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Evaluation of carnivory in inland Jomon hunter–gatherers based on nitrogen isotopic compositions of individual amino acids in bone collagen

Yuichi I. Naito [a,*], Yoshito Chikaraishi [b], Naohiko Ohkouchi [b], Minoru Yoneda [a,c]

[a] Department of Integrated Biosciences, Graduate school of Frontier Sciences, University of Tokyo, Kasiwanoha 5-1-5, Kashiwa, Chiba 277-8562, Japan
[b] Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan
[c] The University Museum, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

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ABSTRACT

Archaeological studies including stable carbon and nitrogen isotope analyses of bone collagen from human remains have suggested their heavy dependence on terrestrial foods during the Jomon period in the inland central region in Japan. However, it is not easy to quantitatively evaluate the extent of carnivory for archaeological human remains based on the bulk collagen chemistry, because of variable ^{15}N-enrichment factor along the trophic step and background isotopic variations in ecosystems. In order to overcome these problems and more precisely evaluate diets of prehistoric humans who strongly adapted to terrestrial environment, in this study we applied nitrogen isotope analysis of individual amino acids in bone collagen to two inland human populations in the Jomon period. Our results suggest that the two populations were predominantly dependent on the C_3-plant-based terrestrial ecosystem and consumed little aquatic resources. Furthermore, their mean trophic positions (2.7 for both cases) are closer to that of the fox (2.8–3.0) rather than those of pure herbivores (2.0–2.2), and show little change over time. These results are the first evidence that inland Jomon populations may have had more carnivorous diets than is traditionally considered.

1. Introduction

The Jomon cultural entity began in the Late Pleistocene as the Incipient Jomon culture, which made one of the oldest known pottery in ca.16,000 BP (Nakamura et al., 2001), and was followed by the Initial Jomon period (ca. 10,000–6000 BP) in the early Holocene, which was represented by more sedentary and hunting–fishing–gathering societies (Habu, 2004; Imamura, 1996). Generally speaking, based on the archaeological evidences such as nut shell remains and stone tool compositions, it is considered that the use of plant foods intensified with time and reached a climax during the Middle Jomon period (ca. 5000–4000 BP) especially in the inland central region in Japan. This period also witnessed a marked increase in the number of pit dwellings, suggesting pronounced population growth in this region (Imamura, 1996; Koyama, 1978). However, shortages of plant foods may have caused the decrease of the inland sites by the time of the Late Jomon period (ca. 4000–3000 BP) (Imamura, 1996).

Based on the high density of sites, decrease of stone tools for hunting and so on, Fujimori (1970) hypothesized that agricultural activity was practiced in the inland central region during the Middle Jomon period. This hypothesis stirred up controversy about Jomon subsistence, although it gained few supports by Japanese archaeologists partly due to the lack of cultivated plant remains (e.g. Matsui and Kanehara, 2006). However, even though many Jomon sites in other regions provide evidences of possible cultivation of plants, it is generally assumed that Jomon peoples did not practice agriculture because the meaning of agriculture in Japanese prehistory is almost equivalent with the wet rice agriculture, making the debates complicated (Crawford, 2011, 2008; Matsui and Kanehara, 2006).

Apart from the problem in terminology, stable isotope analysis of bulk samples for human remains provides another way to derive quantitative information about their subsistence. The stable isotope ratios are reported with reference to international standards (VPDB in the case of carbon, and atmospheric air [AIR] in the case of nitrogen) and expressed in the notation "δ (delta)" as follows: $\delta^{13}C$ or $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1000$ (‰), where R represents $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Based on the isotopic composition of bone

* Corresponding author. Present address: Universität Tübingen, Institut für Geowissenschaften, Biogeologie, Hölderlinstr. 12, D-72074 Tübingen, Germany. Tel.: +49 (0)7071 29 74687; fax: +49 (0)7071 29 5059.
E-mail addresses: yuichi.naito@ifg.uni-tuebingen.de, ynaito0825@gmail.com (Y.I. Naito).

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collagen, some pioneering studies evaluated the extent of carnivory for Jomon human remains from Kitamura site located in the inland central region (e.g. Akazawa et al., 1993; Minagawa and Akazawa, 1993). They demonstrated that: i) the Kitamura humans did not consume large amount of C_4 cereals such as foxtail millet as evidenced by low $\delta^{13}C$ values of bone collagen (around −19.5‰); and ii) they relied more than 70% of dietary proteins on C_3 plants as evidenced by low $\delta^{15}N$ values (around 6.4‰). However, those estimations need more careful consideration on reference faunal materials as human food source because they did not use local reference materials in their evaluations. Indeed, faunal remains from this region show very low $\delta^{15}N$ values (e.g. +3.0 ± 1.0‰ for herbivores and +3.4 ± 0.5‰ for omnivores; Yoneda, 2012), leaving a possibility that the Kitamura humans might be less plant-dependent. Methodological difficulties associated with those estimations will be explained below.

Nitrogen isotopic composition is the most frequently used tool to estimate the trophic position (TP) that is useful indicators of the extent of carnivory in terrestrial species. This technique is based on the well founded ^{15}N-enrichment that occurs between a prey and its consumer. The empirical mean isotopic differences of 3.0–3.4‰ between them are often cited. These values were evaluated based on the isotope analysis of whole animal body (DeNiro and Epstein, 1981; Post, 2002), bone collagen (Schoeninger and Deniro, 1984) and various kinds of body tissues (Minagawa and Wada, 1984). It is generally assumed that the ^{15}N-depleted urea excreted as the main byproduct during amino acid metabolism accounts for the ^{15}N-enriched nitrogen pool that is incorporated into animal tissues (Ambrose, 1991, 1986; Ambrose and DeNiro, 1986; Minagawa and Wada, 1984; Steele and Daniel, 1978).

However, it is also pointed out that the precise estimation of animal trophic position by bulk collagen $\delta^{15}N$ measurements entails some difficulties: (i) the characterization of $\delta^{15}N$ value for past plant foods (primary producers) is difficult because plant tissue degrades faster than animal hard tissues such as bones, and because the value of primary producers could vary as a function of local environmental conditions (e.g. Heaton, 1987; Mariotti et al., 1980; Stevens and Hedges, 2004); (ii) relatively large uncertainties have been reported for the ^{15}N-enrichment factors between a prey and its consumer not only for animals (Ambrose, 2000; DeNiro and Epstein, 1981; Hare et al., 1991; Hobson and Clark, 1992; McCutchan et al., 2003; Robbins et al., 2005; Sponheimer et al., 2003) but also for humans (Hedges et al., 2009; O'Connell et al., 2012; Yoshinaga et al., 1996). For the analyses of bone collagen, several studies recommended to use the range of the ^{15}N-enrichment factor of +3‰ (possibly +2‰) to +5‰ rather than average ones (Bocherens and Drucker, 2003; Hedges and Reynard, 2007). In the case of omnivorous species, including humans, further problem can occur because the consumption of freshwater resources may elevate the $\delta^{15}N$ value of a species, leading to the overestimation of its trophic position in a terrestrial ecosystem. These problems make it difficult to precisely evaluate the extent of a species' carnivory using the isotopic analysis of bulk bone collagen (see the review by Hedges and Reynard, 2007).

