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The effect of trophic level on individual amino acid δ15N values in a terrestrial ruminant food web

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ABSTRACT
Bulk collagen δ^{15}N analysis is widely used to investigate past diet and trophic positions, but these values average the δ^{15}N values of the constituent amino acids. Compound–specific isotope analysis of amino acids (AAs) can help elucidate the complex metabolic effects underpinning bulk δ^{15}N values. Although trophic level effects on individual AA δ^{15}N values have been investigated in aquatic and terrestrial invertebrate food webs, most archaeological applications involve terrestrial herbivores, hence a greater understanding of these effects between diet and consumer in this food chain is required. The North Wyke Farm Platform provided baseline nitrogen isotope information for cattle grazing on a *Lolium perenne*-dominated pasture. Bulk dentine δ^{15}N values show a shift expected for a one trophic level increase, but obscure insight into the underlying metabolic processes that cause this change in value. However, determination of AA δ^{15}N values of hydrolysable plant protein and cattle tooth dentine clarifies the trophic effect on consumer AA δ^{15}N values. The observed trophic shift in the studied system is different from previously studied food webs, with a trophic enrichment factor, based on the δ^{15}N values of glutamate and phenylalanine, of 4.0‰ compared to 7.6‰ commonly used in ecological and archaeological studies. This emphasises the need to understand the trophic shifts in the particular food web being investigated in order to apply isotopic investigations in archaeological contexts.

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Nitrogen isotopes; controlled diet; dentine collagen; *Lolium perenne*; cattle

Introduction

The nitrogen isotopic signature of an organism's tissues is related to the δ^{15}N value of its diet, with consumer ^{15}N generally being enriched by ca. 3-5‰ relative to diet (DeNiro and Epstein 1981, Minagawa and Wada 1984, Schoeninger and DeNiro 1984). The enrichment is believed to result from nitrogen isotopic fractionations involving the transamination and deamination of nitrogen-containing biosynthetic intermediates in the consumer. The diet-consumer enrichment has been used to determine the trophic position of organisms within a food web: if the δ^{15}N values of the primary producers at the base of the food web and of the consumer are known, then the trophic level can be calculated based on the number of diet-consumer fractionations between the consumer and producer δ^{15}N values. Although the average trophic Δ^{15}N value, i.e. the difference between diet and consumer δ^{15}N values, of 3.4‰ is often used for trophic level calculations, a wide range of trophic enrichment Δ^{15}N values have been documented, ranging from -2.1‰ to + 9.2‰ (DeNiro and Epstein 1981, Post 2002, McCutchan et al. 2003, Vanderklift and Ponsard 2003, Spence and Rosenheim 2005, Caut et al. 2009).

Interpretations of bulk isotopic values in ecology and archaeology may be hindered by an incomplete understanding of the complex biochemical pathways and mechanisms which control nitrogen stable isotope ratios. For example, manuring of crops consumed by prehistoric humans or their animals may lead to an apparent trophic level effect (Bogaard et al. 2007). Aridity (Heaton 1987) and nutritional stress (Hobson et al. 1993) can also lead to changes in nitrogen isotope ratios. Investigation of nitrogen stable isotope values at the individual amino acid (AA) level can improve understanding of the isotope signals of various nitrogen-containing biomolecules, and allows access to stable isotope information inaccessible to bulk methods. For example, manuring has been shown to cause a consistent increase in δ^{15}N values across all AAs (Styring et al. 2014), which distinguishes this from trophic level effects where δ^{15}N values are seen to vary much more widely at the AA level, reflecting isotopic fractionations at specific points in protein biosynthetic pathways (e.g. Hare et al. 1991, McClelland and Montoya 2002).

Additionally, differences in animal physiology can have a significant effect on AA δ^{15}N values. For example, while excess nitrogen in mammals is mainly

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excreted as urea, some reptiles such as tortoises can excrete both urea and uric acid. The different metabolic pathways involved in producing uric acid are thought to be the cause of the lower glutamate $\delta^{15}N$ values observed in these animals compared to mammals (Styring et al. 2010). A similar difference has been seen in penguins, which can directly excrete excess N as ammonia as well as uric acid (McMahon et al. 2015).

McClelland and Montoya (2002) determined $\delta^{15}N$ values of AAs from organisms in a marine food web. They observed that for some AAs, such as glutamic acid (Glu) and aspartic acid (Asp) – the 'trophic group' AAs – an increase in trophic level leads to a large enrichment in their $\delta^{15}N$ values. For others, such as phenylalanine (Phe), glycine (Gly), serine (Ser) and tyrosine (Tyr) – the 'source group' AAs – $\delta^{15}N$ values remain essentially unchanged (Popp et al. 2007). Similar findings have been observed for terrestrial food webs (Chikaraishi et al. 2011), although the AAs in each group vary (Figure 1).

The differences in nitrogen isotope fractionations between different classes of organisms discussed above are believed to be due to differences in the metabolic pathways of these AAs (see O'Connell 2017). Transamination reactions, which involve breaking and formation of C-N bonds and therefore introduce kinetic isotope effects, result in the newly formed AAs being depleted in ^{15}N, while the remaining AA pool will become enriched. The $\delta^{15}N$ values of AAs involved in a greater number of transamination reactions will be more affected. For example, Phe is an essential AA in mammals, and is converted to Tyr as the major initial metabolic step (Bender 1975), a process that does not involve breaking or making a C-N bond. On the other hand, Glu plays a central role in AA biosynthesis, as the major initial metabolic step involves a transamination reaction, with the amino group donated to α–ketoacids to form other amino acids. Glu is also involved in nitrogen excretion, where deamination of Glu is the first step of urea formation (Bender 1975).

These differences between source group and trophic group amino acids have been used in trophic level studies to estimate the trophic positions of organisms using the following equation:

$$TL_{Glx-Phe} = \frac{\Delta^{15}N_{Glx-Phe} + \beta}{TEF} + 1\% \qquad \text{(Eq.1)}$$

Where $\Delta^{15}N_{Glx\text{-}Phe}$ is the difference in $\delta^{15}N$ values of Glx and Phe in the tissue being studied, β is the difference in $\delta^{15}N$ values of Glx and Phe in the primary producers of the food chain, and TEF (the trophic enrichment factor, also known as the trophic discrimination factor, TDF) is the ^{15}N-enrichment of Glx relative to Phe with increasing trophic level. Based on AA $\delta^{15}N$ values in marine photoautotrophs and consumers, Chikaraishi et al. (2009) observed little variability in the determined β value of -3.4‰ and TEF of 7.6‰. However, these values are not universal across all taxa, or between different trophic level shifts. The value of β depends upon the ecosystem being studied, with β values of + 8.4‰ in C_3-based terrestrial systems, or -0.4‰ in C_4 systems, determined by Chikaraishi et al. (2010). Although these values were also found to correctly predict the trophic position of plants and insects in a terrestrial food web (Chikaraishi et al. 2011), studies of carnivorous aquatic species, such as

