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The influence of varying proportions of terrestrial and marine dietary protein on the stable carbon-isotope compositions of pig tissues from a controlled feeding experiment

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ABSTRACT

In recent years, it has become evident that limitations exist in our ability to meaningfully assess palaeodiet using stable isotope compositions. These limitations in part arise because many of the fundamental assumptions about tissue-diet relationships are poorly understood. In order to redress this deficiency, a controlled feeding experiment was undertaken to define the impact of terrestrial- vs. marine-derived dietary protein consumption on consumer tissue carbon isotopic compositions ($\delta^{13}C$). Two generations of pigs were raised on one of five feeds with varying proportions of terrestrial (soy) and marine (fish meal) protein. A comprehensive range of tissues and fluids from 49 pigs was submitted for $\delta^{13}C$ analysis.

The observed tissue–whole diet and tissue–dietary protein carbon isotopic offsets were found to be highly dependent on the percentage of marine protein in diet. We suggest that the trend in $\delta^{13}C$ offsets most likely derives from the increased routing of non-essential amino acids, especially glycine, with the increasing proportion of marine protein in the diet. These findings demonstrate that solely using bulk $\delta^{13}C$ compositions not only masks considerable information about diet, but may also lead to erroneous representations of marine and terrestrial resource consumption in the past.

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Introduction

Of central importance in many archaeological investigations are patterns of resource use through time and across geographic regions, particularly during environmentally- or culturally-mediated periods of dietary change, such as the postulated shift away from marine resource consumption during the Mesolithic-Neolithic transition across Europe (Richards et al., 2003; Tauber et al., 1981). Stable carbon-isotope ($\delta^{13}C$) analysis of human and faunal remains has been used for over three decades to investigate palaeodiet. These determinations make valuable contributions to our understanding of resource exploitation in the past where differential access and exploitation can have important implications for socio-political and economic behaviour. In ecologically-complex areas where numerous resources are available for exploitation, stable isotopic compositions are similarly used to evaluate relative consumption of different kinds of food, for example, Mesoamerica (e.g. Emery et al., 2000; Scherer et al., 2007; Warinner et al., 2013; White, 2005; White et al., 1993) or the western coast of South America (e.g. Finucane et al., 2006; Gil et al., 2011; Knudson et al., 2015; Tomczak, 2003; Webb et al., 2013; Yesner et al., 2003).

Early stable isotope research (DeNiro & Epstein, 1976, 1978) determined that there was a strong correlation between the stable isotopic compositions of various body tissues and diet. It was hypothesised that bone mineral $\delta^{13}C$ values (bioapatite; $\delta^{13}C_{sc}$) should reflect the $\delta^{13}C$ values of dietary carbohydrates, lipids, and, to a lesser extent, protein, whereas bone protein $\delta^{13}C$ values (collagen; $\delta^{13}C_{col}$) should most strongly reflect dietary protein (Krueger & Sullivan, 1984). Numerous subsequent feeding experiments explored the influence of varying the $\delta^{13}C$ values of different dietary macronutrients, as well as the impact of different relative proportions of dietary protein, carbohydrates and lipids on the relationships among $\delta^{13}C_{sc}$, $\delta^{13}C_{col}$, $\delta^{13}C_{whole\ diet}$ and $\delta^{13}C_{dietary\ protein}$ (e.g. Ambrose & Norr, 1993; Howland et al., 2003; Jim et al., 2006; Krueger & Sullivan, 1984; Passey et al., 2005; Tieszen & Fagre, 1993). These studies further investigated the process of carbon routing from dietary macronutrients, and proposed that a reasonably straightforward linear mixing model best explained

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the relationship between $\delta^{13}C_{sc}$ and $\delta^{13}C_{whole\ diet}$ values. Ultimately, they also confirmed that collagen $\delta^{13}C$ values reflect the $\delta^{13}C$ of dietary protein when diet is protein-sufficient (Ambrose & Norr, 1993; Chisholm et al., 1982; Tieszan & Fagre, 1993).

In recent years, however, it has become increasingly evident that there are limitations associated with our ability to meaningfully assess palaeodiet using stable carbon-isotope compositions. In part this is because of the complexity of questions now being investigated, but largely because many of the fundamental assumptions about tissue-diet relationships are poorly understood (Hedges, 2004). Beyond constraints on interpretation imposed by the archaeological record, attempts to refine the predictive ability of isotopic compositions have included the applications of an index to estimate degree of meat consumption ($\Delta^{13}C_{ap-col}$), using multiple light isotope proxies, including nitrogen and sulphur (e.g. Druker & Bocherens, 2004; Hedges & Reynard, 2007; Müldner & Richards, 2005; Nehlich et al., 2010, 2011; Reynard & Tuross, 2015; Richards et al., 2003; Sayle et al., 2013). New approaches using stable strontium ($\delta^{88}Sr$), calcium ($\delta^{44}Ca$), iron ($\delta^{56}Fe$), copper ($\delta^{65}Cu$) and zinc ($\delta^{66}Zn$) isotopes from mineralized tissues have also been applied (e.g. Jaouen et al., 2013; Knudson et al., 2010; Reynard et al., 2007). Meta-analyses of published isotopic datasets, incorporating isotopic data from archaeological humans and fauna, as well as controlled feeding studies, have enabled reassessment of many of the earlier dietary models and the outcomes of feeding experiments. Drawing on these data, increasingly sophisticated mathematical models have been developed and applied to the problem of archaeological human palaeodietary reconstruction (e.g. Fernandes et al., 2012, 2014; Froehle et al., 2012; Kellner & Schoeninger, 2007; Newsome et al., 2004). Models, although useful, are unfortunately limited by a lack of knowledge about dietary routing, tissue-diet relationships, and fractional contributions of different macronutrients (e.g. dietary amino acids, fatty acids, etc.) to different tissues. Moreover, many of these models have been adapted from ecological research in which ecosystems and ecological niches are well-constrained in terms of natural isotopic variability and dietary resource exploitation. However, models appropriate for use in these contexts are unlikely to be uncritically applicable to human palaeodietary studies. Crucially, human food choice is more than a function of the relative quantities of various edible resources in the environment; indeed, edibility is understood to be culturally-defined, as are food preparation and consumption methods (e.g. cooking or assembling meals), which can impact the quality of dietary macronutrients and how they are absorbed. Similarly, dietary heterogeneity resulting from socioeconomic factors and differential food access (e.g. status, economic role, trade and exchange, age, or gender) further complicates palaeodietary reconstruction. This suite of potential environmental, social and economic influences on human dietary choice creates a complex context in which to reconstruct palaeodiet. A thorough understanding of the biological and metabolic factors influencing the relationship between consumed food and the biochemical/metabolic record archived in archaeological and faunal remains will thus add much-needed rigour to palaeodietary reconstruction, and mitigate many of the uncertainties associated with elucidating resource exploitation in the past.

With these considerations in mind, a controlled feeding study was undertaken led by the University of Bristol. Two successive generations of pigs were fed one of five feeds containing varying proportions of terrestrial (soy) and marine (fish meal) protein. Pigs were chosen since they are widely recognised as an excellent physiological analogue for humans although there are differences in gestation time, average number of offspring per pregnancy and time to reach physical and sexual maturity (*inter alia* Heinritz et al., 2013; Litten-Brown et al., 2010; Sullivan et al., 2001; Swindle et al., 2012). The overarching goal of this study is to improve the understanding of consumer tissue isotopic composition for palaeodietary reconstruction, particularly the exploitation of marine resources. Here, our objectives are to elucidate tissue-tissue and tissue-diet carbon isotopic discrimination under different conditions of dietary protein consumption ranging from 100% terrestrial protein to 100% marine-derived protein. A comprehensive range of tissues and fluids including bone collagen (femoral and rib), muscle (femoral and loin), liver, blood, plasma, milk, hair, and faeces from 49 pigs from the first and second generation of the study were analysed for their $\delta^{13}C$ compositions and a preliminary set of collagen and muscle tissues have been analysed for their individual amino acid $\delta^{13}C$ ($\delta^{13}C_{AA}$) values. The $\delta^{13}C$ compositions of pig feeds, archived throughout the study, provide a defined dietary carbon isotopic baseline.

Theoretical considerations for palaeodietary reconstruction

The reconstruction of palaeodiet using stable isotope analysis is based on the well-tested assumption that tissue isotopic composition reflects the isotopic composition of consumed food (Ambrose, 1993). There are systematic differences in isotopic composition between tissue and food, and among different tissues within the body (Ambrose, 1993; DeNiro & Epstein, 1978). Here we define differences between tissues and diet and different tissues using a capital delta notations where $\Delta^{13}C_{X-Y} = \delta^{13}C_x - \delta^{13}C_y$. These tissue-diet and tissue-tissue offsets are a result of isotopic fractionation, the differential partitioning of isotopes between phases in a reaction (e.g. ingested food → consumer tissue)

caused by the mass differences among isotopes of the same element (Urey, 1947). Isotopic data are used to assess relative contributions to diet of isotopically distinct foods by comparing tissue isotopic compositions to a food web model that describes the variability in isotopic compositions of local food resources (i.e. terrestrial and marine fauna and plant material; Ambrose, 1993; Kellner & Schoeninger, 2007).

