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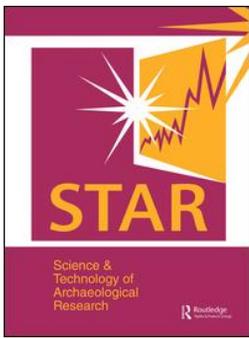
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## Sulphur-isotope compositions of pig tissues from a controlled feeding study

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### ABSTRACT

Sulphur-isotope determinations are becoming increasingly useful for palaeodietary reconstruction, but knowledge of isotopic discrimination between diet and various tissues remains inadequate. In this study, we explore the sensitivity of  $\delta^{34}\text{S}_{\text{tissue}}$  values to changes in  $\delta^{34}\text{S}_{\text{diet}}$  values, sulphur isotopic discrimination between diet and consumer, and the potential impact of terrestrial vs. marine protein consumption on these discrimination offsets. We present new  $\delta^{34}\text{S}$  values of bone collagen, muscle, liver, hair, milk and faeces from ten mature sows, ten piglets and fifteen adolescent pigs from a controlled feeding study. The  $\delta^{34}\text{S}_{\text{tissue}}$  values were found to co-vary with the  $\delta^{34}\text{S}_{\text{diet}}$  values, the  $\delta^{34}\text{S}_{\text{tissue}} - \delta^{34}\text{S}_{\text{diet}}$  isotopic offsets ( $\Delta^{34}\text{S}_{\text{tissue-diet}}$ ) are small but consistent, and dietary protein source does not systematically alter the  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  isotopic discrimination. The outcomes of this study are of particular relevance to questions that are difficult to resolve using carbon and nitrogen stable isotopes alone, and will also be useful in regions where terrestrial, freshwater, and marine resources could have all potentially contributed to human diet.

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Sulphur isotopes; isotopic discrimination; palaeodiet

## Introduction

In the last decade, improvements in isotope ratio mass spectrometry (IRMS) and associated sample introduction instrumentation have enabled high throughput analysis of biological tissues for their sulphur isotopic compositions ( $\delta^{34}\text{S}$ ). This technological improvement has resulted in an increase in the use of sulphur stable isotope analyses for palaeodietary reconstruction, migration, and ecological research. For example,  $\delta^{34}\text{S}$  values have been used to distinguish between marine and terrestrial resource consumption, assess the interaction between terrestrial and estuarine environments, disentangle terrestrial, freshwater and marine resource contributions to diet in complex ecosystems, and to elucidate migration and residential proximity to the sea (*inter alia*, Arneson and MacAvoy, 2005; Craig et al., 2006; Howcroft et al., 2012; Nehlich, 2015; Nehlich et al., 2010, 2011; Oelze et al., 2012a, 2012b; Privat et al., 2007; Richards et al., 2001, 2003; Sayle et al., 2013; Valenzuela et al., 2011; Wilson et al., 2007).

Typically, it is assumed that there is minimal sulphur isotopic fractionation between source and consumer relative to the large scale of environmental variation in  $\delta^{34}\text{S}$  values (i.e.  $\Delta^{34}\text{S}_{\text{tissue-diet}} \approx 0\text{‰}$ , Peterson et al., 1985; Richards et al., 2003). There are, however, few controlled feeding studies involving animals that can be reliably used to make inferences about

tissue – diet and tissue – tissue isotopic discrimination for humans. Studies using, for example, invertebrates (e.g., McCutchan et al., 2003), fish (e.g., Barnes and Jennings, 2007) and ruminants (e.g., Tanz and Schmidt, 2010) as animal models, although valuable in their field, are of limited use for human palaeodietary reconstruction. More relevant feeding experiments using pigs (Gonzalez-Martin et al., 2001), horses (Richards et al., 2003) and mice (Arneson and MacAvoy, 2005) have been hampered by small sample sizes or few replicates per dietary group, and have not included the investigation of archaeologically-relevant tissues (i.e., bone collagen). With the increasing use of sulphur isotopic analysis for palaeodiet reconstruction in complex ecosystems, further research using a larger sample sizes, a more appropriate animal model, and multiple consumer tissues and excreta is needed. This will enable a more complete understanding of sulphur incorporation into different tissues in the body.

A controlled feeding study was undertaken through the University of Bristol with the objective of investigating the impact of terrestrial vs. marine-derived dietary protein consumption on isotopic discrimination between different tissues, and between tissues and diet. Two generations of pigs were fed one of five feeds with varying proportions of terrestrial (soy) and marine (fish meal) protein. The overarching goal of

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this project was to improve the understanding of consumer tissue isotopic compositions for multiple isotopic proxies, and in doing so, enable increasingly refined palaeodietary reconstructions in complex ecosystems ( $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ ,  $^{87}\text{Sr}/^{86}\text{Sr}$  and  $\delta^{88}\text{Sr}$ ; Webb et al., 2016; Webb et al., 2017; Lewis et al., 2017).

Here, we explore the sensitivity of  $\delta^{34}\text{S}_{\text{tissue}}$  values to changes in  $\delta^{34}\text{S}_{\text{diet}}$  values, sulphur isotopic discrimination between diet and consumer, and the potential impact of terrestrial vs. marine protein source on these discrimination offsets. We present  $\delta^{34}\text{S}$  values of bone collagen, muscle, liver, hair, milk and faeces from ten mature sows, ten piglets and fifteen adolescent pigs from the controlled feeding experiment.