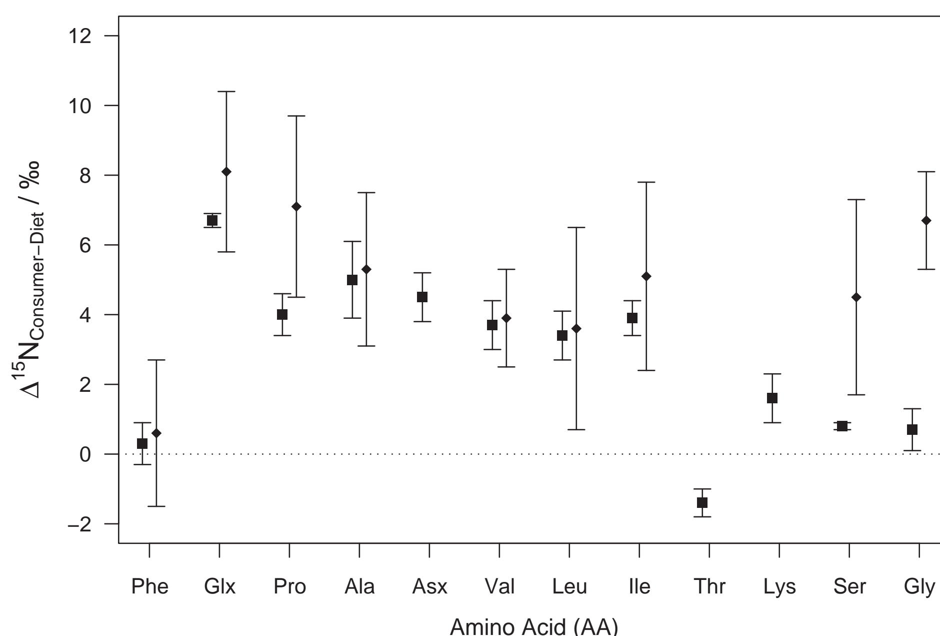

Figure 1. Differences between consumer and diet amino acid $\delta^{15}N$ values in an aquatic (squares, n = 2; McClelland and Montoya 2002), and terrestrial invertebrate (diamonds, n = 4; Chikaraishi et al. 2011), food webs. Error bars represent ± 1 standard deviation of multiple samples; $\Delta^{15}N_{Consumer-Diet} = \delta^{15}N_{Consumer} - \delta^{15}N_{Diet}$.

harbour seals (Germain et al. 2013), fishes (Hoen et al. 2014), and penguins (McMahon et al. 2015), revealed markedly lower TEFs of between 2 and 5‰. TEF values may also be affected by diet quality and dietary protein content, as higher protein diets can lead to lower TEFs (Chikaraishi et al. 2015, Nielsen et al. 2015, McMahon and McCarthy 2016).

While to date these sorts of trophic level studies, involving both the consumers and their diet, have been performed in aquatic food webs (e.g. McClelland and Montoya 2002, Pakhomov et al. 2004, Chikaraishi et al. 2007, Chikaraishi et al. 2009, Hoen et al. 2014), microbial communities (Steffan et al. 2015), and terrestrial invertebrate food webs (Chikaraishi et al. 2011, and see Ohkouchi et al. 2017 for a recent review of AA nitrogen isotope sudies), there have been very few studies into terrestrial vertebrate food webs, which include human infants (Romek et al. 2013), mice (Steffan et al. 2015), and bears (Nakashita et al. 2011). No studies have yet been performed for ecologically and archaeologically important ruminant herbivore food webs. Critically, ruminants have markedly different digestive systems to previously studied species, as well as different macronutrient contributions to their diet. Ingested protein can be classified as rumen undegradable protein, which passes through the rumen and is digested in the abomasum, and rumen degradable protein, which is degraded to ammonium via amino acids in the rumen. The latter is used for protein biosynthesis by rumen microbes, although some rumen microbes can directly utilise amino acids and peptides. These proteins are later digested, providing a protein source for the host ruminant (Figure 2). Consequently, ruminants have no essential amino acid requirements through microbial amino acid anabolism. Approximately 50-80% of ruminant protein is obtained from rumen microbial protein (Ørskov 1982), and therefore the dietary AAs are likely to undergo different trophic enrichment effects than non-ruminants which do not utilise microbial protein from the rumen.

The lack of information regarding the nitrogen tropic level effect in these common domestic herbivores constitutes a major gap in our knowledge of the isotope systematic of primary producers at the base of the human terrestrial food chain. Such information is vital to investigations of ancient food webs involving humans and their major food animals. In order to redress this, herein, we investigate the trophic level effect in ruminant animals for the first time at the amino acid level. For our investigation, we sampled the tissues of steers of *Bos taurus* grazing within a controlled agricultural experiment, wherein the animals are raised solely on pastures and fodders of defined plant composition and origin, and the plants on which these animals were fed, thereby offering a unique opportunity to study the nitrogen isotopic interrelationships between animal diet and tissues at the AA level.

Materials and Methods

Plant and animal tissue sampling strategy

Plants and animal (*Bos taurus*) tissues for this study were sourced from the BBSRC National Capability - North Wyke Farm Platform, Rothamsted Research, Devon, UK (www.rothamsted.ac.uk/north-wyke-farm-platform). This is a highly controlled farm-scale, long term experiment, which aims to address sustainable ruminant livestock production systems through a unique level of instrumentation and measurement. The farm platform comprises of three 20 ha

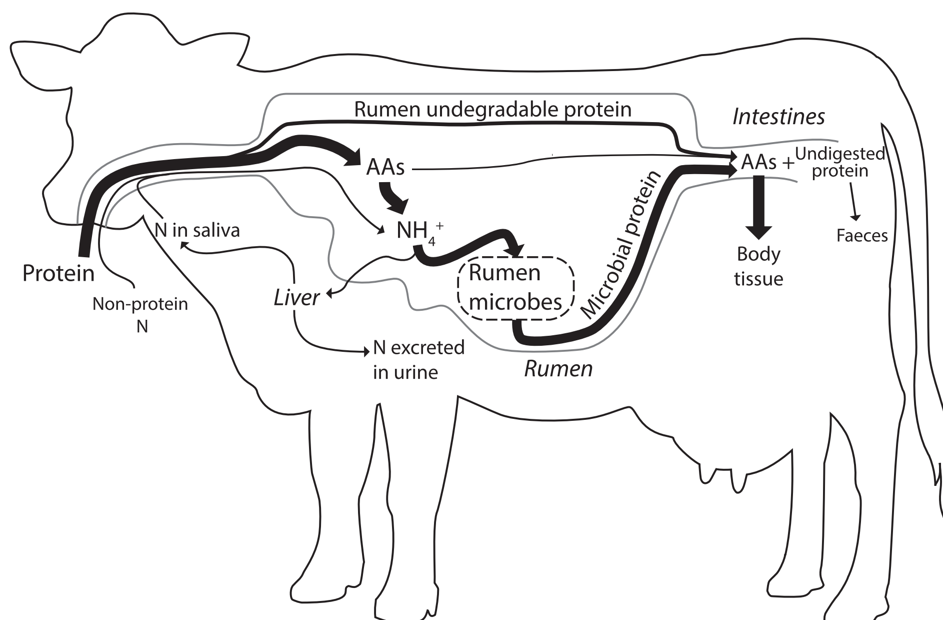

Figure 2. Nitrogen metabolism in ruminants, adapted from Satter and Roffler (1975).

self-contained grassland farms: (i) a 'control' permanent pasture, (ii) a grass: white clover pasture, and (iii) a pasture reseeded with innovative grass species, selected for their nutritional or environmental qualities. Each farm pasture system is managed as beef cattle (30 per system) and sheep (75 ewes and their lambs per system) enterprises with a high degree of control over inputs (fertiliser, labour, veterinary drugs etc.) and assessment of farm nutrient fluxes through hydrologically isolated field catchments to measure loss into water (at a high temporal resolution; every 15 min) and Eddy covariance towers to assess air quality. The key concept of the Farm Platform is to determine management pathways for sustainable ruminant livestock production through the assessment of social (meat quality, animal welfare, biodiversity), economic (input costs, livestock production) and environmental (loss of nutrients to air and water) metrics and their associated trade-offs.

All the plant and animal specimens for this study were collected from the 'control' permanent pasture, which was dominated by perennial ryegrass (*Lolium perenne*). Eight hand-plucked specimens of *L. perenne* were collected in October 2015. These samples were collected from points along transects across three fields comprising the 20 ha control pasture, using a 5 cm diameter corer, and the foliar tissue was separated from the roots and soil. The specimens were analysed individually to determine the natural variability in plant δ^{15}N values.

