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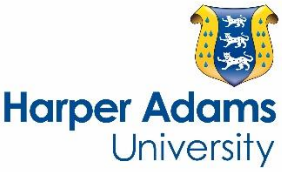
DOI: 10.1080/20548923.2015.1133121



Webb, E.C., Stewart, A., Miller, B., Tarlton, J. and Evershed, R.P. 2016. Age effects and the influence of varying proportions of terrestrial and marine dietary protein on the stable nitrogen-isotope compositions of pig bone collagen and soft tissues from a controlled feeding experiment. *STAR: Science & Technology of Archaeological Research,* 2 (1), pp. 54-66.

STAR: Science & Technology of Archaeological Research

ISSN: (Print) 2054-8923 (Online) Journal homepage: http://www.tandfonline.com/loi/ysta20

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To cite this article: Emily C. Webb, Alan Stewart, Bevis Miller, John Tarlton & Richard P. Evershed (2016) Age effects and the influence of varying proportions of terrestrial and marine dietary protein on the stable nitrogen-isotope compositions of pig bone collagen and soft tissues from a controlled feeding experiment, STAR: Science & Technology of Archaeological Research, 2:1, 54-66, DOI: 10.1080/20548923.2015.1133121

To link to this article: http://dx.doi.org/10.1080/20548923.2015.1133121

Science and Technology of Archaeological Research

Research Article

Age effects and the influence of varying proportions of terrestrial and marine dietary protein on the stable nitrogen-isotope compositions of pig bone collagen and soft tissues from a controlled feeding experiment

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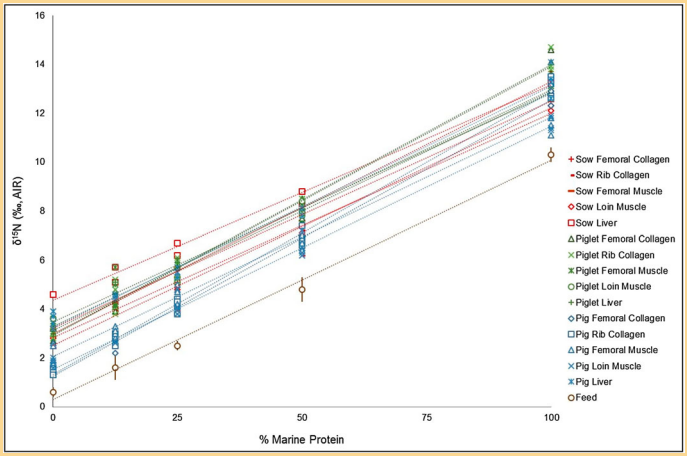
Abstract In this study, femoral collagen, rib collagen, femoral muscle, loin muscle and liver samples from sows, piglets and pigs raised in a controlled feeding study are analysed for their nitrogen-isotope compositions. The objectives of this research are to investigate the relationship between tissue and dietary $\delta^{15}N$ values across age categories under controlled feeding and housing conditions, and to assess tissue ^{15}N-enrichment relative to diet when pigs of different ages are consuming terrestrial, marine, or mixed terrestrial-marine dietary protein. There is a strong linear relationship between all tissue $\delta^{15}N$ values and the amount of marine protein consumed, but the $\delta^{15}N$ values do not become consistently elevated for all individuals consuming the same diet until at least 25% of the dietary protein source is marine-derived. Adolescent pigs also had consistently lower $\delta^{15}N$ values than either piglets or sows consuming the same diet for collagen and muscle, which is most likely caused by the differences in growth rate among the age categories. Further, for some tissues and animals, a linear relationship between the amount of marine protein consumed and the $\Delta^{15}N_{Tissue\ -\ Whole\ Diet}$ offset was also observed. We suggest that this variability results from both age-associated growth rates and differential incorporation of amino acids from terrestrial and marine dietary protein into rapidly growing tissue.



Keywords nitrogen isotopes; nitrogen balance; palaeodietary reconstruction; trophic levels; isotopic discrimination

Received 1 July 2015; **accepted** 5 November 2015

Statement of significance:

Nitrogen-isotope compositions are widely used in archaeological and ecological research to better understand producer-consumer relationships and to determine consumer diet. The isotopic offset between producer and consumer is commonly treated as constant across species, conspecifics and tissues within the same organism. Metabolic factors, such as age, stress, and illness, as well as abiotic factors (e.g., aridity and manuring), can all influence the tissue $\delta^{15}N$ values and the relationship between tissue and whole diet isotopic compositions. This research elucidates the relationships among trophic

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DOI: 10.1080/20548923.2015.1133121

level offsets, growth rate, and dietary protein source by determining nitrogen-isotope compositions of collagen, muscle and liver across three age categories of pigs reared in a multigenerational controlled feeding study. We evaluate the sensitivity of δ^{15}N values to incremental changes in marine protein consumption, the impact of growth rate on tissue isotopic compositions, and identify potential differences in trophic offset associated with the type of dietary protein consumed.

Introduction

Stable nitrogen-isotope analysis (δ^{15}N) is widely used in archaeology and ecology to investigate diet and food web relationships in both modern and extinct contexts. A better understanding of the fundamental interaction of age, diet and nitrogen metabolism and their influences on consumer tissue nitrogen-isotope compositions is essential to improving palaeodietary and ecological interpretations. Although measured nitrogen-isotope compositions for various plants and animals may vary among ecosystems, consumer tissues will always be ^{15}N-enriched relative to their diets (Ambrose 1993, 2000). This systematic ^{15}N-enrichment between producers and consumers is used to assess trophic level or relative position in the food web. There is a general consensus that trophic offsets are relatively consistent among species (Δ^{15}N ≈ +3–5‰[1]), but there is variability among different groups of producers and consumers, and across different tissues and organs in the body (*inter alia* Ambrose 2000; Ambrose and DeNiro 1986; Bocherens and Druker 2003; DeNiro and Epstein 1981; Hedges and Reynard 2007; Minagawa and Wada 1984; O'Connell et al. 2012; Sealy et al., 1987). Variability in Δ^{15}N offsets associated with age, growth rate and other factors influencing metabolism has also been observed (*inter alia* Ambrose 2000; Hobson and Clark 1992; Minigawa and Wada 1984; Mizutani et al. 1992; Roth and Hobson 2002; Sutoh et al. 1987; Yoneyama et al. 1983). Metabolism has an important impact on tissue δ^{15}N values, and the isotopic composition of tissues formed during periods of perturbed nitrogen balance are often aberrantly ^{15}N–enriched or –depleted relative to expected tissue nitrogen-isotope compositions based on dietary intake. In healthy individuals, the rate of protein synthesis equals the rate of protein breakdown and loss (Waterlow 1999). Rapid tissue growth, injury repair, infection, or inadequate diet can push the body into a state of positive or negative nitrogen balance, which alters the relationship between the isotopic compositions of newly forming tissues and diet (i.e., the Δ^{15}N offset; Hobson et al. 1993; Schoeller 1999). This outcome has been exploited to investigate numerous pathological conditions and perturbations in physiological or metabolic states in both archaeological and modern clinical contexts (Fuller et al. 2004, 2005; Katzenberg and Lovell 1999; Mekota et al. 2006, 2009; Petzke et al. 2006; Olsen et al. 2014; Williams et al. 2011). Moreover, abiotic factors, including aridity, manuring and, in archaeological or palaeoecological research, diagenetic alteration of collagen, can also exert an often-unquantifiable influence on the δ^{15}N values and thus any assessment of Δ^{15}N offsets (Bogaard et al. 2007; Fraser et al. 2011; Heaton et al. 1986; Hobson et al. 1993). Because of the dynamic nature of nitrogen metabolism and the numerous environmental and intrinsic factors that impact both tissue δ^{15}N values and trophic offsets, targeted studies performed under controlled feeding and rearing conditions are vital if the impact of dietary and metabolic influences is to be disentangled. With this in mind, we undertook a controlled feeding study using pigs as an animal model for humans. The research presented here elucidates the relationships among trophic level offsets, growth rate, and the nature of dietary protein source, which act simultaneously to mediate tissue nitrogen-isotope compositions.