### Sulphur isotope systematics

There are four stable isotopes of sulphur,  $^{32}\text{S}$  (>94.93%),  $^{33}\text{S}$  (0.76%),  $^{34}\text{S}$  (4.29%) and  $^{36}\text{S}$  (0.02%) (Rosman and Taylor 1997). For stable S isotope analysis, the ratios of the most abundant isotopes  $^{34}\text{S}$  and  $^{32}\text{S}$  are measured and reported as the comparative measure  $\delta^{34}\text{S}$  using the delta notation relative to Vienna Canyon Diablo Troilite (Robinson, 1995). Bedrock average  $\delta^{34}\text{S}$  values vary widely and depend on rock type, age and dominant valence state of the sulphur, and may range from  $\sim -19$  to  $+30$  ‰ (Faure, 1977; Peterson and Fry, 1987). Terrestrial plants acquire sulphur predominantly from soil, most of which is in the form of sulphate derived from bedrock weathering (Krouse et al., 1991). Atmospheric sulphur can, however, constitute up to 35% of plant sulphur, acquired through wet deposition (i.e., sea spray or acid rain) and dry deposition (i.e.,  $\text{SO}_2$  gas; Krouse et al., 1991). Plant sulphur is then stored largely in biomolecules, of which approximately 90% are sulphur-containing amino acids (Blair, 1979). As plant sulphur-isotope compositions vary based on bedrock geology and proximity to the ocean, global terrestrial plant  $\delta^{34}\text{S}$  values range widely from  $\sim -30$  to  $+35$  ‰ (Coplen et al., 2002). Quantifying terrestrial plant isotopic fractionation is complicated by contributions of sulphur with a different isotopic composition from atmospheric deposition. There is, however, a tendency for organic sulphur in plants to be  $^{34}\text{S}$ -depleted by  $\sim 1$  to  $2$  ‰ relative to local sulphur sources. Ocean water sulphate ( $\text{SO}_4^{2-}$ ) is isotopically uniform with a  $\delta^{34}\text{S}$  value of  $\sim +20$  ‰; consequently, marine primary producers typically have  $\delta^{34}\text{S}$  values ranging from  $\sim +17$  to  $+21$  ‰ (Coplen et al., 2002; Nehlich et al., 2011). Marine algae are  $^{34}\text{S}$ -depleted relative to the sulphate source by  $\sim 1$  to  $2$  ‰. Higher trophic level plants typically have no or small differences in  $\delta^{34}\text{S}$  values compared to local sulphur in the environment (Mekhtieva et al., 1976; Peterson and Fry, 1987).

The sulphur-isotope compositions of consumer tissues reflect the  $\delta^{34}\text{S}$  values of consumed food, which

are in turn controlled by the  $\delta^{34}\text{S}$  values of bedrock, atmospheric sulphur (modified by sea spray) and sulphur altered through soil microbial processes. Sulphur is an essential element for animals, and is found in the amino acids cysteine, methionine and taurine, as well as some vitamins and cofactors. Unlike plants, animals cannot directly assimilate and metabolise  $\text{SO}_4^{2-}$  to form amino acids, they must ingest sufficient quantities of organic sulphur-containing compounds (Nehlich 2015). As such, methionine (Met) is an essential amino acid, and is the only sulphur-containing amino acid in bone collagen protein (five residues per 1000; Eastoe, 1955). Cysteine, a nonessential amino acid and the dominant sulphur-containing amino acid in hair (112 residues per 1000), can be biosynthesised from methionine and serine or assimilated directly from diet (Valkovic, 1977).

Sulphur isotopic discrimination effects between animal tissues and diet and among tissues in the same animal are generally observed to be small. Variable tissue – diet and tissue – tissue offsets have been observed in mice and horses, typically ranging from  $\pm 0.1$  to  $\pm 1.0$  ‰. Arneson and MacAvoy (2005) observed inter-tissue differences in the degree of  $^{34}\text{S}$ -enrichment or depletion relative to diet for mice, and Richards et al. (2003) determined that tail hair keratin was  $^{34}\text{S}$ -depleted relative to diet ( $\sim -1.0$  ‰) for horses ( $n=1$ ). Arneson and MacAvoy (2005) also compared sulphur isotopic compositions for mice consuming a terrestrial protein-based diet (casein, beet sucrose and soybean oil) and mice consuming a marine protein-based diet (fish meal, cane sucrose and soybean oil). They determined that mice tissues were slightly  $^{34}\text{S}$ -depleted relative to the terrestrial protein diet and slightly  $^{34}\text{S}$ -enriched relative to the marine protein diet. Richards et al. (2003) further postulated that a diet low in digestible protein or deficient in sulphur-containing amino acids may induce a larger tissue – diet isotopic offset due to incorporation of endogenous  $^{34}\text{S}$ -enriched sulphur into growing tissues.

### Controlled feeding experiment

The controlled feeding study was completed at Harper Adams University College (Shropshire, UK), and a full suite of tissues, fluids and excreta were archived. All pigs drank from the same local water source (on site borehole), and each pig was fed one of five diets with known protein source composition: (i) 100% terrestrial-derived (soy), (ii) 87.5% terrestrial/12.5% marine (fish meal), (iii) 75% terrestrial/25% marine, (iv) 50% terrestrial/50% marine, and (v) 100% marine-derived. In the first generation, five groups of gilts (female pigs that have not yet reproduced) were fed one of the above diets from weaning until sacrifice. All first generation pigs were artificially inseminated and a subset of second generation pigs was sacrificed at weaning.