The North Wyke Farm Platform recruits 30 weaned Hereford-Friesian × Charolais cattle (balanced number of heifers and steers) to each of the three farm pasture systems each year from its commercial farm. The cattle sampled for this study had been grazed only in the control pasture as described above. Therefore, these cattle have a well-defined, natural diet with no formulated animal feeds or pellets, which would otherwise affect the isotopic composition of consumers. The left mandibular third molars (M3) of five steers were collected after the animals were slaughtered at 22 months of age (Confirmation Class R and Fat Class 4L). Formation of these teeth begins at ca. 12 month of age, and the crown is complete by *ca.* 24 months (Brown et al. 1960). Coming up to slaughter weight gain is mainly in the form of fat (known as finishing, to meet fat class and confirmation grades for the market), rather than rapid growth of muscle and bone, and therefore there will be no high turnover of nitrogen, as in the case of rapidly growing infant animals, during the period of formation of these teeth. To ensure there is no variation in the isotopic composition across this period, three of these teeth were sequentially sampled at six points along the growth axis of each tooth. Once formed, dentine in teeth is not remodelled, and therefore the collagen preserves the isotopic composition of the period of formation.

Analytical procedures

Plant leaves and stems were washed with double-distilled water (DDW), lyophilised and ground to a powder. Lipids were extracted from the powdered samples with chloroform/methanol (2:1 *v/v*, 2 × 3 mL) by ultrasonication. Dentine from the left mandibular third molar was collected as a powder, using a modelling drill with a diamond abrasive drill bit.

Hydrolysis of proteins and derivatisation of AAs

AA *N*-acetyl isopropyl (NAIP) ester derivatives were prepared according to established protocols (Corr et al. 2007, Styring et al. 2012). Briefly, norleucine was added as an internal standard to ca. 10 mg of dentine or 25 mg of freeze-dried plant material. For dentine, demineralisation of the inorganic fraction and hydrolysis of the collagen was achieved in one step by heating with acid (6 M HCl, 5 mL; 100°C, 24 h). The solutions were blown to dryness under nitrogen. Plant tissues were hydrolysed (6 M HCl, 5 mL; 100°C, 24 h) and allowed to cool before centrifugation (1700 ×g; 10 min). The hydrolysates were transferred to clean culture tubes with 0.1 M HCl (2 mL), and the solutions blown to dryness under nitrogen.

AAs were separated from other compounds using Dowex 50WX8 ion-exchange resin. The AAs were converted to their isopropyl esters by addition of a mixture of isopropanol and acetyl chloride (4:1 *v/v*, 1 mL; 100°C, 1 h). Reagents were evaporated under a gentle stream of N_2 (40°C). AA isopropyl esters were then treated with a mixture of acetone, triethylamine and acetic anhydride (5:2:1 v/v/v, 1 mL; 60°C, 10 min). Reagents were removed under a gentle stream of N_2 at room temperature, then 1 mL saturated NaCl solution added, and NAIP esters extracted into ethyl acetate (3 × 3 mL). The solvent was evaporated under a gentle stream of N_2 at room temperature. AA NAIP ester derivatives were redissolved in ethyl acetate for analysis.

Asparagine and glutamine are converted into aspartic acid and glutamic acid respectively during hydrolysis. The δ^{15}N value of Asx therefore combines the N of aspartate and the amino N of asparagine, while the δ^{15}N value of Glx is a mean of the N of glutamate and the amino N of glutamine.

Instrumental analyses

AAs were identified by GC-FID by comparison with AA standards, and quantified by comparison with a known amount of norleucine internal standard. Their δ^{15}N values were determined by GC-C-IRMS as described in Styring et al. (2012) with a modified GC method, using DB-35 capillary column (30 m × 0.32 mm internal diameter; 0.5 µm film thickness;

Agilent Technologies, UK) and the oven temperature of the GC held at 40°C for 5 min before programming at 15°C min^{-1} to 120°C, then 3°C min^{-1} to 180°C, then 1.5°C min^{-1} to 210°C and finally 5°C min^{-1} to 270°C and held for 1 min. A Nafion drier removed water and a cryogenic trap removed CO_2 from the oxidised and reduced sample. Isotopic compositions are expressed using the delta scale as follows: $\delta^{15}N = R_{sample} / R_{standard} - 1$, where R is the $^{15}N/^{14}N$ ratio, and the standard is atmospheric N_2 (AIR). All $\delta^{15}N$ values are reported relative to reference N_2 of known isotopic composition, introduced directly into the ion source in four pulses at the start and end of each run. Each reported $\delta^{15}N$ value is the mean of triplicate determinations. A standard mixture of AAs of known $\delta^{15}N$ values was analysed every three runs to ensure acceptable instrument performance.

Bulk $^{15}N/^{14}N$ analysis was performed by sample combustion in a Flash 112 elemental analyser (Thermo Quest, Milan) linked under continuous flow with a $Delta_{plus}XP$ mass spectrometer (Thermo-Finnigan, Bremen). Isotope ratios were calculated as $\delta^{15}N$ versus atmospheric N_2 by comparison with standards calibrated against IAEA-N-1 and N-2. The precision (1σ) among replicates of a quality control standard was 0.3‰ for $\delta^{15}N$ analysed in 9 separate runs.

Results

Bulk $\delta^{15}N$ values were determined for plant tissues and dentine, and were shown to range from 3.1‰ to 7.8‰ for the former, and 9.3‰ to 10.0‰ for the latter. The variability of the bulk tissue $\delta^{15}N$ values between samples were of the same magnitude as in those of the individual AAs (see below), with a standard deviation of 1.8‰ for bulk plant tissue $\delta^{15}N$ values, and 0.3‰ for bulk dentine $\delta^{15}N$ values.

Typical gas chromatograms for plant protein and collagen are shown in Figure 3. These match well with the expected AA distributions, based on the total hydrolysable amino acid content of *Lolium perenne* (Yeoh and Watson 1982), and of type I collagen (Eastoe 1955) and *Bos taurus* genome predictions (Table 1). The nitrogen isotopic compositions of 12 AAs from plant protein and 13 AAs from collagen were determined by GC-C-IRMS and are given in Table 2. These AAs represent 90% of collagen AAs in cattle, and 85% of total hydrolysable AAs in *Lolium perenne*, accounting for 75% and 74% of AA nitrogen in the plants and animals, respectively. Although Hyp accounts for about 11% of AAs in collagen, it is synthesised as a post-translational modification of Pro, leading to very similar $\delta^{15}N$ values in collagen Pro and Hyp. Hyp is present at very low abundance in plants.

In the figures AAs are ordered according to their metabolic relationships in plants, as described in Styring, Fraser et al. (2014). Phe has a distinct metabolic pathway from the other AAs; Glx and Pro are closely related since the amino group of Pro comes from Glu; the amino group of Ala comes from Glu or γ-aminobutyric acid; the N from Asx can be exchanged with