Here we present new data of nitrogen isotopic composition of individual amino acids in human remains from the two archaeological sites, Tochibara rock shelter site (ca. 8300–8600 BP, Initial Jomon period) and the Kitamura site (5000–3000 BP, Middle to Late Jomon period), in the inland central region (Nagano prefecture), Japan. This relatively new approach was applied to the archaeological specimens in order to overcome the difficult situation mentioned above because its usefulness in the evaluation of animal trophic positions has been well demonstrated (see Material and methods for detailed information). The goal of this study is to test the hypothesis about their very much heavy reliance on plants and refine understanding of Jomon subsistence in the inland region. Both of the two sites are among a few archaeological sites where many human remains were found in this region, providing us a unique opportunity to perform the isotope analysis.

2. Material and methods

2.1. Archeological samples

The Tochibara rock shelter site (ca. 8300–8600 BP, Initial Jomon period; Yoneda et al., 2002a) and the Kitamura site (ca. 5000–3000 BP, Middle to Late Jomon period) are inland archaeological sites in Nagano Prefecture, Central Honshu, Japan (see Yoneda et al., 2002a, for detailed information about the location and cultural context of the Tochibara site). In total, 190 human skeletal remains were unearthed from the Kitamura site in 1988–1989 (Archaeological Research Center of Nagano Prefecture, 1993). Nearly all of the identified faunal remains at this site are wild boar or deer, and most are burned and fragmented. The carbon and nitrogen isotopic compositions of the bulk bone collagen were measured in the human remains from the Tochibara (Yoneda et al., 2002a) and Kitamura sites (Akazawa et al., 1993; Minagawa and Akazawa, 1993; Yoneda et al., 1996). However, as we note in introduction, it is still unclear how dependent they were on plant versus animal proteins.

In this study, the human bone collagen samples from Tochibara ($n = 4$) used in a previous study were subsampled (Yoneda et al., 2002a). The fox (*Vulpes vulpes*, $n = 3$), Asiatic black bear (*Ursus thibetanus*, $n = 1$), boar (*Sus scrofa*, $n = 2$), serow (*Capricornis crispus*, $n = 1$), hare (*Lepus brachyurus*, $n = 1$), and deer (*Cervus nippon*, $n = 1$) bone collagen samples and freshwater shellfish (*Margaritifera laevis*, $n = 1$) from the Tochibara site were newly prepared for amino acid $\delta^{15}N$ analysis in this study. Bone collagen from human, and bone and dentine collagen from deer and boar collected at the Kitamura site were also newly prepared. Because the formation of mammal dentine collagen begins since they are the pre-born child and this tissue shows no turnover through their lives, the isotopic composition of dentine collagen may reflect a formation period different from that of bone collagen.

2.2. Sample preparation and instrumental analysis

Bone and dentin collagens were extracted using the methods described by Longin (1971) and Yoneda et al. (2002b). Weight percent of collagen in bone and dentine samples (%OM, organic matter) was recorded (we call the extracted organic matter "collagen" in this paper). For the extraction of shell protein, surface contaminants were initially removed by brushing and ultrasonic cleaning. After crushing, 1.5–2.0 g of the shell powder was reacted with H_2O_2 in water at 80 °C to remove any adherent soil organic matter. The powder was then slowly demineralized in 12 M HCl (Naito et al., 2010).

Bulk collagen samples (ca. 0.25–0.50 mg) were analyzed for their stable carbon and nitrogen isotope ratios using an elemental analyzer/isotope ratio mass spectrometry (EA/IRMS) system consisting of a Carlo Erba NA1500 elemental analyzer, a Finnigan MAT ConFlo II interface, and a Finnigan MAT 252 isotope ratio mass spectrometer. Samples were calibrated to $\delta^{13}C$ (−19.6‰ vs VPDB) and $\delta^{15}N$ values (+10.1‰ vs AIR) of an isotopic reference material (L-Alanine distributed from SI Science Co., Ltd.). Based on the standard deviations of replicate analyses of the reference material measured with unknown samples, the reproducibility of each measurement was estimated to be better than ±0.2‰ for $\delta^{13}C$ and ±0.4‰ for $\delta^{15}N$.

Amino acids were prepared according to the modified procedure of Metges et al. (1996). Standards, reagents, and solvents were

purchased from Wako Pure Chemical Industries Ltd. All the solvents were of dioxin analysis grade. The collagen and demineralized shell samples were subjected to HCl hydrolysis, followed by the derivatization. Carboxyl and amino groups in amino acid molecules must be derivatized to increase the volatility during the nitrogen isotope measurements. Additional cation-exchange column chromatography was applied only to the shell sample after hydrolysis to isolate the amino acids, because abundant calcium ions can obstruct the quantitative pivaloyl/isopropyl derivatization of amino acids (Takano et al., 2009). Derivatization of amino acid was performed by the following steps: dried hydrolysates were dissolved in ~0.2 ml of esterification reagent (thionyl chloride solution in isopropanol; 1:4, v/v) and heated for 2 h at 110 °C; the product was dried under a gentle stream of nitrogen, dissolved in ~0.2 ml acylation reagent (pivaloyl chloride/dichloromethane; 1:4, v/v) and heated at 110 °C for 2 h; after the addition of ~0.2 ml of *n*-hexane/dichloromethane (3:2, v/v) and the same volume of distilled water, liquid–liquid extraction of the organic solvent layer was performed and repeated at least three times; the extract was evaporated with a gentle stream of nitrogen and the residue dissolved in dichloromethane for sample injection. It is certain that there is no isotopic fractionation in nitrogen during the preparation of the amino acid derivatives and the nitrogen isotope analysis by GC/C/IRMS (Chikaraishi et al., 2010a) as well as the cation exchange chromatography (Takano et al., 2010, 2009).

The nitrogen isotopic compositions of the individual amino acids were measured by gas chromatography/combustion/IRMS (GC/C/IRMS) using an Agilent Technologies 6890 GC coupled to a Thermo Fisher Scientific Deltaplus XP IRMS via combustion and reduction furnaces. The amino acid derivatives were injected into the GC column using a Gerstel PTV injector 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 1000 °C and 550 °C, respectively (Chikaraishi et al., 2007). The GC was equipped with an Ultra-2 capillary column (50 m × 0.32 mm i.d., 0.52 μm film thickness; Agilent Technologies). The GC oven temperature was programmed as follows: isothermal hold at 40 °C for 5 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}, subsequently holding isothermally at 220 °C for 13 min. Carrier gas (He) flow rate through the GC column was 1.3 ml min^{-1}. CO_2 generated in the combustion furnace was eliminated by a liquid nitrogen trap. Standard mixtures of eight amino acids with known $\delta^{15}N$ values were injected into the GC/C/IRMS every 4 or 5 runs to confirm the reproducibility of the isotope measurements. The analytical errors (1σ) of the standards were better than ±0.5‰ for a minimum sample size of >30 ng N (Chikaraishi et al., 2011). All reported $\delta^{15}N$ values for glutamic acid include a contribution from the α-amino group of glutamine because glutamine is converted to glutamic acid during acid hydrolysis.

