## EXPRESS LETTER

# Refinement of reconstructed ancient food webs based on the nitrogen isotopic compositions of amino acids from bone collagen: A case study of archaeological herbivores from Tell Ain el-Kerkh, Syria

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We determined the stable nitrogen isotopic composition ($\delta^{15}$N) of amino acids in bone collagen from samples of three archaeological herbivores (cattle, sheep, and goats), collected from the Tell Ain el-Kerkh Neolithic site in Syria. Bulk collagen $\delta^{15}$N values exhibited significant differences between the three species (by up to 3.2‰), and were strongly correlated with those of glycine ($R^2 = 0.87$), the most abundant amino acid in bone collagen. On the other hand, the $\delta^{15}$N values of two other minor amino acids (glutamic acid and phenylalanine) in the different samples were within narrow ranges (0.9‰ and 0.5‰, respectively), and exhibited either weak or no correlation with those of bulk collagen. The trophic position estimated by the $\delta^{15}$N values of glutamic acid and phenylalanine (2.0 ± 0.1) is consistent with that of herbivores. These results suggest that the $\delta^{15}$N values of bulk bone collagen may vary among herbivores, partly on account of their differing amino acid compositions, whereas the trophic position of different herbivores is faithfully preserved in the $\delta^{15}$N values of glutamic acid and phenylalanine.

### INTRODUCTION

The stable nitrogen isotopic composition ($\delta^{15}$N) of bulk bone collagen has been widely used in archaeological studies as a chemical tool for accessing the diets and life styles of ancient animals and humans (e.g., Schoeninger and DeNiro, 1984; Yoneda et al., 2004). The approach is based on the isotopic discrimination that occurs during assimilation/dissimilation processes; on average, animal tissues are enriched in $^{15}$N by ~3.4‰ relative to their prey (e.g., DeNiro and Epstein, 1981; Minagawa and Wada, 1984).

However, in the case of Neolithic Near East, where agriculture and pastoralism originally developed, significant differences in the $\delta^{15}$N values of bulk bone collagen (>2.4‰) amongst different herbivore species from a single site have been frequently observed (e.g., Lösch et al., 2006; Pearson et al., 2007), even though the trophic position (TP) of the herbivores should be equivalent to that

of a primary consumer (i.e., TP = 2.0). For example, at Geoktchik Depe, an Iron Age site in Turkmenistan, Bocherens et al. (2005) reported that $\delta^{15}$N values in cattle were enriched by 5.0‰ relative to those in goats, a value which is greater than the ~3.4‰ difference attributed to isotopic discrimination between different trophic levels; thus, these isotopic data are not consistent with the fact that both cattle and goats are herbivores. This contradiction has generally been explained by the fact that bulk $\delta^{15}$N values reflect not only the trophic position, but also temporal and spatial variations in the isotopic composition of feed plants in the environment (Hartman, 2011) and physiological effects (e.g., the degree of nitrogen efflux to urine and feces) on $^{15}$N-enrichment in each herbivore (Sponheimer et al., 2003). These issues generate ambiguity when interpreting the isotopic data.

On the other hand, analyses of the nitrogen isotope values of amino acids have recently been applied in a number of ecological studies of modern animals (e.g., Ohkouchi et al., 2013) and in a few studies of ancient animal and human remains (e.g., Naito et al., 2010; Styring et al., 2010). This approach is based on a significant difference in the trophic isotopic discrimination of

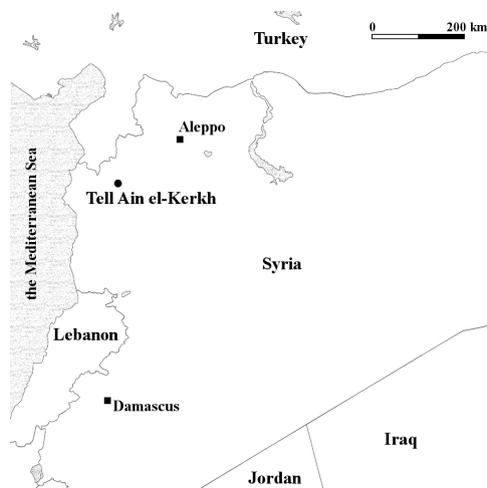*Corresponding author (e-mail: 106310@ib.k.u-tokyo.ac.jp)