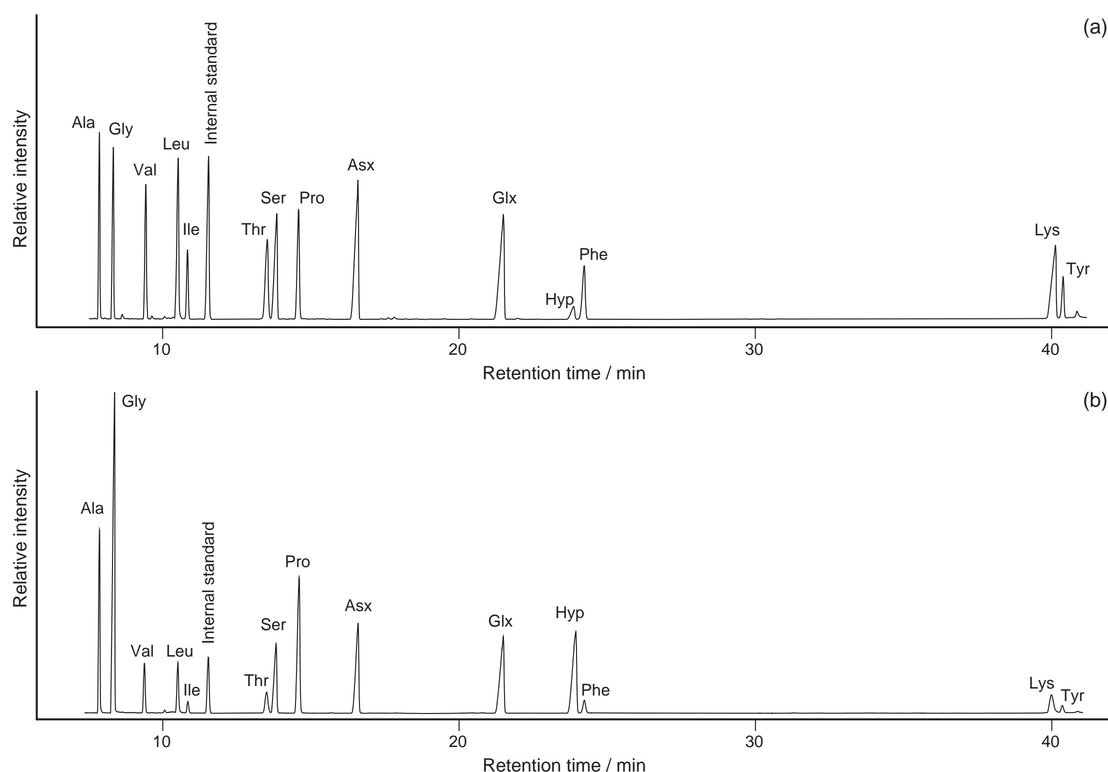

Figure 3. Typical gas chromatograms for (a) plant protein AAs and (b) collagen AAs, showing the different AA distributions between diet and consumer tissues.

Table 1. AA concentrations for plant protein and dentine collagen as percentages of total hydrolysable AAs.

	% total hydrolysable AA													
Sample	Phe	Tyr	Glx	Pro	Hyp	Ala	Asx	Val	Leu	Ile	Thr	Lys	Ser	Gly
Plants	4.5	2.9	14.6	7.0	0.2	9.3	12.2	6.6	9.8	2.7	7.4	9.3	6.0	7.4
	4.2	2.7	15.0	7.2	0.4	8.9	12.3	6.9	9.6	2.6	6.9	9.7	6.4	7.2
	4.1	2.4	16.0	7.1	0.3	9.1	12.5	6.8	9.4	2.6	7.1	8.9	6.6	7.3
	4.2	2.7	13.7	7.2	0.3	9.1	11.8	7.2	9.5	2.5	7.1	9.9	6.6	8.2
	4.3	2.8	15.2	6.9	0.3	8.6	12.3	6.8	9.7	2.7	6.7	10.3	6.4	7.1
	4.1	2.5	14.0	7.1	0.3	9.7	11.9	7.0	9.3	2.6	7.9	8.7	6.8	8.0
	4.5	2.8	14.5	6.8	0.2	9.4	13.6	6.5	9.5	2.5	6.8	8.7	6.2	7.9
	4.1	2.5	14.8	6.8	0.2	9.2	13.1	6.3	9.4	2.4	7.7	9.7	6.4	7.3
Mean	**4.3**	**2.7**	**14.7**	**7.0**	**0.3**	**9.2**	**12.5**	**6.8**	**9.5**	**2.6**	**7.2**	**9.4**	**6.4**	**7.5**
	(0.2)	**(0.2)**	**(0.7)**	**(0.2)**	**(0.1)**	**(0.3)**	**(0.6)**	**(0.3)**	**(0.2)**	**(0.1)**	**(0.4)**	**(0.6)**	**(0.2)**	**(0.4)**
Dentine collagen	1.2	0.7	11.7	13.1	10.2	9.5	8.9	4.9	3.6	0.8	3.0	3.6	6.3	22.5
	1.1	0.6	11.8	13.4	10.2	9.8	8.9	4.2	3.3	0.7	3.1	3.7	6.4	22.9
	1.1	0.5	11.9	14.0	10.2	10.2	8.8	3.6	3.0	0.5	2.6	3.7	6.3	23.7
	0.9	0.4	11.0	13.4	11.4	10.9	8.9	3.6	2.7	0.6	2.5	2.4	6.2	24.9
	0.9	0.5	11.6	13.6	12.1	9.2	9.6	3.2	2.8	0.6	2.7	2.3	6.5	24.3
Mean	**1.1**	**0.5**	**11.6**	**13.5**	**10.8**	**9.9**	**9.0**	**3.9**	**3.1**	**0.6**	**2.8**	**3.2**	**6.3**	**23.7**
	(0.1)	**(0.1)**	**(0.3)**	**(0.3)**	**(0.8)**	**(0.6)**	**(0.3)**	**(0.6)**	**(0.3)**	**(0.1)**	**(0.2)**	**(0.6)**	**(0.1)**	**(0.9)**

Note: Standard deviations are displayed in brackets.

many AAs, including Glu and Ala; Val, Leu and Ile are branched-chain AAs; and Gly and Ser can be biosynthesised from each other.

The plant AA $\delta^{15}N$ values varied from -3.1‰ for Gly to 13.8‰ for Phe, reflecting the isotopic fractionations caused by the differences in individual AA routing and metabolism (Figure 4(a)). A similarly wide range in collagen AA $\delta^{15}N$ values was observed, from -4.8‰ for Thr to 14.5‰ for Leu. Variability in $\delta^{15}N$ values between individual plant samples was larger than between collagen, with the standard deviation across the 8 samples ranging from 1.3‰ for Lys to 2.2‰ for Ile, while standard deviations of collagen AAs range from 0.3‰ to 1.1‰. Trophic enrichment in $\delta^{15}N$ values from plant protein AAs to collagen ($\Delta^{15}N_{consumer\text{-}diet}$) vary for each AA, ranging from -6.9‰ to 10.3‰ (Table 2), and are displayed in Figure 4 (b). Phe and Lys have $\Delta^{15}N_{consumer\text{-}diet}$ values of 1.4 ± 1.6‰ and 0.2 ± 1.4‰ respectively. These values are within one standard deviation of 0‰, and therefore Phe and Lys fit their classification as source group AAs. All others showed a trophic enrichment to some extent, except threonine, which shows a consumer $\delta^{15}N$ value 6.9‰ depleted relative to diet.

The sequentially sampled teeth show little variation in AA $\delta^{15}N$ values across the growth axis of the teeth, with a maximum standard deviation of 1.1‰ for Gly, and standard deviations for $\Delta^{15}N_{Glx\text{-}Phe}$ below 0.5‰ (Figure 5).

Discussion

The relatively large standard deviations about the mean plant bulk $\delta^{15}N$ values and individual AA $\delta^{15}N$ values reported here, of *ca.* 1.6‰ for each AA, reflect the natural variations in nitrogen isotope values

Table 2. AA and bulk tissue $\delta^{15}N$ values for plant protein and dentine collagen, where TL is the trophic level calculated by Eq. 1, using the previously published values of $\beta = +8.4‰$ and TEF = +7.6‰, and $\Delta^{15}N_{Consumer-Diet} = \delta^{15}N_{Consumer} - \delta^{15}N_{Diet}$.