2.3. Trophic position estimates based on nitrogen isotopic compositions of individual amino acids

In laboratory feeding experiments and field ecological studies, following principles were reported: $\delta^{15}N_{Glu}$ ($\delta^{15}N$ of glutamic acid) increases greatly from prey to consumer (+8.0 ± 1.1‰) while $\delta^{15}N_{Phe}$ ($\delta^{15}N$ of phenylalanine) shows little change (+0.4 ± 0.4‰), in both aquatic ecosystems (Chikaraishi et al., 2009, 2007; McClelland and Montoya, 2002) and terrestrial ecosystems (Chikaraishi et al., 2011); there are distinctive difference in $\Delta^{15}N_{Glu-Phe}$ ($\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$) value in primary producer among algae and cyanobacteria (+3.4‰), C_3-plant (−8.4‰) and C_4-plant (+0.4‰) (Chikaraishi et al., 2010b; Fig. 1). Based on these principles, the following equations that quantify the trophic positions of organisms in three types of ecosystems were established:

$$TP_{AA} = \left(\left[\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 3.4\right]/7.6\right) + 1 \quad \text{for aquatic ecosystems;} \tag{1}$$

$$TP_{AA} = \left(\left[\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4\right]/7.6\right) + 1 \quad \text{for } C_3 - \text{plant} - \text{based ecosystems,} \tag{2}$$

$$TP_{AA} = \left(\left[\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - 0.4\right]/7.6\right) + 1 \quad \text{for } C_4 - \text{plant} - \text{based ecosystems,} \tag{3}$$

where TP_{AA} indicates the amino-acid-based trophic position. These equations have an advantage over the traditional isotopic method based on bulk protein in that they do not require the

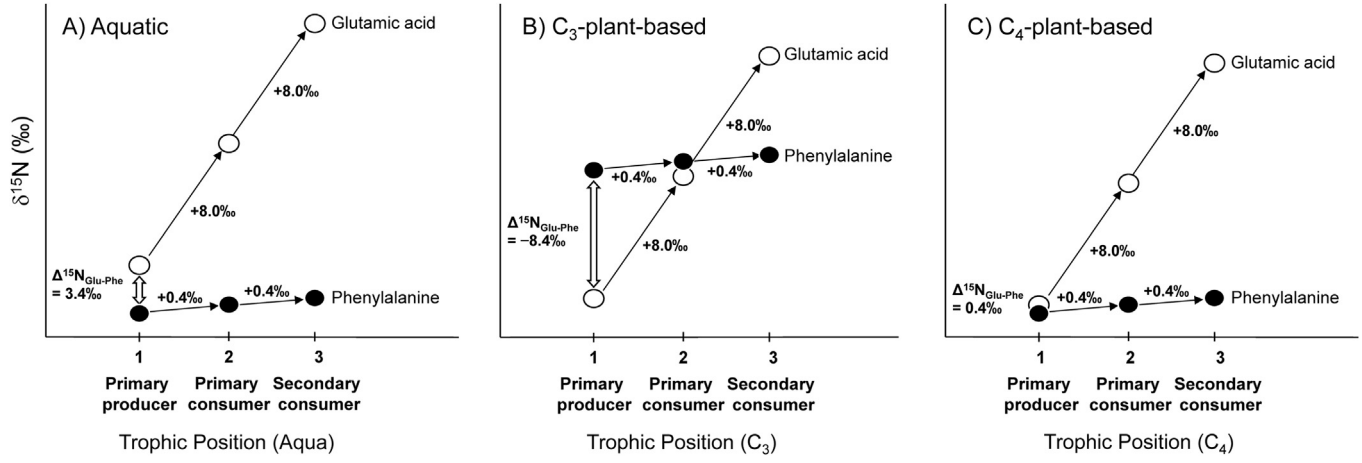

Fig. 1. Schematic illustration of the relationship between trophic position (TP) of organisms in an ecosystem and their nitrogen isotopic composition of glutamic acid and phenylalanine (modified after Chikaraishi et al., 2011). The $\Delta^{15}N_{Glu-Phe}$ ($\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$) value in primary producer varies among algae and cyanobacteria (+3.4‰), C_3-plant (−8.4‰) and C_4-plant (+0.4‰) (Chikaraishi et al., 2010b).

characterization of the $\delta^{15}N$ values for a pure type of primary producer (any of algae and cyanobacteria, C_3 plants or C_4 plants) because they utilize the isotopic spacing between two amino acids within a single organism. Therefore, by focusing on these specific amino acids, we could more precisely estimate the trophic position of each organism and reconstruct foodweb structure.

3. Results

3.1. Diagenetic effects on the isotopic composition of bulk bone collagen and amino acids

As described in previous study (Yoneda et al., 1996), all specimens from the Kitamura site, except one faunal remain, showed the C/N ratio (3.7–10.7) outside the biological range for bone collagen (2.9–3.6; DeNiro, 1985; Table 1). The degraded collagen with higher C/N ratios had lower carbon content (Fig. 2). Changes in the C/N ratios in the faunal specimens showed higher $\delta^{15}N$ and lower $\delta^{13}C$ values compared with samples with biological C/N ratios (Table 1).

It should be noted that the samples in poorly preserved bone collagen with altered C/N ratios (3.7–10.7) and %OM values (<1.0%) exhibited $\delta^{15}N$ values of glutamic acid and phenylalanine similar to those of samples in a better state of preservation (Fig. 3). No significant differences were observed in $\delta^{15}N$ values for both glutamic acid (Fig. 3a and b) and phenylalanine (Fig. 3c and d) between animals (boar and deer) with altered C/N ratios and the same animal species with C/N ratios within the biological range (2.9–3.6) (*U*-test, $P > 0.1$). Thus even samples with the altered C/N ratios could be available in the $\delta^{15}N$ analysis of amino acids, at least for glutamic acid and phenylalanine.

3.2. Nitrogen isotopic composition of individual amino acids

As a general trend, valine, proline, glutamic acid, and hydroxyproline exhibited relatively high $\delta^{15}N$ values, whereas glycine and serine exhibited low values for the human and faunal samples in this study (Fig. 4; individual data are shown in Inline Supplementary Table S1 and S2). Serine, glutamic acid, phenylalanine and hydroxyproline showed relatively consistent $\delta^{15}N$ values between the collagen samples with high and low C/N ratios. Considering the $\delta^{15}N$ analytical error (ca. 0.5‰), these isotopic differences between better and poorly preserved samples (up to 1.1‰ for serine) wer virtually negligible. The relatively higher $\delta^{15}N$ values of alanine, valine, leucine and isoleucine of the boar samples as well as alanine, glycine, valine, leucine, proline and hydroxyproline of the deer samples seem to explain higher $\delta^{15}N_{bulk}$ values in the significantly degraded collagens compared to those of well-preserved ones (Fig. 4c). On the other hand, the two human populations showed similar amino acid $\delta^{15}N$ values except the significant differences in valine (*U*-test, $P < 0.01$) and proline (*U*-test, $P < 0.05$).

Inline supplementary Table S1 can be found online at http://dx.doi.org/10.1016/j.jas.2013.03.012.

Inline supplementary Table S2 can be found online at http://dx.doi.org/10.1016/j.jas.2013.03.012.