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, (iv) 50% terrestrial/50% marine, and (v) 100% marine-derived (fish meal). In the first generation, five groups of gilts were fed one of the above diets from weaning until sacrifice. All pigs were artificially inseminated and the second generation pigs were fed exclusively on one of the five diets from weaning until sacrifice in adolescence. All pig feeds were manufactured by Parnutt Foods, Ltd. (Sleaford, UK). Diet formulations are presented in Table 1. 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 two to four pigs, the second generation 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. Crucially, all five diets were nutritionally equivalent, with 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 (1st generation), 19 piglets (2nd generation, aged four weeks) and 39 pigs (2nd generation, aged ≥160 and ≤ 190 days) were reared and slaughtered over the course of the study.

Here, femoral collagen, rib collagen, femoral muscle, loin muscle and liver samples from sows, piglets and pigs are analysed for their nitrogen-isotope compositions. The overarching goal of this

[1]Herein, Δ^{15}N is defined as $\delta^{15}N_{Tissue} - \delta^{15}N_{Whole\ Diet}$.

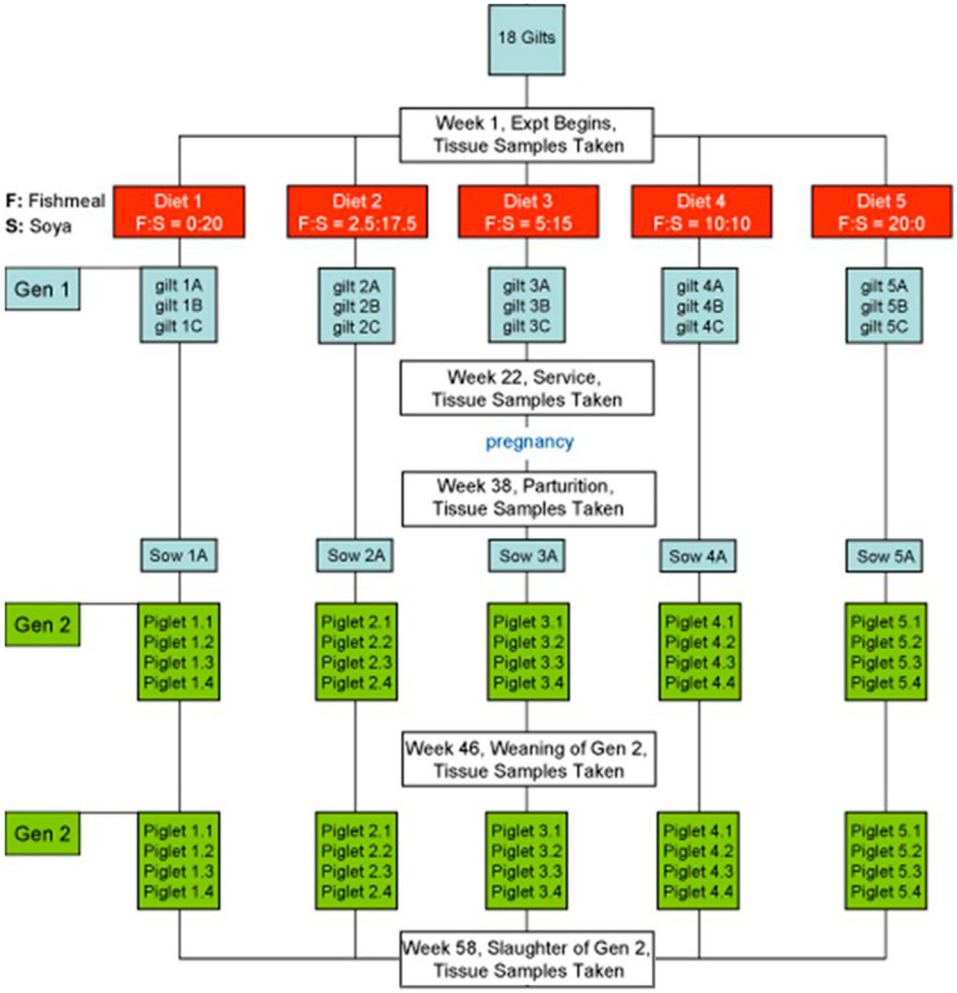

Figure 1 Organisation of the controlled feeding study.

Table 1 Diet formulations and nitrogen-isotope compositions

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
	0:20[1]	2.5:17.5	5:15	10:10	20:0
$\delta^{15}N \pm 1\sigma$ (‰, AIR)	+0.6±0.2	+1.6±0.5	+2.5±0.2	+4.8±0.5	+10.3±0.3
			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

[1] ratio of marine to terrestrial protein where protein constitutes 20% of diet.

controlled feeding study is to improve the understanding of consumer tissue isotopic composition for palaeodietary reconstruction, particularly the exploitation of marine resources in the past. More specifically, our objectives in this paper are to investigate the relationship between tissue and dietary $\delta^{15}N$ values across age categories under controlled feeding and housing conditions, and to assess ^{15}N-enrichment of collagen, muscle and liver relative to diet when pigs are consuming terrestrial, marine, or mixed terrestrial-marine dietary protein. We explore the relationship between tissue nitrogen-isotope compositions ($\delta^{15}N_{Tissue}$) and the amount of marine protein consumed, the sensitivity of tissue $\delta^{15}N$ values to incremental changes in marine protein consumption, and the relationship between the % marine protein consumed and the $\Delta^{15}N_{Tissue - Whole\ Diet}$ offset. We speculate that the results of this study are related to both age-associated growth rates and differential incorporation of amino acids from terrestrial and marine dietary protein into rapidly growing tissue.