	$\delta^{15}N_{AA}$ (‰)													$\delta^{15}N_{Bulk}$	TL
Sample	Phe	Glx	Pro	Hyp	Ala	Asx	Val	Leu	Ile	Thr	Lys	Ser	Gly	(‰)	
Plants	11.5	6.8	6.8	n.d.	5.3	7.5	6.8	2.9	5.4	6.1	3.8	1.9	-0.1	4.4	1.5
	13.0	8.9	8.7	n.d.	6.9	8.8	7.5	3.6	5.4	5.1	4.9	0.2	-0.4	n.d.	1.6
	10.8	4.7	5.5	n.d.	4.2	6.1	5.5	1.0	2.4	2.8	2.8	-2.5	-2.6	3.1	1.3
	8.4	4.9	4.7	n.d.	4.0	6.2	5.6	1.1	3.5	3.3	2.6	-1.5	-3.1	3.1	1.6
	13.8	9.6	11.1	n.d.	8.8	11.1	10.0	6.7	10.0	7.2	6.4	2.7	2.8	7.8	1.6
	10.1	6.2	5.8	n.d.	5.0	7.2	6.1	2.6	4.8	4.6	3.1	-0.4	-1.5	4.1	1.6
	10.5	6.9	6.6	n.d.	5.8	7.6	6.9	3.1	5.6	4.7	4.3	0.0	-0.6	5.0	1.6
	11.4	5.4	7.0	n.d.	4.5	7.2	5.7	2.8	n.d.	2.7	2.0	-0.9	-2.2	n.d.	1.3
Mean	**11.2**	**6.7**	**7.0**	**n.d.**	**5.6**	**7.7**	**6.8**	**3.0**	**5.3**	**4.6**	**3.7**	**-0.1**	**-1.0**	**4.6**	**1.5**
	(1.6)	**(1.7)**	**(1.9)**		**(1.5)**	**(1.5)**	**(1.4)**	**(1.7)**	**(2.2)**	**(1.5)**	**(1.3)**	**(1.6)**	**(1.8)**	**(1.8)**	
Dentine collagen	12.2	11.6	10.8	10.6	8.4	10.1	9.4	9.6	9.6	-1.8	3.8	6.6	8.9	9.3	2.0
	13.0	11.9	10.6	11.0	9.1	10.7	10.8	11.3	11.0	-1.6	4.7	7.3	9.2	9.3	2.0
	12.8	12.4	10.7	11.0	9.0	11.3	11.6	10.8	10.7	-1.0	3.6	7.7	9.7	9.6	2.1
	12.4	11.9	10.0	10.4	9.2	10.6	12.4	11.4	10.9	-4.8	3.3	7.3	8.3	10.0	2.0
	12.7	12.3	11.0	11.2	10.2	10.9	9.9	9.5	8.6	-2.6	4.0	6.7	10.6	9.5	2.1
Mean	**12.6**	**12.0**	**10.6**	**10.8**	**9.2**	**10.7**	**10.8**	**10.5**	**10.2**	**-2.4**	**3.9**	**7.1**	**9.3**	**9.5**	**2.0**
	(0.3)	**(0.3)**	**(0.3)**	**(0.3)**	**(0.6)**	**(0.4)**	**(1.1)**	**(0.8)**	**(0.9)**	**(1.3)**	**(0.5)**	**(0.4)**	**(1.6)**	**(0.3)**	
$\Delta^{15}N_{Consumer\text{-}Diet}$	1.4	5.4	3.6	n.d.	3.6	3.0	4.1	7.5	4.9	-6.9	0.2	7.2	10.3	4.9	

Note: Standard deviations are displayed in brackets. n.d. = not determined.

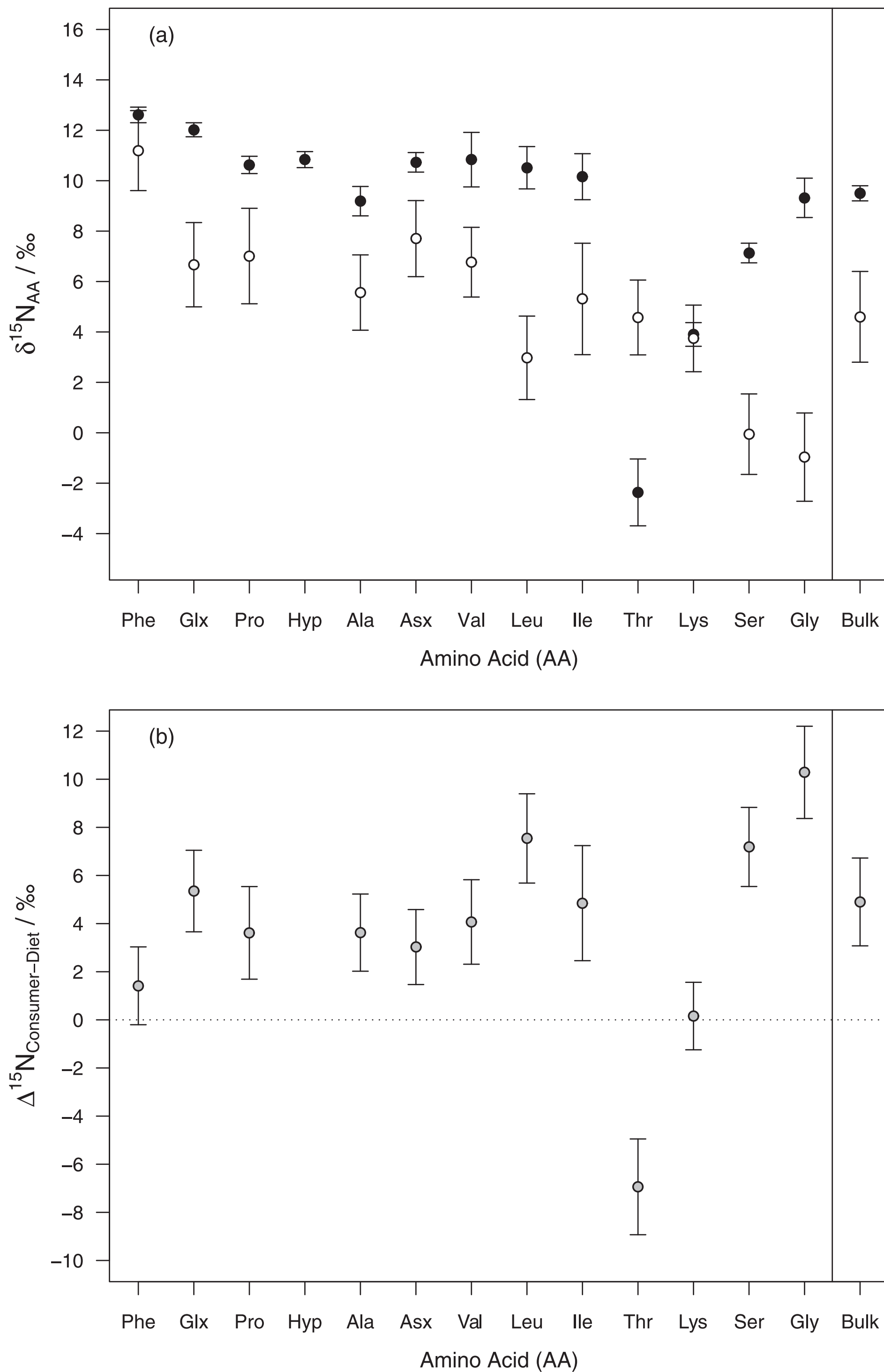

Figure 4. (a) Mean δ^{15}N values of each AA for plant protein (open circles) and cattle dentine collagen (closed circles). Error bars represent ± 1 standard deviation of multiple samples. (b) Differences between cattle and diet δ^{15}N values for each AA, where $\Delta^{15}N_{Consumer\text{-}Diet} = \delta^{15}N_{Consumer} - \delta^{15}N_{Diet}$.

between the individual plants at different locations across the control pasture fields. The variation in bulk and individual AA δ^{15}N values of the dentine is much smaller, i.e. *ca.* 0.6‰ for each AA, because animal tissue does not immediately incorporate the isotopic composition of the diet, but integrates the isotope compositions of many plants consumed over the period of tissue formation. Due to this averaging, therefore, the variation between collagen bulk and AA δ^{15}N values of the individual cattle is expected to be smaller than for a smaller number of plants collected across the fields.

The bulk $\Delta^{15}N_{consumer\text{-}diet}$ value, of 4.9‰, is in agreement with the range of 3-5‰ expected for a one trophic level shift, which by itself does not allow insight into the underlying metabolic processes that cause this change in value.