Most specimens, except freshwater shellfish, showed consistent $\delta^{15}N$ values with respect to phenylalanine, suggesting that they could belong to the same food-chain (see Fig. 4). The difference in $\delta^{15}N_{Glu}$ values between the fox and pure herbivores with C/N ratios of 2.9–3.6 was 6.4‰. A similar $\delta^{15}N_{Glu}$ difference (6.5‰) was also observed between the fox and deer at Kitakogane shell midden, the coastal Jomon site in Hokkaido, further supporting the isotopic integrities of those archaeological materials (Naito et al., 2010).

3.3. Trophic position estimates for the faunal remains

TP_{AA} values of the faunal samples were estimated by applying equations (1) for the freshwater shellfish and both (2) and (3) for the terrestrial species, respectively (Table 2). While the estimated TP_{AA} values of the pure herbivores (deer, serow, and hare) were confined to a range of 2.0–2.2 based on the equation (2) for C_3-

Table 1
$\delta^{13}C$ and $\delta^{15}N$, C/N ratio, %C, %N and %OM of the human and faunal bulk bone and dentin collagen from the Tochibara and the Kitamura archaeological sites.

Archaeological site	Original ID	Common name (*Species name*)	$\delta^{13}C_{Bulk}$	$\delta^{15}N_{Bulk}$	%C	%N	C/N	%OM
Tochibara	KA-4	Human	−19.7	7.6	45.1	15.8	3.3	7.54%
Tochibara	KA-7	Human	−19.8	7.1	46.2	16.2	3.3	8.19%
Tochibara	KA-8	Human	−19.8	6.9	46.0	16.7	3.2	11.51%
Tochibara	KA-10	Human	−19.8	7.4	45.3	16.5	3.2	10.15%
Tochibara	KA-A-25	Fox (*Vulpes vulpes*)	−18.2	7.8	43.1	15.6	3.2	15.02%
Tochibara	KA-A-26	Fox (*Vulpes vulpes*)	−20.2	5.5	44.4	15.9	3.3	14.63%
Tochibara	KA-A-27	Fox (*Vulpes vulpes*)	−17.9	7.5	43.3	15.7	3.2	6.29%
Tochibara	KA-A-3	Bear (*Ursus thibetanus*)	−20.1	3.2	43.1	15.7	3.2	7.79%
Kitamura	SB-560	Boar (*Sus scrofa*)	−21.1	3.8	43.7	14.5	3.5	2.14%
Tochibara	KA-A-35	Boar (*Sus scrofa*)	−20.4	4.6	45.2	15.8	3.3	6.06%
Tochibara	KA-A-37	Boar (*Sus scrofa*)	−19.6	3.0	28.7	9.2	3.6	5.79%
Tochibara	KA-A-6	Deer (*Cervus nippon*)	−21.6	2.0	43.3	15.7	3.2	15.79%
Tochibara	KA-A-20	Serow (*Capricornis crispus*)	−20.0	3.4	43.4	15.5	3.3	8.56%
Tochibara	KA-A-40	Hare (*Lepus brachyurus*)	−23.5	2.4	42.5	14.8	3.4	11.80%
Samples with C/N atomic ratio >3.6								
Kitamura	SH-508	Human	−22.6	6.1	38.6	9.6	4.7	0.26%
Kitamura	SH-573	Human	−22.0	7.5	24.0	4.4	6.4	0.31%
Kitamura	SH-735	Human	−21.5	ND	24.8	3.8	7.5	0.04%
Kitamura	SH-743	Human	−20.9	8.1	31.3	5.4	6.7	0.12%
Kitamura	SH-775	Human	−20.7	5.8	37.3	9.9	4.4	0.42%
Kitamura	SH-803	Human	−20.3	5.5	42.3	12.4	4.0	0.94%
Kitamura	SH-1177	Human	−24.4	4.4	29.3	4.2	8.1	0.18%
Kitamura	SH-1200	Human	−21.1	7.1	33.0	6.3	6.1	0.18%
Kitamura	SH-1203	Human	−21.1	ND	29.2	3.2	10.7	0.02%
Kitamura	EKM (E) L7 No.84	Boar (*Sus scrofa*)	−21.5	ND	29.9	6.2	5.6	0.60%
Kitamura	SH-977	Boar (*Sus scrofa*)	−23.9	6.4	38.6	6.1	7.4	0.04%
Kitamura	SH-698	Deer (*Cervus nippon*)	−22.6	7.2	34.8	8.2	5.0	0.35%
Kitamura	SH-745	Deer (*Cervus nippon*)	−22.2	3.1	41.4	13.0	3.7	2.44%

ND, not determined.

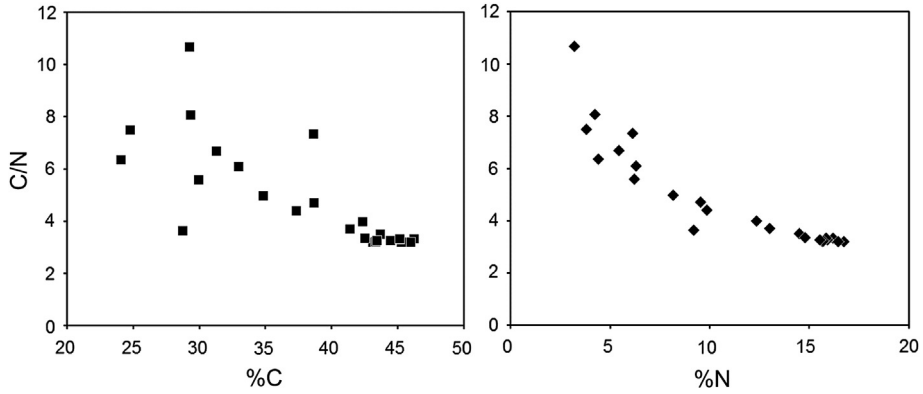

Fig. 2. C/N atomic ratio and weight% of carbon and nitrogen in bone collagen.

plant-based ecosystem, the estimates by equation (3) for C_4-plant-based ecosystem ranged from 0.8 to 1.1 that were obviously different from the biologically expected trophic position (i.e. ~2). Considering the standard deviation (1σ) of the accuracy (= [actual TP] − [TP_{AA} value]) of 0.1–0.2 units estimated for the variety of plant and fauna in controlled feeding experiments and field observations, the slight deviation up to 0.2 in the former estimates is virtually negligible (Chikaraishi et al., 2011). This result means that these pure herbivores fed almost exclusively C_3 plants. Trophic positions of the boar and Asiatic black bear were estimated to be 2.0–2.3, whereas that of the fox was 2.8–3.0. Equation (1) indicates a TP_{AA} value of 1.9 for the freshwater shellfish. This estimate is consistent with their feeding behavior primarily filtering suspended materials, such as algae, from the water (Bauer and Wächtler, 2001).

4. Discussion

4.1. Diagenetic effects on the isotopic composition of bulk bone collagen and amino acids

The observed relationships between elemental concentrations and C/N ratio in collagen suggest that the changes in the C/N ratio were not caused by an increase in the carbon content of the degraded collagen, but mainly by a reduction in nitrogen content (see Fig. 2). The relatively lower $\delta^{13}C$ and higher $\delta^{15}N$ values of the boar and deer collagen with C/N ratio outside the biological range are consistent with the observation in significantly degraded archaeological bone collagen (DeNiro, 1985; Yoneda et al., 2004) and inoculation experiment of soil bacteria onto collagen (Balzer et al., 1997; Grupe et al., 2000; Turban-Just and Schramm, 1998).