The remaining second generation pigs were fed exclusively on one of the five diets from weaning until sacrifice in adolescence, i.e., when the growth rate plateaued. Pig growth and performance did not vary systematically with diet group, nor were there any statistically significant differences in birth weight, weaning weight, average gain per day or total weight gain (Webb et al., 2017). 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 which have only consumed the experimental diet (including sow milk from the same dietary group), and only one dietary variable – the ratio of terrestrial to marine protein – was manipulated. Moreover, all five diets were nutritionally equivalent, with a constant 20% protein contribution to whole diet, which eliminates variability in isotopic data and tissue – diet isotopic offsets associated with low ( $\leq 5\%$ ) or high ( $\sim 70\%$ ) protein consumption. In total, ten sows (first generation), 19 piglets (aged four weeks) and 39 pigs (second generation, aged 160–190 days) were reared and slaughtered over the course of the study.

Pig feed  $\delta^{34}\text{S}$  values are used to establish isotopic baselines of dietary intake for each of the five diet groups. As a result, the expected differences between marine and terrestrial sulphur isotopic compositions, the bulk  $\delta^{34}\text{S}$  values of the five feeds are expected to vary based on the ratio of marine to terrestrial protein in the experimental diets. Both soymeal and fishmeal are excellent sources of organic sulphur, and a small methionine additive was included in all feeds ( $\leq 0.2\%$  by weight). Other ingredients in the experimental diets may also contribute small amounts of sulphur, corn and tapioca which are present in the formulations for dietary energy also contain small amounts of protein. Thus, internal sulphur recycling caused by inadequate sulphur intake, which was observed by Richards et al. (2003), is not expected to be an important influence on the sulphur isotopic results reported herein.

## Methodology

Ten first generation sows (two per diet group), ten piglets (two per diet group), and fifteen second generation pigs (three per diet group) were selected for isotopic analysis. Femoral bone collagen, femoral muscle and liver samples were analysed for their stable sulphur isotopic compositions for all thirty-five pigs. Milk ( $n=9$ ; sows only), hair ( $n=10$ ; piglets only) and faeces ( $n=15$ ; pigs only) were also analysed. Prior to use, all glassware was washed with Decon 90 and solvent-rinsed before furnacing at  $450^\circ\text{C}$  for 4 h. Aluminium foil and disposable gloves were used to handle samples.

Bone collagen was extracted using a modified Longin (1971) method. For each sample, a section of

bone was taken using a hacksaw or rotary tool (Dremel tool, 3000JB with diamond cutting wheel). The bone was mechanically defleshed using a scalpel, and a silicon carbide burr was used to remove all trabecular bone and  $\sim 0.5$  mm of surface bone. Each sample ( $\sim 500$  mg) was then crushed using a pestle and mortar, and lipids were extracted using 2:1 *v/v* chloroform:methanol solution ( $3 \times 8$  mL solvent solution,  $3 \times 20$  min ultrasonication). Collagen was extracted by soaking in 0.5M hydrochloric acid (HCl) until the bone fragments were entirely soft (typically 48–72 h at room temperature). Extracted collagen was solubilised in  $10^{-3}\text{M}$  HCl at  $75^\circ\text{C}$  for 48 h, filtered (E-Zee filters, 60–90  $\mu\text{m}$ ), and freeze-dried for more than 24 h.

For each soft tissue sample, a cross-section of tissue was removed using a new scalpel blade. For soft tissues ( $\sim 2$  g), faeces (1 g), hair (10 mg) and milk (1 ml) samples were freeze dried for  $>24$  h, lipids were extracted using 2:1 *v/v* chloroform:methanol solution ( $3 \times 8$  mL solvent solution,  $3 \times 20$  min ultrasonication). For all samples, homogenised aliquots were then weighed into tin capsules ( $\sim 11.0$  mg for collagen and  $\sim 2.0$  mg for all other samples) for isotopic analysis. Samples of all four lots of feed (2 g per aliquot) were analysed to assess variation in feed isotopic composition over the course of the feeding study. Homogenised feed samples were weighed into tin capsules ( $\sim 11$  mg) for isotopic analysis.

Combustion and  $\delta^{34}\text{S}$  values were attained using an Elementar (Hanau, Germany) Pyrocube elemental analyser (EA) interfaced with an Isoprime (Stockport, UK) VisION IRMS. The EA uses “purge and trap” chromatography to reliably separate the analyte  $\text{SO}_2$  from other evolved gases even where the carbon: sulphur ratio is very high, as is the case with bone collagen. Several reference materials were used to ensure accuracy: IAEA silver sulphide ( $\text{Ag}_2\text{S}$ ) isotope reference materials S1 (which defines the  $\delta^{34}\text{S}_{\text{VCDT}}$  scale, Krouse and Coplen, 1997;  $-0.3$  ‰), and S2 ( $+22.62$  ‰) and S3 ( $-32.49$  ‰), which bracket most of the natural range of sulphur-isotope compositions. Laboratory standard MSAG2 (a solution of methanesulfonamide and gelatin) was used to check linearity, and both MSAG2 and M1 (methionine; Sigma-Aldrich) were used to check for drift throughout the period of the experiments (Werner and Brand, 2001). The silver sulphides were also used to calculate sulphur content. Finally, for collagen, methodological reproducibility was assessed through duplicate collagen preparation and analysis for 10% of samples, and was  $\pm 0.2$  ‰.

