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Identification of an optimal sampling position for stable isotopic analysis of bone collagen of extinct moa (Aves: Emeidae)

RICHARD N. HOLDAWAY*
Palaecol Research Ltd, P.O. Box 16-569, Christchurch 8042, New Zealand
School of Biological Sciences, University of Canterbury, Christchurch 8140, New Zealand

DAVID J. HAWKE
School of Applied Sciences and Allied Health, Christchurch Polytechnic Institute of Technology, P.O. Box 540, Christchurch 8140, New Zealand

MICHAEL BUNCE
Ancient DNA Laboratory, School of Biological Sciences and Biotechnology, Murdoch University, 6150 Perth, WA, Australia

MORTEN E. ALLENTOFT**
School of Biological Sciences, University of Canterbury, Christchurch 8140, New Zealand

Abstract Stable isotopic ($\delta^{13}C$; $\delta^{15}N$) analysis of bone collagen and other refractory biological materials is a mainstay of palaeoecological research, but comparability between individuals depends on homogeneity within the sample specimens. Long bones of extinct New Zealand moa display lines of arrested growth that reflect prolonged development over several years, leading to potential systematic inhomogeneity in stable isotopic enrichment within the bone. We tested whether the isotopic content within a *Euryapteryx curtus* tibiotarsus is homogeneous by measuring $\delta^{15}N$ and $\delta^{13}C$ values in 6 adjacent 1cm-diameter cortical bone cores arranged along the bone axis from each of the proximal and distal ends. We then measured isotopic ratios in 5 radial slices of a core from the mid-shaft of a *Pachyornis elephantopus* tibiotarsus to see if there was any depth (ontogenetic) effect at a single sampling point. The $\delta^{13}C$ value increased with distance from the proximal bone end, but neither $\delta^{13}C$ nor $\delta^{15}N$ values in samples from the distal end of the bone were correlated with position. Within mid-shaft cortical bone, the $\delta^{13}C$ value decreased with depth but $\delta^{15}N$ values were constant. Sampling the entire depth of cortical bone from the caudal surface at the distal end of the tibiotarsus, if feasible, therefore provides a spatially homogenous material, free of maturation effects on stable isotopic composition. If for any reason that position cannot be sampled, the outer (radial) layer at the mid-shaft can be substituted.

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Keywords bone collagen; carbon-13; *Euryapteryx curtus*; moa; nitrogen-15; *Pachyornis elephantopus*; stable isotopes

INTRODUCTION

Moa (Aves: Dinornithiformes) comprise 3 families of large flightless birds endemic to New Zealand that were driven to extinction by human hunting and anthropogenic habitat change early in the 15th century C.E. (Holdaway & Jacomb 2000). The various moa taxa (Bunce *et al.* 2009) occupied most habitats in pre-human New Zealand, from dry forest-shrubland mosaics to rain forest, and subalpine shrubland and fellfield (Worthy & Holdaway 2002). Preserved gut contents suggest that moa were browsers, consuming mostly fruit,

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*Correspondence: *turnagra@clear.net.nz*
**Present address: Ancient DNA Laboratory, School of Biological Sciences & Biotechnology, Murdoch University, 6150 Perth, WA, Australia

leaves, and twigs of forest trees, shrubs, and lianes (Worthy & Holdaway 2002; Lee *et al*. 2010).

The study of habitat, food web structure, and environment of extinct animals is underpinned by stable isotope analyses, especially the use of $\delta^{13}C$ and $\delta^{15}N$ measurements of preserved organic materials (Koch 2007). Bone collagen is favoured to provide an isotopic signal time-averaged over much of the animal's life (e.g., Ambrose & DeNiro 1986; Bocherens *et al*. 1997; Drucker *et al*. 2008). The approach works well for mammals and almost all birds, and most other rapidly-growing vertebrates, because their bone is continuously remodelled during ontogeny and as adults. Tissues such as teeth and feathers are not subject to remodelling and provide time-related information such as diet seasonality (e.g., Kohn *et al*. 1998) and diet during feather growth (e.g., Hawke & Holdaway 2009). The presence of metabolically active material laid down at different stages means that the sampling position and protocol must be chosen carefully to ensure comparability among individuals (Newsome *et al.* 2007).