The $\delta^{13}C$ composition of any proteinaceous tissue is a weighted average of the $\delta^{13}C$ compositions of both its essential and non-essential amino acids ($\delta^{13}C_{AA}$). Essential amino acids (e.g. threonine, valine, methionine, isoleucine, leucine, histidine, lysine, and phenylalanine) cannot be generated by the body and therefore must be ingested in sufficient quantities. Non-essential amino acids (e.g. asparagine/aspartic acid, hydroxyproline, glutamic acid/glutamate, serine, glycine, alanine, proline and arginine) can be assimilated with minimal modification from a dietary source, or may be synthesised *de novo* using components drawn from the body's biochemical pools (Ambrose, 1993; Schwarcz, 2000). As a result, tissue essential amino acid $\delta^{13}C$ values are expected to closely approximate dietary essential amino acid $\delta^{13}C$ values due to direct routing, that is, $\delta^{13}C_{tissue\ AA} - \delta^{13}C_{diet\ AA} \approx 0‰$. Non-essential amino acid $\delta^{13}C$ values, however, may show evidence of both direct routing and biosynthesis.

The controls of the relative proportion of direct routing vs. *de novo* synthesis for non-essential amino acids is dependent on both the total protein in the diet and the amount of a given amino acid in the diet. In a high protein diet direct routing is more likely to occur, since it is more energetically efficient to assimilate non-essential amino acids than it is to synthesise them and one would expect $\delta^{13}C_{tissue\ AA}$ to approximate $\delta^{13}C_{diet\ AA}$ (Corr et al., 2005; Jim et al., 2006; Jones, 2002; Schwarcz, 2000; Umbarger, 1978). Conversely when the diet is protein poor *de novo* synthesis dominates and a stronger relationship to whole diet $\delta^{13}C$ values rather than to the corresponding dietary amino acid $\delta^{13}C$ value is expected. A final caveat is that whilst it is convenient to discuss total dietary protein the metabolic effects are driven at the amino acid level. Thus for a diet that may be poor in overall protein but rich in a particular individual non-essential amino acid, say glycine, *de novo* synthesis may dominate for most non-essential amino acids but glycine will be directly routed.

Although this variability in essential and non-essential amino acid metabolism and its influence on amino acid isotopic compositions is subsumed within the sampling resolution for this study, these processes underlie "bulk"[1] tissue $\delta^{13}C$ compositions, and their impact may be detectable at the bulk protein sampling resolution under controlled feeding conditions.

Based on numerous controlled feeding and well-constrained wild faunal studies, it is generally assumed that bone collagen $\delta^{13}C$ values are elevated by ~+5‰ relative to whole diet, and by 0 to +1‰ relative to dietary protein (Ambrose & Norr, 1993). The $\delta^{13}C$ values of other proteinaceous tissues, including muscle, blood and plasma are ~1‰ higher than whole diet, whereas liver and faeces $\delta^{13}C$ values are generally moderately reduced relative to diet (Sponheimer et al., 2003, 2006). Many experimental studies show considerable variability in the relationships between collagen and whole diet and collagen and dietary protein. Larger tissue–diet offsets are typically attributed to experimentally-controlled isotopic differences between the "energy" (non-protein) and protein components of diet, and to variable contributions to bone collagen from dietary energy vs. dietary protein (explored in Froehle et al., 2010, 2012; Kellner & Schoeninger, 2007). Early work determined that, by varying the energy and protein component isotopic compositions, it was possible to induce tissue-diet offsets ranging from ~−2 to +10‰ (e.g. Ambrose & Norr, 1993; Young, 2002). Moreover, although the $\delta^{13}C_{col}$ values do predominately reflect dietary protein $\delta^{13}C$ values, direct routing of protein from diet to tissue can occur, indicating that non-essential amino acids can be assimilated directly rather than biosynthesised from non-protein carbon skeletons. At higher levels of protein consumption, dietary amino acids are routed more or less directly from diet to tissue with minimal modification. When protein consumption is low, however, carbon drawn from dietary carbohydrates and lipids can constitute ~49–58% of the carbon in collagen. If that small amount of consumed protein is from a marine resource, with the remainder of the diet derived from plant foods, tissue $\delta^{13}C$ values may be quite low, obscuring the marine carbon contribution. In contrast, protein-rich diets that incorporate both marine and terrestrial protein resources may overestimate marine protein consumption due to preferential routing of dietary marine protein to collagen. Preliminary models suggest that a contribution of up to 20% marine protein to diet may cause tissue isotopic changes of only +0.3 to +1.8‰, depending on the overall protein content of diet and thus on the importance of routing versus biosynthesis of non-essential amino acids (Hedges, 2004).

Methodology

Controlled feeding study

The pigs were raised at Harper Adams University (Shropshire, UK), and a full suite of tissues, fluids and excreta was archived (Figure 1). Each pig was fed one of five diets of known dietary protein source composition: (i) 100% terrestrial-derived (soy), (ii) 87.5% terrestrial/12.5% marine, (iii) 75% terrestrial/25% marine,

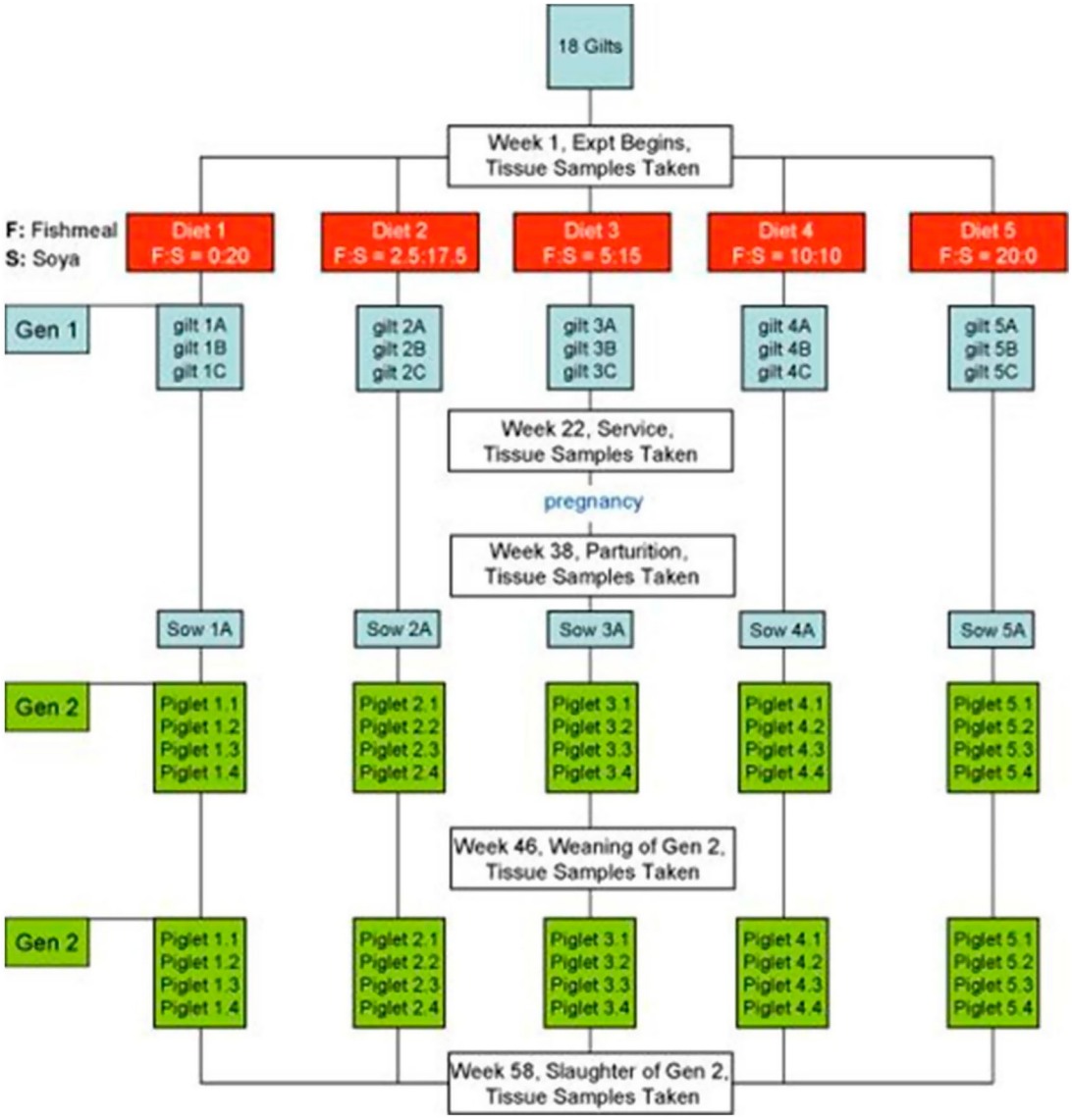

Figure 1. Flowchart illustrating the structure of the controlled feeding study.

(iv) 50% terrestrial/50% marine, and (v) 100% marine-derived (fish meal). All five diets were nutritionally equivalent, that is, there was no difference in the amount or quality of dietary protein, carbohydrates or lipids among feeds. The experiment was run over two successive generations, in the first generation, five groups of gilts were fed one of the above diets from weaning until sacrifice. All gilts were artificially inseminated and the second generation pigs were fed exclusively on one of the five diets from weaning until sacrifice in adolescence. Five tonnes of each feed was produced from the same batches of ingredients by Parnutt Foods, Ltd. (Sleaford, UK) and held in cold storage until required. Diet formulations are presented in Table 1. Four subsamples of each feed were taken as batches were released from storage to confirm homogeneity.