*Fig. 1. Location of Tell Ain el-Kerkh.*

two common amino acids: in the transition from prey to consumer, the $\delta^{15}N$ value of glutamic acid ($\delta^{15}N_{Glu}$) increases by $+8.0 \pm 1.1‰$, while that of phenylalanine ($\delta^{15}N_{Phe}$) increases by only $+0.4 \pm 0.4‰$ (Chikaraishi *et al*., 2009). Therefore, the $\delta^{15}N$ values of phenylalanine in animals mainly reflect those of the plants upon which they feed, which in turn depend on the growth environment (e.g., temperature and precipitation). In contrast, the trophic position (TP) of animals can be estimated by differences in the $\delta^{15}N$ values of glutamic acid and phenylalanine. This method does not require information on the $\delta^{15}N$ values of primary producers as the $\delta^{15}N$ baseline of the food web.

Recently, we successfully collected bone samples of three archaeological herbivores (cattle, sheep, and goats) from the Neolithic Tell Ain el-Kerkh site in Syria. We used the herbivore samples to attempt to resolve the contradiction between the differences in the $\delta^{15}N$ values of bulk collagen in the different species. The isotopic analysis of amino acids directly verifies whether these herbivores lived in isotopically distinct environments, or were characterized by different $^{15}N$-enrichment factors. Moreover, the observed results will provide an opportunity to further evaluate the method of using amino acid $\delta^{15}N$ data for reconstructing ancient diets.

## MATERIALS AND METHODS

Tell Ain el-Kerkh is a Neolithic settlement site located 75 km southwest of Aleppo, northwest Syria (Fig. 1). At the site, remains of animals such as domesticated cattle, sheep, and goats, and several wild animals have been collected from late Neolithic layers (Tsuneki *et al*., 2006). This study examined faunal bone collagen from three domestic herbivorous animals: cattle (*Bos* sp., $n = 3$),

sheep (*Ovis* sp., $n = 2$), and goats (*Capra* sp., $n = 3$); the specimens were excavated from a site which is dated to the Pottery Neolithic period (6600–6100 BC) (Tsuneki, 2010).

Collagen samples (i.e., gelatin consisting mainly of collagen) were extracted from bones by gelatinization, following Yoneda *et al*. (2002). Approximately 0.5 g of dried bone fragments were first cleaned by brushing and ultrasonication. After removing humic and fulvic acids by soaking in 0.2 M NaOH for 8 h, the bones were washed with Milli-Q$^{TM}$ water. Cleaned and freeze-dried bone samples were ground to a fine powder. Hydroxyapatite was removed from the powdered bone by mixing with 1 M HCl in cellulose tubes. The remains were heated in Milli-Q$^{TM}$ water at 90°C for 12 h to extract the gelatin. After gelatinization, the dissolved gelatin was filtrated and freeze-dried to obtain collagen samples. The $\delta^{15}N$ values of the bulk collagen samples were determined by an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS; Flash 2000 EA coupled to a MAT 253 IRMS, Thermo Fisher Scientific). The analytical precision ($1\sigma$) based on replicate analyses of reference alanine was $<0.2‰$. The purity of the collagen samples was evaluated on the basis of the carbon and nitrogen content (%C and %N, respectively) in the extracted collagen samples. The C/N ratio was in the range of 2.9–3.6 (DeNiro, 1985) and extracted gelatin yields were >1% (Ambrose, 1993). The samples were considered as acceptable for the purposes of this study.

Amino acids were extracted from approximately 2 mg of the collagen samples by 12 M HCl hydrolysis at 110°C for 12 h, and were then derivatized for the isotopic analysis, following Chikaraishi *et al*. (2010). The hydrolyzed samples were derivatized using thionyl chloride/2-propanol (1/4, v/v) at 110°C for 2 h and subsequently using pivaloyl chloride/dichloromethane (1/4, v/v) at 110°C for 2 h. After derivatization, the derivatives of the amino acids were extracted by liquid-liquid extraction with *n*-hexane/dichloromethane (3/2, v/v) and distilled water. The $\delta^{15}N$ values of the amino acids were determined using a gas chromatograph-isotope ratio mass spectrometer (GC-IRMS; Agilent 6890GC coupled to a ThermoFinnigan Delta$^{plus}$XP IRMS via combustion at 950°C and reduction furnaces at 550°C) (Chikaraishi *et al*., 2010). The derivatives of amino acids were injected using a programmable temperature vaporizing (PTV) injector (Gerstel) into an HP Ultra-2 capillary column (length 50 m; i.d. 0.32 mm; film thickness 0.52 $\mu$m; Agilent Technologies). The carrier gas (He) flow rate was controlled using a constant flow mode at 1.4 ml min$^{-1}$. Standard mixtures of 10 amino acids (alanine, glycine, valine, leucine, norleucine, aspartic acid, methionine, glutamic acid, phenylalanine, and hydroxylproline; SI Science Co., Ltd.) with known $\delta^{15}N$ values were analyzed.