Methods

Ten first generation sows, 19 piglets, and 20 adolescent pigs were selected for isotopic analysis. Bone collagen (femur and rib, reflecting slow and fast biochemical turnover respectively), muscle (femoral and loin) and liver samples were analysed for their stable nitrogen isotopic compositions. 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 3000JB with diamond cutting wheel SC545). Samples were taken from the femoral shaft or rib body. 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. 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 each soft tissue sample, a cross-section of tissue was removed using a new scalpel blade and freeze-dried for more than 48 h. Lipids were extracted using 2:1 v/v chloroform: methanol solution (3×8 mL solvent solution, 3×20 min sonication), and tissues were mechanically homogenised using fine scissors. For all tissue samples, homogenised aliquots were then weighed into tin capsules (~0.7±0.1 mg) for isotopic analysis. Twenty 1 g feed samples (four from each diet) were crushed to homogenise, and aliquots 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 4 h. Aluminium foil and disposable gloves were used to handle samples.

All isotopic analyses were performed using a Flash HT elemental analyser interfaced with a Thermo Electron DeltaPlus 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 mean $\delta^{15}N$ value obtained over all analytical sessions was -4.5 ±0.3‰, which compares well with the accepted value of -4.5‰.

Results

All results are reported as mean ±one standard deviation [range] unless otherwise stated. Growth performance was monitored throughout the study. Sow weight did not change appreciably during the controlled feeding study, and was 195±15 kg during pregnancy (adjusted downward to compensate for total litter weight), 203±15 kg when the piglets were weaned, and 205±13 kg at slaughter. Pig growth performance was monitored from birth to slaughter for all second generation animals (i.e., piglets and pigs). Taken as a proxy for growth rate, the average gain per day for piglets from birth to slaughter at four weeks of age was 183±55 g day^{-1}, and 624±90 g day^{-1} for pigs from birth to slaughter at >160 days of age (Figure 2). The average gain per day does not differ across diet categories (Kruskal-Wallis, p=0.409), but does increase significantly with age (Spearman's rank correlation test, ρ=0.831, p<0.01).

All feeds were held in cold storage until required for use. Subsamples (lots 1 through 4) were taken for analysis as batches of feed were released from storage to confirm homogeneity. Lots 1 through 4 of each pig feed are isotopically equivalent for all five diets (Kruskal-Wallis, p=0.976; Table S1). The differences in the mean $\delta^{15}N$ values of diets 1 through 5 are statistically significant as expected (Kruskal-Wallis, p=0.001), and are as follows: diet 1: +0.6±0.2‰ [0.4‰], diet 2: +1.6±0.5‰ [1.1‰], diet 3: +2.5±0.2‰ [0.3‰], diet 4: +4.8±0.5‰ [0.9‰], and diet 5: +10.3±0.3‰ [0.6‰]. The dietary protein sources, i.e., soymeal (+0.2‰) and/or fish meal (+11.4‰), are the dominant sources of nitrogen in the diet, and the $\delta^{15}N$ values increase linearly as expected based on the ratio of terrestrial to marine protein in diet.

All nitrogen-isotope compositions are reported in Table 2 and Figure 3. Intra-individual variation was assessed by comparing the isotopic compositions of paired rib and femoral collagen and loin and femoral muscle samples for each animal (e.g., $\delta^{15}N_{femoral}$

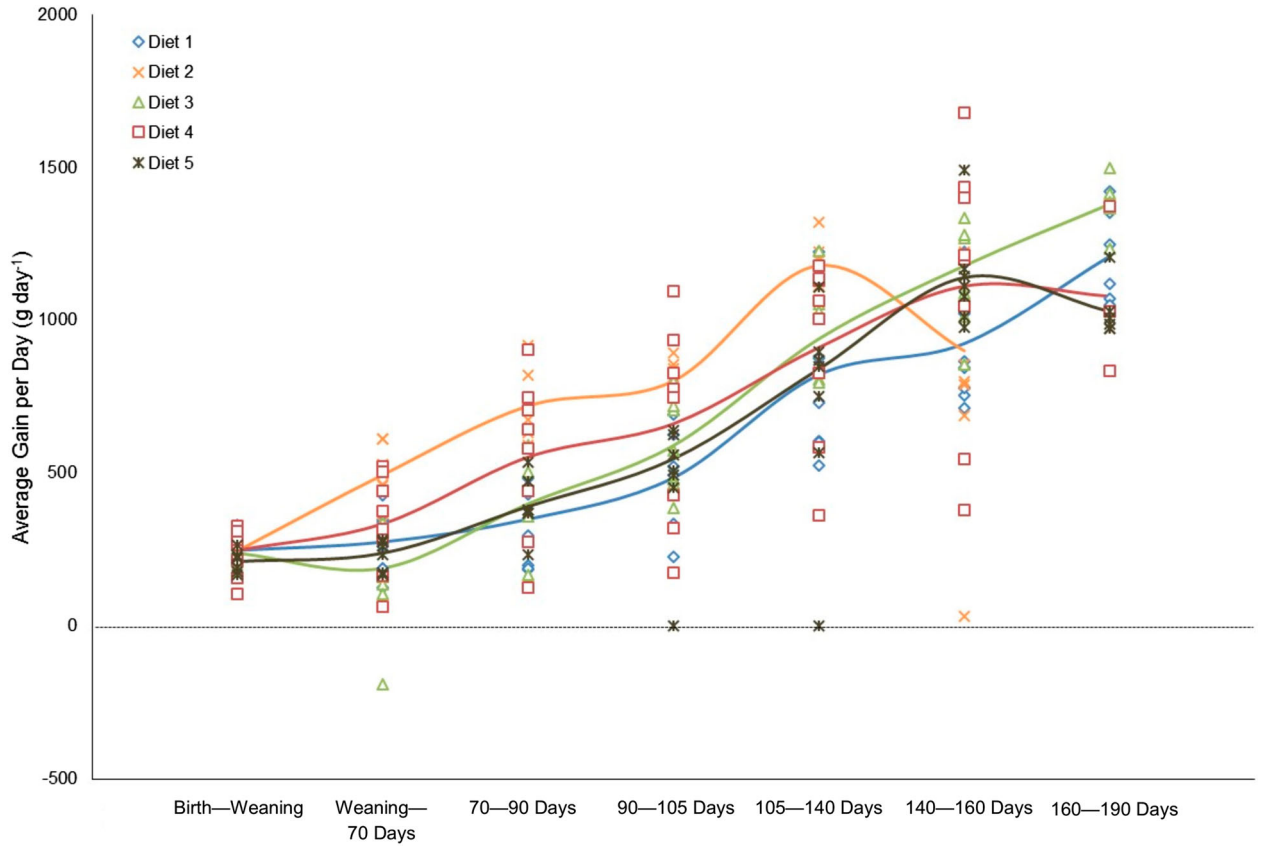

Figure 2 Average daily gain for second generation pigs from birth to slaughter.