This study was explicitly designed to address limitations recognized in earlier feeding studies, particularly difficulties associated with sample size, differential tissue turnover, and nutritional stress. As such, each diet group is represented by several pigs, all of which have only consumed a single experimental diet (including sow milk from the same dietary group), and only one dietary variable – the ratio of terrestrial to marine protein – was changed. All five diets have a constant 20% protein contribution to whole diet, which eliminates variability in isotopic data and tissue-diet offsets associated with low (≤5%) or high (~70%) protein consumption. In total, 10 sows (first generation), 19 piglets (second generation, aged four weeks) and 39 pigs (second generation, aged >160 days) were reared and sacrificed over the course of the study.

Laboratory methods

Ten first generation sows, 19 piglets, and 20 second generation pigs were selected for isotopic analysis. Bone collagen (femur and rib), muscle (femoral and loin) and liver samples were analyzed for their $\delta^{13}C$ compositions. Milk ($n = 10$; sows only) and hair ($n = 10$; piglets only) samples were also analyzed. Plasma

Table 1. Diet formulations and carbon-isotope compositions.

	Diet 1 0:20[a]	Diet 2 2.5:17.5	Diet 3 5:15	Diet 4 10:10	Diet 5 20:0
$\delta^{13}C_{\text{whole diet}} \pm 1\sigma$ (‰, VPDB)	−25.0 ± 0.2	−24.7 ± 0.2	−24.9 ± 0.2	−24.8 ± 0.2	−24.5 ± 0.3
Est. $\delta^{13}C_{\text{dietary protein}}$ (‰, VPDB)[b]	−25.6	−25.0	−24.3	−23.1	−20.5
	Weight (%)				
Major dietary carbon sources					
"Provasoy" Soy flour	27.2	23.8	20.4	13.6	0.0
Fish meal	0.0	2.6	5.2	10.4	20.8
Native starch	35.1	36.6	38.1	41.1	46.0
Tapioca	17.5	17.5	17.5	17.5	17.5
Corn starch	10.0	10.0	10.0	10.0	10.0
Soya oil	3.1	2.6	2.2	1.3	0.5
Trace nutrients and feed components					
Dicalcium phosphate	2.0	1.8	1.5	1.0	0.0
Vitacell	1.8	1.9	2.0	2.2	2.6
Nutripur	1.0	1.0	1.0	1.0	1.0
Sow vitamin supplement	0.5	0.5	0.5	0.5	0.5
Chalk	0.5	0.5	0.5	0.4	0.4
Salt	0.3	0.3	0.3	0.2	0.0
Lysine	0.3	0.2	0.2	0.2	0.1
Potassium sorbate	0.2	0.2	0.2	0.2	0.2
Methionine	0.2	0.2	0.2	0.2	0.1
Threonine	0.2	0.2	0.1	0.1	0.1
Citric acid	0.1	0.1	0.1	0.1	0.1
Choline chloride	0.1	0.1	0.1	0.1	0.1
Pigortek	0.02	0.02	0.02	0.02	0.02
Tryptophan	0.02	0.02	0.02	0.03	0.05
BHT antioxidant	0.02	0.02	0.02	0.02	0.02

[a]Ratio of marine to terrestrial protein where protein constitutes 20% of diet.
[b]$\delta^{13}C$ dietary protein values were estimated using equation 1.

(sampled at 70 and 140 days of age), blood, faeces and urine samples were analyzed as available. Bone collagen was extracted using a modified Longin (1971) method. For each femur or rib sample, a section of bone was taken using a hacksaw or rotary tool (Dremel tool, 3000JB with diamond cutting wheel SC545). The bone was mechanically defleshed using a scalpel, freeze-dried, and a rotary tool with a silicon carbide burr was used to remove all trabecular bone and ~0.5 mm of surface bone. Each sample was then crushed and sieved, retaining approximately 450 mg of ≥212 μm and ≤1 mm fragments for collagen extraction. Lipids were extracted using 2:1 v/v chloroform: methanol solution (3 × 8 mL solvent solution, 3 × 20 min sonication). Collagen was extracted by soaking in 0.5 M hydrochloric acid (HCl) until bone chips were entirely soft. Extracted collagen was solubilised in 10^{-3} M HCl at 75°C for 48 h, filtered (E-Zee filters, 60–90 μm), and freeze-dried for ≥24 h.

For soft tissue samples, a cross section of the tissue was removed using a new scalpel blade. Soft tissues, faeces and milk were then freeze dried for more than 48 hours and then lipid extracted using a 2:1 v/v chloroform: methanol solution (3 × 8 mL solvent solution, 3 × 20 min sonication). Fluid samples were freeze dried and hair samples were cleaned of contaminant lipids and other organics by soaking in 2:1 v/v chloroform: methanol solution (2 × 8 mL for >24 h). For all samples, homogenised aliquots were then weighed into tin capsules (~0.70 ± 0.1 mg) for isotopic analysis. Twenty homogenised feed samples were weighed into tin capsules (~1.4 ± 0.1 mg) for isotopic analysis. Prior to use, all glassware was washed with Decon 90 and solvent-rinsed before furnacing at 450° C for four hours. Aluminium foil and disposable gloves were used to handle samples.

All bulk isotopic analyses were performed using a Flash HT elemental analyser interfaced with a Thermo Electron Delta^{Plus} XP mass spectrometer at the Natural Environment Research Council Life Science Mass Spectrometry Facility in East Kilbride, Scotland. For collagen, methodological reproducibility was determined through duplicate collagen preparation and analysis for 10% of samples and was ± 0.1‰. Analytical reproducibility for all samples was assessed by repeated analyses of 10% of samples, and was also ±0.1‰. Analytical precision was monitored throughout using USGS-40, and the average $\delta^{13}C$ value obtained over all analytical sessions was −26.3 ± 0.1‰, which compares well with the accepted value of −26.4‰ (Brand et al., 2014).

$\delta^{13}C$ determinations on individual amino acids from the 5 diet feeds, 10 sow collagen and 10 sow muscle samples were made by LC-IRMS using a Thermo-Finnigan Surveyor HPLC coupled to a Delta V IRMS via an LC-Isolink (Krummen et al., 2004). Amino acids are separated using a PrimeStep A column (250 mm × 3.2 mm, 5 μm particle size, 100 Å pore size; SIELC Technologies Ltd., Prospect Heights, IL, USA) following the method of Dunn et al. (2011). Repeated analysis of an amino acid mixture gives reproducibility within ±0.5‰ of the EA-IRMS value.

Results

Growth performance

All results are presented as average ± 1σ [range] unless otherwise noted and all growth performance data are summarised in Table 2. There were no significant health issues throughout the production stages, and there was no evidence of nutritional stress or delayed growth among diet groups, which is reflected in the average weight gain per day and total weight gain.

Sows were sacrificed on 11th February 2011. The average sow weight during pregnancy (adjusted downward to compensate for total litter weight) was 195 ± 15 kg, and was 203 ± 15 kg when the piglets were weaned. The average weight at slaughter was 205 ± 13 kg. All piglets were born between 29th August and 5th September 2010. A subset of piglets (three to five per diet group) were sacrificed at approximately four weeks of age on 30th September 2010. The average birth weight for all piglets was 1.4 ± 0.2 kg, and did not differ significantly among sow diet groups (Kruskal-Wallis, $p = 0.371$). Similarly, there was no difference in weaning weight (6.4 ± 1.5 kg), total gain (4.9 ± 1.4 kg) or average gain per day (183 ± 55 g day^{-1}) among piglets from different diet groups (Kruskal-Wallis, $p > 0.36$ for all tests).

The 39 second generation pigs were sacrificed on either 8th February or 9th March 2011 at ~160 or ~190 days of age, respectively. The average birth weight for all pigs was 1.5 ± 0.3 kg [0.7 to 2.2 kg], and differed significantly across the five diets (Kruskal-Wallis, $p = 0.017$), but the difference in weight among diet groups was no longer apparent at weaning. The average weaning weight was 14.6 ± 2.9 kg [7.3 to 20.0 kg], and did not vary significantly with maternal diet (Kruskal-Wallis, $p = 0.261$). Similarly, the total weight gain from weaning until slaughter (107.8 ± 13.9 kg [59.0 to 138.2 kg]) did not differ among the five diet groups (ANOVA, $p = 0.466$). The average gain per day was 624 ± 90 g day^{-1} [316 to 752 g day^{-1}], and also did not differ among the five diet groups (ANOVA, $p = 0.274$). Finally, the average slaughter weight was 109.3 ± 13.8 kg [60.6 to 139.6 kg], and did not differ among pigs consuming different diets (ANOVA, $p = 0.480$).