Table 1. The $\delta^{15}N$ values (mean $\pm 1\sigma$ ‰) of bulk bone collagen, glycine, glutamic acid, phenylalanine, poline, hydroxyproline, the weighted mean of the five amino acids, and the estimated trophic positions ($TP_{Glu/Phe}$) for the cattle ($n = 3$), sheep ($n = 2$), and goats ($n = 3$)

| Sample | Cattle | Sheep | Goats |
|---|---|---|---|
| Bulk collagen | $7.5 \pm 0.7$ | $6.3 \pm 0.8$ | $4.3 \pm 1.2$ |
| Glycine | $6.6 \pm 0.5$ | $4.6 \pm 0.7$ | $2.1 \pm 1.2$ |
| Glutamic acid | $9.5 \pm 0.1$ | $8.7 \pm 1.5$ | $8.4 \pm 1.0$ |
| Phenylalanine | $9.5 \pm 0.3$ | $9.0 \pm 0.4$ | $9.5 \pm 0.6$ |
| Proline | $10.5 \pm 0.6$ | $10.1 \pm 2.8$ | $9.9 \pm 1.8$ |
| Hydroxyproline | $9.3 \pm 1.0$ | $8.6 \pm 1.3$ | $7.4 \pm 0.9$ |
| Weighted mean | $8.2 \pm 0.4$ | $7.3 \pm 1.3$ | $5.4 \pm 1.5$ |
| $TP_{Glu/Phe}$ | $2.1 \pm 0.0$ | $2.1 \pm 0.1$ | $2.0 \pm 0.1$ |



Fig. 2. Relationship between the $\delta^{15}N$ values of bulk bone collagen and the estimated trophic positions ($TP_{Glu/Phe}$) of the three herbivores. The dashed line indicates a $TP_{Glu/Phe}$ value of 2.0.

The analytical precision ($1\sigma$) on replicate analyses of reference amino acids was <0.5‰ for samples including more than 2 nmol N for each amino acid.

We measured the $\delta^{15}N$ values of five amino acids (glycine, proline, glutamic acid, phenylalanine, and hydroxyproline), because these amino acids are relatively abundant in the collagen (Eastoe, 1955) and always show baseline resolution on GC-IRMS chromatograms. The trophic position ($TP_{Glu/Phe}$) was estimated from the $\delta^{15}N$ values of glutamic acid ($\delta^{15}N_{Glu}$) and phenylalanine ($\delta^{15}N_{Phe}$), using the following equation (Chikaraishi et al., 2010):

$$TP_{Glu/Phe} = [(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + 8.4)/7.6] + 1.$$

The error of the estimated trophic position is 0.20 ($1\sigma$; for details, see Chikaraishi et al., 2010). We use the equation for C3 plants-based food webs, because these animals mainly consumed C3 plants at the studied period.

## RESULTS AND DISCUSSION

The $\delta^{15}N$ values of bulk collagen were 7.5 ± 0.7‰ (mean ± $1\sigma$) for cattle, 4.3 ± 1.2‰ for goats, and 6.3 ± 0.8‰ for sheep (Table 1). The Kruskal-Wallis test to compare the $\delta^{15}N$ values of each animal suggested a significant difference ($p$-value < 0.05) in the $\delta^{15}N$ values of bulk collagen among cattle, sheep, and goats ($\chi^2 = 6.25$, $p = 0.044$). These results support previous findings. For example, it was reported from other Neolithic sites in the Near East that cattle bone collagens are enriched in $^{15}N$ relative to values in caprine animals (Lösch et al., 2006; Pearson et al., 2007). We conclude therefore that the $\delta^{15}N$ values of bulk collagen in herbivores in a closed region may exhibit a specific ordered relationship with some factor.