The agreement in $\delta^{15}N$ values for both glutamic acid and phenylalanine between animals (boar and deer) with altered C/N ratios and those with non-altered ratios is compatible with the theoretical consideration that diagenetic processes of proteins do not affect the isotopic composition of the non-exchangeable nitrogen that forms the peptide bond (Ohkouchi and Takano, in press). Although early diagenetic processes (e.g. internal aminolysis at the N-terminal position of proteins and the deamidation of glutamine and asparagine) related to proteins and amino acids could eventually cause substantial changes in nitrogen isotopic compositions of bulk proteins or peptides, none of those reactions involve changes in the isotopic composition of the peptide-bond-forming nitrogen. Hence, even after a protein or peptide undergoes significant diagenetic alteration of its overall organic matter

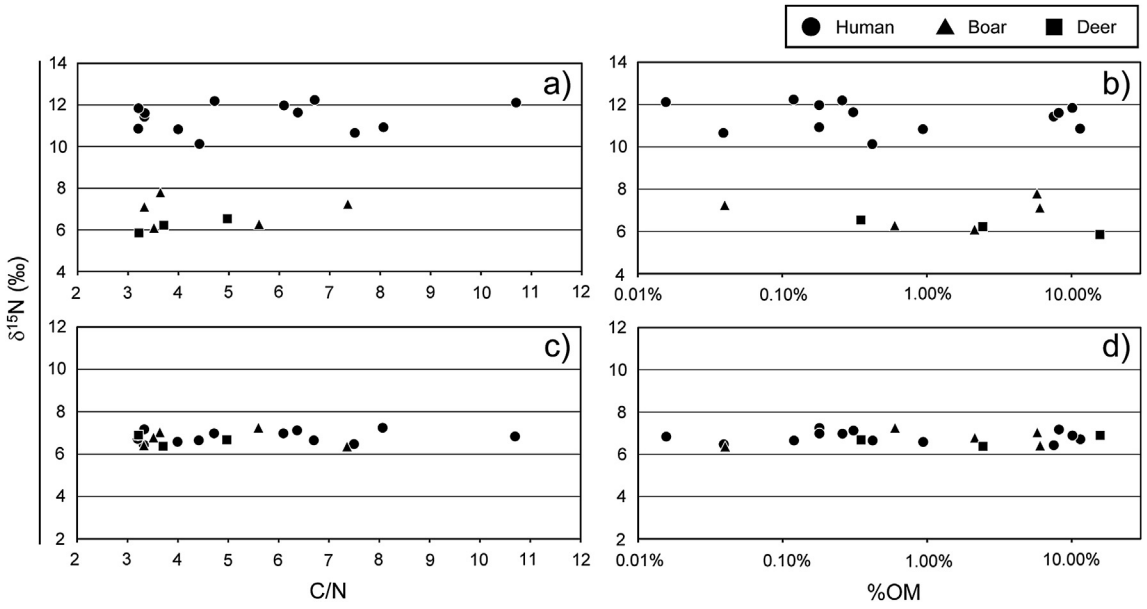

Fig. 3. Nitrogen isotopic compositions of glutamic acid (a and b) and phenylalanine (c and d) of the humans and selected faunal species against C/N ratio of bone collagen and %OM.

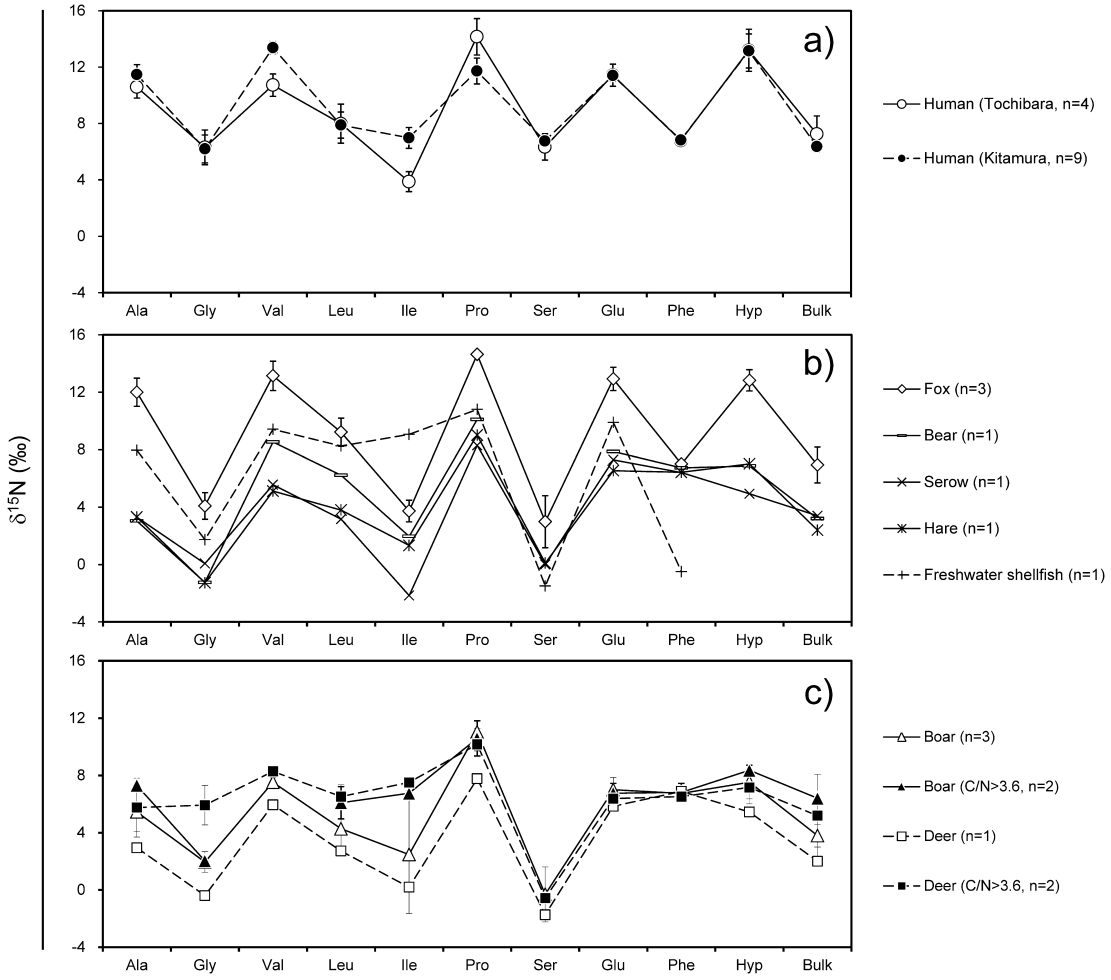

Fig. 4. Nitrogen isotopic compositions of individual amino acids from, a) the Tochibara and the Kitamura humans, b) faunal species other than deer and boar, and c) deer and boar. Black symbols indicate samples with C/N ratio >3.6. See the text for abbreviations of each amino acid.

preservation, its C/N atomic ratio, and its amino acid composition, the isotopic composition of the peptide-bond-forming nitrogen could still reflect its original isotopic signature unless ambient proteins and/or peptides have contaminated.