$_{collagen}$– $\delta^{15}N_{rib\ collagen}$). The $\delta^{15}N$ values are not statistically significantly different for either collagen or muscle for any age category. The mean difference between femoral and rib collagen were +0.7±0.7‰, -0.1±0.3‰ and -0.2±0.2‰ for sows, piglets and pigs, respectively. For femoral and loin muscle, the mean difference was +0.4±0.3‰ for sows, +0.0±0.1‰ for piglets and +0.4±0.4‰ for pigs. These differences do not greatly exceed analytical error and reproducibility for these data, and are thus not considered interpretively significant. Inter-individual variation was assessed by comparing the isotopic compositions of the different tissues of animals of the same age consuming the same diet. Here, absolute ranges are used to better characterise the potential influence of idiosyncratic metabolic variation on the range of tissue isotopic compositions possible for organisms consuming the same diet. For sow collagen, the differences in the $\delta^{15}N$ values range from as small as ±0.2 to as large as ±1.2‰ for animals consuming the same diet. Similarly, the differences range from ±0.5 to ±1.4‰ for piglet collagen and ±0.3 to ±0.9‰ for pig collagen. Sow muscle and liver $\delta^{15}N$ values range from ±0.0 to ±0.4‰ among animals consuming the same diet. The differences are considerably larger for piglets and pigs, ranging from ±0.3 to ±1.5‰ and ±0.1 to ±1.0‰ for piglet muscle and liver, and from ±0.1 to ±2.1‰ and ±0.2 to ±1.5‰ for pig muscle and liver.

The pig $\delta^{15}N$ values are generally ^{15}N-depleted relative to both sows and piglets for collagen and muscle, but the liver $\delta^{15}N$ values overlap for all three age categories (Figure 4). Although the absolute $\delta^{15}N$ values did not differ significantly among age categories for any tissue, the $\Delta^{15}N_{\ Tissue\ -\ Whole\ Diet}$ [2] offsets were statistically significantly different for some comparisons (Table 3). For each tissue, a Kruskal-Wallis test was used to determine if any significant differences existed among the three age categories, and when differences were identified, paired Mann-Whitney tests were used to determine which pairs differed from each other. A Bonferroni correction was applied to limit Type I errors associated with repeated pairwise comparisons. The Kruskal-Wallis tests determined that statistically significant differences exist for all tissues. The pairwise Mann-Whitney comparisons revealed that these differences are not systematic (Figure 5). Sow and piglet $\Delta^{15}N_{\ Tissue\ -\ Whole\ Diet}$ offsets differ for rib collagen and loin muscle, sow and pig $\Delta^{15}N_{\ Tissue\ -\ Whole\ Diet}$ offsets differ for femoral collagen, femoral muscle and loin muscle, and piglet and pig $\Delta^{15}N_{\ Tissue\ -\ Whole\ Diet}$ offsets are statistically significantly different for all tissues except liver.

[2]The $\Delta^{15}N_{\ Tissue\ -\ Whole\ Diet}$ offset was calculated by subtracting the mean whole diet $\delta^{15}N$ value specific to the diet group from the individual $\delta^{15}N$ value for each tissue.

Table 2: Nitrogen-isotope compositions of sow, pig and piglet tissues

	Diet Group	$\delta^{15}N$ Femoral Collagen (‰, AIR)	$\delta^{15}N$ Rib Collagen (‰, AIR)	$\delta^{15}N$ *Collagen* *(‰, AIR)*	$\delta^{15}N$ Femoral Muscle (‰, AIR)	$\delta^{15}N$ Loin Muscle (‰, AIR)	$\delta^{15}N$ *Muscle* *(‰, AIR)*	$\delta^{15}N$ *Liver* *(‰, AIR)*
Sows								
F17	1	+3.1	+2.4	**+2.8**	+3.1	+2.8	**+3.0**	**+4.6**
F18	1	+3.4	+3.1	**+3.3**	+3.6	+3.3	**+3.5**	**+4.6**
F14	2	+4.0	+3.9	**+4.0**	+4.2	+4.2	**+4.2**	**+5.7**
F13	2	+4.3	+4.5	**+4.4**	+4.3	+4.1	**+4.2**	**+5.1**
F10	3	+5.3	+5.1	**+5.2**	+5.3	+5.0	**+5.2**	**+6.7**
F12	3	+5.6	+3.9	**+4.8**	+5.7	+4.8	**+5.3**	**+6.2**
F5	4	+8.2	+7.2	**+7.7**	+7.6	+7.0	**+7.3**	**+8.3**
F8	4	+7.8	+6.2	**+7.0**	+8.2	+7.1	**+7.7**	**+8.8**
F2	5	+13.1	+12.7	**+12.9**	+12.5	+12.3	**+12.4**	**+13.4**
F3	5	+13.7	+12.5	**+13.1**	+12.5	+12.1	**+12.3**	**+13.2**
Piglets								
W15	1	+3.2		**+3.2**				
W1839	1	+3.3	+3.3	**+3.3**	+3.7	+3.6	**+3.7**	**+3.0**
W1846	1	+3.0	+3.0	**+3.0**	+3.9		**+3.9**	**+3.7**
W1853	1	+2.7	+2.8	**+2.8**	+3.2		**+3.2**	**+3.4**
W1766	2	+4.7	+4.8	**+4.8**	+5.0	+5.1	**+5.1**	**+5.0**
W1767	2	+4.7	+5.2	**+5.0**	+5.7		**+5.7**	**+5.1**
W1800	2	+3.9	+4.1	**+4.0**	+4.2		**+4.2**	**+4.2**
W1822	2	+4.1	+3.8	**+4.0**	+4.6		**+4.6**	**+4.1**
W1804	3	+5.7	+5.1	**+5.4**	+5.7		**+5.7**	**+5.1**
W34	3	+5.3	+5.3	**+5.3**	+5.1	+5.1	**+5.1**	**+5.3**
W43	3	+5.7	+5.8	**+5.8**	+5.5	+5.3	**+5.4**	**+4.9**
W49	3	+5.7	+6.1	**+5.9**	+6.0		**+6.0**	**+5.9**
W1789	4	+7.7	+8.0	**+7.9**	+7.5		**+7.5**	**+7.8**
W1793	4	+8.4	+8.5	**+8.5**	+8.3	+8.4	**+8.4**	**+8.0**
W21	4	+8.5	+8.5	**+8.5**	+8.0	+8.0	**+8.0**	**+7.5**
W28	4	+8.0	+8.0	**+8.0**	+7.9	+7.8	**+7.9**	**+7.3**
W1834	5	+14.6	+14.7	**+14.7**	+13.1	+13.2	**+13.2**	**+13.2**
W1835	5	+14.1	+13.8	**+14.0**	+12.8		**+12.8**	**+13.1**
W51	5	+13.6	+13.9	**+13.8**	+13.1		**+13.1**	**+13.2**
Pigs								
226	1	+1.6	+1.6	**+1.6**	+2.6	+1.8	**+2.2**	**+3.9**
227	1	+1.8	+1.5	**+1.7**	+2.5	+1.9	**+2.2**	**+3.4**
231	1	+1.5	+1.4	**+1.5**	+1.8	+1.6	**+1.7**	**+3.7**
232	1	+1.4	+1.3	**+1.4**	+1.9	+2.0	**+2.0**	**+3.2**
266	2	+2.7	+3.0	**+2.9**	+2.8	+2.7	**+2.8**	**+4.6**
267	2	+2.6	+2.8	**+2.7**	+3.3	+2.6	**+3.0**	**+4.4**
268	2	+2.2	+2.5	**+2.4**	+3.0	+2.7	**+2.9**	**+4.5**
271	2	+2.5	+2.9	**+2.7**	+3.1	+2.6	**+2.9**	**+4.5**
243	3	+4.2	+4.4	**+4.3**	+4.8	+4.1	**+4.5**	**+5.7**
244	3	+4.2	+4.3	**+4.3**	+4.2	+4.0	**+4.1**	**+5.5**
247	3	+4.1	+4.1	**+4.1**	+4.7	+3.8	**+4.3**	**+5.3**
248	3	+3.8	+3.8	**+3.8**	+5.0	+3.8	**+4.4**	**+5.5**
233	4	+6.8	+6.9	**+6.9**	+6.7	+6.3	**+6.5**	**+8.1**
238	4	+6.7	+7.4	**+7.1**	+6.4	+6.2	**+6.3**	**+8.1**
239	4	+6.4	+6.7	**+6.6**	+6.9	+6.5	**+6.7**	**+7.2**
241	4	+6.5	+6.6	**+6.6**	+7.0	+6.2	**+6.6**	**+7.8**
255	5	+12.8	+12.7	**+12.8**	+11.5	+11.9	**+11.7**	**+13.3**
256	5	+12.3	+12.6	**+12.5**	+11.1	+11.3	**+11.2**	**+12.6**
258	5	+12.9	+13.2	**+13.1**	+13.2	+11.8	**+12.5**	**+14.1**
260	5	+12.9	+13.5	**+13.2**	+11.8	+11.4	**+11.6**	**+13.4**