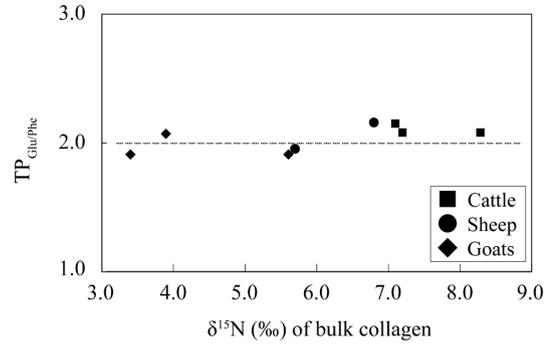We determined the nitrogen isotopic composition of various amino acids to confirm whether the herbivores exhibit large differences in the $\delta^{15}N$ values of different amino acids (Table 1). The largest variation in $\delta^{15}N$ values was observed in the $\delta^{15}N$ values of glycine in cattle (6.6 ± 0.5‰), followed by those in sheep (4.6 ± 0.7‰) and those in goats (2.1 ± 1.2‰); this pattern is similar to that observed in bulk collagen values. We conclude that the $\delta^{15}N$ values of glycine are significantly different among cattle, sheep, and goats ($\chi^2 = 6.25$, $p = 0.044$). In contrast, the $\delta^{15}N$ values of other amino acids, such as glutamic acid and phenylalanine, exhibit low variability (7.4–9.4‰ and 9.6–10.1‰, respectively). Proline and hydroxyproline show overlapping $\delta^{15}N$ ranges in the different herbivores, suggesting no significant differences in the $\delta^{15}N$ values of these animals. Thus, not all amino acids exhibit large differences in $\delta^{15}N$ values in the different herbivores.

Despite the large differences in the $\delta^{15}N$ values of bulk collagen in different herbivores, the $TP_{Glu/Phe}$ values of the herbivores were similar, and were estimated to be 2.1 ± 0.0 for cattle, 2.0 ± 0.1 for sheep, and 2.1 ± 0.1 for goats (Fig. 2); this pattern is consistent with the expected trophic position of these herbivores as certain primary consumers (i.e., TP = 2.0). Moreover, the $\delta^{15}N$ values of phenylalanine were similar amongst these herbivores (Table 1). Because the $\delta^{15}N$ values of phenylalanine show little variation with respect to the trophic level of animals (Chikaraishi et al., 2010), the results indicate that these herbivores likely consumed feeds with similar $\delta^{15}N$ values. This finding is consistent with the observation that these herbivores were domestic animals raised around a settlement. Thus, small variations observed in the $\delta^{15}N$ values of phenylalanine among cattle, sheep, and goats suggest that large differences in the $\delta^{15}N$ values of bulk collagen among herbivores are not caused by isotopically different feed sources.

The relative abundances of glycine, proline, glutamic acid, phenylalanine, and hydroxyproline were 26.2, 10.1,
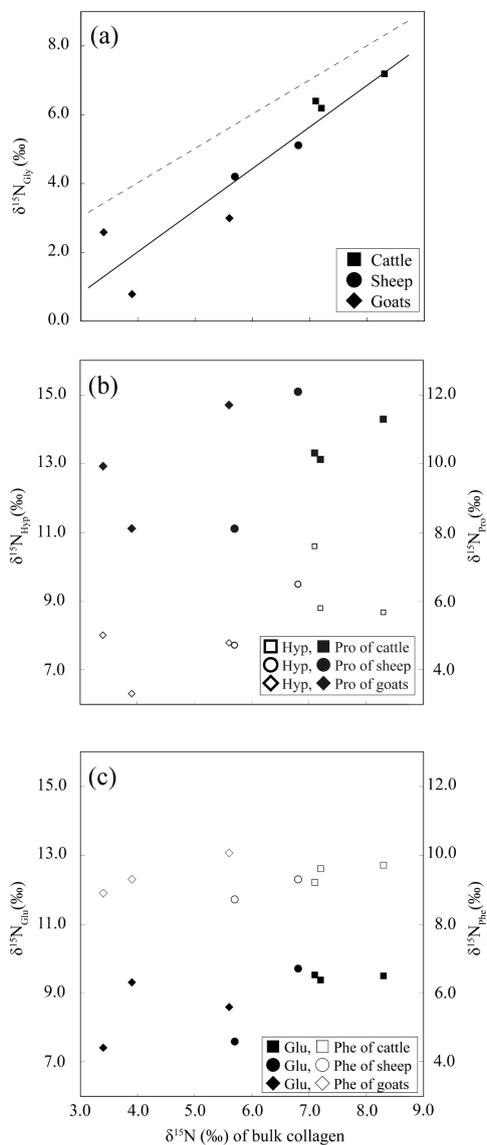
*Fig. 3. Relationships between the $\delta^{15}N$ values of bulk bone collagen and (a) glycine (the solid line shows the result of a linear regression of $\delta^{15}N_{bulk}$ vs. $\delta^{15}N_{Gly}$, and the dashed line indicates a 1:1 relationship between the two variables), (b) hydroxyproline (Hyp) and proline (Pro), and (c) Glutamic acid (Glu) and phenylalanine (Phe) of the three herbivores.*