## Results

All isotopic compositions and offsets are reported as mean  $\pm$  standard deviation [range] unless otherwise stated. The average feed  $\delta^{34}\text{S}$  values for diets 1 through 5 are:  $+7.6 \pm 0.5$ ,  $+9.3 \pm 0.2$ ,  $+10.4 \pm 0.8$ ,  $+11.6 \pm 0.2$  and

+15.6 ± 0.5 ‰. There is a strong positive linear relationship between diet and tissue  $\delta^{34}\text{S}$  values for all tissues and for faeces (Pearson's correlation tests,  $p < 0.05$ ). The  $\delta^{34}\text{S}_{\text{diet}}$  values for the 100% terrestrial and 100% marine diets are somewhat lower than pure soy (+10.86 ± 0.4 ‰) and fishmeal (+20.74 ± 0.75 ‰), suggesting sulphur contributions from a secondary source in the diet (e.g., corn or tapioca, which are both potential sources of dietary methionine) or from the methionine supplement, although the latter constitutes a comparatively small feed component it does have a  $\delta^{34}\text{S}$  value less than that of the soy or fish meal ( $\leq 0.2\%$  by weight;  $\delta^{34}\text{S} = 2.2$  ‰). The  $\delta^{34}\text{S}_{\text{diet}}$  values nonetheless increase linearly as the proportion of marine

protein increases from 0 to 100%, suggesting a strong influence of protein source on  $\delta^{34}\text{S}_{\text{diet}}$  values.

The collagen, muscle, liver and milk  $\delta^{34}\text{S}$  values, reported in Tables 1–3 and presented in Figure 1, are lower than the corresponding  $\delta^{34}\text{S}_{\text{diet}}$  values. In contrast, the  $\delta^{34}\text{S}_{\text{hair}}$  values are systematically  $^{34}\text{S}$ -enriched compared to diet, and the  $\delta^{34}\text{S}_{\text{faeces}}$  values are also generally high compared to the  $\delta^{34}\text{S}_{\text{diet}}$  values (Figures 1b and 1c, respectively). For femoral collagen, femoral muscle and liver, the three tissues common to all age groups, the  $\delta^{34}\text{S}$  values were compared. There were no statistically significant differences in  $\delta^{34}\text{S}_{\text{tissue}}$  values associated with age for these three tissues (Kruskal-Wallis,  $p > 0.1$ ).

**Table 1.** Sulphur-isotope compositions of sow tissues.

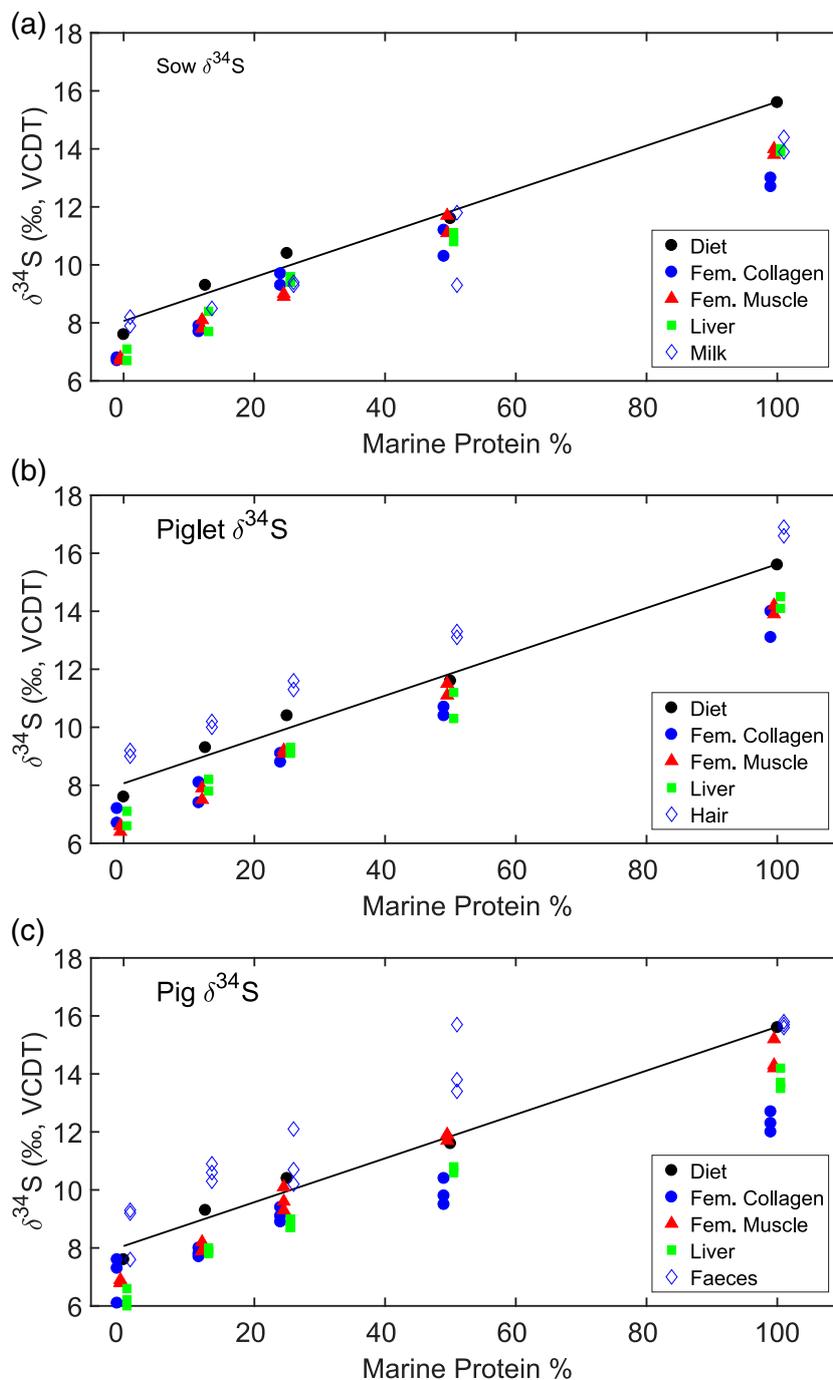
Sample	Marine Protein %	Femoral Collagen		Femoral Muscle		Liver		Sample	Marine Protein %	Milk	
		$\delta^{34}\text{S}$ (‰, VCDT)	%S	$\delta^{34}\text{S}$ (‰, VCDT)	%S	$\delta^{34}\text{S}$ (‰, VCDT)	%S			$\delta^{34}\text{S}$ (‰, VCDT)	%S
F17	0	6.7	0.2	6.8	0.9	7.1	0.9	F17	0	7.9	0.5
F18	0	6.8	0.2	6.7	0.9	6.7	0.7	F18	0	8.2	0.6
F13	12.5	7.7	0.2	7.8	0.9	8.4	0.8	F13	12.5	8.5	0.6
F14	12.5	7.9	0.2	8.1	0.9	7.7	0.7				
F10	25	9.3	0.2	9	0.9	9.6	0.9	F10	25	9.4	0.6
F12	25	9.7	0.2	8.9	0.9	9.4	0.8	F12	25	9.3	0.5
F5	50	10.3	0.2	11.1	0.9	10.8	0.9	F6	50	9.3	0.4
F8	50	11.2	0.2	11.7	1	11.1	0.8	F8	50	11.8	0.5
F2	100	13	0.2	13.8	1	14	0.8	F2	100	13.9	0.5
F3	100	12.7	0.2	14	0.9	13.9	0.9	F3	100	14.4	0.7