Pig feed isotopic compositions

Lots 1 through 4 of each pig feed are isotopically equivalent for all five diets (Kruskal-Wallis, $p = 0.288$; Table S1). The differences in $\delta^{13}C$ compositions of diet 1 through 5 are likewise not statistically significant (Kruskal-Wallis, $p = 0.275$). The average $\delta^{13}C$ values over the five diets are: diet 1: −25.0 ± 0.2‰ [0.3‰], diet 2: −24.7 ± 0.2‰ [0.4‰], diet 3: −24.8 ± 0.2‰ [0.5‰], diet 4: −24.8 ± 0.2‰ [0.4‰], and diet 5: −24.5 ± 0.3‰ [0.5‰]. Thus, although the dietary protein source was qualitatively different for each diet, all pigs were consuming feed with the same overall $\delta^{13}C$ composition (−24.7 ± 0.2‰). The $\delta^{13}C$ value of the fish meal and soymeal dietary protein sources were determined to be −20.5‰ and −25.6‰, respectively.

Tissue isotopic compositions

Collagen

Collagen $\delta^{13}C$ values were determined for both femoral and rib bone collagen for 10 sows, 19 piglets[2] and 20 pigs (Tables S2-S4). The average rib collagen $\delta^{13}C$ values are −20.8 ± 1.5‰ [4.5‰] for sows, −20.0 ± 1.4‰ [5.0‰] for piglets and −20.2 ± 1.5‰ [4.7‰] for pigs. The average femoral bone

Table 2. Pig growth performance summary.

	Birth Weight (kg)[a]	Weaning Weight (kg)[a]	Slaughter Weight (kg)[a]	Total Gain (kg)[a]	Average Daily Gain (g day^{-1}) [1]
Piglets					*Birth – Slaughter*
Diet 1 0:20[b]	1.6 ± 0.3 [0.6]		6.0 ± 1.1 [2.1]	4.4 ± 0.9 [1.7]	161 ± 32 [63]
Diet 2 2.5:17.5	1.4 ± 0.2 [0.3]		6.1 ± 2.5 [5.0]	4.7 ± 2.5 [4.7]	174 ± 93 [174]
Diet 3 5:15	1.4 ± 0.3 [0.7]		7.4 ± 0.9 [5.2]	6.0 ± 0.7 [1.4]	223 ± 44 [103]
Diet 4 10:10	1.5 ± 0.1 [0.3]		6.6 ± 0.9 [2.0]	5.1 ± 0.9 [1.9	189 ± 32 [71]
Diet 5 20:0	1.2 ± 0.2 [0.4]		5.1 ± 0.9 [1.2]	3.9 ± 0.6 [0.8]	141 ± 21 [30]
Pigs					*Weaning – Slaughter*
Diet 1 0:20[a]	1.7 ± 0.3 [0.7]	15.0 ± 2.9 [8.8]	106.3 ± 11.1 [35.4]	104.6 ± 11.1 [35.6]	586 ± 76 [229]
Diet 2 2.5:17.5	1.6 ± 0.3 [0.8]	15.3 ± 1.5 [5.3]	108.3 ± 7.0 [17.4]	106.7 ± 7.0 [17.2]	667 ± 44 [108]
Diet 3 5:15	1.4 ± 0.2 [0.6]	14.2 ± 1.9 [5.2]	118 ± 12.6 [35.0]	116.6 ± 12.7 [35.3]	654 ± 49 [135]
Diet 4 10:10	1.6 ± 0.2 [0.5]	15.2 ± 4.6 [12.8]	106.1 ± 24.0 [73.4]	104.6 ± 23.9 [73.4]	623 ± 162 [436]
Diet 5 20:0	1.2 ± 0.3 [0.9]	12.8 ± 2.0 [6.0]	108.8 ± 6.0 [17.4]	107.7 ± 6.0 [17.9]	587 ± 35 [108]

[a]All data are reported as average ± 1σ [range].
[b]Ratio of marine to terrestrial protein where protein constitutes 20% of diet.

collagen $\delta^{13}C$ values are −20.2 ± 1.4‰ [4.1‰] for sows, −19.6 ± 1.2‰ [4.2‰] for piglets and −22.6 ± 1.4‰ [4.1‰] for pigs. Within each age category, rib and femoral bone collagen are not statistically significantly different (Mann-Whitney, sows $p = 0.247$; piglets $p = 0.327$; pigs $p = 0.512$). Similarly, across age categories, neither rib nor femoral bone collagen $\delta^{13}C$ values are significantly different (Kruskal-Wallis, $p = 0.227$ for femoral collagen and $p = 0.274$ for rib collagen). As such, an average collagen $\delta^{13}C$ value was determined for each pig, and all age categories are pooled for the remainder of this paper (Figure 2). We caution however, that although there are no interpretively or statistically important differences among sows, piglets and pigs at the bulk carbon-isotope level for collagen, this determination may not hold true at the compound-specific amino acid level of analysis.

Muscle and liver

Muscle $\delta^{13}C$ values were determined for both femoral and loin muscle samples for 10 sows, 19 piglets[3] and 20 pigs. The average femoral muscle $\delta^{13}C$ values are −22.3 ± 1.4‰ [3.9‰] for sows, −22.3 ± 1.2‰ [3.6‰] for piglets and −22.6 ± 1.4‰ [4.1‰] for pigs. The average loin muscle $\delta^{13}C$ values are −22.3 ± 1.4‰ [3.9‰] for sows, −22.1 ± 1.0‰ [3.3‰] for piglets and −22.5 ± 1.4‰ [4.2‰] for pigs. Liver $\delta^{13}C$ values were determined for 10 sows, 19 piglets and 20 pigs, and are −23.2 ± 1.1‰ [3.4‰], −22.9 ± 1.0‰ [3.3‰] and −23.7 ± 1.2‰ [3.9‰], respectively. Within each age category, femoral and loin muscle are not statistically significantly different (Mann-Whitney, sows $p = 0.912$; piglets $p = 0.367$; pigs $p = 0.620$). Across age categories, femoral and loin muscle $\delta^{13}C$ values are similarly statistically indistinguishable (Kruskal-Wallis, $p = 0.305$ for femoral muscle and $p = 0.336$ for loin muscle). Thus, for all pigs with both femoral and loin muscle isotopic data, an average $\delta^{13}C$ value is used for the remainder of this paper. Liver $\delta^{13}C$ values are statistically different between piglets and pigs (Mann-Whitney, $p = 0.010$), but the absolute difference in $\delta^{13}C$ is only 0.8‰ and this is not considered interpretively significant (Figure 2). Other inter-age category comparisons do not reveal statistically significant differences.

Blood, plasma, milk, hair and faeces

For the second generation pigs only, blood and plasma samples were collected and analysed for their $\delta^{13}C$ composition. The average blood $\delta^{13}C$ value was −23.7 ± 1.4‰ [4.0‰]. The average plasma $\delta^{13}C$ value at 70 days of age was −23.0 ± 1.2‰ [3.2‰], and −23.3 ± 1.3‰ [3.8‰] at 140 days of age; these paired samples do not have statistically different $\delta^{13}C$ values (Mann-Whitney, $p = 0.567$). The average sow milk $\delta^{13}C$ value was −22.5 ± 1.0‰ [2.9‰]. Piglet hair had an average $\delta^{13}C$ value of −20.4 ± 1.2‰ [3.2‰]. Faeces ($n = 15$) samples were analysed as available from second generation pigs, and are generally lower in $\delta^{13}C$ relative to tissues. The average $\delta^{13}C$ value was −25.1 ± 0.7‰ [2.3‰] and is very similar to that of the feed (Figure 2).

Differences across diet groups for all tissues and samples

Statistical comparison of all eight tissues and samples (collagen, muscle, liver, milk, hair, faeces, blood and plasma) has determined that there are differences in $\delta^{13}C$ values among tissues (Kruskal-Wallis, $p = 0.000$; Figure 2). Collagen and hair $\delta^{13}C$ values do not differ significantly, but both tissues are significantly different from the other six tissues and samples. Faeces are similarly distinct from all other tissues and samples. Liver, muscle, milk, blood and plasma are all statistically similar.

The $\Delta^{13}C_{tissue\ -\ tissue}$, $\Delta^{13}C_{tissue\ -\ whole\ diet}$ and $\Delta^{13}C_{tissue-dietary\ protein}$ isotopic offsets. Tissue–tissue, tissue–whole diet and tissue–dietary protein isotopic offsets for all pigs and tissues are presented in Table 3, Figure 3 and Figure 4, respectively. With the exception of $\Delta^{13}C_{tissue\ -\ faeces}$, there are no consistent, significant linear trends in tissue–tissue isotopic offsets from diets 1 to 5. There are some weak linear trends for the tissue-tissue isotopic offsets, for example, in the average $\Delta^{13}C_{muscle\ -\ milk}$ isotopic offsets which range from −0.3‰ (Diet 2) to +1.0‰ (Diet 5). However, these trends only narrowly exceed the analytical reproducibility associated with the isotopic measurements and the magnitude of the fully propagated error associated with replicated pigs consuming the same diet (± 0.5‰ across all diet groups). As faeces $\delta^{13}C$ values closely reflect whole diet $\delta^{13}C$ values, there is a strong linear relationship between pig tissues and faeces, wherein the $\Delta^{13}C_{tissue\ -\ faeces}$ isotopic offset increases with greater contributions of marine protein to total dietary protein.