Considering the plant protein AAs in turn, Glx, Pro, Ala and Asx all have similar δ^{15}N values, ranging between 5.6 ± 1.5‰ for Ala and 7.7 ± 1.5‰ for Asx,

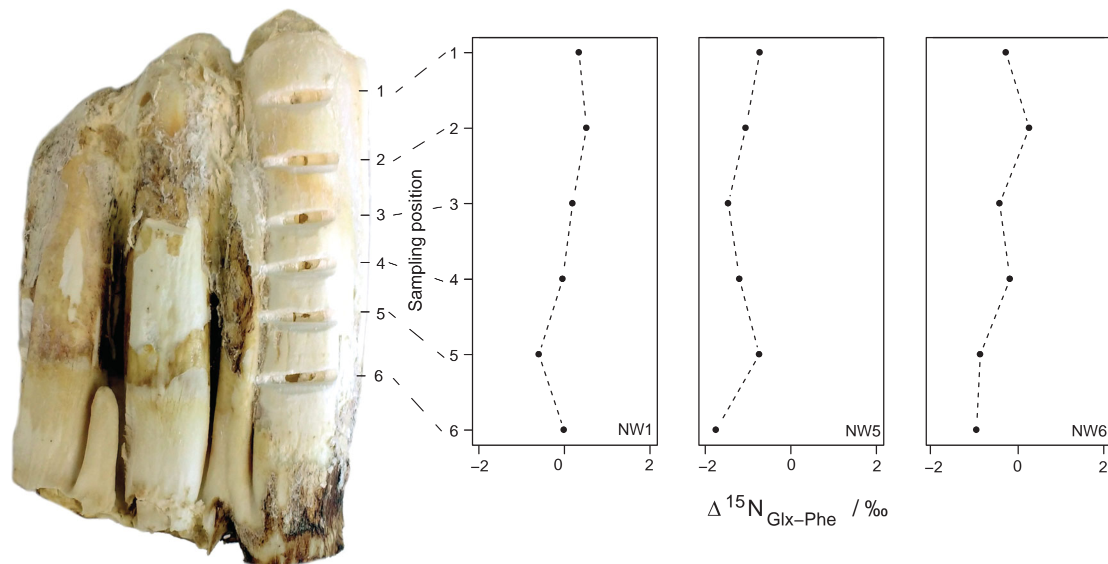

Figure 5. Variation of dentine AA $\Delta^{15}N_{Glx\text{-}Phe}$ values in sequentially sampled teeth. Six dentine samples were taken per tooth, with sampling position 1 closest to the crown of the tooth, formed earlier in the animal's life, and position 6 closest to the enamel-root junction, formed later. $\Delta^{15}N_{Glx-Phe} = \delta^{15}N_{Glx} - \delta^{15}N_{Phe}$.

In the dentine they also show similar trophic enrichments between plant proteins and collagen, of between 5.4 ± 1.7‰ for Glx and 3.0 ± 1.6‰ for Asx. Glx and Asx are of central importance in nitrogen cycling in plants and animals through transamination reactions. Ala is formed by a transamination reaction of glutamate or aspartate with pyruvate, leading to slightly lower $\delta^{15}N$ values in Ala relative to Glx and Asx. Pro is formed by the cyclisation of Glu, and as this cyclisation involves the formation of a C-N bond, Pro is expected to be ^{15}N depleted relative to Glx. This is indeed the case in collagen, where Pro and Hyp constitute 22% of amino acids so a high net production of Pro is required, and Pro is depleted by 1.4‰ relative to Glx. However, there is less demand for Pro in plant protein; its contribution to leaf protein AAs is less than that of Glu and Gln, leading to little fractionation as there is likely to be reversibility in this transaminase reaction. Hyp is formed by a post-translational modification of Pro in collagen, by prolyl hydroxylase. No C-N bonds are involved in this reaction, so there is no fractionation, therefore Hyp and Pro have essentially the same $\delta^{15}N$ values within all dentine samples, with a mean difference of 0.2 ± 0.5‰.

The branched-chain AAs (BCAAs) Val, Leu and Ile are biosynthesised in plants from their corresponding α-keto acids using two different branched-chain AA aminotransferase enzymes. Valine-pyruvate aminotransferase catalyses the biosynthesis of both Val and Ile, with the amino group coming from Ala, while the amino group of Leu comes from Glx catalysed by leucine aminotransferase. As the $\delta^{15}N$ values of Val and Ile are similar, at 6.7 ± 1.4‰ and 5.3 ± 2.2‰ respectively, while Leu is lower at 3.0 ± 1.7‰, suggesting that these enzymes discriminate against ^{15}N to different degrees.

In mammals, the BCAAs are all essential AAs, and therefore the same pattern in $\delta^{15}N$ values may be expected as in the dietary plants. Although the rumen microbes are able to biosynthesise all 22 AAs (the 20 standard AAs plus selenocysteine and pyrrolysine), the C skeleton of Leu and Ile are difficult to biosynthesise *de novo* (Atasoglu and Guliye 2004), and are likely to be preferentially obtained from the diet, as confirmed by Lee et al. (2014) who showed comparable intake to duodenal flow of both Leu and Ile in cattle on high forage diets, so there is unlikely to be any fractionation between diet and rumen microbes. However, the $\delta^{15}N$ values of all three BCAAs are essentially the same in collagen, at ca. 10‰. The reason for this is not clear, however, the first step in BCAA catabolism is reversible deamination to their α-keto acids and Glu, which may allow some equilibration of their $\delta^{15}N$ values, before the irreversible catabolic step.

Phe and Lys are both source group AAs in this system, i.e. there is little nitrogen fractionation between the diet and the consumer tissue (1.4 ± 1.6‰ and 0.2 ± 1.4‰ respectively). Phe and Lys are also difficult for rumen bacteria to biosynthesise *de novo* (Atasoglu and Guliye 2004), and so these AAs are routed to the cattle via the rumen microbes with little or no fractionation. The first major step in Phe metabolism in mammals is hydroxylation to Tyr (Bender 1975). As this does not involve breaking or forming a C-N bond, there is no fractionation involved. In contrast, the first step in Lys metabolism is a condensation reaction with α–ketoglutarate, involving the ε–amino group. However, Lys is an essential AA, and is a limiting AA in cattle (Merchen and Titgemeyer 1992), and therefore dietary Lys is routed directly to tissue protein with no fractionation.

The depletion in the $\delta^{15}N$ value of Thr in the cattle collagen relative to plant protein is consistent with previous studies, where negative $\delta^{15}N$ values in collagen threonine have been previously observed in pigs (Hare et al., 1991), and seems to be related to trophic level, as the depletion is greater in marine mammals (Styring et al. 2010), likely due to the larger number of trophic levels in marine ecosystems. The reasons behind this ^{15}N-depletion are as yet unclear, although the hypothesis proposed by Hare et al. (1991), that threonine catabolism proceeds with an inverse kinetic isotope effect, has been ruled out (Wallace and Hedges 2016).

The largest $\Delta^{15}N_{consumer\text{-}diet}$ value, of 10.3 ± 1.9‰, was observed for Gly. While this AA has previously been considered to be source group (McClelland and Montoya 2002, Popp et al. 2007, Steffan et al. 2015), this large trophic enrichment clearly indicates that it is a trophic group AA in this system. Similarly, the closely metabolically related Ser, also previously thought of as a source group AA, shows a large trophic enrichment of 7.2 ± 1.6‰. This is in agreement with the findings of a meta-analysis of AA $\delta^{15}N$ trophic fractionation by McMahon and McCarthy (2016), which found that trophic enrichments of Gly and Ser vary depending on the system being studied.

Gly is a major constituent of collagen, comprising approximately one third of collagen AA, so it is likely that much of this results from *de novo* biosynthesis rather than direct incorporation from the diet. Gly is biosynthesised from Ser, which is in turn formed by amination of phosphohydroxypyruvic acid derived from the glycolysis pathway. The amine group is transferred to Ser from Glu by the enzyme phosphoserine transaminase, leading to Ser being ^{15}N depleted relative to Glu. However, as the biosynthesis of Gly from Ser does not involve breaking or forming a C-N bond, it would be expected that Ser and Gly have similar $\delta^{15}N$ values. Gly can also be formed by the catabolism of Thr via 2-amino-3-ketobutyrate. Again, there is no C-N bond breaking or forming, so it may be expected that there should be no change in $\delta^{15}N$ value. However, it has been shown that Thr incorporated into body tissue is ^{15}N depleted in consumers relative to their diet, and therefore any Thr being converted to Gly would be ^{15}N enriched. As previously mentioned, Gly and Ser are source group amino acids in aquatic ecosystems. This suggests that in marine systems the diet supplies adequate Gly and Ser, so that extensive *de novo* biosynthesis is not required, and therefore there is little ^{15}N fractionation between diet and consumer.