Spearman's rank correlation tests were used to assess the statistical significance of the relationships between % marine protein in diet and both measured $\delta^{15}N$ values and $\Delta^{15}N_{\text{Tissue - Whole Diet}}$ offsets. As expected, there were strong linear relationships between $\delta^{15}N_{Tissue}$ and % marine protein for all tissues and age categories. The relationship between $\Delta^{15}N_{\text{Tissue - Whole Diet}}$ offsets and % marine protein was, for most tissues, not statistically significant. Important exceptions were the statistically significant correlations determined for piglet femoral (ρ=0.861, p=0.000) and rib (ρ=0.722, p=0.001) collagen and pig femoral (ρ=0.904, p=0.000) and rib (ρ=0.940, p=0.000) collagen.

Discussion

As expected, there are strong linear relationships between the nitrogen-isotope composition of the feed, the % marine protein in feed, and the nitrogen-isotope compositions of all tissues for all age categories. Differences from one diet group to the next are not, however, always large enough to be interpretively meaningful at low levels of marine protein

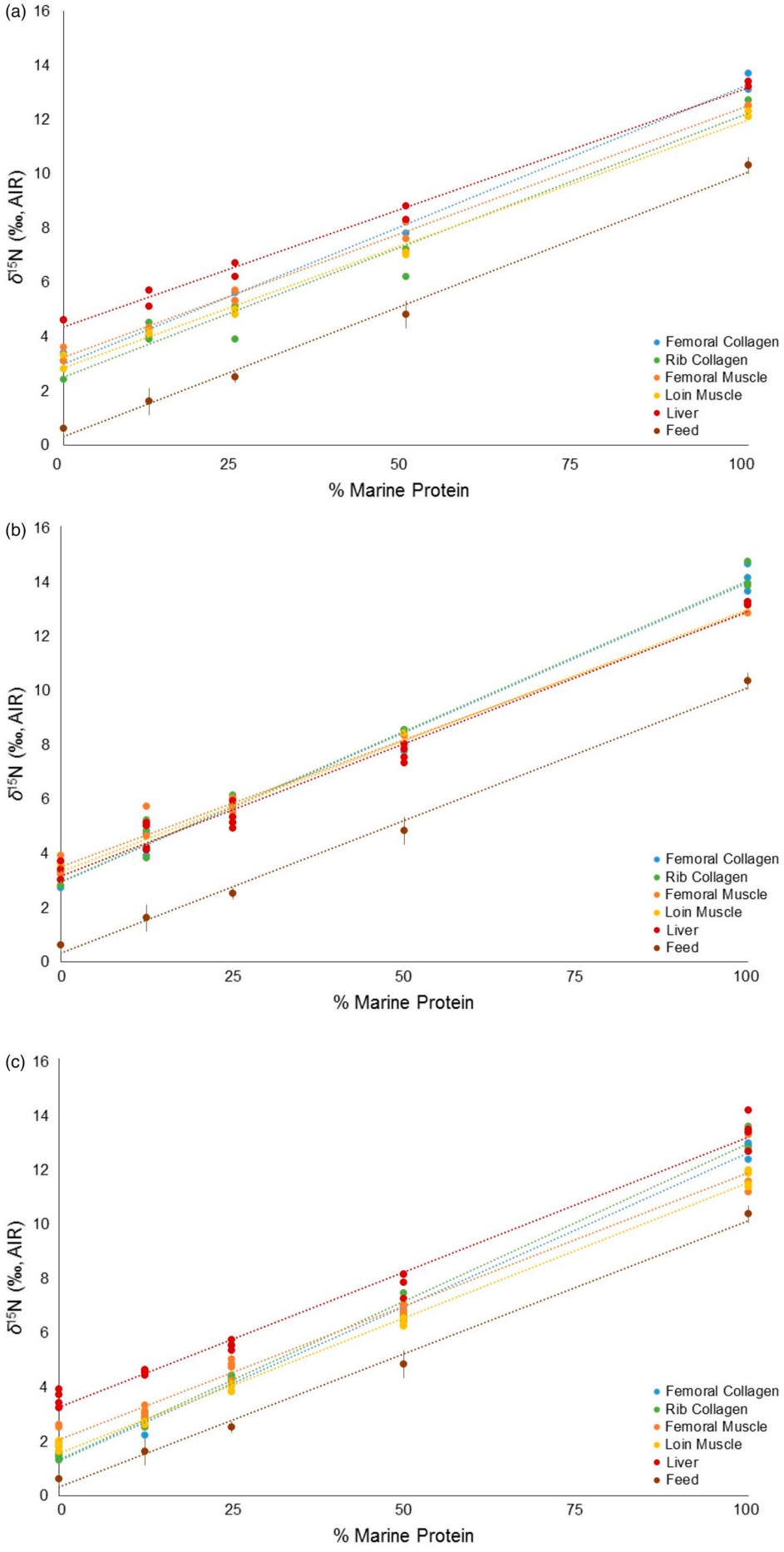

Figure 3 Nitrogen-isotope compositions for (a) sows, (b) piglets and (c) pigs.