Similar problems of age-related differences between different parts of the same organ can also be present in bones of moa, which were slow-growing, long-lived birds. Moa leg bones display cortical growth marks (lines of arrested growth, LAGs) and grow incrementally at the ends as in theropod dinosaurs (Horner & Padian 2004; Padian *et al*. 2004; Turvey *et al*. 2005), and not from sub-terminal plates as in mammals. The bone material is therefore laid down over several years, in moa in the family Emeidae at least 10 years, with protracted morphological reshaping (Turvey & Holdaway 2005; Turvey *et al*. 2005), and the bird's habitat (and hence the stable isotopic composition of its food materials) and physiology could change over that time. If different moa individuals are not sampled consistently from the same region of the same bone, measurement of stable isotopic ratios from bone collagen laid down at different periods in the animal's life could result, and hence not be comparable.

We tested the hypothesis that the stable isotopic composition ($\delta^{13}C$, $\delta^{15}N$) of moa leg bones varies systematically with position along, and with depth within, the bone. We sought a location within a bone which would yield repeatable isotopic measurements between individuals. We chose a major leg bone because these 3 bones (femur, tibiotarsus, tarsometatarsus) are the most commonly preserved parts of the moa skeleton and hence provide the largest sample sizes in a collection. In addition, the bone material of these elements, and particularly of their shafts, is usually dense and retains the best-preserved biomolecules. Of the 3, the tarsometatarsus, being formed by the fusion of longitudinal and lateral fusion of tarsals and metatarsals, is most prone to damage and has thin walls. The femur is far more robust, but is comparatively short and its complex structure means that sampling is difficult without damaging morphological structures of phylogenetic or ecomorphological importance. The tibiotarsus is both long and robust, and the important morphological features are more localised. We therefore sampled tibiotarsi of 2 individual adult emeid moas (one each of *Pachyornis elephantopus* and *Euryapteryx curtus*). The remains of these species are present in many eastern South I deposits (e.g., Worthy & Holdaway 1996, 2002; Allentoft *et al.* 2010) making ecological studies feasible, and their tibiotarsi are large enough (>600 mm long; 50-60 mm wide) to be sampled from positions at each end and at the mid-shaft.

MATERIALS AND METHODS

We sampled from 2 tibiotarsi of adult moa retrieved from pond-excavation debris at Bell Hill Vineyard (Waikari, North Canterbury, South I; see map in Allentoft *et al.* 2010) in 2001. They were identified and sexed genetically, using strict ancient DNA protocols, one being from a female *Pachyornis elephantopus* and the other from a female *Euryapteryx curtus* (Allentoft *et al*. 2010). Sampling was conducted as in Allentoft *et al*. (2009) and PCR amplification of mitochondrial Control Region DNA was performed in a dedicated ancient DNA laboratory at Murdoch University (Perth, Australia) using moa-specific primers (262F/441R) as described in Bunce *et al*. (2003). The 242 basepair PCR-products were sequenced in both directions and queried against the GenBank database for accurate species identification.

To test whether $\delta^{13}C$ and $\delta^{15}N$ values varied along the tibiotarsus, adjacent cores of cortical bone were removed in a zig-zag pattern along the proximal and distal caudal surfaces of the *E. curtus* bone (Fig. 1). Six cores were removed from each end using a hollow, diamond-coated bit (Diamond Drill Bit & Tool Co., Omaha, Nebraska, USA; model DT-516; OD, 12.9 mm; ID, 10.9 mm) in a hand-held electric drill, run at a medium speed. Once extracted from the plug bit, each core was divided longitudinally into quarter segments, using a chisel, so each of the 4 resulting prisms of bone included the full depth range of the core. Opposite quarters were submitted for duplicate collagen extraction and isotopic analysis.

The *P. elephantopus* tibiotarsus was used to investigate whether isotopic enrichment varied with depth within the cortical bone of the shaft. Five depth slices, each approximately equivalent to 2 layers delineated by LAGs, were cut using a