As established, muscle, plasma, blood, liver and milk are not statistically different, nor are collagen and hair. In contrast, the tissue–whole diet isotopic offsets vary significantly for all tissues across diet groups for all three age categories, ranging, for example, from +3.6 ± 0.1‰ to +7.0 ± 0.4‰ for pig collagen from diet 1 to 5 (Figure 3). These offsets are also significantly linearly correlated with the percentage of marine protein in diet. Spearman's correlation tests determined that $p = 0.000$ for all comparisons with ρ values ranging from 0.832 to 0.991 (fully reported in Table S5). The magnitude of change in $\Delta^{13}C_{faeces\ -\ whole\ diet}$ offset is smaller than that observed for other tissues and samples, but there is nonetheless a linear trend apparent in the data.

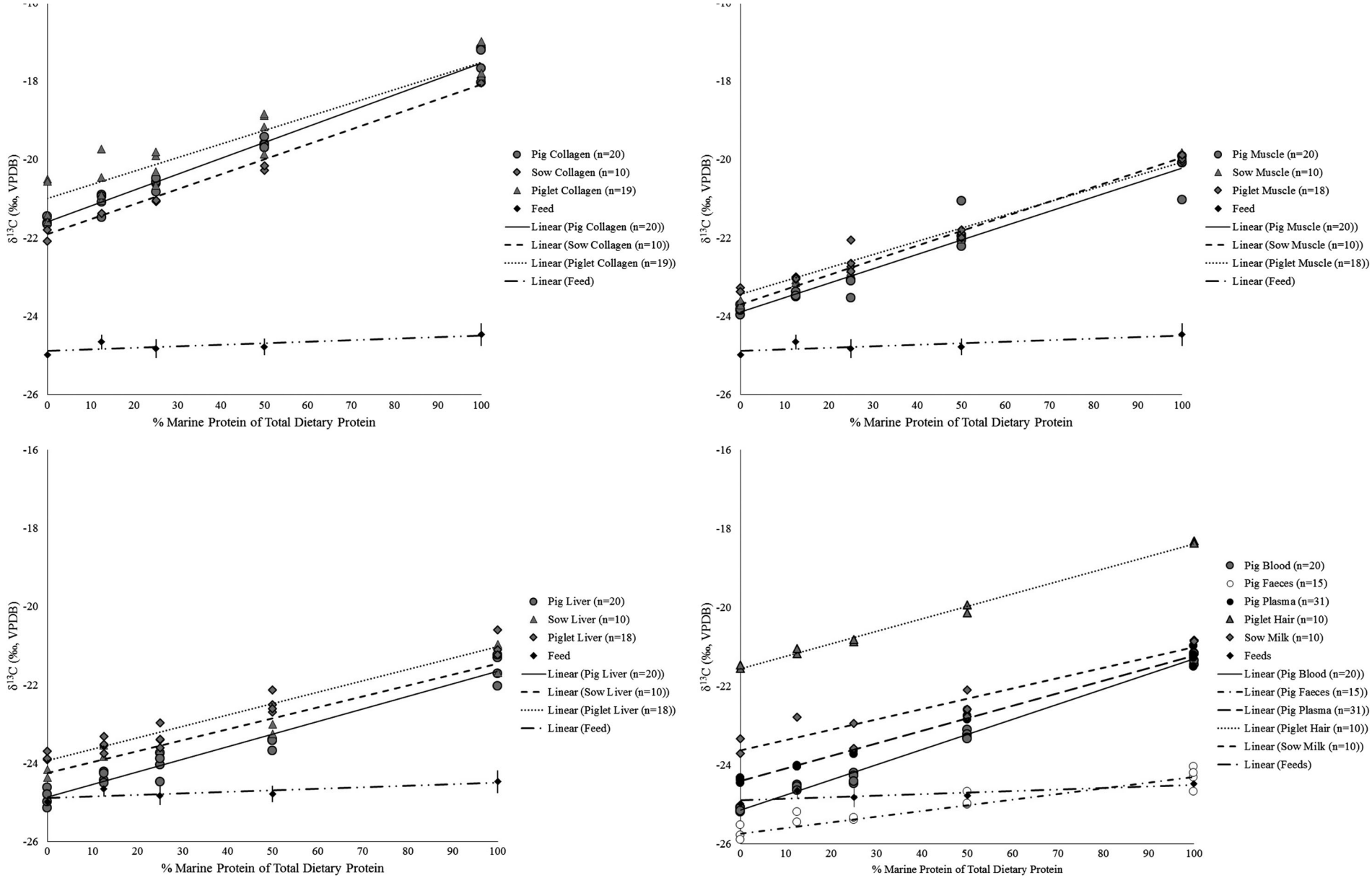

Figure 2. The $\delta^{13}C$ values of all tissues for all pigs discussed herein. For feed samples, the error bars represent one standard deviation about the average value. Note that collagen and muscle carbon-isotope compositions are average values for rib and femoral collagen and loin and femoral muscle, respectively.

Table 3. Tissue–tissue isotopic offsets.

	Diet 1 Average $\delta^{13}C$ (‰, VPDB)	Diet 2 Average $\delta^{13}C$ (‰, VPDB)	Diet 3 Average $\delta^{13}C$ (‰, VPDB)	Diet 4 Average $\delta^{13}C$ (‰, VPDB)	Diet 5 Average $\delta^{13}C$ (‰, VPDB)	Average ± 1σ [range] (‰, VPDB)
Sows						
$\delta^{13}C_{collagen} - \delta^{13}C_{muscle}$	+1.7	+1.9	+1.8	+1.8	+1.9	+1.8 ± 0.1 [0.2]
$\delta^{13}C_{collagen} - \delta^{13}C_{liver}$	+2.3	+2.5	+2.6	+2.9	+3.3	+2.7 ± 0.4 [1.0]
$\delta^{13}C_{collagen} - \delta^{13}C_{milk}$	+1.6	+1.6	+2.2	+2.3	+2.9	+2.1 ± 0.5 [1.3]
$\delta^{13}C_{muscle} - \delta^{13}C_{liver}$	+0.6	+0.6	+0.8	+1.1	+1.5	+0.9 ± 0.4 [0.9]
$\delta^{13}C_{muscle} - \delta^{13}C_{milk}$	−0.1	−0.3	+0.4	+0.5	+1.0	+0.3 ± 0.5 [1.3]
$\delta^{13}C_{liver} - \delta^{13}C_{milk}$	−0.7	−0.9	−0.4	−0.6	−0.5	−0.6 ± 0.2 [0.5]
Piglets						
$\delta^{13}C_{collagen} - \delta^{13}C_{muscle}$	+2.3	+2.5	+2.4	+2.7	+2.4	+2.5 ± 0.1 [0.4]
$\delta^{13}C_{collagen} - \delta^{13}C_{liver}$	+2.8	+3.0	+3.2	+3.3	+3.4	+3.1 ± 0.2 [0.6]
$\delta^{13}C_{collagen} - \delta^{13}C_{hair}$	+0.5	+0.6	+0.7	+0.8	+0.8	+0.7 ± 0.1 [0.4]
$\delta^{13}C_{muscle} - \delta^{13}C_{liver}$	+0.5	+0.5	+0.8	+0.6	+1.0	+0.7 ± 0.2 [0.5]
$\delta^{13}C_{hair} - \delta^{13}C_{muscle}$	+1.8	+1.9	+1.7	+1.9	+1.6	+1.8 ± 0.1 [0.3]
$\delta^{13}C_{hair} - \delta^{13}C_{liver}$	+2.3	+2.4	+2.5	+2.4	2.6	+2.5 ± 0.1 [0.3]
Pigs						
$\delta^{13}C_{collagen} - \delta^{13}C_{muscle}$	+2.3	+2.4	+2.6	+2.2	+2.8	+2.4 ± 0.2 [0.5]
$\delta^{13}C_{collagen} - \delta^{13}C_{liver}$	+3.3	+3.2	+3.4	+3.9	+4.1	+3.6 ± 0.4 [0.8]
$\delta^{13}C_{collagen} - \delta^{13}C_{blood}$	+3.6	+3.5	+3.7	+3.6	+3.8	+3.6 ± 0.1 [0.3]
$\delta^{13}C_{collagen} - \delta^{13}C_{plasma}$	+2.8	+2.9	+3.0	+3.0	+3.7	+3.1 ± 0.3 [0.9]
$\delta^{13}C_{collagen} - \delta^{13}C_{faeces}$	+4.2	+4.2	+5.1	+5.3	+5.5	+4.9 ± 0.6 [1.3]
$\delta^{13}C_{muscle} - \delta^{13}C_{liver}$	+1.0	+0.9	+0.8	+1.9	+1.3	+1.2 ± 0.4 [1.1]
$\delta^{13}C_{muscle} - \delta^{13}C_{blood}$	+1.3	+1.1	+1.1	+1.4	+1.0	+1.2 ± 0.2 [0.4]
$\delta^{13}C_{muscle} - \delta^{13}C_{plasma}$	+0.5	+0.6	+0.5	+0.9	+1.0	+0.7 ± 0.2 [0.5]
$\delta^{13}C_{muscle} - \delta^{13}C_{faeces}$	+1.9	+1.9	+2.5	+3.1	+4.0	+2.7 ± 0.9 [2.2]
$\delta^{13}C_{liver} - \delta^{13}C_{blood}$	+0.2	+0.2	+0.3	−0.2	−0.3	+0.0 ± 0.3 [0.6]
$\delta^{13}C_{liver} - \delta^{13}C_{plasma}$	−0.5	−0.3	−0.4	−0.7	−0.3	−0.5 ± 0.2 [0.4]
$\delta^{13}C_{liver} - \delta^{13}C_{faeces}$	+0.9	+1.0	+1.6	+1.4	+2.7	+1.5 ± 0.8 [1.9]
$\delta^{13}C_{blood} - \delta^{13}C_{plasma}$	−0.7	−0.5	−0.7	−0.5	+0.0	−0.5 ± 0.3 [0.7]
$\delta^{13}C_{blood} - \delta^{13}C_{faeces}$	+0.6	+0.7	+1.3	+1.7	+3.0	+1.5 ± 1.0 [2.4]
$\delta^{13}C_{plasma} - \delta^{13}C_{faeces}$	+1.4	+1.3	+2.0	+2.2	+3.1	+2.0 ± 0.7 [1.8]

The $\delta^{13}C$ values for dietary protein for diets 1 through 5 were estimated using balance equation. The $\delta^{13}C_{dietary\ protein}$ values are thereby estimated to be −25.6, −25.0, −24.3, −23.1 and −20.5‰ for diets 1 through 5. The linear relationships between % marine protein and $\Delta^{13}C_{tissue\ -\ dietary\ protein}$ isotopic offsets for collagen, muscle, liver, milk, hair and faeces are statistically significant, however the $\delta^{13}C_{tissue} - \delta^{13}C_{dietary\ protein}$ values do vary among the tissues (Figure 4).