4.2. Trophic position estimates for the human remains

The isotopic patterns described above suggest that little protein from non-C_3-plant-based terrestrial ecosystems, such as the C_4-

Table 2
TP_{AA} estimation of the faunal samples from the two archaeological sites. TP_{AA} (Aq), (C_3) and (C_4) indicate the estimated values based on the equations (1)–(3) in the text, respectively.

Archaeological site	Original ID	Common name (*Species name*)	TP_{AA} (Aq)	TP_{AA} (C_3)	TP_{AA} (C_4)
Tochibara	KA-A-25	Fox (*Vulpes vulpes*)	–	2.8	1.6
Tochibara	KA-A-26	Fox (*Vulpes vulpes*)	–	2.9	1.8
Tochibara	KA-A-27	Fox (*Vulpes vulpes*)	–	3.0	1.8
Tochibara	KA-A-3	Bear (*Ursus thibetanus*)	–	2.3	1.1
Tochibara	KA-A-35	Boar (*Sus scrofa*)	–	2.2	1.0
Tochibara	KA-A-37	Boar (*Sus scrofa*)	–	2.2	1.0
Kitamura	SB-560	Boar (*Sus scrofa*)	–	2.0	0.9
Kitamura	*EKM (E) L7 No.84	Boar (*Sus scrofa*)	–	2.0	0.8
Kitamura	*SH-977	Boar (*Sus scrofa*)	–	2.2	1.1
Tochibara	KA-A-6	Deer (*Cervus nippon*)	–	2.0	0.8
Kitamura	*SH-698	Deer (*Cervus nippon*)	–	2.1	0.9
Kitamura	*SH-745	Deer (*Cervus nippon*)	–	2.1	0.9
Tochibara	KA-A-20	Serow (*Capricornis crispus*)	–	2.2	1.1
Tochibara	KA-A-40	Hare (*Lepus brachyurus*)	–	2.1	1.0
Tochibara	70167, T9-S16	Shellfish (*Margaritifera laevis*)	1.9	–	–

Asterisks (*) indicate samples with C/N ratio >3.6.

plant-based ecosystem and the freshwater ecosystem, contributed to the human diets because: i) the pure herbivores from the two sites fed C_3 plants in the ecosystem; ii) the $\delta^{15}N_{Phe}$ signature were quite consistent and convergent among the terrestrial species as well as the humans, meaning that they all shared the same nitrogen source (i.e. C_3 plants); iii) the $\delta^{13}C$ values in the bulk collagen were low (<−20‰, except fox); iv) the $\delta^{15}N_{Phe}$ value for shellfish (−0.5‰) was quite different from those of the terrestrial species (>+6‰) (Fig. 5). The $\delta^{15}N_{Phe}$ value of the freshwater shellfish is consistent with the fact that the nitrate in natural mountain streams in Japan can have $\delta^{15}N$ values of less than 0‰ (Koba et al., 1997; Osaka et al., 2010).

Although fish bones and freshwater shellfish (*M. laevis*) were recovered from the Tochibara site (Nishizawa, 1982), these freshwater species would not have played significant roles as protein sources in the diets of the Tochibara human remains in this study. Because artificially perforated shells of the *M. laevis* are rare and their use as an ornament is unlikely, they might be consumed as a minor accompanying dish or fallback food. Therefore, equation (2) was used to estimate the TP_{AA} values for these human populations, which were 2.7–2.8 (2.7 on population average) for the Tochibara site and 2.6–2.8 (2.7 on average) for the Kitamura site, respectively (Table 3). Importantly, there is no significant difference in the estimated TP_{AA} of the Tochibara and Kitamura human populations (*U*-test, $P > 0.1$). This suggests that the extent of their reliance on animal foods as a protein source should not differ greatly between the two populations.

If we apply the same equation (2) to the published amino acid $\delta^{15}N$ data for prehistoric humans in the cape region in South Africa (Styring et al., 2010), results show $TP_{AA} = 2.1$ and 2.7 for the two "Inland C_3 Consumers". In addition, the herbivores (sheep and steenbok) with low $\delta^{13}C$ values (−18.5‰ to −17.9‰) show the TP_{AA}

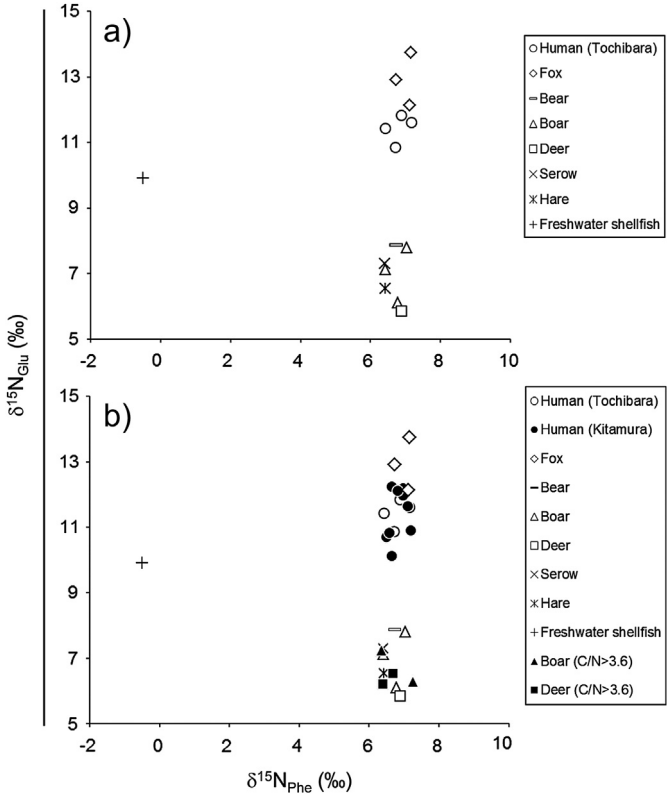

Fig. 5. Nitrogen isotopic compositions of glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$) of a) the samples with C/N ratio of 2.9–3.6 and b) all samples in this study. Black symbols indicate samples with C/N ratio >3.6.

Table 3
TP_{AA} estimation and basic morphological data of the two human populations. The morphological data of Tochibara and Kitamura human remains were according to Kohara et al. (2011) and Archaeological Research Center of Nagano Prefecture (1993), respectively.