As described herein, and shown in Figure 4, there is a wide variation in $\delta^{15}N$ values between the individual AAs in both plants and cattle, as well as a large variation in individual trophic enrichments, which are not apparent by the use of a single bulk tissue $\delta^{15}N$ value. Nitrogen metabolism in plants and animals involves a combination of AA biosynthesis and catabolism, protein metabolism, ingestion, digestion, and nitrogen excretion processes. The $\delta^{15}N$ values of tissues are controlled by the exact combination of these processes, and investigation at the compound specific level reveals information that is obscured through use of bulk values, such as the unexpected depletion in Thr $\delta^{15}N$ values with increased trophic level.

Using the trophic level equation (Eq. 1) with the previously published values of β = +8.4‰ and TEF = +7.6‰, the mean trophic positions of the plants and cattle in this study were calculated as 1.5 and 2.0, respectively. These values therefore overestimate the trophic position of the primary producers, although correctly predict the tropic position of the cattle. In this study, the difference between Glx and Phe $\delta^{15}N$ values in the primary producers (i.e. β) is +4.5‰, while the enrichment in the $\delta^{15}N$ values of Glx relative to Phe with increasing trophic level (i.e. TEF) is +4.0‰.

The $\Delta^{15}N_{consumer\text{-}diet}$ value of Glx, of 5.4 ± 1.7‰ for this terrestrial higher plant-ruminant food web, is markedly lower than previously reported values of 8‰ for aquatic and invertebrate terrestrial food webs (Chikaraishi et al. 2009, Steffan et al. 2013). The underlying reason why a lower $\Delta^{15}N_{consumer\text{-}diet}$ value has been observed here lies in the fact that ruminants are able to hydrolyse and recycle urea N as ammonia or amino N, by transfer to the rumen via saliva and from the blood (Huntington 1986). As urea is depleted in ^{15}N in consumers relative to diet (Steele and Daniel 1978), this recycling will decrease the trophic enrichment of AAs into which this N is incorporated, compared to organisms which cannot recycle nitrogen. This also explains why the TEF is lower in this study than previously reported studies in non-ruminant food webs. Critically, this finding emphasises that TEF values are not transferable between food webs, especially where organisms possess contrasting metabolisms.

Conclusions

This investigation used plant and animal tissues from a controlled agricultural experiment to provide baseline information relating to the expression of natural abundance stable nitrogen isotope values in a vertebrate terrestrial food web. The increase in bulk tissue $\delta^{15}N$ values with trophic level was within the range of that seen in other studies. This investigation is the first to consider the effect of trophic level on $\delta^{15}N$ values of AAs of a ruminant species and its well-defined diet. The study has resulted in a number of important observations:

(i) Trophic enrichment factor values based on AAs are not transferrable between all food webs and ecosystems as had been previously hypothesised. For archaeological and ecological applications,

the TEF values for the food web must be known for accurate trophic position estimations.

(ii) Phenylalanine and lysine have been shown to be source group AAs in this system, i.e. their $\delta^{15}N$ values change little with increased trophic level.

(iii) The $\delta^{15}N$ values of the other AAs were found to change with increased trophic level, including glycine and serine. This is in contrast to studies in aquatic ecosystems in which glycine and serine are source group AAs.

(iv) These differences from other analysed food webs are likely due to the different digestive physiology of ruminants, namely their symbiotic relationship with rumen microbes and their ability to recycle N from urea via saliva.

There is therefore a need to extend these investigations to include other ecologically and archaeologically important terrestrial vertebrate animals raised on well controlled diets or feeding experiments, in order to apply these sorts of trophic position studies to other food webs.

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Author contribution statement

IPK and RPE conceived and designed the experiment, IPK performed the experiments and analysed the data, IPK and RPE wrote the manuscript, MRFL commented on the manuscript and provided access to the biological samples.

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References

Atasoglu, C. and A. Y. Guliye 2004. "Use of stable isotopes to measure de novo synthesis and turnover of amino acid-C and -N in mixed micro-organisms from the sheep rumen in vitro." *British Journal of Nutrition* 91 (2): 253-261.

Bender, D. A. 1975. *Amino Acid Metabolism*. London, John Wiley & Sons.

Bogaard, A., T. H. E. Heaton, P. Poulton and I. Merbach 2007. "The impact of manuring on nitrogen isotope ratios in cereals: archaeological implications for reconstruction of diet and crop management practices." *Journal of Archaeological Science* 34 (3): 335–343.

Brown, W. A. B., P. V. Christofferson, M. Massler and M. B. Weiss 1960. "Postnatal tooth development in cattle." *American Journal of Veterinary Research* 21: 7–34.

Caut, S., E. Angulo and F. Courchamp 2009. "Variation in discrimination factors (Δ15N and Δ13C): the effect of diet isotopic values and applications for diet reconstruction." *Journal of Applied Ecology* 46 (2): 443–453.

Chikaraishi, Y., Y. Kashiyama, N. O. Ogawa, H. Kitazato and N. Ohkouchi 2007. "Metabolic control of nitrogen isotope composition of amino acids in macroalgae and gastropods: implications for aquatic food web studies." *Marine Ecology Progress Series* 342: 85–90.

Chikaraishi, Y., N. O. Ogawa, H. Doi and N. Ohkouchi 2011. "$^{15}N/^{14}N$ ratios of amino acids as a tool for studying terrestrial food webs: a case study of terrestrial insects (bees, wasps, and hornets)." *Ecological Research* 26 (4): 835–844.

Chikaraishi, Y., N. O. Ogawa, Y. Kashiyama, Y. Takano, H. Suga, A. Tomitani, H. Miyashita, H. Kitazato and N. Ohkouchi 2009. "Determination of aquatic food-web structure based on compound-specific nitrogen isotopic composition of amino acids." *Limnology and Oceanography: Methods* 7 (11): 740–750.

Chikaraishi, Y., N. O. Ogawa and N. Ohkouchi 2010. Further evaluation of the trophic level estimation based on nitrogen isotopic composition of amino acids. *Earth, Life and Isotopes*. N. Ohkouchi, I. Tayasu and K. Koba. Kyoto, Kyoto University Press: 37–51.

Chikaraishi, Y., S. A. Steffan, Y. Takano and N. Ohkouchi 2015. "Diet quality influences isotopic discrimination among amino acids in an aquatic vertebrate." *Ecology and Evolution* 5 (10): 2048–2059.

Corr, L. T., R. Berstan and R. P. Evershed 2007. "Optimisation of derivatisation procedures for the determination of $\delta^{13}C$ values of amino acids by gas chromatography/combustion/isotope ratio mass spectrometry." *Rapid Communications in Mass Spectrometry* 21 (23): 3759–3771.

DeNiro, M. J. and S. Epstein 1981. "Influence of diet on the distribution of nitrogen isotopes in animals." *Geochimica et Cosmochimica Acta* 45 (3): 341–351.

Eastoe, J. E. 1955. "The amino acid composition of mammalian collagen and gelatin." *Biochemical Journal* 61 (4): 589–600.

Germain, L. R., P. L. Koch, J. Harvey and M. D. McCarthy 2013. "Nitrogen isotope fractionation in amino acids from harbor seals: implications for compound-specific

trophic position calculations." *Marine Ecology Progress Series* 482: 265–277.

Hare, P. E., M. L. Fogel, T. W. Stafford, A. D. Mitchell and T. C. Hoering 1991. "The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins." *Journal of Archaeological Science* 18 (3): 277–292.

Heaton, T. H. E. 1987. "The $^{15}N/^{14}N$ ratios of plants in South Africa and Namibia: relationship to climate and coastal/saline environments." *Oecologia* 74 (2): 236–246.

