TPs ($\approx 2.1$ and $2.2$, respectively) similar to those of the bone or adult tooth collagen for another individual of the same species (see SOM Table S4), indicating their herbivorous feeding habits with little signal of the suckling effect (see SOM Table S4).

5. Discussion

5.1. The mammoth $\delta^{15}N$ values

The cause of the $^{15}N$ enrichment in mammoth collagen compared to that of coeval large herbivores has been explained through physiological adaptation to aridity (Ambrose and DeNiro, 1986), consumption of mature grasses from disturbed areas (Bocherens, 2003), or coprophagy (Clementz et al., 2009). Recent work showing that aridity impacts on the $\delta^{15}N$ values of plants rather than on the fractionation between plants and herbivores (Murphy and Bowman, 2006; Hartman, 2011), and that elephants do not present unusual nitrogen isotopic fractionation when fed the same plant food as horse and rhinoceros (Kuitems et al., 2012), supports the hypothesis that mammoths consumed plants with higher $\delta^{15}N$ values than other herbivores. Moreover, the recent description of late mammoth populations with $\delta^{15}N$ values as low as those of horses in Ukraine and that of horses with $\delta^{15}N$ values as high as those of mammoth in southwestern Germany 35–25 kyr ago is further evidence for a dietary cause of the unusually high $\delta^{15}N$ values in mammoths (Drucker et al., 2014, 2015). Interestingly enough, a recent study based on the same method as the present one, performed on mammoth steppe fauna from Alaska and Yukon, found the same position of the mammoth compared to coeval herbivores and carnivores, i.e., an herbivore feeding on plants with high $\delta^{15}N$ values (Schwartz-Narbonne et al., 2015). Therefore, mammoths at both ends of the mammoth steppe ecosystem had a

Figure 3. a) Carbon and nitrogen isotopic composition of bulk collagen, and b) nitrogen isotopic composition of glutamic acid ($\delta^{15}N_{\text{Glu}}$) and phenylalanine ($\delta^{15}N_{\text{Phe}}$) in collagen of the Spy Neanderthals and associated fauna. Solid, dotted, and dashed lines in Figure 3b indicate theoretical lines for the $\delta^{15}N_{\text{Glu}}$ and $\delta^{15}N_{\text{Phe}}$ values of organisms with TP (Terrestrial) = 1, 2, and 3, respectively (see Fig. 2b). Abbreviations: TP = trophic position; T = deciduous tooth. Values in Figure 3a are quoted from the literature (Bocherens et al., 1997, 2011; Semal et al., 2009; SOM Table S3).

Figure 4. A cluster analysis based on $\delta^{15}N_{\text{Glu}}$ and $\delta^{15}N_{\text{Phe}}$ values of the prey animals for the Neanderthals. a) Cluster of the prey animals, b) $\delta^{15}N_{\text{Glu}}$ and $\delta^{15}N_{\text{Phe}}$ values of herbivorous prey species with grouping of the values according to the cluster defined through the cluster analysis. Estimates of $\delta^{15}N_{\text{Glu}}$ and $\delta^{15}N_{\text{Phe}}$ values for the Neanderthal plant diets are shown together as ‘plants for Neanderthal’ (see text). Bars indicate one standard deviation (1s).
similar ecological niche, different from the other large herbivores. This confirms the original hypothesis that the high bulk collagen δ15N value of mammoth was linked to a high δ15N value in its forage instead of a physiological feature of this species (Bocherens, 2003).