Preliminary $\delta^{13}C_{AA}$ results. Preliminary $\delta^{13}C$ results for individual amino acids from the 5 diet feeds, 10 sow collagen and 10 sow muscle samples are presented in Figure 5 and supplementary table 6. $\delta^{13}C_{AA}$ values for individuals on replicated diets are typically within 0.75 ‰ of each other and so are combined as an average $\delta^{13}C_{AA}$ value for each feeding group. For essential amino acids, $\Delta^{13}C_{AA\ tissue\ -\ AA\ whole\ diet} \approx 0$‰ as expected, since these amino acids must be directly routed from dietary protein. Non-essential amino acids in low marine protein feed groups show an increased $\Delta^{13}C_{AA\ tissue\ -\ AA\ whole\ diet}$ reflecting their mixed direct assimilation *vs. de novo* synthesis origins. As the marine protein content of the diet increases $\Delta^{13}C_{AA\ tissue\ -\ AA\ whole\ diet}$ for non-essential amino acids trends towards 0‰. Across all feeds and tissues there is a trend towards increasing $\delta^{13}C_{AA}$ with increasing marine protein content which is indicative of the marine origin of these proteins.

Discussion

Differences among individuals and tissues

The similarity in $\delta^{13}C$ compositions across age categories for commonly-sampled tissues (i.e. collagen, muscle and liver) is not unexpected. All pigs consumed one of the controlled feeding study diets during periods of significant growth or for a prolonged period of time (i.e. sows and pigs). Piglets, although slaughtered at a young age and likely still consuming sow milk, would also have been consuming pelleted feed. Sow milk production typically peaks 21 days post-partum and the volume of milk produced after that time is insufficient to meet the nutritional requirements of growing piglets (Patience et al., 1995). Thus, it is expected that piglets were consuming pelleted feed *ad libitum* for at least one week before slaughter, which, coupled with a rapid growth rate and high tissue turnover, would obscure any potential milk-related ^{13}C-depletion in piglet tissues relative to sow tissues.

Among pigs from all age categories consuming the same diet, tissue $\delta^{13}C$ values were very similar, with standard deviations of ±0.1 to ±0.7‰ (Table 3). These low inter-individual differences suggest that, among healthy organisms consuming an identical, nutritionally-adequate diet, individual metabolic differences do not introduce significant variability into bone collagen, muscle, liver, blood, faeces, hair, milk or plasma $\delta^{13}C$ compositions. This outcome further suggests that small differences in $\delta^{13}C$ values among individuals may be interpretively significant.

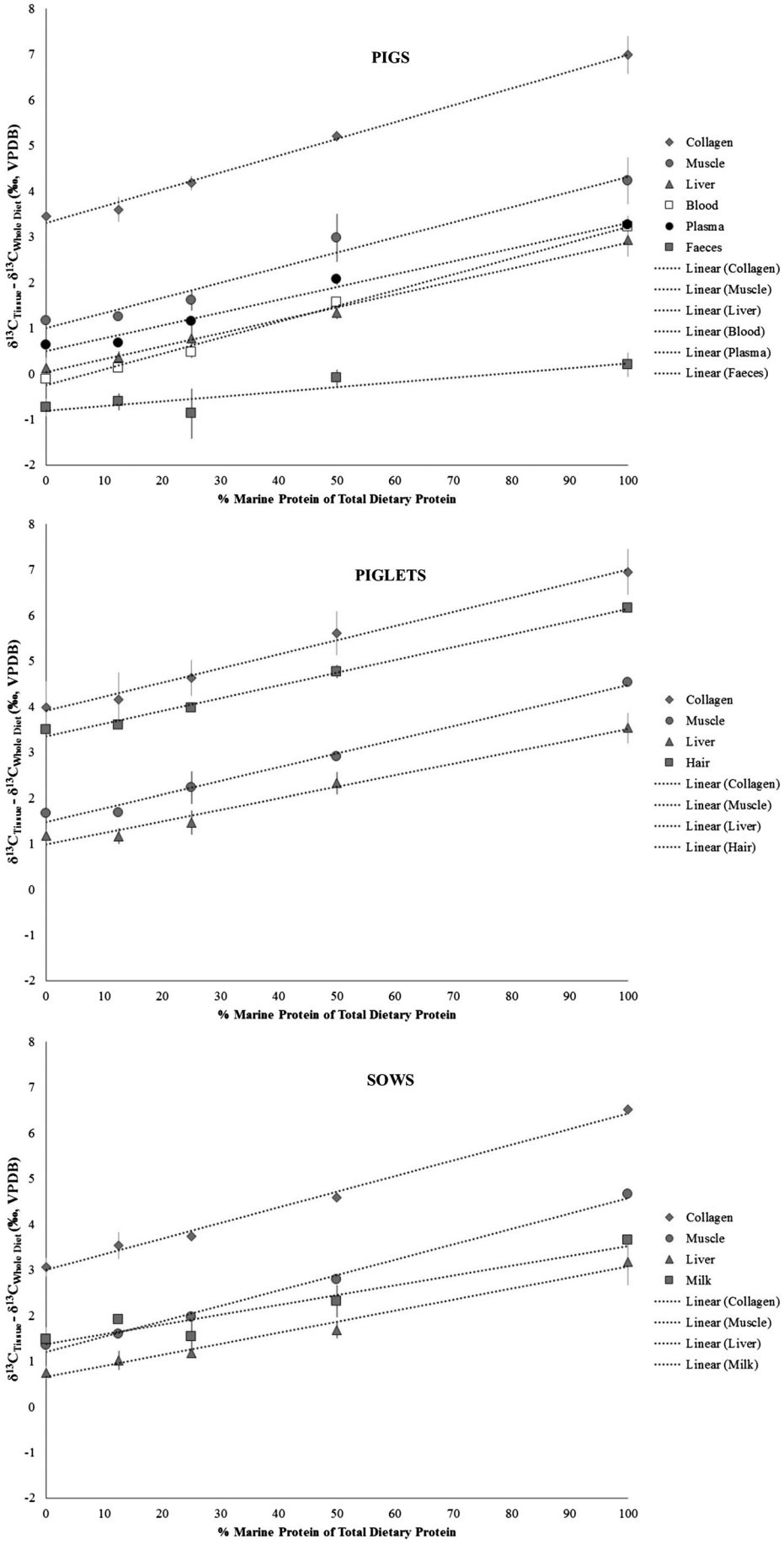

Figure 3. The $\delta^{13}C_{tissue} - \delta^{13}C_{whole\ diet}$ isotopic offsets for all tissues for all pigs discussed herein. The error bars represent one standard deviation about the average value.