Hobson, K. A., R. T. Alisauskas and R. G. Clark 1993. "Stable-Nitrogen Isotope Enrichment in Avian Tissues Due to Fasting and Nutritional Stress: Implications for Isotopic Analyses of Diet." *The Condor* 95 (2): 388–394.

Hoen, D. K., S. L. Kim, N. E. Hussey, N. J. Wallsgrove, J. C. Drazen and B. N. Popp 2014. "Amino acid ^{15}N trophic enrichment factors of four large carnivorous fishes." *Journal of Experimental Marine Biology and Ecology* 453: 76–83.

Huntington, G. B. 1986. "Uptake and transport of nonprotein nitrogen by the ruminant gut." *Federation Proceedings* 45 (8): 2272–2276.

Lee, M. R. F., V. J. Theobald, N. Gordon, M. Leyland, J. K. S. Tweed, R. Fychan and N. D. Scollan 2014. "The effect of high polyphenol oxidase grass silage on metabolism of polyunsaturated fatty acids and nitrogen across the rumen of beef steers." *Journal of Animal Science* 92 (11): 5076–5087.

McClelland, J. W. and J. P. Montoya 2002. "Trophic relationships and the nitrogen isotopic composition of amino acids in plankton." *Ecology* 83 (8): 2173–2180.

McCutchan, J. H., W. M. Lewis, C. Kendall and C. C. McGrath 2003. "Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur." *Oikos* 102 (2): 378–390.

McMahon, K. W. and M. D. McCarthy 2016. "Embracing variability in amino acid δ15N fractionation: mechanisms, implications, and applications for trophic ecology." *Ecosphere* 7 (12): e01511–n/a.

McMahon, K. W., M. J. Polito, S. Abel, M. D. McCarthy and S. R. Thorrold 2015. "Carbon and nitrogen isotope fractionation of amino acids in an avian marine predator, the gentoo penguin (*Pygoscelis papua*)." *Ecology and Evolution* 5 (6): 1278–1290.

Merchen, N. R. and E. C. Titgemeyer 1992. "Manipulation of amino acid supply to the growing ruminant." *Journal of Animal Science* 70 (10): 3238–3247.

Minagawa, M. and E. Wada 1984. "Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age." *Geochimica et Cosmochimica Acta* 48 (5): 1135–1140.

Nakashita, R., Y. Suzuki, F. Akamatsu, Y. I. Naito, M. Sato-Hashimoto and T. Tsubota 2011. "Ecological application of compound-specific stable nitrogen isotope analysis of amino acids: A case study of captive and wild bears." *Researches in Organic Geochemistry* 27: 73–79.

Nielsen, J. M., B. N. Popp and M. Winder 2015. "Meta-analysis of amino acid stable nitrogen isotope ratios for estimating trophic position in marine organisms." *Oecologia*: 1–12.

O'Connell, T. C. 2017. "'Trophic' and 'source' amino acids in trophic estimation: a likely metabolic explanation." *Oecologia* 184 (2): 317–326.

Ohkouchi, N., Y. Chikaraishi, H. G. Close, B. Fry, T. Larsen, D. J. Madigan, M. D. McCarthy, K. W. McMahon, T. Nagata, Y. I. Naito, N. O. Ogawa, B. N. Popp, S. Steffan, Y. Takano, I. Tayasu, A. S. J. Wyatt, Y. T. Yamaguchi and Y. Yokoyama 2017. "Advances in the application of amino acid nitrogen isotopic analysis in ecological and biogeochemical studies." *Organic Geochemistry*.

Ørskov, E. R. 1982. *Protein nutrition in ruminants*. London, Academic Press.

Pakhomov, E. A., J. W. McClelland, K. Bernard, S. Kaehler and J. P. Montoya 2004. "Spatial and temporal shifts in stable isotope values of the bottom-dwelling shrimp *Nauticaris marionis* at the sub-Antarctic archipelago." *Marine Biology* 144 (2): 317–325.

Popp, B. N., B. S. Graham, R. J. Olson, C. C. S. Hannides, M. J. Lott, G. A. López-Ibarra, F. Galván-Magaña and B. Fry 2007. Insight into the Trophic Ecology of Yellowfin Tuna, *Thunnus albacares*, from Compound-Specific Nitrogen Isotope Analysis of Proteinaceous Amino Acids. *Stable Isotopes as Indicators of Ecological Change*. E. D. Todd and T. W. S. Rolf. Cambridge, MA, USA, Academic Press. 1: 173–190.

Post, D. M. 2002. "Using stable isotopes to estimate trophic position: models, methods, and assumptions." *Ecology* 83 (3): 703–718.

Romek, K. M., M. Julien, M. Frasquet-Darrieux, I. Tea, I. Antheaume, R. Hankard and R. J. Robins 2013. "Human baby hair amino acid natural abundance ^{15}N-isotope values are not related to the ^{15}N-isotope values of amino acids in mother's breast milk protein." *Amino Acids* 45 (6): 1365–1372.

Satter, L. D. and R. E. Roffler 1975. "Nitrogen Requirement and Utilization in Dairy Cattle." *Journal of Dairy Science* 58 (8): 1219–1237.

Schoeninger, M. J. and M. J. DeNiro 1984. "Nitrogen and carbon isotopic composition of bone collagen from marine and terrestrial animals." *Geochimica et Cosmochimica Acta* 48 (4): 625–639.

Spence, K. O. and J. A. Rosenheim 2005. "Isotopic enrichment in herbivorous insects: a comparative field-based study of variation." *Oecologia* 146 (1): 89–97.

Steele, K. W. and R. M. Daniel 1978. "Fractionation of nitrogen isotopes by animals: a further complication to the use of variations in the natural abundance of ^{15}N for tracer studies." *Journal of Agricultural Science* 90 (1): 7–9.

Steffan, S. A., Y. Chikaraishi, C. R. Currie, H. Horn, H. R. Gaines-Day, J. N. Pauli, J. E. Zalapa and N. Ohkouchi 2015. "Microbes are trophic analogs of animals." *Proceedings of the National Academy of Sciences* 112 (49): 15119–15124.

Steffan, S. A., Y. Chikaraishi, D. R. Horton, N. Ohkouchi, M. E. Singleton, E. Miliczky, D. B. Hogg and V. P. Jones 2013. "Trophic Hierarchies Illuminated via Amino Acid Isotopic Analysis." *PLOS ONE* 8 (9).

Styring, A. K., R. A. Fraser, A. Bogaard and R. P. Evershed 2014. "The effect of manuring on cereal and pulse amino acid $\delta^{15}N$ values." *Phytochemistry* 102: 40–45.

Styring, A. K., A. Kuhl, T. D. I. Knowles, R. A. Fraser, A. Bogaard and R. P. Evershed 2012. "Practical considerations in the determination of compound-specific amino acid $\delta^{15}N$ values in animal and plant tissues by gas chromatography-combustion-isotope ratio mass spectrometry, following derivatisation to their *N*-acetyl isopropyl esters." *Rapid Communications in Mass Spectrometry* 26 (19): 2328–2334.

Styring, A. K., J. C. Sealy and R. P. Evershed 2010. "Resolving the bulk $\delta^{15}N$ values of ancient human and animal bone collagen via compound-specific nitrogen isotope analysis of constituent amino acids." *Geochimica et Cosmochimica Acta* 74 (1): 241–251.

Vanderklift, M. A. and S. Ponsard 2003. “Sources of variation in consumer-diet $\delta^{15}N$ enrichment: a meta-analysis.” *Oecologia* 136 (2): 169–182.

Wallace, C. J. A. and R. E. M. Hedges 2016. “Nitrogen isotopic discrimination in dietary amino acids: The threonine anomaly.” *Rapid Communications in Mass Spectrometry* 30 (22): 2442–2446.

Yeoh, H.-H. and L. Watson 1982. “Taxonomic variation in total leaf protein amino acid compositions of grasses.” *Phytochemistry* 21 (3): 615–626.