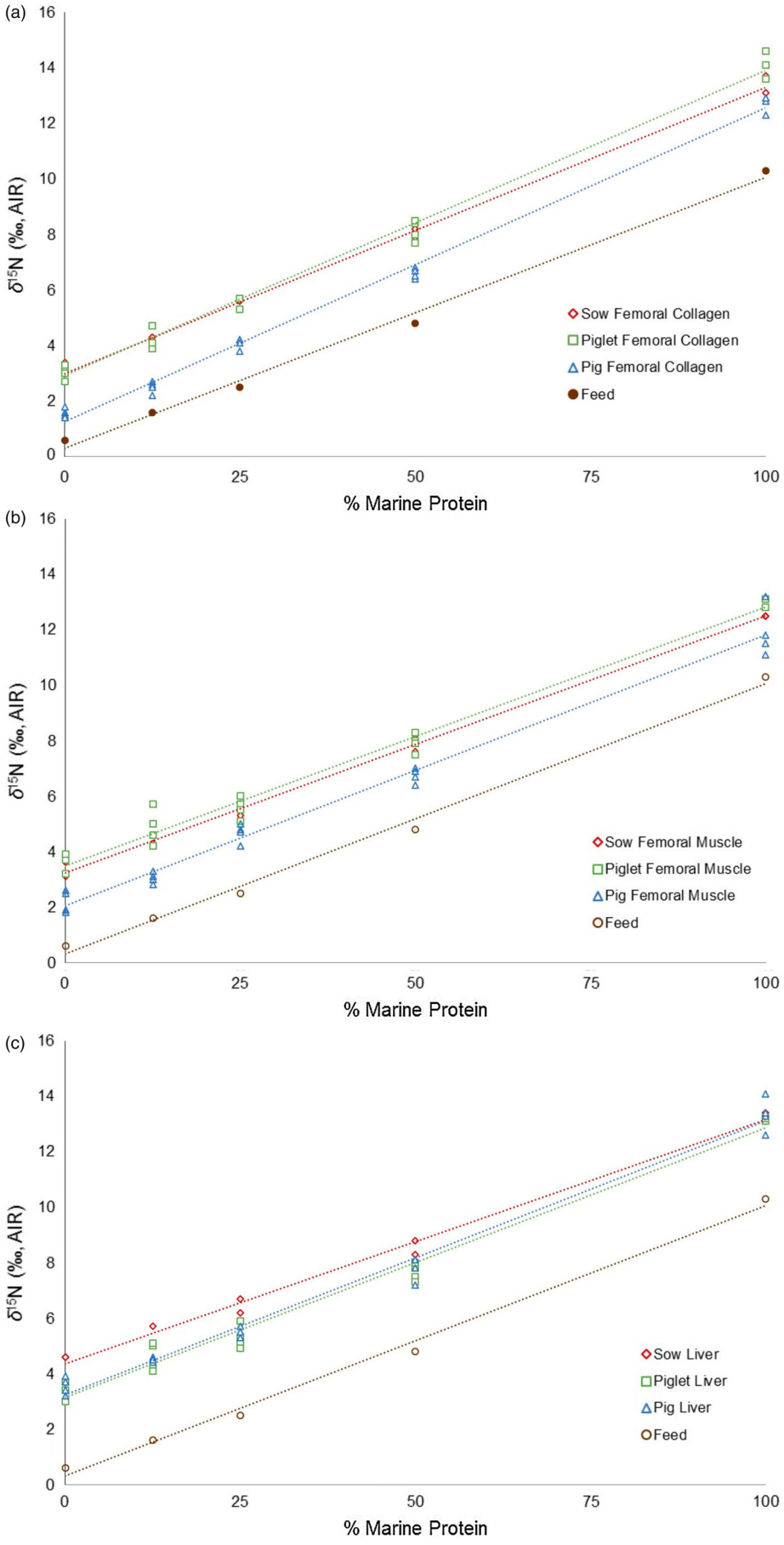

Figure 4 Comparisons of (a) femoral collagen, (b) femoral muscle, and (c) liver nitrogen-isotope compositions across age categories.

Table 3: The $\Delta^{15}N_{Tissue - Whole\ Diet}$ Offsets for Sows, Piglets and Pigs[1]

	Collagen (‰, AIR)		Muscle (‰, AIR)		Liver (‰, AIR)
	Femoral Collagen	Rib Collagen	Femoral Muscle	Loin Muscle	
Diet Group 1					
Sow	+2.7±0.2	+2.2±0.5	+2.8±0.4	+2.5±0.4	+4.0±0.0
Piglet	+2.5±0.3	+2.4±0.3	+3.0±0.4	+3.0	+2.8±0.4
Pig	+1.0±0.2	+0.9±0.1	+1.6±0.4	+1.2±0.2	+3.0±0.3
Diet Group 2					
Sow	+2.6±0.2	+2.6±0.4	+2.7±0.1	+2.6±0.1	+3.8±0.4
Piglet	+2.8±0.4	+2.9±0.6	+3.3±0.6	+3.5	+3.0±0.5
Pig	+0.9±0.2	+1.2±0.2	+1.5±0.2	+1.1±0.1	+2.9±0.1
Diet Group 3					
Sow	+3.0±0.2	+2.0±0.8	+3.0±0.3	+2.4±0.1	+4.0±0.4
Piglet	+3.1±0.2	+3.1±0.5	+3.1±0.4	+2.7±0.1	+2.8±0.4
Pig	+1.6±0.2	+1.7±0.3	+2.2±0.3	+1.4±0.2	+3.0±0.2
Diet Group 4					
Sow	+3.2±0.3	+1.9±0.7	+3.1±0.4	+2.3±0.1	+3.8±0.4
Piglet	+3.4±0.4	+3.5±0.3	+3.1±0.3	+3.3±0.3	+2.9±0.3
Pig	+1.8±0.2	+2.1±0.4	+2.0±0.3	+1.5±0.1	+3.0±0.4
Diet Group 5					
Sow	+2.6±0.4	+1.8±0.1	+1.7±0.0	+1.4±0.1	+2.5±0.1
Piglet	+3.8±0.5	+3.8±0.5	+2.7	+2.9	+2.9±0.1
Pig	+2.4±0.3	+2.7±0.4	+1.6±0.9	+1.3±0.3	+3.1±0.6

[1]All offsets reported as mean ± one standard deviation in ‰ for n>2.

consumption for all individuals, i.e., between animals consuming 0 and 12.5% or 12.5 and 25% marine protein (Figure 3). This overlap suggests lower sensitivity to less than ~25% marine protein contribution to total dietary protein for bulk protein $\delta^{15}N$ values. Low sensitivity of isotopic compositions to the consumption of proportionally small amounts of marine protein has been proposed by other researchers in the past, notably Hedges et al. (2004). Intra-individual variation among co-forming same tissues (i.e., rib and femoral collagen) is generally less than 1‰, and often less than 0.5‰. Because all animals were fed the same diet throughout the study, there was no expectation that, for example, differential rates of collagen turnover between ribs and femora would influence nitrogen-isotope compositions.

Inter-individual differences in the $\delta^{15}N$ values for the same tissues among animals consuming the same diet from the same age category can be used to assess the influence of individual metabolic variability on group-level trends. Healthy conspecific animals consuming the same diet are expected to have very similar $\delta^{15}N$ values for the same tissues. We determined that inter-individual variability was generally low, but did range larger than ±1.5‰ for some tissues. Younger animals (pigs and piglets) tended to have greater inter-individual variability than sows. For piglets, this variability may be due to differing contributions of sow milk to individual piglet diets, resulting in a less well-constrained dietary protein source for these animals. Pigs, however, had been weaned for several months at the time of slaughter and were consuming a common feed. Thus, the reduced inter-individual variability for sows is most likely a result of age-related differences in growth and metabolism compared to pigs and piglets. We caution, however, that the small number of sows in each diet group (n=2) may be affecting the apparent homogeneity observed for this age category.