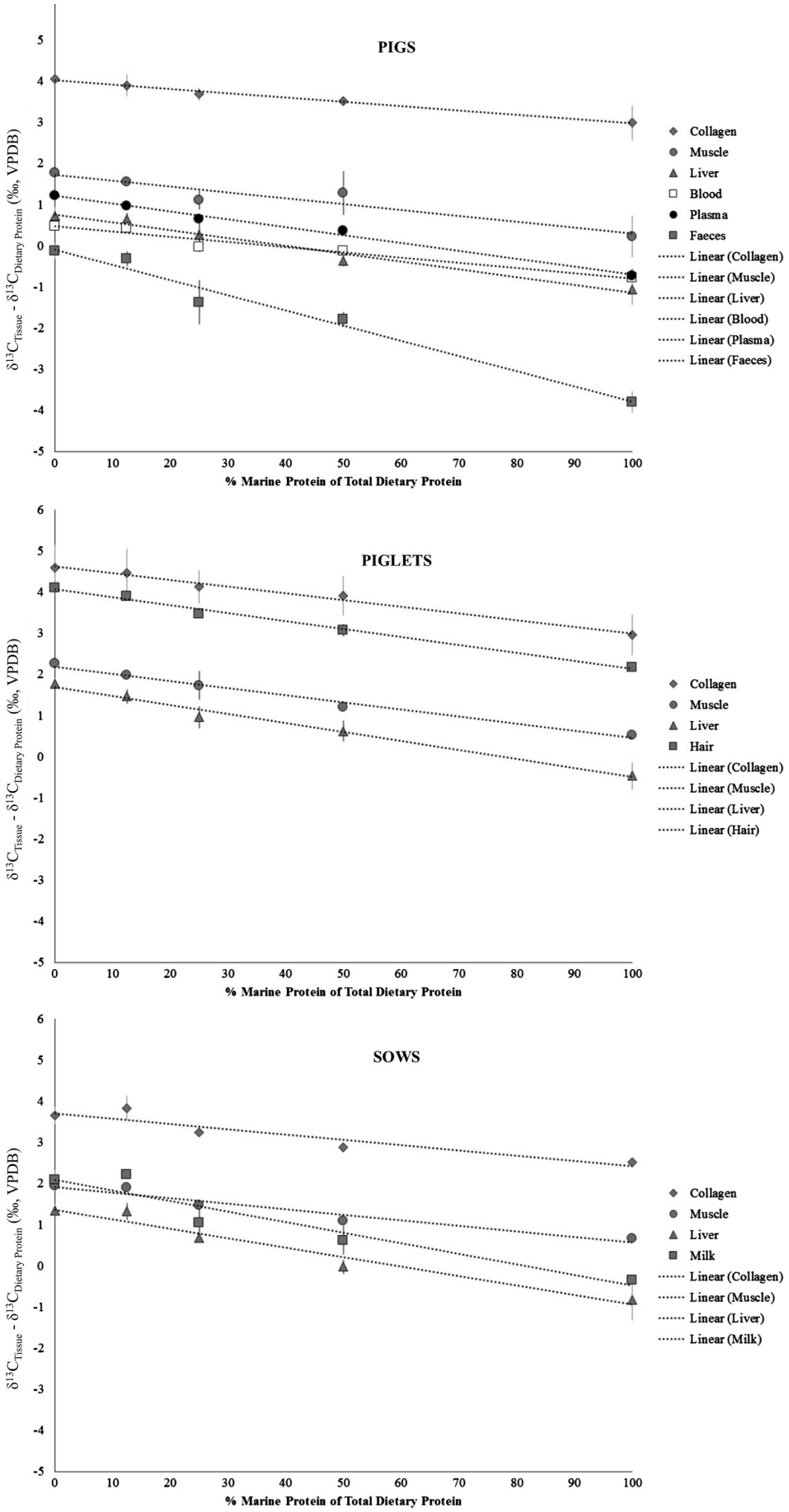

Figure 4. The $\delta^{13}C_{tissue} - \delta^{13}C_{dietary\ protein}$ isotopic offsets for all tissues for all pigs discussed herein. The error bars represent one standard deviation about the average value.

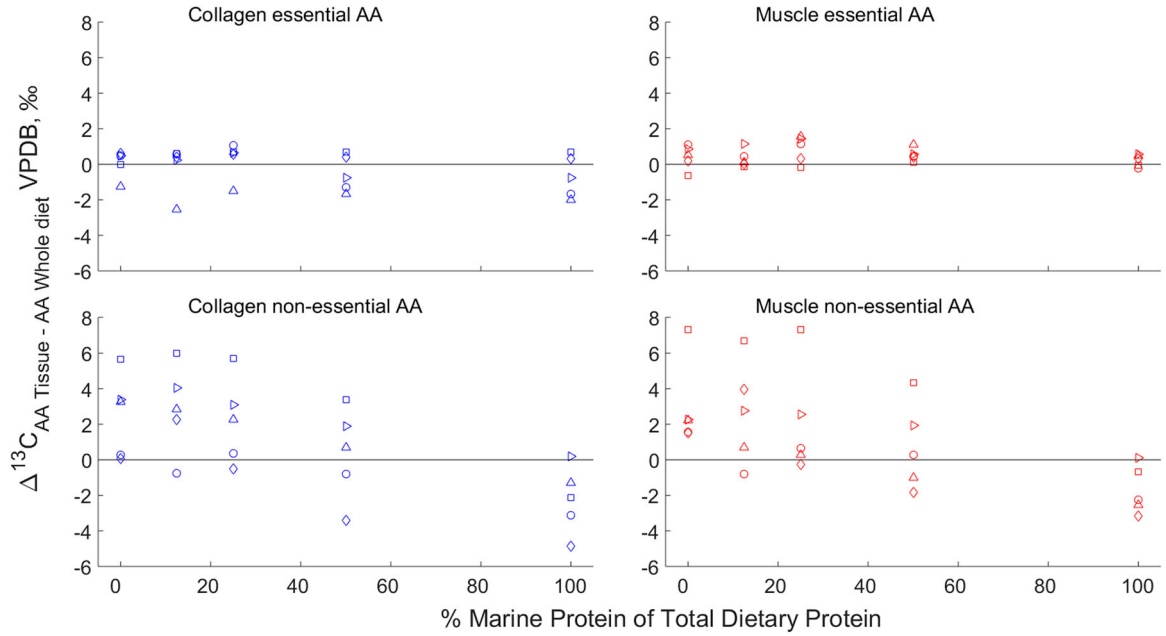

Figure 5. $\Delta^{13}C_{\text{tissue AA – whole diet AA}}$ for essential and non-essential amino acids from sow bone collagen and muscle samples. For essential amino acids phenylalanine (◇), lysine (□), threonine (△), valine (○) and leucine/isoleucine (▷) are shown for non-essential amino acids aspartic acid (◇), glutamic acid (□), glycine (△), alanine (○) and proline (▷) are shown.

Although variable bone turnover rates may induce differences in tissue isotopic compositions intra-skeletally if diet has changed over several years (Cox & Sealy, 1997; Hedges et al., 2007; Hill & Orth, 1998; Taylor et al., 2013), our comparison of the femoral and rib collagen data support the contention that there is no inherent $\delta^{13}C$ difference of the protein component of different bones given a constant dietary input. Similarly, co-forming femoral and loin muscle do not differ isotopically, further supporting the hypothesis that intra-tissue differences across an organism are minimal. As a result of the comparatively fast turnover of blood, plasma and liver (i.e. several days), these samples will reflect short-term diet. Diet did not change throughout the experiment and, as expected, there is good agreement among the $\delta^{13}C$ values of these tissues, and between replicate plasma samples taken mid-study and shortly before slaughter. Similarly, faeces $\delta^{13}C$ also reflect short-term diet (i.e. a few days). Faeces $\delta^{13}C$ values were remarkably consistent among pigs consuming the same diet, ranging from ±0.3 to ±0.6 ‰ across the five diet groups, and there is also good agreement between faeces and whole diet $\delta^{13}C$ values (+0.2 ± 0.3‰). This trend is readily observable in our data because diet did not change during the pig's lifetime, but a similar relationship between whole diet and faeces has been suggested for other species in wild contexts (Codron et al., 2007; Sponheimer et al., 2003).

As expected, different tissue types have different $\delta^{13}C$ compositions. These differences most likely result from the impact of tissue-specific rates tissue growth and of the rate of reaction on carbon-isotope fractionation, wherein the $\delta^{13}C$ values of fast-growing tissues would thus be expected to be more similar to those of whole diet. Further, bulk tissue isotope compositions are essentially weighted averages of the $\delta^{13}C$ values of the individual amino acids that constitute the protein(s) making up the tissue. Individual amino acids can vary significantly in their $\delta^{13}C$ values; for example, isotopic ranges of greater than 20‰ have been determined under controlled feeding conditions for pig collagen amino acids (Hare et al., 1991; Howland, 2003; Jones, 2002). Thus, the $\delta^{13}C$ composition of the various tissues and fluids sampled here are also expected to be moderately different because the dietary $\delta^{13}C_{AA}$ values vary among the five different feeds due to the changing dietary protein source. Tissues are relatively ^{13}C-enriched as follows: $\delta^{13}C_{collagen} \approx \delta^{13}C_{hair} > \delta^{13}C_{muscle} \approx \delta^{13}C_{milk} \approx \delta^{13}C_{blood} \approx \delta^{13}C_{plasma} > \delta^{13}C_{liver} > \delta^{13}C_{faeces} \approx \delta^{13}C_{diet}$.

Relationships between tissues, whole diet, and dietary protein carbon-isotope compositions

The $\delta^{13}C$ offsets between each tissue and the whole diet $\delta^{13}C$ composition of the corresponding pig feed were examined. As expected, the $\delta^{13}C$ values of all tissues and faeces increased as the proportion of marine protein in diet increased. The overall change is small from 0 to 100% marine protein contribution (e.g. ~+3.5‰ for collagen), but, again, this is not unexpected given the relatively small difference between the terrestrial (soy meal, −25.6‰) and marine (fish meal, −20.5‰) end members for this study. Contrary to the widely-held assumption that tissue – whole

diet fractionation would be similar for all diets, we determined that the $\Delta^{13}C_{tissue\ -\ whole\ diet}$ values vary widely relative to the isotopic compositions of tissues and whole diet. For example, the overall change in $\delta^{13}C_{collagen}$ values for pigs was +4.7‰ from diet 1 to diet 5 (−21.8 to −17.1‰, Supplementary Table 4), and the tissue – whole diet offset ranged from +2.8 to +7.2‰ (Supplementary Table 5), however, whole diet $\delta^{13}C$ remains essentially unchanged across all diets. The $\Delta^{13}C_{tissue\ -\ whole\ diet}$ values thus appear to be highly sensitive to marine protein consumption. In the context of archaeological and ecological research, this degree of variability is large enough to make dietary reconstruction considerably less informative. Without making *a priori* assumptions about the likely proportions of marine vs. terrestrial protein intake, relating the $\delta^{13}C_{tissue}$ values to the isotopic composition of various dietary resources in the local food web becomes problematic.

