Quantifying variability in stable carbon and nitrogen isotope ratios within the skeletons of marine mammals of the suborder Canifomia

Casey T. Clark[a,b,*], Lara Horstmann[a], Nicole Misarti[b]

[a] College of Fisheries and Ocean Sciences, University of Alaska Fairbanks, P.O. Box 757220, Fairbanks, AK 99775-7220, USA
[b] Water and Environmental Research Center, University of Alaska Fairbanks, P.O. Box 755860, Fairbanks, AK 99775-5860, USA

A B S T R A C T

Stable isotope ratios of bone collagen are commonly used to investigate foraging and movement of human and animal populations. This technique is especially valuable for archaeological and paleoecological applications, as bones are among the few tissues that are commonly preserved in archaeological and assemblages. Selection of skeletal elements for stable isotope analysis is typically driven by sample sizes and convenience, with the assumption that each bone is equally likely to be representative of the entire skeleton. This study investigated the degree of variability in stable carbon and nitrogen isotope ratios (δ^{13}C and δ^{15}N) within the skeletons of individual marine mammals to determine whether any systematic differences in δ^{13}C and δ^{15}N exist among skeletal elements. We measured δ^{13}C and δ^{15}N in paired crania and mandibles from 11 Pacific walruses (*Odobenus rosmarus divergens*), as well as representative elements from the skeletons of three marine mammals: an adult ringed seal (*Pusa hispida*, n = 10), a juvenile seal of the genus *Phoca* (*Phoca* sp., n = 9), and an adult sea otter (*Enhydra lutris*, n = 8). Differences among the walrus cranium/mandible pairs were not significant, mostly falling within analytical error. Variability across the skeletons of the seals and sea otter was greater, exceeding 1.0‰ in some cases. Hierarchical cluster analysis indicated systematic differences within all three skeletons, with the distal appendicular bones (metatarsal, phalanx, calcaneus) separating from the rest of the skeleton in the two seals, and the scapula and vertebra distinct from all other bones in the sea otter. Removing these bones from analysis greatly reduced overall variability in all three animals. Further study is required to determine whether the patterns observed in this study are consistent across individuals and taxa as sample sizes increase.

1. Introduction

Stable isotope analysis is a powerful tool for investigating animal diet, movement, and physiology (Hobson, 1999; Kelly, 2000). This technique is particularly useful for paleoecological and archaeological studies, where feeding habits and movements of animal and human populations cannot be directly observed and must instead be reconstructed from preserved or fossil remains (Schoeninger and Moore, 1992). Stable carbon and nitrogen isotope ratios of bone collagen are widely used for such reconstructions, as bones are among the few animal parts commonly recovered from archaeological and paleontological sites. Applications of stable isotope analysis of zooarchaeological assemblages include reconstructions of human and animal diet (e.g., Hilderbrand et al., 1996; Katzenberg and Weber, 1999; Richards and Hedges, 1999; Szpak et al., 2012), food web structure (e.g., Misarti et al., 2009; Bocherens et al., 2015), and environmental change (e.g., Ambrose and DeNiro, 1989; Zangrando et al., 2014; Commendador and Finney, 2016), as well as investigations of human and animal

distribution, movement, and dispersal patterns (e.g., Sealy et al., 1995; Barberena et al., 2009; Lamb et al., 2014). Analysis of bone collagen can thus provide information about vertebrate diet and movements across thousands of years.

Though bone collagen is commonly used in archaeology and paleoecology, relatively little work has been done to investigate the degree to which stable isotope ratios vary within the skeletons of individual animals. Bone turnover rates vary with age and type of bone (cortical vs. trabecular), thus stable isotope ratios might be expected to vary accordingly among skeletal elements (Snyder et al., 1975; Klepinger, 1984; Libby et al., 1995; Sealy et al., 1995; Lamb et al., 2014). For the sake of convenience, however, it is commonly assumed that the stable isotope ratios of collagen extracted from any single bone will be representative of the entire skeleton, an assumption that is loosely supported by the work of DeNiro and Schoeninger (1983). Decisions about which skeletal elements to use for stable isotope analysis are typically driven by the availability and preservation of elements within an assemblage (Jørkov et al., 2009); however, if systematic

differences in stable isotope ratios do exist within the skeletons of individual animals, these differences are important to consider when selecting elements for analysis and reconstructions.

The purpose of this study was to quantify variability of stable isotope ratios among the bones of individual, free-ranging marine Caniformia. To accomplish this, we analyzed the stable carbon and nitrogen isotope ratios of bone collagen extracted from the crania and mandibles of Pacific walruses (*Odobenus rosmarus divergens*), as well as from a variety of bones selected to represent the skeletons of two phocid seals and a sea otter (*Enhydra lutris*). The resulting estimates of variability within the skeletons of individual animals, as well as the identification of systematic differences in stable carbon and nitrogen isotope ratios among skeletal elements, will improve future studies by providing researchers with a better understanding of intra-skeletal stable isotope variability. Furthermore, these data will allow for the exclusion of skeletal elements that are not representative of the entire skeleton.

2. Materials and methods

Eleven Pacific walrus cranium/mandible pairs and representative elements from three marine mammal skeletons (n = 8–10) were on loan from the University of Alaska Museum, Fairbanks, AK. Skeletons used for this study were from an adult ringed seal (*Pusa hispida*), a juvenile seal of the genus *Phoca*, and an adult sea otter. Representative bones were selected from all parts of the skeleton including the skull (cranium/mandible), axial skeleton (vertebra, rib), and appendicular skeleton (scapula, innominate, humerus, femur, metatarsal, calcaneus and/or phalanx). All samples were from animals that died after 1930, thus were historical or modern and not of archaeological origin.

Bones were sampled using handheld cutting tools, and ~0.4 g of bone was used for collagen extractions, which were carried out according to the methods described by Misarti et al. (2009), as modified from Matheus (1995). Briefly, bones were cleaned in a sonic bath, then lipids were extracted by soaking bone in 2:1 choloroform:methanol for eight hours. Hydrochloric acid was used to remove the mineral component of the bone. The organic component was then gelatinized in a mildly acidic solution at 65 °C, filtered through a 0.45 μm filter to remove any insoluble particles and non-collagen organic compounds, and freeze dried to produce purified collagen. A subsample of 0.2–0.4 mg of collagen was then submitted for stable isotope analysis. Collagen was extracted from walrus crania and mandibles once. Three replicate subsamples were cut from the bones of the seals and sea otter to incorporate some of the variability in stable isotope ratios of collagen within each bone. Replicate subsamples were taken from locations directly adjacent to one another and collagen was extracted from each subsample separately. The phalanx of the ringed seal and the metatarsal and rib of the *Phoca* sp. could only be subsampled and collagen extracted twice due to limitations in the amount of available material.