It is generally assumed that tissues from animals consuming the same dietary protein source will have nitrogen-isotope compositions that are similarly ^{15}N-enriched due to isotopic fractionation (i.e., via the trophic effect mechanism) relative to diet. Both essential and non-essential amino acids can be directly routed from dietary protein and, with minimal isotopic alteration, incorporated into growing tissue. Non-essential amino acids can also be metabolized by the body and used along with other diet-derived molecules (e.g., carbon from carbohydrates) to synthesise amino acids. Amino acids synthesised through a series of enzyme-catalysed reactions (e.g., repeated transamination reactions) will be ^{15}N-enriched relative to whole diet. Pigs instead have consistently lower $\delta^{15}N$ values for collagen and muscle than either piglets or sows, even though animals from all three age categories were consuming the same feed. Excluding the liver $\delta^{15}N$ values, the mean difference between sow and pig $\delta^{15}N$ values is +1.0‰, and +1.5‰ for piglets and pigs. In contrast, the mean sow – piglet difference in $\delta^{15}N$ values is -0.6‰. Pig collagen and muscle $\delta^{15}N$ values are therefore closer to the $\delta^{15}N_{Whole\ Diet}$ values than either piglet or sow collagen or muscle. Early studies (e.g., birds – Hobson and Clark 1992; mussels – Minigawa and Wada 1984; birds – Mizutani et al. 1992; cattle – Sutoh et al. 1987; rats – Yoneyama et al. 1983) did not clearly identify differences in nitrogen-isotope composition associated with age in the absence of dietary change, but subsequent work (e.g., Ambrose, 2000; Roth and Hobson, 2002) has revealed some association between age and unexpected shifts in tissue

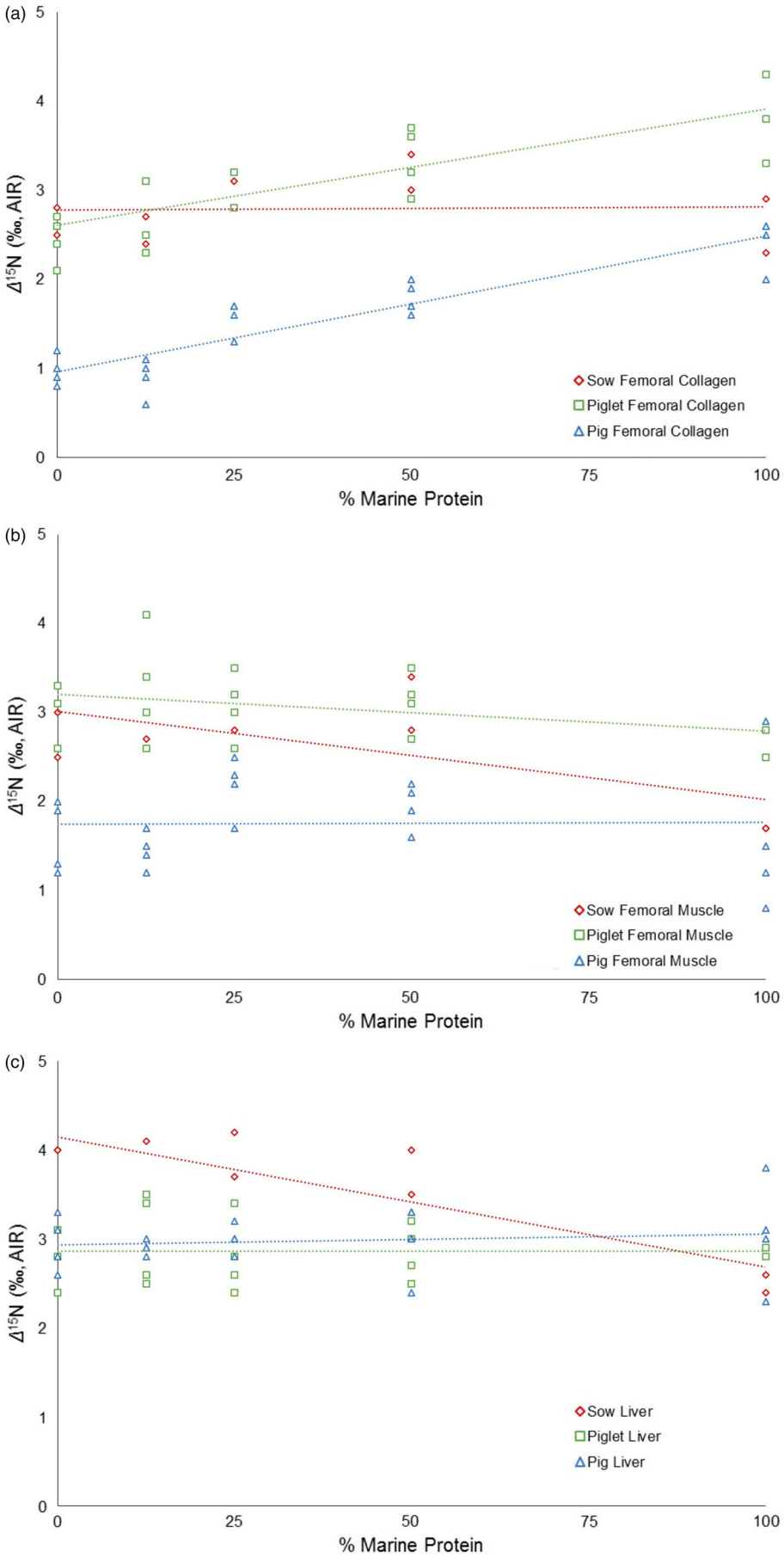

Figure 5 Comparison of $\Delta^{15}N_{Tissue - Whole\ Diet}$ offsets across age categories for (a) femoral collagen, (b) femoral muscle and (c) liver.

nitrogen-isotope compositions. Roth and Hobson (2002) determined that juvenile foxes had consistently higher $\delta^{15}N_{Tissue}$ values than adult foxes raised on the same diet (~+0.3‰), which was attributed to a higher rate of protein synthesis and catabolism associated with rapid growth. Ambrose (2000) determined that, for rats, the $\delta^{15}N$ values of hair and collagen increased with age, whereas flesh $\delta^{15}N$ values decreased, although few of these relationships achieved statistical significance.