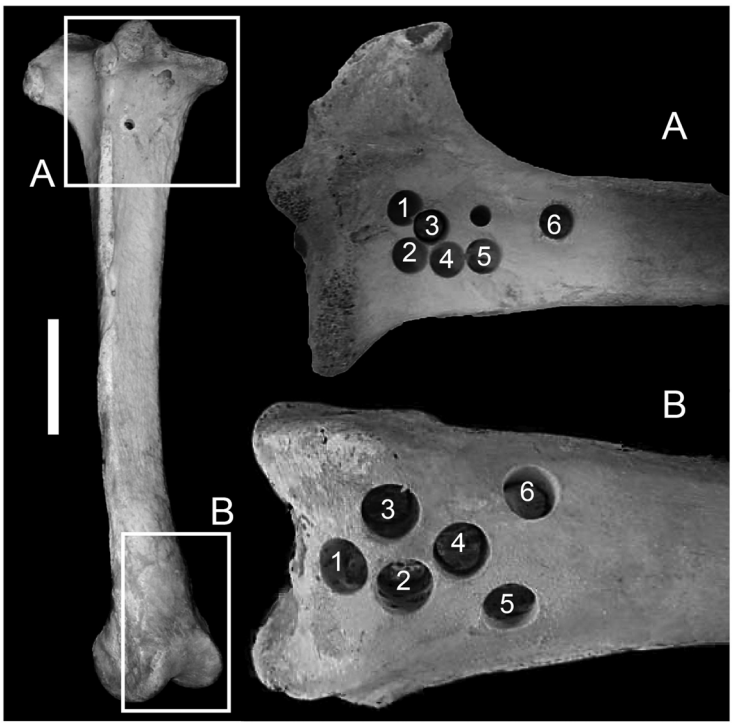

Fig. 1. Left: *Euryapteryx curtus* tibiotarsus, caudal view, showing positions of sampling areas (scale bar: 100 mm). Right: A, proximal end samples; B, distal end samples. Core position numbers correspond to sequences shown in Fig. 2.

Dremel® abrasive disk cutter from a block of bone from near the middle of the shaft. The depth slices were identified and separated under a dissection microscope.

Collagen was extracted using the procedure described in Holdaway & Beavan (1999), with variations as noted. Briefly, cleaned, crushed bone was demineralised with 0.5M HCl at 4°C then rinsed to neutral pH in MilliQ® water. The crude collagen extract was gelatinised at 80°C in 0.001M HCl for 24-48 h. After filtering, the gelatinised extract was ultra-filtered (Amicon® 30kDa) and freeze-dried.

Isotopic results are reported in δ notation, with units of per mil (‰):

$$\delta^{H}X = [(R_{SAMPLE} / R_{STANDARD} - 1)] \times 1000$$

where X is C or N, H is the heavy isotope of the element (^{13}C or ^{15}N), R_{SAMPLE} is the ratio of the heavy isotope to the light isotope in the sample, and $R_{STANDARD}$ is the corresponding ratio for the reference standard. For $\delta^{13}C$, the reference standard is the Vienna Pee Dee Belemnite; for $\delta^{15}N$, the reference standard is atmospheric nitrogen. Analysis was carried out using a continuous flow Europa Geo 20/20 isotope ratio mass spectrometer by IsoTrace New Zealand Ltd (Dunedin, New Zealand). IAEA standard EDTA ($\delta^{13}C$, -38.3‰; $\delta^{15}N$, -0.9‰) was used as the laboratory working standard. Analytical precision, based on repeated laboratory standard analysis, was ±0.0-0.1‰ for $\delta^{13}C$ and ±0.2-0.3‰ for $\delta^{15}N$ depending on analytical run.

Differences between samples at different positions at the distal and proximal ends of the bone were tested for each isotope using Spearman's Rank correlation and Mann-Whitney *U* tests. Homogeneity within the distal end of the bone was tested by 1-way ANOVA of sample duplicates. The relationship between magnitudes of $\delta^{13}C$ and $\delta^{15}N$ and depth from bone surface (decreasing ontogenetic age) was assessed using linear correlation against number of LAGs from the bone surface.

The 2 tibiotarsi are held by RNH; they will be returned to the landowners on completion of the research programme.

RESULTS

Isotopic measurements, carbon and nitrogen content (w/w), and C:N molar ratios (as preservation quality indicators), along with laboratory standards, are given in Table 1. Values of $\delta^{13}C$ in the *E. curtus* tibiotarsus increased significantly with increasing distance from the proximal end of the bone (*c.* 0.8‰; $P = 0.017$, Spearman $r = 0.94$; Fig. 2), but did not vary significantly with distance from the distal end ($P = 0.92$, Spearman $r = 0.091$). For $\delta^{15}N$, there was no significant correlation with

Table 1. Stable isotopic ($\delta^{13}C$; $\delta^{15}N$) values and carbon and nitrogen composition (% m/m) of bone gelatin samples from *Euryapteryx curtus* (proximal, distal) and *Pachyornis elephantopus* (radial) tibiotarsi. Distal and proximal samples were analysed as opposite quarters of each sample plug; each quarter was analysed in duplicate. The overall means ("Mean") shown were used in statistical analyses and presented in Fig. 2. Analysis of control material given at bottom of table.