5.2. Trophic position estimates for the Spy Neanderthals

Based on Eq. (1), the three Neanderthals showed TP of 2.7–2.8 (Table 1). These values were similar or slightly lower than that of the wolf (2.9). It is important to note that these trophic relationships among predators are not visible in the bulk collagen data, where the Neanderthals showed a much higher δ15N value of bulk collagen than did the wolf and hyenas (Fig. 3a). Previously, this Neanderthal high δ15N has been interpreted as being due to their strong reliance on mammoths, which also show higher δ15N values compared with other herbivores (Bocherens et al., 2005, 2013). However, the amino acid isotope data in this study do not necessarily indicate a high mammoth contribution to the Neanderthal diet, especially as the SPY-92b and SPY-430a specimens showed much lower δ15Nphe values than the mammoth, suggesting that they obtained their nitrogen from prey with similarly lower δ15Nphe values on average (see Fig. 3b; more quantitative discussion will be given later). Considering that δ15N of phenylalanine produced in the river ecosystem should strongly reflect that of nitrate generally ranging as low as −1 to 5.5‰ (Nestler et al., 2011), significant dietary contributions of freshwater animals to the Neanderthal diet would be unlikely. The Neanderthals’ TPs were lower than those of humans from an archaeological Mesolithic site in France who exploited and consumed substantial amounts of freshwater food resources (Naito et al., 2013b), again suggesting little, if any, access to such resources.

5.3. Prey preferences and niche separation among predators

A variation in TP estimates as well as in δ15Nphe among the Neanderthal individuals suggests some differences in their prey preference. We quantified this prey preference using the SIAR dual-isotope mixing model based on δ15Nglu and δ15Nphe data (AA model). Judging from the median of prey contributions, among the seven prey species, the AA model predicts that SPY-94a (Spy I) relatively evenly consumed every prey, while SPY-92b and SPY-430a (Spy II) preferred bovine and possibly horse and reindeer as well (Fig. 5a). The SIAR mixing model based on isotope data of the clusters of prey animals and plant diets produced dietary contributions of each cluster to the predators (see Fig. 4); the AA model indicates that SPY-94a (Spy I) relied relatively evenly on every cluster, while the other individual (Spy II) relied more on Cluster A1 (horse, bovine, and reindeer), probably due to their low δ15Nphe (Fig. 5b).

An important implication based on the above calculation is that up to ~20% of dietary protein for the Spy Neanderthals could originate from plant foods (Fig. 5). However, the calculation of prey proportions assumes trophic enrichment factors (TEFs) that might vary due to a variety of factors, especially for glutamic acid (around 8‰), animal physiology (Styrga et al., 2010) and dietary components (Chikaraishi et al., 2015; McMahon et al., 2015a,b), among others. It has been already suggested in bulk δ15N studies that dietary protein quality and quantity (Robbins et al., 2005; Florin et al., 2011), physiology, and health (Reitsema, 2013) could affect consumer’s TEF. Therefore, it must be kept in mind that the calculation of prey proportion in predators’ diet is sensitive to the TEF values assumed. Though the TP estimations based on the TEF of glutamic acid and phenylalanine have been largely successful if limited in terrestrial environments (see Introduction; Chikaraishi et al., 2010, 2011; Naito et al., 2013a,b; Stefan et al., 2013), further studies are clearly needed to reveal appropriate TEFs and associated variations especially for animals in higher trophic positions (e.g., apex predators).
question remaining is whether or not the observed 0.1–0.2 unit difference in the TP between the wolf and the Neanderthals can be reasonably attributed to their food differences, especially in the light of plant consumption.

In agreement with recent findings, our results suggest that Neanderthal subsistence strategies did not merely involve hunting and scavenging, as once believed. Although only three samples from possibly two individuals were analysed, this novel approach provides a promising development in the study of the Neanderthal diet at sites where collagen is well-preserved. With a growing body of data on nitrogen isotopes in amino acids, a clearer picture of the diversity of the subsistence strategies of this extinct hominin is beginning to emerge.

Acknowledgements

The authors acknowledge D. Bonjean (Centre archéologique de la grotte Scladina) and N. Conard (Prehistoric Archaeology, Tubingen) for discussions. They also thank N.O. Ogawa, Y. Takano, M. Kaneko, H. Suga, and Y. Sasaki of the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) and Y. Iwashita of The University of Tokyo for discussions and their assistance with the laboratory work. This study was financially supported by the JSPS Postdoctoral Fellowships for Research Abroad (20120329) to Y.I.N. and Grant-in-Aid for Scientific Research from MEXT to N.O.

Supplementary Online Material

Supplementary online material related to this article can be found at http://dx.doi.org/10.1016/j.jhevol.2016.01.009.

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