Stable carbon and nitrogen isotope ratios of collagen samples were analyzed by the Alaska Stable Isotope Facility at the Water and Environmental Research Center, University of Alaska Fairbanks, using a Costech ECS 4010 elemental analyzer and ThermoScientific Conflo IV, interfaced with a ThermoScientific Delta V mass spectrometer. Stable isotopic compositions were calibrated relative to Vienna Pee Dee Belemnite and atmospheric nitrogen gas (air) scales using USGS40 and USGS41. Results were reported in parts per thousand (‰) using δ notation. A commercially available peptone standard (No. P-7750 Bovine based protein. Sigma Chemical Company, lot #76f-0300; δ^{13}C: − 15.8‰, δ^{15}N: 7.0‰) was analyzed as a check standard after every 10 samples to measure uncertainty. Precision of these analyses was determined to be ± 0.2‰ for both δ^{13}C and δ^{15}N, based on repeated measurements of this check standard across all analytical runs (n = 48). Measurements were accurate to within less than ± 0.01‰ for both δ^{13}C and δ^{15}N, based on differences between observed and known values of the check standard. Collagen yield (percent of dry bone weight) and sample composition (weight percent carbon, weight

percent nitrogen, and C/N ratio) were assessed to evaluate the quality of the collagen samples.

Differences among crania and mandibles were examined using linear regression analyses. The structure of the data did not allow for parametric comparisons of variability within the skeletons of the seals and sea otter, thus this information was summarized using primarily descriptive statistics. Pooled standard deviations were calculated for measures that averaged variability across all three skeletons to account for differences in the number of skeletal elements analyzed for each individual. Systematic differences in stable isotope ratios within the skeleton were investigated using Ward's method of hierarchical clustering, an agglomerative method that generates clusters based on smallest squared Euclidean distances between cluster centers. All statistical analyses were conducted using R version 3.2.3 (R Core Team, 2014) with RStudio version 1.0.136 (RStudio Team, 2015).

3. Results

Stable carbon and nitrogen isotope ratios of walrus crania exhibited linear correlations with those of mandibles from the same individuals (δ^{15}N: $F_{1,9}$ = 153.8, P ≤ 0.001; δ^{13}C: $F_{1,9}$ = 86.6, P < 0.001). These correlations had slopes close to one, y-intercepts close to zero, and explained the majority of the variability in the data (δ^{13}C: R^2 = 0.90; δ^{15}N: R^2 = 0.94), indicating that δ^{13}C and δ^{15}N values of the one skeletal element (cranium/mandible) from an individual walrus can be used to accurately predict values of the other element and that these values are essentially identical (Fig. 1). The mean differences (± 1 SD) between the two bones were 0.0 ± 0.2‰ for δ^{13}C and 0.0 ± 0.3‰ for δ^{15}N. The maximum differences between the cranium and mandible of an individual walrus were 0.2‰ for δ^{13}C and 0.4‰ for δ^{15}N (Table 1).

Variability in δ^{13}C and δ^{15}N was low across multiple skeletal elements from each individual marine mammal. The mean range (± 1 pooled SD) of stable carbon and nitrogen isotope ratios for the three animals was 0.9 ± 0.6‰ for δ^{13}C and 0.9 ± 0.4‰ for δ^{15}N. Stable carbon and nitrogen isotope ratios from all ringed seal elements exhibited a maximum range of 1.2‰ for δ^{13}C and 1.3‰ for δ^{15}N within a single replicate analysis. The range of all values across all three replicate analyses was 1.6‰ for δ^{13}C and 1.5‰ for δ^{15}N, and the range of the mean values of the three replicate analyses was 0.6‰ for δ^{13}C and 1.1‰ for δ^{15}N (Table 2). Hierarchical cluster analysis indicated the presence of two groups within the ringed seal bones, with the metatarsal, phalanx, and calcaneus grouping separately from the rest of the bones in the skeleton (Fig. 2). With these bones removed from analysis, stable carbon and nitrogen isotope ratios from the ringed seal exhibited a maximum range of 0.9‰ for δ^{13}C and 0.8‰ for δ^{15}N within a single replicate analysis. The range of all values across all three replicate analyses with these bones removed was 1.2‰ for both δ^{13}C and δ^{15}N, and the range of mean values across all three replicate analyses was 0.2‰ for δ^{13}C and 0.5‰ for δ^{15}N (Table 2).

The stable carbon and nitrogen isotope ratios of the juvenile seal of the genus *Phoca* exhibited a maximum range of 0.7‰ for δ^{13}C and 1.3‰ for δ^{15}N within a single analytical replicate analysis. The range of all values across all three replicate analyses was 0.9‰ for δ^{13}C and 1.4‰ for δ^{15}N, and the range of the mean values across the three replicate analyses was 0.6‰ for δ^{13}C and 1.2‰ for δ^{15}N (Table 3). Hierarchical cluster analysis also indicated the presence of two groups in the bones of the juvenile seal, with the metatarsal and phalanx grouping separately from the rest of the skeleton (Fig. 2). With these bones removed from analysis, stable carbon and nitrogen isotope ratios from this animal exhibited a maximum range of 0.6‰ for δ^{13}C and 0.8‰ for δ^{15}N within a single replicate analysis. The range of all values across all three replicate analyses with these bones removed was 0.8‰ for δ^{13}C and 0.9‰ for δ^{15}N, and the range of mean values across all three replicate analyses was 0.4‰ for δ^{13}C and 0.7‰ for δ^{15}N (Table 3).
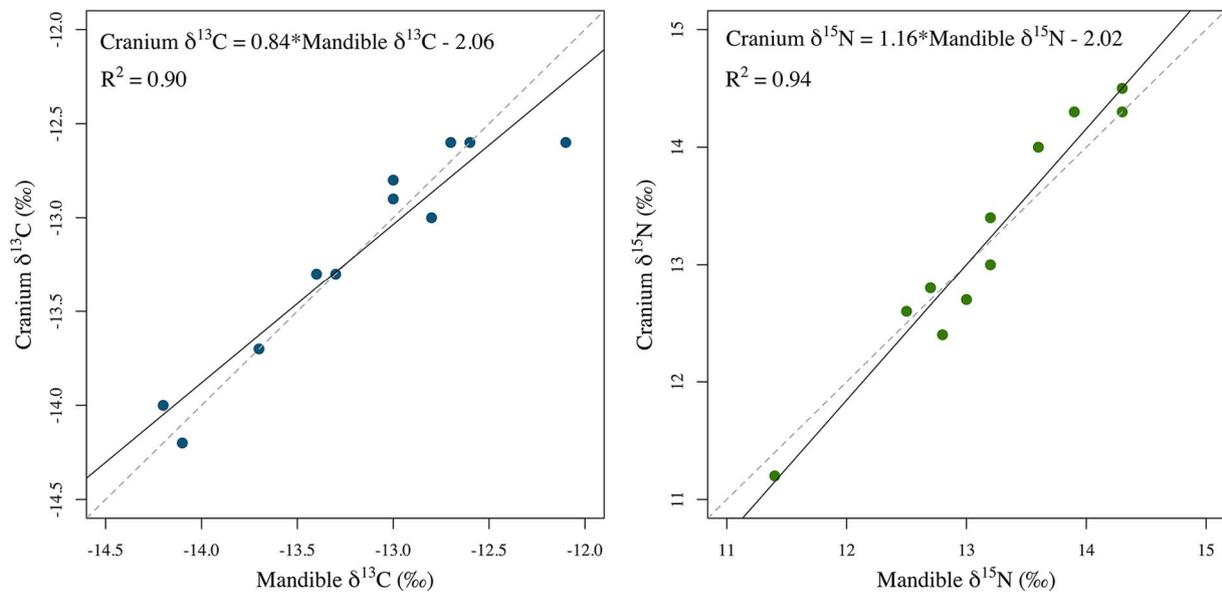
Fig. 1. Linear regressions of the cranium and mandible $\delta^{13}C$ (left) and $\delta^{15}N$ (right) values of 11 Pacific walruses. High R^2 values (≥ 0.90) indicate that $\delta^{13}C$ and $\delta^{15}N$ values of the one skeletal element (cranium/mandible) from an individual walrus can be used to accurately predict the $\delta^{13}C$ or $\delta^{15}N$ of the other element. Slopes close to one indicate this correlation is approximately 1:1 (dashed line) for $\delta^{13}C$ and $\delta^{15}N$.