**Table 2.** Sulphur-isotope compositions of piglet tissues.

Sample	Marine Protein %	Femoral Collagen		Femoral Muscle		Liver		Hair	
		$\delta^{34}\text{S}$ (‰, VCDT)	%S						
W1839	0	6.7	0.2	6.6	0.9	7.1	0.7	9	3.3
W1846	0	7.2	0.2	6.4	0.8	6.6	0.8	9.2	3.9
W1766	12.5	7.4	0.2	7.5	0.9	7.8	0.8	10	3.7
W1767	12.5	8.1	0.2	7.9	0.9	8.2	0.7	10.2	4.1
W34	25	9.1	0.1	9.2	1	9.3	0.9	11.3	3.9
W43	25	8.8	0.2	9.1	0.9	9.1	0.8	11.6	3.9
W21	50	10.7	0.1	11.5	0.9	11.2	0.8	13.3	3.8
W28	50	10.4	0.2	11.1	1	10.3	0.8	13.1	3.3
W1834	100	13.1	0.1	13.9	0.8	14.1	0.8	16.9	6.5
W1835	100	14	0.1	14.2	0.8	14.5	0.7	16.6	3.7

**Table 3.** Sulphur-isotope compositions of pig tissues. Samples 2-MIX and 5MIX represent faeces combined from multiple individuals on diets 2 and 5 respectively.

Sample	Marine Protein %	Femoral Collagen		Femoral Muscle		Liver		Sample	Marine Protein %	Faeces	
		$\delta^{34}\text{S}$ (‰, VCDT)	%S	$\delta^{34}\text{S}$ (‰, VCDT)	%S	$\delta^{34}\text{S}$ (‰, VCDT)	%S			$\delta^{34}\text{S}$ (‰, VCDT)	%S
226	0	6.1	0.2	6.8	1	6.6	0.7	226	0	9.3	0.4
227	0	7.3	0.2	6.9	1	6.2	0.8	231	0	9.2	0.4
231	0	7.6	0.2	6.8	0.9	6	0.8	232	0	7.6	0.2
266	12.5	7.8	0.2	7.9	0.9	8	0.8	268	12.5	10.6	0.4
267	12.5	8	0.2	7.9	0.8	7.9	0.9	2-MIX	12.5	10.3	0.4
268	12.5	7.7	0.2	8.2	0.9	7.8	0.9	243	25	10.9	0.4
243	25	9.4	0.2	9.6	1	9	0.8	244	25	10.2	0.4
244	25	9.1	0.2	10.1	1	8.7	0.8	248	25	10.7	0.4
248	25	8.9	0.2	9.3	1	8.7	0.8	237	50	12.1	0.4
238	50	9.5	0.2	11.7	0.9	10.8	0.8	238	50	13.4	0.4
239	50	9.8	0.2	11.9	0.9	10.6	0.7	241	50	13.8	0.4
241	50	10.4	0.2	11.9	0.9	10.7	0.9	255	100	15.7	0.5
255	100	12	0.3	14.3	0.9	13.7	0.9	258	100	15.6	0.4
258	100	12.3	0.2	15.2	1	13.5	0.8	260	100	15.7	0.4
260	100	12.7	0.2	14.2	0.9	14.2	0.9	5-MIX	100	15.8	0.5



**Figure 1.** Sulphur isotopic compositions of (a) sow, (b) piglet and (c) pig tissues and samples. Linear regressions are least squares fit to diet  $\delta^{34}\text{S}$ , analytical precision is smaller than symbols.