Archaeological site	Period	Original ID	Sex	Age	TP_{AA} (C_3)
Tochibara	Initial Jomon	KA-4	Female	Adult	2.8
Tochibara	Initial Jomon	KA-7	Female	Adult	2.7
Tochibara	Initial Jomon	KA-8	Male	Adult	2.7
Tochibara	Initial Jomon	KA-10	Female	Adult	2.8
Average					**2.7**
Kitamura	Middle Jomon	*SH-735	Female	25–30	2.7
Kitamura	Middle Jomon	*SH-775	Male	30–40	2.6
Kitamura	Middle Jomon	*SH-803	Male	25–30	2.7
Kitamura	Middle Jomon	*SH-1203	Female	Adult	2.8
Average					**2.7**
Kitamura	Late Jomon	*SH-508	Male	50–60	2.8
Kitamura	Late Jomon	*SH-573	Female	20–25	2.7
Kitamura	Late Jomon	*SH-743	Male	50–60	2.8
Kitamura	Late Jomon	*SH-1177	Female	~25	2.6
Kitamura	Late Jomon	*SH-1200	Female	50–55	2.8
Average					**2.7**

Asterisks (*) indicate samples with C/N ratio >3.6.

of 2.0–2.3 based on the same equation, suggesting that this method still work well in the environment very different from prehistoric Japan. These observations suggest that the equation could detect some variations in TP among prehistoric humans not only in Japan but also in another region.

4.3. Quantitative evaluation of carnivory among the inland humans

In order to quantitatively evaluate the extent of carnivory for the humans, we developed several models based on the (I) TP_{AA} value, (II) $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values and (III) $\delta^{13}C_{Bulk}$ and $\delta^{15}N_{Bulk}$ values and compared them (Table 4). First of all, we used the isotopic compositions only for the animal and human samples having C/N ratio of bulk bone collagen within 2.9–3.6 in these models. Thus, the following comparison of estimates between the models is made on the Tochibara human population and we focus on the population averages of the above values for simplicity.

In the Model (I), the dietary animal protein contribution (*Fa*: here we define it as [animal protein]/([animal protein] + [plant protein]); the "animal" does not include freshwater species) to their diets can be estimated by the following equation, considering the variation in the TP_{AA} values for the herbivorous prey species (2.0–2.3), the monotonous TP_{AA} values for plants (1.0), and the TP_{AA} of the diets of these humans (i.e., 1.7 on average) (Fig. 6):

$$Fa(\%) = (TP_{AA}[\text{human}] - 2)/(TP_{AA}[\text{animal}] - 1) \times 100. \quad (4)$$

The equation suggests that the dietary animal protein contributions for these human populations were 54–70% on population averages (Table 5). Bearing the uncertainty of approximately 0.2 units associated with the TP_{AA} estimates in mind (Chikaraishi et al., 2011), the *Fa* values involve 17–24% of associate errors (minimum and maximum estimates of the *Fa* for mean TP_{AA} of the humans are 54 ± 17% and 70 ± 24%, respectively).

In the Models (II) and (III), we incorporated the uncertainties associated with the ^{15}N-enrichment factor from diets to bone collagen and the mean isotopic compositions of the animals and plants in the simulation using the SIAR model (Parnell et al., 2010; see Table 4). Following in part the approach in Bocherens et al. (2006), isotopic compositions of the animal meat and plants were estimated (Fig. 7). We considered that: the known $\delta^{13}C$ difference between muscle and bone collagen in animal is 3‰ (Bocherens and Mariotti, 2002); the isotopic enrichment factors from diet to consumer bone collagen are +5‰ for $\delta^{13}C_{bulk}$ (Bocherens and Drucker,

Table 4
TP_{AA} value and mean isotopic compositions of the Tochibara human remains and their food sources, and isotopic enrichment factors from diet to consumer bone collagen used in the comparison between models. For the calculation of the isotopic compositions of animal meat, the mean value for bear, deer, boar, serow and hare were used. Uncertainties (1σ) associated with the mean values are also shown.

	Model (I)				Model (II)				Model (III)			
Food source	TP_{AA}	1σ	TP_{AA}	1σ	$\delta^{15}N_{Glu}$	1σ	$\delta^{15}N_{Phe}$	1σ	$\delta^{13}C_{Bulk}$	1σ	$\delta^{15}N_{Bulk}$	1σ
Animal	2	0.2	2.3	0.2	6.9	0.8	6.7	0.3	−23.9	1.3	3.2	0.9
Plant	1	0.2	1	0.2	−1.0	1.4	6.3	0.5	−25.4	0.7	0.4	1.4
Δ_{d-co}[a]	–	–	–	–	8.0	1.1	0.4	0.4	5.0	–	3.4	1.1
Human (Tochibara)	2.7	–	2.7	–	11.4	–	6.8	–	−19.8	–	7.3	–
Human diet	1.7	–	1.7	–	3.4	1.1	6.4	0.4	−24.8	–	3.9	1.1

[a] Isotopic enrichment factor from diets to bone collagen.

2003), +3.4 ± 1.1 for $\delta^{15}N_{bulk}$ (Minagawa and Wada, 1984), +8.0 ± 1.1 for $\delta^{15}N_{Glu}$ and +0.4 ± 0.4 for $\delta^{15}N_{Phe}$ (Chikaraishi et al., 2011, 2009; McClelland and Montoya, 2002); isotopic compositions of plant foods for humans could be estimated from those of boars based on the analogy in diets. Both of the Models (II) and (III) suggested >50% dietary animal protein contribution in the human diet (Table 5). As shown in the box plots of the simulated proportion of food sources, the Model (II) produced the estimate more precisely than the Model (III), supporting the usefulness of the approach based on $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values (Fig. 8).

Although it is difficult to derive a simple conclusion about the Kitamura human diets based on the $\delta^{13}C$ and $\delta^{15}N$ values of bulk bone collagen, the proximities in the TP_{AA}, $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values between the Kitamura and Tochibara human remains suggest a similarity between their diets. The >50% dietary animal protein contribution (i.e. <50% dietary plant protein contribution) estimated in this study is not consistent with the previous estimate (>80% plant protein contribution) for the diet of the Kitamura humans based on $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen (Minagawa and Akazawa, 1993). The substantial difference between the estimates may be related to the uncertainties such as the ^{15}N-enrichment factor and the representative isotopic composition of the end-member food sources. For example, if we use the $\Delta^{15}N_{prey-consumer}$ of 3.0‰ as well as the $\delta^{13}C$ and $\delta^{15}N$ values presented in Minagawa and Akazawa (1993) for the human remains and the potential dietary sources at the Kitamura site, the IsoSource program (distributed by Phillips and Gregg, 2003) produces a plant protein contribution of 32–80%. Alternatively, if we assume that $\Delta^{15}N_{prey-consumer}$ is 4.0‰ and the conditions given above for the other variables apply, the same program calculates a plant protein contribution of 64–86%. Therefore, the estimation of the percentage contribution made by plant protein is very sensitive to the ^{15}N-enrichment factor between a prey and its consumer, and could vary significantly with slight changes in this factor.

As we noted in introduction, the representative isotopic composition of food sources could also affect the quantitative estimation. Minagawa and Akazawa (1993) used 4.4‰ as the representative $\delta^{15}N$ value of terrestrial animals for all prehistoric Japanese populations, while the faunal samples analyzed in this study show the lower values (3.8‰ of one boar from the Kitamura site and 3.2 ± 0.9‰ for the herbivores and omnivores from the Tochibara site). Yoneda (2012) also shows low $\delta^{15}N$ values of +3.0 ± 1.0‰ for herbivores and +3.4 ± 0.5‰ for omnivores from Tochibara site. These slight differences in representative faunal $\delta^{15}N$ values are not insignificant in the estimation of meat dependency because the difference in $\delta^{15}N$ values between terrestrial plants and animals is small, suggesting the importance to use appropriate reference materials.