The stable carbon and nitrogen isotope ratios of the sea otter exhibited a maximum range of 1.9‰ for $\delta^{13}C$ and 0.8‰ for $\delta^{15}N$ within a single replicate analysis. The range of all values across all three replicate analyses was 1.9‰ for $\delta^{13}C$ and 0.9‰ for $\delta^{15}N$, and the range of mean values across all three replicate analyses was 1.0‰ for $\delta^{13}C$ and 0.6‰ for $\delta^{15}N$ (Table 4). Hierarchical cluster analysis again indicated the presence of two groups within the sea otter bones; however, this time the scapula and vertebra separated from the rest of the skeleton (Fig. 2). With these bones removed from analysis, stable carbon and nitrogen isotope ratios from this animal exhibited a maximum range of 0.7‰ for $\delta^{13}C$ and 0.4‰ for $\delta^{15}N$ within a single replicate analysis. The range of all values across all three replicate analyses with these bones removed was 1.1‰ for $\delta^{13}C$ and 0.5‰ for $\delta^{15}N$, and the range of the mean values across all three replicate analyses was 0.3‰ for $\delta^{13}C$ and 0.2‰ for $\delta^{15}N$ (Table 4).

The degree of variability in both $\delta^{13}C$ and $\delta^{15}N$ across repeated analyses for some of the individual skeletal elements was unexpectedly large. For most elements, this variability was close to instrumental error (± 0.2‰); however, at least one bone in each of the three skeletons had a standard deviation of $\delta^{13}C$ or $\delta^{15}N$ of 0.4‰ or greater. In the seals, $\delta^{15}N$ tended to be more variable across the three replicate analyses (*P. hispida*: 1 pooled SD = 0.4‰; *Phoca* sp.: pooled SD = 0.4‰) than $\delta^{13}C$ (*P. hispida*: 1 pooled SD = 0.2‰; *Phoca* sp.: 1 pooled SD = 0.3‰). The opposite was true for the sea otter ($\delta^{15}N$: 1 pooled SD = 0.2‰; $\delta^{13}C$: 1 pooled SD = 0.4‰). There were no obvious

patterns in which skeletal elements exhibited the most or least variability in $\delta^{13}C$ and $\delta^{15}N$.

Assessment of collagen yield and sample composition indicated good recovery of collagen from all bone samples, ranging from 13.9 to 32.1% of dry bone weight (Tables S1-S4). Collagen yield could not be accurately calculated for three samples, including two instances where the original vial broke and an accurate weight could not be obtained (walrus cranium UAM 3382 and walrus mandible UAM 11699), and one instance where some sample was lost during filtering resulting in an artificially low collagen yield of 4.7% (*Phoca* spp. vertebra – extraction 1). Analysis of sample composition indicated high quality collagen was recovered, with weight %C typically ~15%, weight %N typically ~45%, and C/N ratios ranging from 2.9–3.7 (Tables S1-S4).

4. Discussion

Understanding how $\delta^{13}C$ and $\delta^{15}N$ vary within the skeletons of individual animals is important for appropriate design and interpretation of studies that measure these ratios in bone collagen. The results presented here indicate little variability in the stable carbon and nitrogen isotope ratios of bone collagen extracted from different elements within the skeletons of individual, free-ranging marine mammals. This was especially true of the walrus cranium/mandible pairs, for which the differences in $\delta^{13}C$ and $\delta^{15}N$ generally fell within the range of EA-IRMS instrument error (± 0.2‰). Variability among the representative

Table 1
Stable carbon and nitrogen isotope ratios of crania and mandibles from 11 walruses. Specimens included both males and females with age classes ranging from neonate to adult (Neo = neonate, Juv = juvenile, Adu = adult).

	Specimen ID										
	UAM 3382	UAM 7277	UAM 11512	UAM 11513	UAM 11517	UAM 11519	UAM 11684	UAM 11689	UAM 11699	UAM 12079	UAM 16593
Age class	Juv	Adu	Juv	Neo	Juv	Neo	Adu	Juv	Adu	Adu	Adu
Cranium											
$\delta^{13}C$ (‰)	−12.6	−12.9	−12.6	−13.0	−14.0	−12.6	−12.8	−14.2	−13.3	−13.3	−13.7
$\delta^{15}N$ (‰)	11.2	13.0	14.5	14.3	14.0	14.3	12.8	12.4	12.7	12.6	13.4
Mandible											
$\delta^{13}C$ (‰)	−12.7	−13.0	−12.6	−12.8	−14.2	−12.1	−13.0	−14.1	−13.4	−13.3	−13.7
$\delta^{15}N$ (‰)	11.4	13.2	14.3	14.3	13.6	13.9	12.7	12.8	13.0	12.4	13.2

Table 2
Stable carbon and nitrogen isotope ratios for each replicate analysis (Extraction 1, Extraction 2, Extraction 3) of each skeletal element (femur, humerus, scapula, innominate, metatarsal, rib, vertebra, mandible, phalanx, calcaneus) of the ringed seal (*Pusa hispida*). Means (± 1 SD) and ranges of $\delta^{13}C$ and $\delta^{15}N$ across the three replicate analyses of each bone are presented in the last four columns. Within-analysis means (± 1 SD) and ranges of $\delta^{13}C$ and $\delta^{15}N$, both with and without the metatarsal, phalanx, and calcaneus (T, P, C), are presented in the last four rows. Overall mean (± 1 pooled SD of all replicate analyses of each element) $\delta^{13}C$ and $\delta^{15}N$ for the ringed seal, both with and without the metatarsal, phalanx, and calcaneus (M, P, C), are presented in the lower right corner.