The  $\delta^{34}\text{S}$  values for all animals are statistically different across all five diets for collagen, muscle, liver and faeces (Kruskal-Wallis,  $p < 0.05$ ). Milk does not differ significantly across diet groups, hair is different across diet groups but narrowly misses significance (Kruskal-Wallis  $p > 0.1$  and  $p = 0.068$ ). Subsequent pair wise Mann-Whitney U tests for collagen, muscle and liver show that there are significant differences between individual diet groups ( $p < 0.01$ ) suggesting that body tissues can be sensitive to incremental changes in dietary  $\delta^{34}\text{S}$  values.

Intra-individual variation (i.e., the  $\delta^{34}\text{S}_{\text{tissue}} - \delta^{34}\text{S}_{\text{tissue}}$  differences within the same animal) was

determined by comparing collagen, muscle, liver, milk and hair  $\delta^{34}\text{S}$  values. The mean range of intra-individual variation was  $\pm 1.0$  ‰ for sows,  $\pm 2.7$  ‰ for piglets ( $\pm 0.6$  ‰ excluding  $\delta^{34}\text{S}_{\text{hair}}$  values), and  $\pm 1.3$  ‰ for pigs. Inter-individual variation was assessed by comparing the isotopic compositions of the different tissues of animals of the same age consuming the same diet. Absolute ranges were used to better characterise the potential range of tissue sulphur isotopic compositions possible for organisms consuming the same diet. The range of sulphur isotopic compositions among animals consuming the same diet for a specific tissue varied from  $\pm 0.1$  to  $\pm 1.5$  ‰.

**Table 4.** Summary of the mean and 1 SD  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  isotopic offsets for sows, piglets and pigs (‰, VCDT).

	Piglets	Pigs	Sows	All Animals
Femoral Collagen	$-1.4 \pm 0.6$	$-1.7 \pm 1.0$	$-1.4 \pm 0.8$	$-1.5 \pm 0.8$
Femoral Muscle	$-1.2 \pm 0.5$	$-0.7 \pm 0.6$	$-1.1 \pm 0.6$	$-1.0 \pm 0.6$
Liver	$-1.1 \pm 0.4$	$-1.4 \pm 0.4$	$-1.0 \pm 0.4$	$-1.2 \pm 0.4$
Hair	$+1.2 \pm 0.3$			
Faeces		$+0.7 \pm 0.8$		
Milk			$-0.8 \pm 1.0$	

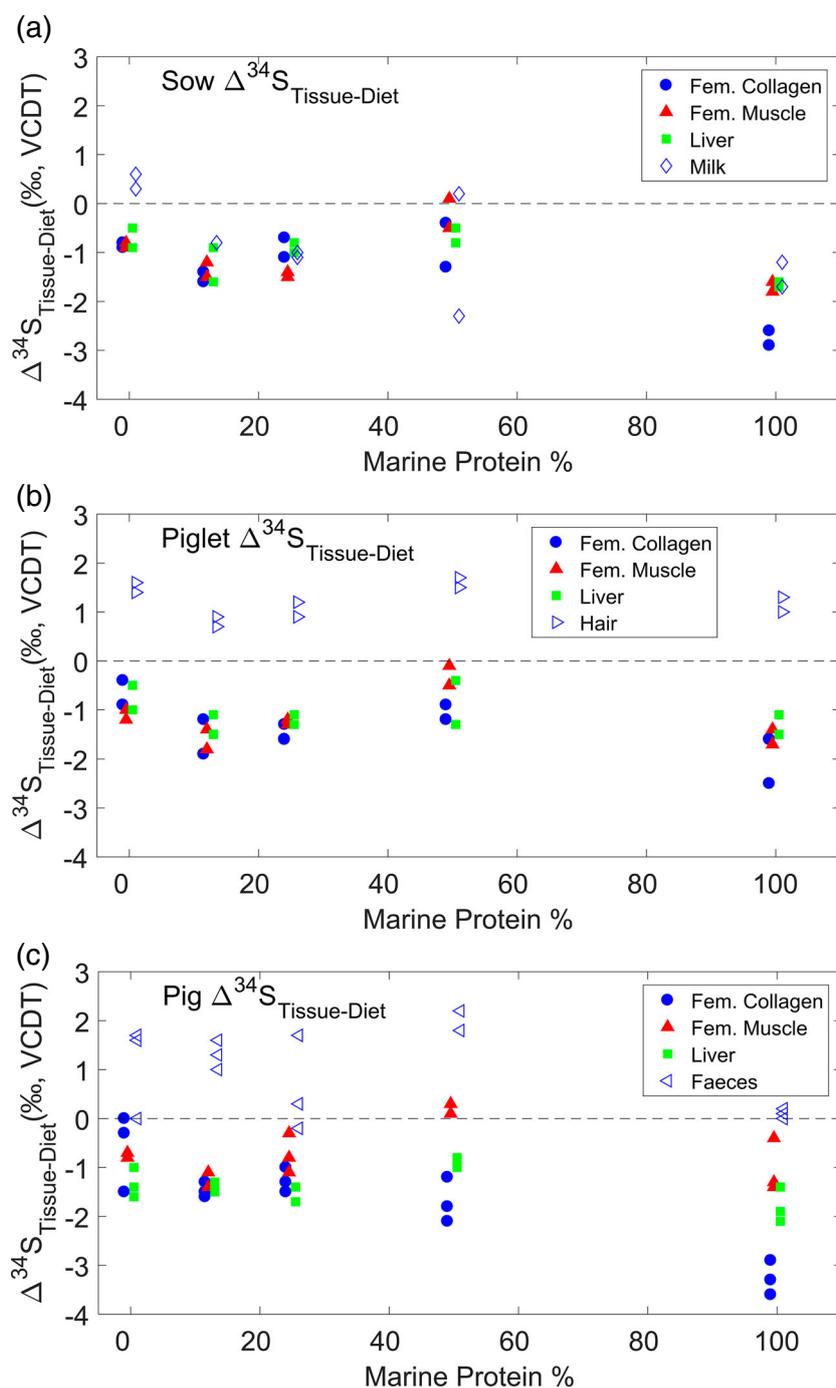
For each tissue, the  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  offsets were compared among age categories, and revealed that the distribution of tissue – diet isotopic offsets is the same across groups of piglets, pigs and sows (Kruskal-Wallis,  $p > 0.05$ ). The mean  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  offsets are summarised