It is possible that the variability in tissue–whole diet offset and its apparent relationship to the consumption of marine protein observed here is, in fact, spurious. Re-assessment of controlled feeding study $\delta^{13}C$ data has clearly demonstrated that when diet is not monoisotopic, that is to say, the energy and protein components have significantly different $\delta^{13}C$ compositions, the $\Delta^{13}C_{collagen\ -\ whole\ diet}$ offset will depend on the specific isotopic composition of the different dietary constituents (Ambrose & Norr, 1993; Froehle et al., 2012). The offset between dietary protein and whole diet ($\Delta^{13}C_{dietary\ protein\ -\ whole\ diet}$) appears to directly influence the $\Delta^{13}C_{collagen\ -\ whole\ diet}$ relationship, regardless of the kind of protein consumed. Thus, when the $\delta^{13}C_{dietary\ protein}$ value is high relative to the $\delta^{13}C_{whole\ diet}$ value, the $\Delta^{13}C_{collagen\ -\ whole\ diet}$ offset is also high and the $\Delta^{13}C_{collagen\ -\ dietary\ protein}$ offset is low. As a result, the isotopic data will seemingly indicate that C_4 plant/marine protein in diet contributes more carbon to collagen than does C_3 plant foods (Froehle et al., 2010). Although this assertion cannot be challenged at the bulk $\delta^{13}C$ level, it nonetheless does not take into consideration differences at the amino acid level. By pooling C_4 plant and marine protein consumers together, this explanation implicitly treats "protein" as a homogenous macronutrient. As previously discussed, different proteins have specific amino acid abundances, and derive their constituent amino acids from different resources in the natural environment. We contend that assuming that all nutritionally-adequate proteins contain the same proportion of bioavailable amino acids with the same range of isotopic variability among individual $\delta^{13}C_{AA}$ values underestimates the actual complexity of different dietary protein sources and their metabolism.

Alternatively, we propose that the relationship between the amount of marine protein in the pig feed and the $\Delta^{13}C_{tissue\ -\ whole\ diet}$ and $\Delta^{13}C_{tissue\ -\ dietary\ protein}$ values results from preferential routing of non-essential amino acids, particularly glycine, from diet to tissue with increasing fish meal consumption (*sensu* Corr et al., 2005). The biosynthesis of non-essential amino acids occurs via enzyme-catalysed reactions and, in some cases, as many as three enzymes are required for synthesis. Biosynthesis can, however, be inhibited by an over-abundance of the end-product, largely because it is more energetically-efficient to make use of available amino acids than it is to make new ones. The enzyme utilised in the first irreversible reaction of glycine synthesis will not be produced in quantity if there is a high concentration of glycine readily available for tissue growth and repair (Schwarcz, 2000; Umbarger, 1978). The bioavailability in the dietary protein source of a particular non-essential amino acid could thus influence the dominance of biosynthesis vs. routing. Glycine constitutes approximately 17% of collagen-carbon and ∼one-third of all amino acid residues in collagen (Herring, 1972). There is a higher concentration of glycine in marine protein than in most kinds of terrestrial protein, including soy,[4] and the $\delta^{13}C$ values of marine glycine are typically higher than those of C_3 or C_4 plants and their consumers (Corr et al., 2005; Fantle et al., 1999; Hare et al., 1991; Howland, 2003; Keil & Fogel, 2001; Young, 2002). Preferential routing of non-essential amino acids from diet is thought to occur simply under conditions of higher protein consumption (e.g. as recently discussed in Fernandes et al. (2012), regardless of the type of protein.

Stable isotopic analysis of amino acids from archaeological human and faunal and controlled feeding study samples provides some support for the preferential routing explanation (e.g. Corr et al., 2005; Howland, 2003; Howland et al., 2003; Jim et al., 2006; Jones, 2002; Young, 2002). For rats fed on diets containing different energy and protein resources (e.g. C_3 carbohydrates and lipids with C_4 protein), Jim et al. (2006) determined that, when dietary protein is present in excess of basic requirements, a non-trivial proportion of non-essential amino acids are routed with minimal fractionation from the protein component of diet (e.g. glycine ∼43%, aspartate ∼28%). It would therefore be expected that tissue non-essential amino acid carbon isotopic compositions would approach the corresponding feed isotopic compositions with higher protein content diets, and this did indeed occur for the controlled feeding study with rats. Crucially, however, the kind of protein (milk casein) was the same for all diets (i.e. only the carbon-isotope compositions were different), so the results of this controlled feeding study (Jim et al., 2006) are not directly applicable here. A subset of carbon isotopic data from the controlled feeding study conducted by Harvard University and the United States Department of

Agriculture (Young, 2002; see also Howland, 2003; Howland et al., 2003) is more relevant to elucidating the mechanism that underlies our findings. A comparison of the $\delta^{13}C_{AA}$ values from two of the diets in that study, specifically, the maize-soybean and maize-fish meal diets, reveals distinct differences in the $\Delta^{13}C_{tissue\ -\ whole\ diet}$ isotopic offsets for glycine. For the exclusively terrestrial protein diet, the $\Delta^{13}C_{Gly\ tissue\ -\ Gly\ whole\ diet}$ offset was +5.2 ± 2.5‰, and was +0.8 ± 2.6‰ for the marine protein diet. Preliminary compound-specific carbon-isotope compositions for muscle and collagen from the 10 sows in this controlled feeding study further demonstrate that the relative predominance of glycine biosynthesis vs. direct routing gradually and linearly increases as the proportion of marine dietary protein increases (Figure 5). We contend that this change in carbon isotopic discrimination indicates a shift from glycine biosynthesis to direct routing caused by the change in the nature of dietary protein, rather than protein quantity or quality, and that it becomes significant after approximately 50% of the dietary protein is marine-derived. The $\Delta^{13}C_{tissue\ AA\ -\ AA\ whole\ diet}$ relationships of other non-essential amino acids (e.g. proline and glutamic acid) also appear to be linearly correlated to the proportion of marine protein in diet. Glycine is likely to have a greater impact on bulk tissue $\delta^{13}C$ values, however, because both the $\Delta^{13}C_{Gly\ tissue\ -\ Gly\ whole\ diet}$ offsets and the absolute $\delta^{13}C_{Gly}$ values change significantly with increasing marine protein consumption. Future compound-specific isotopic analysis of the University of Bristol controlled feeding study pigs will provide further insight into the nature of this proposed change in glycine metabolism through the analysis of multiple tissues and larger numbers of pigs per diet group.

Conclusion

Stable carbon-isotope analysis of archaeological human and animal remains is routinely used to investigate palaeodiet, and is a particularly valuable tool in ecologically-complex regions where many different classes of dietary resources may have been consumed. In recent years, however, it has become increasingly evident that many of the fundamental assumptions about the biochemical relationships between consumer tissues and dietary intake are poorly understood. In this study, we investigated the impact of marine protein consumption on consumer tissue isotopic compositions through a controlled pig feeding study. This study has shown that, when a mixed diet incorporating marine resources is consumed, bulk tissue $\delta^{13}C$ values may be misleading. Further, without considerable *a priori* knowledge about the relative proportions of protein sources consumed, it is difficult to accurately relate tissue carbon-isotope compositions to food resource isotopic data from the natural environment.

We have quantitatively demonstrated that bulk carbon-isotope values must be interpreted with caution when reconstructing palaeodiet and that any attempt at quantifying the proportion of marine/terrestrial resource consumption beyond the broadest level must be very carefully considered indeed. We have shown that even a small change in the amount of marine protein consumed can induce large changes in the relationships between tissue and whole diet and tissue and dietary protein, with comparatively small changes in the $\delta^{13}C_{tissue}$ value. Tissue–whole diet carbon-isotope discrimination changed significantly and was correlated with dietary protein source, specifically with increasing marine protein consumption. This outcome implies that our current understanding of the underlying amino acid isotopic composition and how it relates to the more commonly-used bulk isotopic compositions is poor, and that solely using bulk isotopic compositions not only masks considerable information about diet but may, in fact, present an erroneous picture of resource consumption. Using preliminary amino acid carbon-isotope compositions, we argue that increased routing of non-essential amino acids, especially isotopically-heavy amino acids like glycine, is the primary cause of this dynamic tissue–whole diet offset when marine protein is consumed. Ongoing stable isotope analysis of individual amino acids will determine if this is indeed the mechanism driving the increasing $\Delta^{13}C_{tissue\ -\ whole\ diet}$ isotopic offset.

Notes

1. As opposed to compound-specific isotopic compositions, e.g. of individual amino acids.
2. Excluding piglet W15 for which only femoral bone collagen could be extracted.
3. Loin muscle samples were only available for eight piglets.
4. Fish meal contains approximately 2.3 times as much glycine as soymeal, i.e. 10.2g/16g N vs. 4.5g/16g N, where 16g N ≈ 100g of protein (Jorgensen et al., 1984).

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