Pusa hispida UAM 16603	Extraction 1		Extraction 2		Extraction 3					
Element	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	Mean $\delta^{15}N$ (± 1 SD)	Mean $\delta^{13}C$ (± 1SD)	$\delta^{15}N$ Range	$\delta^{13}C$ Range
Femur	18.4	−15.2	18.0	−15.3	18.3	−15.4	18.2 ± 0.2	−15.3 ± 0.1	0.4	0.2
Humerus	18.1	−15.1	17.6	−15.4	17.9	−15.3	17.9 ± 0.3	−15.3 ± 0.2	0.5	0.3
Scapula	18.2	−15.0	17.7	−15.3	17.8	−16.0	17.9 ± 0.3	−15.4 ± 0.5	0.5	1.0
Innominate	18.4	−15.1	18.0	−15.3	18.3	−15.1	18.2 ± 0.2	−15.2 ± 0.1	0.4	0.2
Metatarsal	19.0	−15.0	18.8	−15.4	19.1	−15.4	19.0 ± 0.2	−15.3 ± 0.2	0.3	0.4
Rib	18.6	−15.6	18.1	−15.3	18.5	−15.3	18.4 ± 0.3	−15.4 ± 0.2	0.5	0.3
Vertebra	18.3	−15.3	18.2	−15.3	18.3	−15.5	18.3 ± 0.1	−15.4 ± 0.1	0.1	0.2
Mandible	18.8	−14.8	18.0	−15.8	18.5	−15.2	18.4 ± 0.4	−15.3 ± 0.5	0.8	1.0
Phalanx	18.9	−14.4	18.3	−15.2	–	–	18.6 ± 0.4	−14.8 ± 0.6	0.6	0.8
Calcaneus	18.8	−15.1	18.9	−15.6	18.7	−15.5	18.8 ± 0.1	−15.4 ± 0.3	0.2	0.5
Cranium	–	–	–	–	–	–	–	–	–	–
Mean (± 1 SD)	18.6 ± 0.3	−15.1 ± 0.3	18.2 ± 0.4	−15.4 ± 0.2	18.4 ± 0.4	−15.4 ± 0.3	Mean $\delta^{15}N$ (± 1 Pooled SD) 18.4 ± 0.4	Mean $\delta^{13}C$ (± 1 Pooled SD) −15.3 ± 0.3		
Range	0.9	1.2	1.3	0.6	1.3	0.9				
Mean (± 1 SD) w/o M,P,C	18.4 ± 0.2	−15.2 ± 0.2	17.9 ± 0.2	−15.4 ± 0.2	18.2 ± 0.3	−15.4 ± 0.3	Mean $\delta^{15}N$ (± 1Pooled SD) 18.2 ± 0.2	Mean $\delta^{13}C$ (± 1Pooled SD) −15.3 ± 0.2		
Range w/o M,P,C	0.7	0.8	0.6	0.5	0.7	0.9				

elements of the complete skeletons was somewhat larger, with mean ranges exceeding instrument error (though typically remaining < 1.0‰) and maximum ranges reaching nearly 2.0‰; however, these maximum ranges likely represent the upper end of the variation that can be expected within the cortical bone of an individual animal, as they incorporate variability associated with collagen extraction and isotope analysis of three separate samples from multiple bones within the skeleton.

Intra-skeletal variability in carbon and nitrogen stable isotope ratios in the present study was similar to that observed in other studies measuring $\delta^{13}C$ and $\delta^{15}N$ of multiple bones from the same individual mammals (Table 5). The mean ranges for both $\delta^{13}C$ and $\delta^{15}N$ were ≤ 1.2‰ in all studies, and were most often ≤ 0.6‰ (Jørkov et al., 2009; Riofrío-Lazo and Aurioles-Gamboa, 2013; Olsen et al., 2014; Webb et al., 2016; Cheung et al., 2017, and for data on variability within fish skeletons see Guiry et al., 2016). The maximum ranges were much larger, with northern elephant seals (*Mirounga angustirostris*) exhibiting a maximum range of > 2.5‰ between the $\delta^{13}C$ of the mandible and maxilla (Riofrío-Lazo and Aurioles-Gamboa, 2013), and human (*Homo sapiens*) femur/rib pairs showing a maximum range of $\delta^{15}N$ of > 2.2‰ (Jørkov et al., 2009). This intra-skeletal variability in stable isotope ratios likely results from differences in turnover rate of bone tissue within the skeleton (Sealy et al., 1995). Bone turnover rate varies with age and bone type (Snyder et al., 1975; Klepinger, 1984; Libby et al., 1995; Hedges et al., 2007), and is also impacted by the mechanical forces acting on the bone (Marotti, 1963). Compact cortical bone turns over much more slowly (humans: 4% per year) than spongy trabecular bone (humans: 28% per year; Manolagas, 2000), thus cortical bone isotope ratios represent average diet from up to 25 years, whereas isotope ratios in trabecular bone represent diet averaged across the most recent ~3–4 years. It is therefore important to consider which type of bone is being sampled for isotopic studies.

Differences in bone turnover rate among skeletal elements may help to explain the systematic differences observed within the skeletons of the seals and sea otter used for this study. In rapidly-growing mammals, some bones may be characterized by negative allometry, i.e., they grow at a slower rate than the skeleton as a whole (Klevezal, 1996), thus these bones would be expected to exhibit slower turnover than other bones in the skeleton. In both seals, hierarchical cluster analysis grouped the distal limb bones (calcaneus, phalanx, metatarsal) separately from the rest of the skeleton (Fig. 2). These bones are small and dense relative to much of the skeleton, consisting primarily of cortical bone; however, they are also subject to a great deal of mechanical stress as they are used, to some extent, for bearing weight on land and as the primary source of propulsion in the water. The impacts of mechanical stress on bone turnover rate might help explain why the isotope ratios of these bones differed from those of the rest of the skeleton (Marotti, 1963). In the sea otter, the scapula and vertebra grouped separately from the other bones, with the vertebra showing a large degree of variability in $\delta^{13}C$ among the three replicate analyses (Fig. 2). Snyder et al. (1975) reported that the vertebra exhibits the fastest turnover of any bone in the human skeleton. Vertebrae consist of a large proportion of trabecular bone and it can be difficult to sample only cortical bone for collagen extraction. It is possible that the three vertebra samples collected from the sea otter contained some trabecular bone (likely in different proportions in each sample). This might explain the great degree of variability among the three replicate analyses for this element. Similarity in turnover rates for the scapula and the vertebra might explain why these elements grouped together; however, this remains uncertain.