in Table 4 and presented in Figure 2. The relationship between the  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  offsets and % marine protein was, for most tissues, not statistically significant (Spearman's rank correlation test,  $p > 0.100$ ), with the notable exception of the pig collagen  $\delta^{34}\text{S}_{\text{tissue}} - \delta^{34}\text{S}_{\text{diet}}$  offsets ( $p = 0.003$ ).

## Discussion

### $\delta^{34}\text{S}_{\text{tissue}} - \delta^{34}\text{S}_{\text{diet}}$ isotopic relationships

There was a strong linear relationship between dietary and consumer tissue  $\delta^{34}\text{S}$  values, which was expected because sulphur is an essential element for animal



**Figure 2.** The  $\delta^{34}\text{S}_{\text{tissue}} - \delta^{34}\text{S}_{\text{diet}}$  isotopic offsets for (a) sows, (b) piglets and (c) pigs, analytical precision is smaller than symbols.

health and growth. In particular, methionine cannot be synthesised by animals, and must be derived from diet (Doyle and Muir, 1979; Walton et al., 1982). The isotopic composition of body methionine should therefore reflect that of dietary protein. Cysteine, the predominant sulphur-containing amino acid in hair, can be assimilated directly from dietary cysteine or can be synthesised from either serine or methionine and thus tissue cysteine  $\delta^{34}\text{S}$  may represent a mixture S from dietary Cys and S from dietary Met and these need not be isotopically identical. Further, cysteine can replace methionine in certain transsulfuration reactions (Finkelstein et al., 1988), however, the influence of the Met – Cys metabolic cycle on consumer tissue isotopic composition is currently unknown (Nehlich, 2015).

The sulphur isotopic offsets between consumer tissues and diet are generally considered to be small and highly variable. A recent synthesis of controlled feeding and archaeological human sulphur isotope studies determined that the average  $\delta^{34}\text{S}_{\text{tissue}} - \delta^{34}\text{S}_{\text{diet}}$  isotopic offset was  $+0.5 \pm 2.4 \text{ ‰}$  [ $-3.2$  to  $+7.3 \text{ ‰}$ ] for fauna, and  $+0.8 \pm 2.5 \text{ ‰}$  [ $-4.9$  to  $+7.0 \text{ ‰}$ ] for archaeological human tissue (using local consumable terrestrial fauna as a proxy for diet; Nehlich, 2015). Here, we determined that the  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  isotopic offsets were somewhat larger and less variable than expected; for example, the  $\delta^{34}\text{S}_{\text{collagen}} - \delta^{34}\text{S}_{\text{diet}}$  isotopic offset was  $-1.5 \text{ ‰}$ , with a standard deviation of  $\pm 0.8 \text{ ‰}$  for all 35 animals (Table 4). The average isotopic offsets, although small, exceed instrumental error and are reasonably systematic between diet and collagen, soft tissues, milk, hair and faeces.

Bone collagen, muscle, liver and milk are all  $^{34}\text{S}$ -depleted relative to diet. This has been observed in other studies (e.g., Arneson and MacAvoy, 2005; Barnes and Jennings, 2007; Tanz and Schmidt, 2010), but our results are less variable. Both the  $\delta^{34}\text{S}_{\text{hair}}$  and  $\delta^{34}\text{S}_{\text{faeces}}$  values are moderately  $^{34}\text{S}$ -enriched compared to diet. Richards et al. (2003) analysed horse hair under controlled feeding conditions and determined that hair keratin was  $^{34}\text{S}$ -depleted relative to diet by  $\sim -1 \text{ ‰}$ . In contrast, we determined a small  $^{34}\text{S}$ -enrichment relative to diet of  $+1.2 \pm 0.3 \text{ ‰}$ . Recycling of body proteins to compensate for a nutritionally inadequate diet, which could induce  $^{34}\text{S}$ -enrichment, is unlikely, since piglets had access to and were consuming both sow milk and pelleted feed. Instead, we suggest that this trend is due to the abundance and  $\delta^{34}\text{S}$  of cysteine in keratin having a dominant influence on the  $\delta^{34}\text{S}$  value of hair rather than methionine. Cysteine S may be directly routed from dietary Cys or the isotopic composition may show some contribution from dietary Met which has been metabolised to Cys via the Met – Cys metabolic cycle (Finkelstein et al., 1988). Either process could lead to the higher  $\delta^{34}\text{S}_{\text{hair}}$  values observed here but crucially the  $\delta^{34}\text{S}_{\text{hair}}$  values will be