Using a larger sample set and an animal model that is more similar to humans in terms of metabolism, nutritional requirements and frame size (Heinritz et al. 2013, Litten-Brown et al. 2010, Sullivan et al. 2001, Swindle et al 2012), we determined that both very young and mature animals had higher $\delta^{15}N$ values for bone collagen and muscle than adolescent pigs did. This difference is most likely a result of increasing growth rate and its influence on nitrogen balance. We propose that sow nitrogen intake was roughly equal to the amount of nitrogen excreted, that is, that these individuals were in nitrogen homeostatic balance. Sows, which had reached their mature weight before slaughter, would have been synthesising new protein largely for tissue maintenance, i.e., for normal tissue turnover at a considerably lower rate than during adolescent growth. The piglets, although they were growing quickly, were not synthesising the same absolute quantity of new protein each day for rapid growth that pigs did; the average gain per day for piglets was 0.18 kg. It is possible that the piglets were likewise in nitrogen homeostasis, or that they were catabolising their body proteins to support tissue growth. The latter explanation is unlikely, since piglets had access to both sow milk and nutritionally-optimised pelleted feed. Pigs, in contrast, were growing very rapidly, synthesising an average of 1.0 to 1.2 kg of new tissue per day on a protein-optimised diet in the month before slaughter. The average gain per day also increased rapidly over the course of the feeding study, ultimately appearing to plateau for some diet groups once an adult body mass was achieved (Figure 2). Increased direct routing of dietary amino acids with limited biosynthesis would be the most energetically-efficient mechanism for maintaining this increasingly rapid growth. This process would be expected to cause smaller $\Delta^{15}N_{Tissue - Whole\ Diet}$ offsets and, as expected, the average $\Delta^{15}N_{Tissue - Whole\ Diet}$ offsets are smaller for pigs than for piglets or sows.

There is also a strong, statistically significant linear relationship between pig and piglet $\Delta^{15}N_{Collagen - Whole\ Diet}$ offsets and % marine protein, which is either weakly correlated or not present for other tissues or for sows. Comparatively slower growth and tissue turnover is the primary difference between collagen and other tissues, and between sows and younger animals. Ambrose (2000) determined that the amount of protein (5% vs. 20%) in diet did not influence the variable $\Delta^{15}N_{Tissue - Whole\ Diet}$ offsets determined for rats. Here, we are investigating a different scenario, in which all diets are nutritionally optimal, but nonetheless differences in the kind of protein consumed (i.e., soymeal vs. fish meal) appear to have some impact on $\Delta^{15}N_{Tissue - Whole\ Diet}$ offsets. It is possible that this trend is a result of an association between high turnover of the body protein pool and consumption of more marine protein. High turnover typically results in selective catabolism of isotopically-light molecules, leading to progressive ^{15}N-enrichment of newly forming tissues relative to dietary protein. That this effect is limited to bone collagen may indicate that this pattern disappears with age as growth rate begins to plateau when either an adult body mass is reached, or due to growth stasis associated with weaning (McCracken et al. 1995). High growth rates are not, however, present solely in diets with higher marine protein contributions, so it is unlikely that growth rate alone is driving this change in $\Delta^{15}N_{Collagen - Whole\ Diet}$ offsets. It is more likely that differences in either the relative proportions of amino acids that are directly routed from dietary protein vs. biosynthesised from whole diet, or in which amino acids are directly routed or biosynthesised, may be influencing the changing $\Delta^{15}N_{Collagen - Whole\ Diet}$ offset. Biosynthesis would result in ^{15}N-enrichment of a non-essential amino acid relative to the same amino acid in dietary protein through various enzyme-catalysed molecular modifications. Although all five diets are nutritionally equivalent, the two dietary protein sources (soy and fish) do not have the same amino acid composition. It is possible that the body metabolises amino acids differently based on the availability of a particular amino acid in the dietary protein source due to, for example, inhibition of an enzymatic pathway by an abundance of the end product. Alternatively, it is possible that we are under-estimating the importance of idiosyncratic differences in metabolism, particularly in rapidly growing juvenile animals. Inter-animal variation in same-tissue $\delta^{15}N$ values was high for both piglets and pigs, so it is possible that a ~1 to 1.5‰ variation in $\Delta^{15}N$ offsets is not significant, and that the apparent linear trends observed are, in fact, spurious.

Conclusion

In order to improve archaeological palaeodietary reconstruction, a more nuanced understanding of the interaction of age, diet and metabolism in producing the nitrogen-isotope compositions of commonly-analysed tissues is essential. Here, our objectives were to investigate the relationship between tissue and dietary $\delta^{15}N$ values across age categories under controlled feeding and housing conditions, and to assess ^{15}N-enrichment of collagen, muscle and liver relative to whole diet when pigs are consuming terrestrial, marine, or mixed terrestrial-marine dietary protein. We determined that the $\delta^{15}N_{Tissue}$ values do not become consistently different

for all individuals from one diet group to the next until at least 25% of the dietary protein source is marine-derived, suggesting low sensitivity to small amounts of marine resource consumption. In the context of human palaeodietary reconstruction, the implication of this result is that marine protein consumption may be undetectable if it constitutes as much as a quarter of dietary protein intake. Understanding resource use and access is a complex but vital question in many archaeological societies. The potential invisibility of a meaningful proportion of a type of food that may, for example, require specialised technology or exchange relationships to access, is not trivial.

The differences in $\delta^{15}N$ values from the same tissues for animals consuming the same diet also varied with age, wherein groups of younger animals from the same diet group differed in their tissue isotopic composition by as much as 1 to 1.5‰. We speculate that this variation reflects individualised metabolic differences among the rapidly growing pigs and piglets. Pigs also had consistently lower $\delta^{15}N_{Tissue}$ values than either piglets or sows and concomitantly smaller $\Delta^{15}N$ offsets, which we attributed to rapid synthesis of large amounts of tissue, indicated by increasing average daily gain throughout the study period. Comparing $\delta^{15}N$ values among animals of different ages would thus require a thorough understanding of growth and development and, of particular relevance for zooarchaeological or palaeontological research, the ability to accurately determine the age of the specimen.

The nitrogen-isotope compositions of human adults, adolescents and juveniles are also frequently compared and, although the timing of human growth and development is distinct from that of the pig, it is possible that similar issues may influence the $\delta^{15}N_{Tissue}$ values of very young juveniles relative to adults, potentially confounded further by pathology-related shifts in nitrogen metabolism. Positive nitrogen balance has been observed during wound healing and tissue repair after trauma, as well as during pregnancy. Here, we clearly demonstrate that a shift in nitrogen balance has a global effect on body nitrogen-isotope compositions, and that the magnitude of the tissue ^{15}N-depletion relative to expected dietary $\delta^{15}N$ values is nontrivial. These results further provide a well-constrained baseline from which to explore disequilibria in nitrogen balance for several tissues at the amino acid level.

Finally, for pig and piglet collagen only, a statistically significant linear relationship between the amount of marine protein consumed and the $\Delta^{15}N_{Tissue\ -\ Whole\ Diet}$ offset was also observed, speculatively attributed to differential incorporation of amino acids from terrestrial and marine dietary protein. Future research investigating compound-specific nitrogen-isotope compositions will elucidate any differences in amino acid metabolism influencing these tissue – whole diet relationships.

Acknowledgements

This work was conducted as part of a responsive mode grant from the UK Natural Environment Research Council (NERC: NE/D004535/1). The authors would like to thank Helen Fewlass and Anika Shain (University of Bristol), and Dr. Jason Newton (NERC Life Sciences and Mass Spectrometry Facility, East Kilbride node).

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