It is important to note that for long-lived animals, differences in stable isotope ratios will only exist within the skeleton if the animal's diet or location change substantially. Animals eating a monotonous diet will have similar isotope ratios across their skeletons, regardless of bone turnover rate (DeNiro and Schoeninger, 1983). Differences in stable isotope ratios of collagen from bones with faster or slower turnover
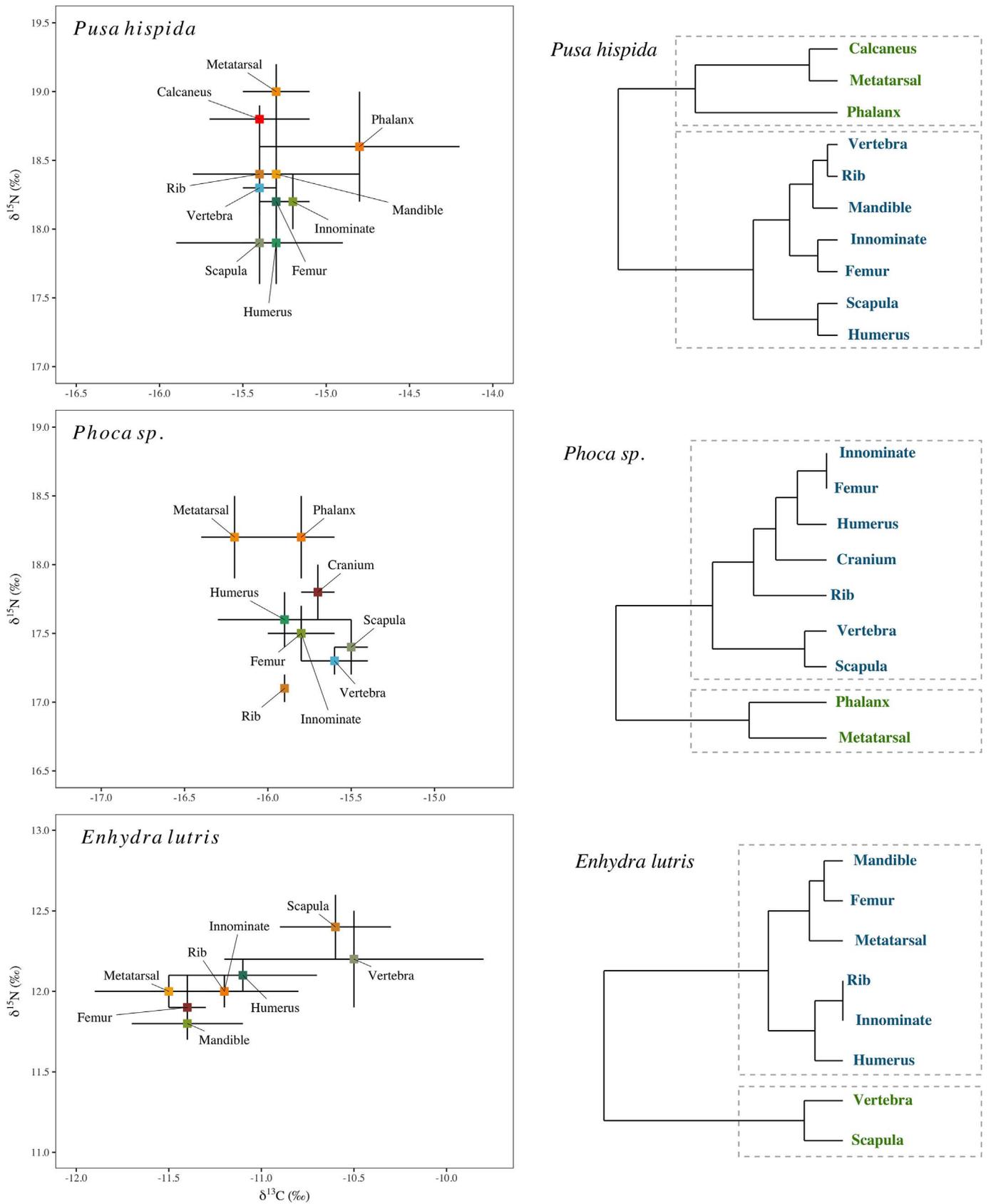
Fig. 2. Mean (± 1 SD) δ¹³C and δ¹⁵N (left) and dendrograms produced from hierarchical cluster analysis (right) of representative elements from the skeletons of an adult ringed seal (top, n = 10), a juvenile seal of the genus *Phoca* (middle, n = 9), and an adult sea otter (bottom, n = 8). Means and standard deviations were calculated across all extractions and analyses of bone collagen. Dashed boxes and colored text on dendrograms indicate separation of groups according to Ward's method of hierarchical clustering.

Table 3
Stable carbon and nitrogen isotope ratios for each replicate analysis (Extraction 1, Extraction 2, Extraction 3) of each skeletal element (femur, humerus, scapula, innominate, metatarsal, rib, vertebra, phalanx, cranium) of the juvenile seal (*Phoca* sp.). Means (± 1 SD) and ranges of $\delta^{13}C$ and $\delta^{15}N$ across the three replicate analyses of each bone are presented in the last four columns. Within-analysis means (± 1 SD) and ranges of $\delta^{13}C$ and $\delta^{15}N$, both with and without the metatarsal and phalanx (T, P), are presented in the last rows. Overall mean (± 1 pooled SD of all replicate analyses of each element) $\delta^{13}C$ and $\delta^{15}N$ for the ringed seal, both with and without the metatarsal and phalanx (M, P), are presented in the lower right corner.

Phoca sp. UAM 87926	Extraction 1		Extraction 2		Extraction 3					
Element	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	Mean $\delta^{15}N$ (± 1 SD)	Mean $\delta^{13}C$ (± 1 SD)	$\delta^{15}N$ Range	$\delta^{13}C$ Range
Femur	17.7	−15.7	17.3	−16.0	17.4	−15.8	17.5 ± 0.2	−15.8 ± 0.2	0.4	0.3
Humerus	17.8	−15.5	17.4	−16.2	17.6	−16.0	17.6 ± 0.2	−15.9 ± 0.4	0.4	0.7
Scapula	17.6	−15.4	17.3	−15.6	17.4	−15.5	17.4 ± 0.2	−15.5 ± 0.1	0.3	0.2
Innominate	17.6	−15.7	17.5	−16.0	17.3	−15.6	17.5 ± 0.2	−15.8 ± 0.2	0.3	0.4
Metatarsal	–	–	18.0	−16.3	18.4	−16.0	18.2 ± 0.3	−16.2 ± 0.2	0.4	0.3
Rib	–	–	17.0	−15.9	17.1	−15.9	17.1 ± 0.1	−15.9 ± 0.0	0.1	0.0
Vertebra	17.4	−15.4	17.3	−15.7	17.2	−15.8	17.3 ± 0.1	−15.6 ± 0.2	0.2	0.4
Mandible	–	–	–	–	–	–	–	–	–	–
Phalanx	18.3	−15.8	17.9	−16.0	18.4	−15.7	18.2 ± 0.3	−15.8 ± 0.2	0.5	0.3
Calcaneus	–	–	–	–	–	–	–	–	–	–
Cranium	17.8	−15.6	17.6	−15.8	17.9	−15.8	17.8 ± 0.1	−15.7 ± 0.1	0.3	0.2
Mean (± 1 SD)	17.7 ± 0.3	−15.6 ± 0.2	17.5 ± 0.3	−15.9 ± 0.2	17.6 ± 0.5	−15.8 ± 0.2	Mean $\delta^{15}N$ (± 1 Pooled SD)	Mean $\delta^{13}C$ (± 1 Pooled SD)		
Range	0.9	0.4	1.0	0.7	1.3	0.5	17.6 ± 0.4	−15.8 ± 0.2		
Mean (± 1 SD) w/o M,P	17.7 ± 0.2	−15.6 ± 0.1	17.3 ± 0.2	−15.9 ± 0.2	17.4 ± 0.3	−15.9 ± 0.2	Mean $\delta^{15}N$ (± 1 Pooled SD)	Mean $\delta^{13}C$ (± 1 Pooled SD)		
Range w/o M,P	0.4	0.3	0.6	0.6	0.8	0.5	17.5 ± 0.2	−15.7 ± 0.2		