dependent on the initial  $\delta^{34}\text{S}$  of dietary cysteine and methionine. If the  $\delta^{34}\text{S}$  of dietary cysteine is greater than that of the bulk diet  $\delta^{34}\text{S}$  and dietary cysteine dominates in the production of hair keratin relative to cysteine metabolised from methionine then this could explain the enrichment in  $^{34}\text{S}$  in our experiment compared to previous studies where  $\delta^{34}\text{S}$  of dietary cysteine may have been lower than bulk diet or dietary methionine leading to  $\delta^{34}\text{S}$  of hair being reduced relative to diet. More detailed examination of the  $\delta^{34}\text{S}$  values of the individual amino acids in the diet and hair and their trophic fractionation (e.g.  $\delta^{34}\text{S}_{\text{Cys Hair}} - \delta^{34}\text{S}_{\text{Cys Diet}}$ ) will elucidate this. A previous study analysing bat faeces also found a small  $^{34}\text{S}$ -enrichment and did not consider it interpretively significant for the reconstruction of dietary sulphur sources (Salvarina et al., 2013). The  $^{34}\text{S}$ -enrichment of faeces suggests that heavy sulphur-containing compounds are preferentially excreted, which would balance the  $^{34}\text{S}$ -depletion of most body tissues.

There are several potentially important metabolic differences among pigs, piglets and sows, specifically, frame size, where piglets are significantly smaller than either pigs or sows, and growth rate, where pigs are synthesising significantly more tissue than either piglets or sows. For commonly sampled tissues (collagen, muscle and liver), there was no difference among the three age categories. This outcome suggests that juvenile and adult organisms metabolise dietary sulphur in the same way when under similar dietary conditions. Differences in isotopic composition associated with these factors has been suggested for hydrogen isotopes (e.g., Kirsanow and Tuross, 2011) and nitrogen isotopes (e.g., Webb et al., 2016), but are not apparent for the sulphur isotopes investigated here.

## 6.2 Impact of dietary protein type

For nearly all tissues and age categories, there is no relationship between the  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  isotopic offsets and the proportion of marine protein in the diet, which is the expected result. There is, however, a statistically significant correlation between the  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  isotopic offsets and % marine protein for pig collagen, primarily driven by diet 5 isotopic offsets (Figure 2c), if diet 5 is removed this is no longer significant ( $p > 0.05$ ). It is possible that the pigs in the diet 5 group were excreting relatively more  $^{34}\text{S}$ -enriched sulphur, but there is no additional evidence to support this conjecture in either the growth performance measurements or in the  $\delta^{34}\text{S}_{\text{faeces}}$  values. Alternatively, the  $\delta^{34}\text{S}$  values may be unevenly influenced by preferential routing of non-marine protein sulphur-containing amino acids from dietary components, such as the methionine dietary supplement or from plant protein. Although there is a limited amount of methionine

present in these non-animal proteins GC-FID analysis of the dietary amino acid composition shows there is ~2.5 times as much methionine in the 100% marine protein diet compared to the 100% terrestrial diet. Again, the absence of any evidence for this change in routing for other pig tissues or for piglet and sow collagen limits further testing of this hypothesis as one would expect to see the same reduction in  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  in the 100% marine protein population in the other age groups. It is more likely that the apparent linear relationship is an artefact of sample size ( $n=2$  or  $n=3$  for each diet group by age) or is emerging from inter-individual variability.

## Conclusions

Sulphur isotopic data are becoming an increasingly useful tool for palaeodietary reconstruction, particularly in regions where terrestrial, freshwater and marine resources may have been consumed. Sulphur isotopic fractionation between diet and various tissues is not, however, well-constrained. Here, we have provided new insights into tissue – diet and tissue – tissue sulphur isotopic discrimination, and assessed the impact of different kinds of dietary protein on metabolic and physiological processes involved in protein synthesis. We determined that there is a strong, linear relationship between tissue and diet  $\delta^{34}\text{S}$  values, and between faeces and diet  $\delta^{34}\text{S}$  values, with moderate inter- and intra-individual variability and moderate sensitivity to incremental changes in dietary  $\delta^{34}\text{S}$  values. The  $\Delta^{34}\text{S}_{\text{tissue-diet}}$  isotopic offsets were generally small, but were consistent for each tissue and not significantly impacted by mixed resource (i.e., marine and terrestrial) dietary protein consumption. Collagen and hair are of particular relevance to archaeological research, and are typically used to investigate diet, ecology and mobility. In this controlled feeding study, collagen and hair keratin were shown not to be isotopically equivalent tissues ( $\delta^{34}\text{S}_{\text{hair}} - \delta^{34}\text{S}_{\text{collagen}} = +2.6 \pm 0.5\text{‰}$ ), but the sulphur isotopic compositions of both tissues co-varied linearly with dietary  $\delta^{34}\text{S}$  values and may therefore be used independently to make inferences about the sulphur-isotope composition of consumed food. Given the recent interest in using sulphur isotopes for ecological and archaeological research, this paper constitutes a timely refinement of the fundamental assumptions underpinning sulphur isotopic fractionation and inter-tissue variability. The outcomes described herein are of relevance to research questions that are difficult to resolve using carbon and nitrogen stable isotope compositions alone, and will also be useful in regions that are ecologically-diverse or had complex trade and exchange networks through which terrestrial, freshwater, and marine resources could have all potentially contributed to human diet.

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