5.9, 1.2, and 8.2 wt%, respectively, and these amino acids account for more than a half of the nitrogen in bone collagen (Eastoe, 1955). The difference between the $\delta^{15}N$ value of bulk collagen and that of the weighted mean of the five amino acids was 0.7 ± 0.7‰ (Table 1), and these values showed a positive correlation (slope = 0.86, $R^2$ = 0.84). However, among these five amino acids, the strongest positive correlation was observed between the $\delta^{15}N$ values of bulk collagen and glycine (slope = 1.2, $R^2$ = 0.87; Fig. 3a). On the other hand, the $\delta^{15}N$ values of pro-

line and hydroxyproline were only weakly correlated with that of bulk collagen ($R^2$ = 0.27 for proline and $R^2$ = 0.46 for hydroxy-proline; Fig. 3b). The $\delta^{15}N$ values of glutamic acid were weakly correlated with those of bulk collagen ($R^2$ = 0.40; Fig. 3c), whereas the $\delta^{15}N$ values of phenyla-lanine showed no correlation with those of bulk collagen ($R^2$ = 0.07; Fig. 3c) and those of glycine ($R^2$ = 0.00). These patterns explain the peculiar differences in the $\delta^{15}N$ values of bulk bone collagen among the herbivores. Because glycine is the most abundant amino acid, normally accounting for 1/4 to 1/3 of the amino acids in bone collagen (e.g., Eastoe, 1955), the $\delta^{15}N$ value of bulk bone collagen could be strongly influenced by that of glycine. Although specific mechanisms controlling the $\delta^{15}N$ values of glycine from animals are unclear at this stage, we consider that the $\delta^{15}N$ value of glycine may mirror differences in the physiological pathway of glycine among these herbivores. Thus, the $\delta^{15}N$ values of bulk bone collagen from archaeological herbivores probably reflect differences in the ^{15}N-enrichment factor among the herbivores rather than $\delta^{15}N$ variations of feed plants in the foraging area. The isotopic signatures of feed plants in several growth area for the herbivores are well preserved in the $\delta^{15}N$ values of phenylalanine, even if the $\delta^{15}N$ values of bulk collagen and glycine are highly variable.

## CONCLUSIONS

Studies of the diets of ancient herbivores have revealed differences in the $\delta^{15}N$ values of bulk bone collagen among different herbivore species, especially in animals consuming mainly terrestrial resources. Although further studies need for a large number of sample sets from different sites, the observed data in Tell Ain el-Kerkh demonstrated that a peculiar difference in the $\delta^{15}N$ values of bulk collagen among archaeological herbivores can be ascribed mainly to variations in the ^{15}N-enrichment factor of several specific amino acids, such as glycine, rather than to isotopic variations of feed plants in the growth environment. In contrast, the $\delta^{15}N$ values of relatively minor amino acids, such as glutamic acid and phenyla-lanine, well preserved the information of the trophic position of the animals. Thus, the $\delta^{15}N$ values of bone collagen are influenced by at least three different factors: physiological, environmental (i.e., by feed plant $\delta^{15}N$ values), and ecological information (i.e., the trophic position of the animals). Moreover, the nitrogen isotopic composition of the different amino acids can resolve the relative contributions of each of these factors. The nitrogen isotopic composition of individual amino acids enhances the resolution and reduces the uncertainty in assessing the diets and life styles of ancient humans and animals. Furthermore, the differences in the $\delta^{15}N$ values of bulk collagen and the ^{15}N-enrichment factor among

mammal herbivores may be useful as potential tracers in the terrestrial food web.

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