Table 4
Stable carbon and nitrogen isotope ratios for each replicate analysis (Extraction 1, Extraction 2, Extraction 3) of each skeletal element (femur, humerus, scapula, innominate, metatarsal, rib, vertebra, mandible) of the sea otter (*Enhydra lutris*). Means (± 1 SD) and ranges of $\delta^{13}C$ and $\delta^{15}N$ across the three replicate analyses of each bone are presented in the last four columns. Within-analysis means (± 1 SD) and ranges of $\delta^{13}C$ and $\delta^{15}N$, both with and without the scapula and vertebra (S, V), are presented in the last rows. Overall mean (± 1 pooled SD of all replicate analyses of each element) $\delta^{13}C$ and $\delta^{15}N$ for the ringed seal, both with and without the scapula and vertebra (S, V), are presented in the lower right corner.

Enhydra lutris UAM7346	Extraction 1		Extraction 2		Extraction 3					
Element	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	Mean $\delta^{15}N$ (± 1 SD)	Mean $\delta^{13}C$ (± 1 SD)	$\delta^{15}N$ Range	$\delta^{13}C$ Range
Femur	11.9	−11.4	12.0	−11.5	11.7	−11.3	11.9 ± 0.2	−11.4 ± 0.1	0.3	0.2
Humerus	12.2	−10.7	12.0	−11.1	12.0	−11.4	12.0 ± 0.1	−11.1 ± 0.4	0.2	0.7
Scapula	12.6	−10.4	12.2	−10.6	12.3	−10.9	12.4 ± 0.2	−10.6 ± 0.3	0.4	0.5
Innominate	12.0	−11.2	12.0	−11.1	12.1	−11.2	12.0 ± 0.1	−11.2 ± 0.1	0.1	0.1
Metatarsal	12.1	−11.1	11.9	−11.8	12.1	−11.5	12.0 ± 0.1	−11.5 ± 0.4	0.2	0.7
Rib	12.0	−10.7	12.0	−11.4	12.1	−11.4	12.0 ± 0.1	−11.2 ± 0.4	0.1	0.7
Vertebra	12.2	−10.3	12.5	−9.9	11.9	−11.2	12.2 ± 0.3	−10.5 ± 0.7	0.6	1.3
Mandible	11.9	−11.0	11.7	−11.5	11.8	−11.6	11.8 ± 0.1	−11.4 ± 0.3	0.2	0.6
Phalanx	–	–	–	–	–	–	–	–	–	–
Calcaneus	–	–	–	–	–	–	–	–	–	–
Cranium	–	–	–	–	–	–	–	–	–	–
Mean (± 1 SD)	12.1 ± 0.2	−10.9 ± 0.4	12.0 ± 0.2	−11.1 ± 0.6	12.0 ± 0.2	−11.3 ± 0.2	Mean $\delta^{15}N$ (± 1 Pooled SD)	Mean $\delta^{13}C$ (± 1 Pooled SD)		
Range	0.7	1.1	0.8	1.9	0.6	0.7	12.1 ± 0.2	−11.1 ± 0.4		
Mean (± 1 SD) w/o S,V	12.0 ± 0.1	−11.0 ± 0.3	11.9 ± 0.1	−11.4 ± 0.3	12.0 ± 0.2	−11.4 ± 0.1	Mean $\delta^{15}N$ (± 1 Pooled SD)	Mean $\delta^{13}C$ (± 1 Pooled SD)		
Range w/o S,V	0.3	0.7	0.3	0.7	0.4	0.4	12.0 ± 0.2	−11.3 ± 0.4		

Table 5
Summary of information from studies that examined $\delta^{13}C$ and/or $\delta^{15}N$ in multiple skeletal elements from individual animals. Information includes name of reference, species studied (human – *Homo sapiens*, Northern elephant seal – *Mirounga angustirostris*, domestic pig – *Sus domesticus*, Pacific walrus – *Odobenus rosmarus divergens*, ringed seal – *Pusa hispida*, seal species of the genus *Phoca*, and sea otter – *Enhydra lutris*), skeletal elements used, sample size (n = # of individuals), mean range (± 1 standard deviation) of $\delta^{13}C$ and $\delta^{15}N$ across all individuals studied, and max range of $\delta^{13}C$ and $\delta^{15}N$ across all individuals studied.

Reference	Species	Elements used	n	Mean range (± 1 SD)		Max range	
				$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Jørkov et al., 2009	Human	Femur, rib	57	0.1 ± 0.1	0.4 ± 0.4	0.5	2.2
Riofrío-Lazo and Aurioles-Gamboa, 2013	Northern elephant seal	Mandible, maxilla	14	1.0 ± 0.6	0.5 ± 0.4	2.7	1.6
Olsen et al., 2014	Human	Fibula, metacarpal, rib	6	0.2 ± 0.1	0.6 ± 0.2	0.3	0.9
Webb et al., 2016	Pig	Femur, rib	48	–	0.3 ± 0.4	–	1.7
Cheung et al., 2017	Human	Femur, fibula	11	1.0 ± 0.8	0.6 ± 0.6	2.3	1.7
Cheung et al., 2017	Human	Femur, radius	6	1.2 ± 0.3	0.5 ± 0.6	1.7	1.6
This study	Pacific walrus	Cranium, mandible	11	0.1 ± 0.1	0.2 ± 0.1	0.5	0.4
This study	Ringed seal, Phoca sp., sea otter	Calcaneus, cranium or mandible, femur, humerus, innominate, phalanx, rib, scapula, metatarsal, vertebra	3	0.7 ± 0.3	0.9 ± 0.3	1.0	1.2

rates would only be expected to occur if the isotope ratios of the food consumed by the animal changed during its life, resulting either from dietary change, geographic movement, or shifting isotopic baselines (Newsome et al., 2010). Physiological changes may also impact stable isotope ratios, but conditions such as severe nutritional stress, illness, etc., are less likely to last for long enough (years) to substantially change bone collagen stable isotope ratios. Stable isotope ratios of bones with different turnover rates can thus be used to gain information about changes that occurred within the life of an animal. For example, Cox and Sealy (1997) reported substantial differences in $\delta^{13}C$ (up to ~12.0‰) and $\delta^{15}N$ (up to ~3.0‰) of skeletal elements with slow turnover rates and those with fast turnover rates from shipwreck survivors that switched from a primarily terrestrial diet, to one incorporating many marine food sources. Lamb et al. (2014) used a similar analysis to reconstruct the diet and geographic movements of King Richard III of England. In the present study, it is possible that some of the variability in $\delta^{13}C$ and $\delta^{15}N$ among the bones of the marine mammal skeletons resulted from dietary changes or geographic movements. This seems particularly likely for the sea otter, which exhibited substantial differences between the stable isotope ratios of the vertebra (rapid turnover rate), and bones such as the humerus and femur (slower turn over) (Snyder et al., 1975; Hedges et al., 2007).