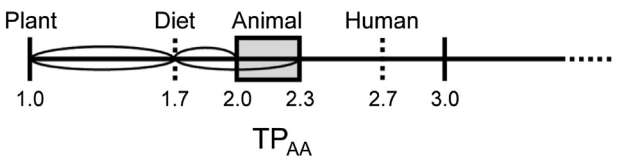

Fig. 6. Schematic illustration of the TP_{AA} for the Tochibara humans, their diets and food sources in the model (I). Dashed vertical lines indicate mean TP_{AA} values for the humans and their diets. Gray box indicates the range of TP_{AA} values for the prey animal species.

4.4. Further considerations of the diets of the inland Jomon populations

Our estimates of 54–70% for the dietary animal protein contribution in the human diets are similar to that of modern US residents (ca. 62%) and higher than that of modern Japanese (ca. 43%) (Food and Agriculture Organization of the United Nations, 2009). Hedges and Reynard (2007) estimated the average dietary animal protein contribution in "developed" and "developing" countries to be 57% and 30%, respectively, based on data published by the FAO. Considering these quantitative data, the dietary animal protein contributions for the inland Jomon populations estimated in this study would suggest their highly carnivorous dietary habits.

The large animal protein contribution to the Kitamura human diet compared with previous estimates might be related to the low frequency of caries teeth (number of caries teeth per total teeth, 0.8–1.0%, according to Shigehara, 1994, and 3.7% according to Temple, 2007). Because tooth caries are generally considered to be associated with the consumptions of carbohydrate-rich foods (Larsen, 1997; Sealy et al., 1992; Turner, 1979), this observation in combination with our estimates may imply that carbohydrate-rich tubers as well as C_4 plants did not significantly contributed to those human diets as not only protein but also energy sources.

It is unlikely that the two human populations studied here consumed very much anadromous fish, such as salmon and trout, or freshwater food resources, although the potential importance of salmon fishing for Jomon subsistence has been noted (Yamanouchi, 1964; Yoneda et al., 2004). Our results reconfirmed that the C_4-plant-based ecosystem did not contributed significantly to the diets of these humans. This is contradictory to the hypothesis that C_4 millets were cultivated in the inland central Honshu region during the Middle Jomon period (Fujimori, 1970). Although proponents of the millet cultivation in this period and region argue that grinding stones excavated from this region were used to grind such millets,

Table 5
Estimates of dietary animal protein contribution for the Tochibara humans produced by the three models.

	Model (I)	
	Min	Max
Estimates (%)	54	70
1σ	17	24
	Model (II)	Model (III)
Maximum likelihood estimates (%)	51	52
95% CI	27–80	19–100

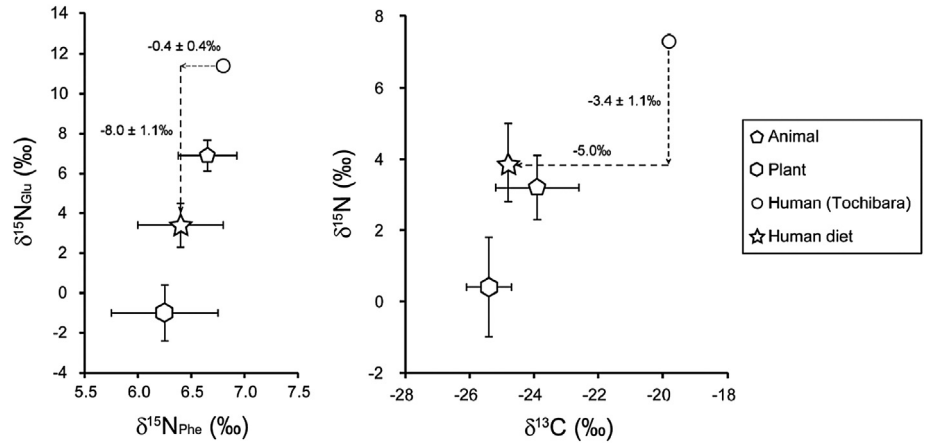

Fig. 7. Dataset used for the comparison of quantitative performance between isotope analysis of individual amino acids (model [II]) and that of bulk bone collagen (model [III]) in the evaluation of dietary animal protein contribution for the Tochibara humans. Open circle indicates mean isotopic compositions of the humans. Open pentagon and hexagon indicate mean isotopic composition of animals (except fox) and plants, respectively. Bars indicate uncertainties associated with the mean values, reflecting standard deviations (1σ) associated with the mean isotopic composition of food sources and the isotopic enrichment factors. For details about the calculation of these values, see the text and Table 4.

the function of this tool has not been fully understood. Furthermore, considering the fact that no archaeobotanical evidence of prehistoric millet cultivation has been found in the region, the presence of agriculture in the Middle Jomon period is not evidenced here. Further isotope analysis of human remains from this region may help critically evaluating these hypotheses.

4.5. Implications for future work

The preservation of archaeological human remains in the inland regions of Japan is generally very poor because of the acidic condition of the soil. However, as demonstrated in this study, archaeological samples with even poor preservation could provide reliable $\delta^{15}N$ values, at least for glutamic acid and phenylalanine. This suggests that it may be still possible to derive useful information even from organic matters with altered C/N ratio in bone or dentine. It seems that further investigations into the relationship between diagenetic effects on the $\delta^{15}N$ of bulk protein and that of individual amino acid will contribute to better understanding of diagenetic processes of bone collagen.

The clear difference in $\delta^{15}N_{Phe}$ values between terrestrial and freshwater animals demonstrated in this study has significant implications for palaeodietary research. This isotopic signature differentiates the amino acid contributions of freshwater ecosystems to human diets, and therefore has the potential to overcome a longstanding problem in dietary reconstruction based on bulk collagen isotopic analyses (problem (iii) discussed in the Introduction). By further testing the utility and robustness of this isotopic tool in modern ecosystems and at archaeological sites, it may be possible to clarify the diet source of ancient human populations with higher resolution.

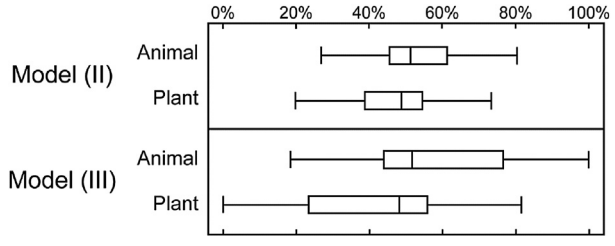

Fig. 8. Box plots of the simulated proportions of the animal and plant foods in the models (II) and (III). Boxes and whiskers indicate credibility intervals of 50% and 95%, respectively. Vertical line inside the box indicates the maximum likelihood proportion of each food source.

5. Conclusions

We reconfirmed that the two inland Jomon populations analyzed in this study were well adapted to a terrestrial C_3-plant-based ecosystem, which is most clearly suggested by the trophic position of terrestrial pure herbivores and the very convergent nitrogen isotopic composition of phenylalanine in the remains of both humans and terrestrial faunal species. The most striking suggestion in this study is the estimated trophic position of the humans (2.7 on average) that could indicate the dietary fraction contributed by animal protein of 54–70%. This estimation is much higher than previously thought.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2013.03.012.

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