Assessment of parameters such as collagen yield and collagen composition is important to assuring the quality of stable isotopic data. In this study, collagen yield, weight %C, weight %N, and C/N ratios all indicated high quality collagen and variability in these values were not related to observed differences in $\delta^{13}C$ and $\delta^{15}N$ among replicate analyses or among skeletal elements (Tables S1-S4). Measurement of these parameters is particularly important for analyses of specimens from zooarchaeological assemblages, as poor preservation and diagenesis may degrade collagen and impact stable isotope ratios (Ambrose, 1990; van Klinken, 1999). Additionally, C/N ratios provide information about whether lipids were effectively removed from the sample during collagen extraction. Failure to remove lipids from bone will result in more negative $\delta^{13}C$ values and may result in underestimation of the dietary contributions of C_4 plants or marine foods (Ambrose, 1990).

Whatever the reason for the systematic differences in $\delta^{13}C$ and $\delta^{15}N$ observed within the skeletons of the seals and sea otter sampled for this study, understanding these differences is important as this information might allow researchers to exclude from analysis any bones that are unlikely to be representative of the skeleton as a whole. Removal of bones that grouped separately from the rest of the skeleton reduced the mean and maximum ranges of $\delta^{13}C$ and $\delta^{15}N$ of all three skeletons to ≤ 1.1‰ (and generally close to ~0.5‰).

Investigations of marine mammal skeletal characteristics require special consideration of how these animals have adapted to life in aquatic environments, and how these adaptations may affect bone structure and growth. For example, the skeleton plays an important role in buoyancy control for marine mammals, acting as ballast to offset the positive buoyancy of lipids in the blubber layer, as well as that of respiratory air and air trapped in fur (Stein, 1989; Fish and Stein, 1991; Taylor, 1994; Coughlin and Fish, 2009). Walrus skulls and mandibles are composed of very thick, dense bone, which helps them remain in a head-down position while foraging in benthic sediments (Fay, 1982). Bowhead whales (*Balaena mysticetus*) exhibit extreme pachyostosis during the first year of life (George et al., 2016). This serves both to offset the flotation provided by the extremely thick blubber layer present at weaning and to act as storage for the materials needed to increase the size of the feeding apparatus by expanding the head and baleen rack. As these animals reach maturity, bone density is greatly diminished and continues to decline throughout life. Such extreme changes in bone physiology and structure would undoubtedly impact bone turnover rate and the length of time represented by stable isotope ratios of bone collagen. Finally, vitamin D levels vary greatly among species, and may be very low in marine mammals, such as walruses, that forage primarily on benthic invertebrates (Kenny et al., 2004). Vitamin D is important for bone health, and deficiency can lead to metabolic bone diseases such as osteoporosis (Holick, 2004). For marine mammals with low vitamin D levels, the process of bone turnover may be faster than for those animals with higher levels such as ringed seals (Kong et al., 2013), which have blubber vitamin D concentrations > 100 times greater than those of walruses (Kenny et al., 2004).

5. Conclusions

The intra-skeletal variability in $\delta^{13}C$ and $\delta^{15}N$ presented here is generally low, falling outside analytical error for the skeletons of the seals and sea otter, but typically remaining below 1.0‰. Variability within the seal skeletons was substantially decreased when distal appendicular elements were removed from analysis. We suggest that, when possible, these bones be excluded from stable isotope analyses. Similarly, removing the scapula and vertebra resulted in much lower variability within the skeleton of the sea otter. These skeletal elements consist of a relatively large proportion of trabecular bone, therefore may exhibit higher turnover rates than other skeletal elements. Efforts should be made when sampling these bones to collect only cortical bone for collagen extraction. Further study will be required to determine if the patterns observed in this study remain consistent across a larger number of individuals and among taxonomic groups.

Acknowledgements

University of Alaska Museum for providing access to the Pacific walrus (UAM3382, UAM7277, UAM11512, UAM11513, UAM11517, UAM11519, UAM11684, UAM11689, UAM11699, UAM16593), ringed seal (UAM16603), *Phoca* spp. (UAM87926), and sea otter (UAM7346) specimens. Special thanks to Courtney Sessum for assisting in the sampling, lab work, and data analyses during the early stages of this project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jasrep.2017.09.007.

References

Ambrose, S.H., 1990. Preparation and characterization of bone and tooth collagen for isotopic analysis. J. Archaeol. Sci. 17, 431–451.

Ambrose, S.H., DeNiro, M.J., 1989. Climate and habitat reconstruction using stable carbon and nitrogen isotope ratios of collagen in prehistoric herbivore teeth from Kenya. Quat. Res. 31, 407–422.

Barberena, R., Francisco Zangrando, A., Gil, A.F., Martínez, G.A., Politis, G.G., Borrero, L.A., Neme, G.A., 2009. Guanaco (*Lama guanicoe*) isotopic ecology in southern South America: spatial and temporal tendencies, and archaeological implications. J. Archaeol. Sci. 36, 2666–2675.

Bocherens, H., Drucker, D.G., Germonpré, M., Laznickova-Galetova, M., Naito, Y.I., Wissing, C., Brůžek, J., Oliva, M., 2015. Reconstruction of the Gravettian food-web at Předmostí I using multi-isotopic tracking (13C, 15N, 34S) of bone collagen. Quat. Int. 359, 211–228.

Cheung, C., Jing, Z., Tang, J., Weston, D.A., Richards, M.P., 2017. Diets, social roles, and geographical origins of sacrificial victims at the royal cemetery at Yinxu, Shang China: new evidence from stable carbon, nitrogen, and sulfur isotope analysis. J. Anthropol. Archaeol. 48, 28–45.

Commendador, A.S., Finney, B.P., 2016. Holocene environmental change in the eastern Snake River plain of Idaho, USA, as inferred from stable isotope analyses of small mammals. Quat. Res. 85, 358–370.

Coughlin, B.L., Fish, F.E., 2009. Hippopotamus underwater locomotion: reduced-gravity movements for a massive mammal. J. Mammal. 90, 675–679.

Cox, G., Sealy, J., 1997. Investigating identity and life histories: isotopic analysis and historical documentation of slave skeletons found on the Cape Town Foreshore, South Africa. Int. J. Hist. Archaeol. 1, 207–224.

DeNiro, M.J., Schoeninger, M.J., 1983. Stable carbon and nitrogen isotope ratios of bone collagen: variations within individuals, between sexes, and within populations raised on monotonous diets. J. Archaeol. Sci. 10, 199–203.

Fay, F.H., 1982. Ecology and biology of the Pacific walrus, Odobenus rosmarus divergens. Illiger. North Am. Fauna 1–279.

Fish, F.E., Stein, B.R., 1991. Functional correlates of differences in bone density among terrestrial and aquatic genera in the family Mustelidae (Mammalia). Zoomorphology 110, 339–345.

George, J.C., Stimmelmayr, R., Suydam, R., Usip, S., Givens, G., Sformo, T., Thewissen, J.G.M., 2016. Severe bone loss as part of the life history strategy of bowhead whales. PLoS One 11, 1–14.

Guiry, E.J., Szpak, P., Richards, M.P., 2016. Effects of lipid extraction and ultrafiltration on stable carbon and nitrogen isotopic compositions of fish bone collagen. Rapid Commun. Mass Spectrom. 1591–1600.

Hedges, R.E.M., Clement, J.G., Thomas, D.L., O'Connell, T.C., 2007. Collagen turnover in the adult femoral mid-shaft: modeled from anthropogenic radiocarbon tracer measurements. Am. J. Phys. Anthropol. 133, 808–816.

Hilderbrand, G., Farley, S., Robbins, C., Hanley, T., Titus, K., Servheen, C., 1996. Use of stable isotopes to determine diets of living and extinct bears. Can. J. Zool. 74, 2080–2088.

Hobson, K.A., 1999. Tracing origins and migration of wildlife using stable isotopes: a review. Oecologia 120, 314–326.

Holick, M.F., 2004. Sunlight and vitamin D for bone health and prevention of auto-immune diseases, cancers, and cardiovascular disease 1–4. Am. J. Clin. Nutr. 80, 1678–1688.

Jørkov, M.L.S., Heinemeier, J., Lynnerup, N., 2009. The petrous bone - a new sampling site for identifying early dietary patterns in stable isotopic studies. Am. J. Phys. Anthropol. 138, 199–209.

Katzenberg, M.A., Weber, A., 1999. Stable isotope ecology and palaeodiet in the Lake Baikal Region of Siberia. J. Archaeol. Sci. 26, 651–659.

Kelly, J.F., 2000. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. Can. J. Zool. 78, 1–27.

Kenny, D.E., O'Hara, T.M., Chen, T.C., Lu, Z., Tian, X., Holick, M.F., 2004. Vitamin D content in Alaskan Arctic zooplankton, fishes, and marine mammals. Zoo Biol. 23, 33–43.

Klepinger, L.L., 1984. Nutritional assessment from bone. Annu. Rev. Anthropol. 13, 75–96.

Klevezal, G.A., 1996. Recording structures of mammals. In: Determination of Age and Reconstruction of Life History. AA Balkema, Rotterdam (315pp).

van Klinken, G.J., 1999. Bone collagen quality indicators for palaeodietary and radio-carbon measurements. J. Archaeol. Sci. 26, 687–695.

Kong, J., Ding, Y., Zhang, C., Fu, Y., Du, J., Lu, C., Dou, X., Chen, Y., Li, Y.C., Zhao, Q., 2013. Severe vitamin D-deficiency and increased bone turnover in patients with hepatitis B from northeastern China. Endocr. Res. 38, 215–222.

Lamb, A.L., Evans, J.E., Buckley, R., Appleby, J., 2014. Multi-isotope analysis demon-strates significant lifestyle changes in King Richard III. J. Archaeol. Sci. 50, 559–565.

Libby, W.F., Berger, R., Mead, J.F., Alexander, G.V., Ross, J.F., 1995. Replacement rates for human tissue from atmospheric radiocarbon. Science 146, 1170–1172.

Manolagas, S.C., 2000. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr. Rev. 21, 115–137.

Marotti, G., 1963. Quantitative studies on bone reconstruction. Acta Anat. (Basel) 52, 291–333.

Matheus, P.E., 1995. Diet and co-ecology of Pleistocene short-faced bears and brown bears in eastern Beringia. Quat. Res. 44, 447–453.

Misarti, N., Finney, B., Maschner, H., Wooller, M.J., 2009. Changes in northeast Pacific marine ecosystems over the last 4500 years: evidence from stable isotope analysis of bone collagen from archeological middens. The Holocene 19, 1139–1151.

Newsome, S.D., Clementz, M.T., Koch, P.L., 2010. Using stable isotope biogeochemistry to study marine mammal ecology. Mar. Mamm. Sci. 26, 509–572.

Olsen, K.C., White, C.D., Longstaffe, F.J., Von Heyking, K., McGlynn, G., Grupe, G., Rühli, F.J., 2014. Intraskeletal isotopic compositions (δ13C, δ15N) of bone collagen: non-pathological and pathological variation. Am. J. Phys. Anthropol. 153, 598–604.

R Core Team, 2014. R: A Language and Environment for Statistical Computing.

Richards, M.P., Hedges, R.E.M., 1999. Stable isotope evidence for similarities in the types of marine foods used by late Mesolithic humans at sites along the Atlantic coast of Europe. J. Archaeol. Sci. 26, 717–722.

Riofrío-Lazo, M., Aurioles-Gamboa, D., 2013. Timing of isotopic integration in marine mammal skull: comparative study between calcified tissues. Rapid Commun. Mass Spectrom. 27, 1076–1082.

RStudio Team, 2015. RStudio: Integrated Development Environment for R.

Schoeninger, M.J., Moore, K., 1992. Bone stable isotope studies in archaeology. J. J. World Prehist. 6, 247–296.

Sealy, J., Armstrong, R., Schrire, C., 1995. Beyond lifetime averages: tracing life histories through isotopic analysis of different calcified tissues from archaeological human skeletons. Antiquity 69, 290–300.

Snyder, W.S., Cook, M.J., Nasset, E.S., Karhausen, L.R., Howells, G.P., Tipton, I.H., 1975. Report of the Task Group on Reference Man. Pergamon Press.

Stein, B.R., 1989. Bone density and adaptation in semiaquatic mammals. J. Mammal. 70, 467–476.

Szpak, P., Orchard, T.J., McKechnie, I., Gröcke, D.R., 2012. Historical ecology of late Holocene sea otters (*Enhydra lutris*) from northern British Columbia: isotopic and zooarchaeological perspectives. J. Archaeol. Sci. 39, 1553–1571.

Taylor, M.A., 1994. Stone, bone or blubber? Buoyancy control strategies in aquatic tet-rapods. In: Mechanics and Physiology of Animal Swimming. Cambridge University Press, Cambridge, UK, pp. 151–161.

Webb, E.C., Stewart, A., Miller, B., Tarlton, J., 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 Sci. Technol. Archaeol. Res. 2, 54–66.

Zangrando, A.F., Panarello, H., Piana, E.L., 2014. Zooarchaeological and stable isotopic assessments on pinniped-human relations in the Beagle Channel (Tierra del Fuego, southern South America). Int. J. Osteoarchaeol. 24, 231–244.