Sample no.	$\delta^{13}C_{VPDB}$ (‰)	Mean	Carbon (%)	$\delta^{15}N_{AIR}$ (‰)	Mean	Nitrogen (%)	Molar C:N
Distal							
DA1	-24.63	-24.5	42.90	9.53	9.7	15.03	3.3
	-24.41		40.60	9.92		14.11	3.4
DB1	-24.52	-24.7	43.29	9.32	9.4	15.02	3.4
	-24.93		43.00	9.39		14.92	3.4
DC1	-24.83	-24.8	44.43	9.35	9.4	14.74	3.5
	-24.78		42.87	9.41		14.86	3.4
DD1	-24.65	-24.7	44.30	9.62	9.6	15.05	3.4
	-24.76		43.10	9.66		14.70	3.4
DE1	-24.69	-24.7	42.63	9.87	9.85	14.88	3.3
	-24.76		42.60	9.83		14.65	3.4
DF1	-24.48	-24.6	42.27	9.46	9.5	15.18	3.3
	-24.67		43.58	9.55		14.92	3.4
Proximal							
PA1	-23.75	-24.0	40.87	9.59	9.4	15.15	3.1
	-24.17		39.88	9.16		14.63	3.2
PB1	-24.41	-24.1	42.26	9.40	9.4	15.04	3.3
	-23.8		38.91	9.48		14.33	3.2
PC1	-24.55	-24.4	42.89	9.14	9.2	14.83	3.4
	-24.26		41.06	9.20		15.18	3.2
PD1	-24.12	-24.3	41.05	9.33	9.4	15.01	3.2
	-24.39		42.35	9.47		14.81	3.3
PE1	-24.4	-24.5	39.45	9.35	9.4	13.97	3.3
	-24.58		42.76	9.39		15.17	3.3
PF1	-24.6	-24.8	41.87	9.37	9.5	15.07	3.2
	-24.91		41.45	9.69		14.88	3.3
Radial							
1AA UF	-23.4		40.3	9.7		14.6	3.2
2AA UF	-24.5		41.4	8.5		14.6	3.3
3AA UF	-24.1		40.8	8.6		14.9	3.2
4AA UF	-25.4		43.2	8.9		15.2	3.3
5AA UF	-25.3		41.0	8.6		14.5	3.3

Control materials	Accepted value (‰)	Measured value (‰)	Standard Deviation	*n*
Proximal/distal samples - measured 18 May 2007				
Carbon (EDTA)	-38.3	-38.3	0.10	3
Nitrogen (EDTA)	-0.9	-0.8	0.04	3
Radial samples - measured 24 Aug 2007				
Carbon (EDTA)	-38.3	-38.3	0.04	3
Nitrogen (EDTA)	-0.9	-0.9	0.11	3

increasing distance from either end of the bone (proximal, $P = 0.30$, Spearman $r = -0.51$; distal, $P = 0.66$, Spearman $r = -0.26$; Fig. 2). The difference between the mean $\delta^{15}N$ value at the proximal end and that from the distal end was nearly significant at the 5% level ($P = 0.074$, Mann Whitney $U = 6.50$). The duplicate $\delta^{13}C$ values were homogeneous within the distal end of the bone, (1-way ANOVA, $P = 0.40$), but the $\delta^{15}N$ duplicates varied more and the differences between $\delta^{15}N$ duplicates were near significance ($P = 0.061$).

The $\delta^{15}N$ value was not correlated with depth within the bone in the *P. elephantopus* tibiotarsus (Fig. 3) ($r^2 = 0.33$, $P = 0.31$, $F = 1.50$, $d.f. = 4$), but $\delta^{13}C$ values declined significantly with depth (decreasing ontogenetic age) ($r^2 = 0.79$, $P = 0.045$, $F = 10.99$, $d.f. = 4$).

DISCUSSION

The $\delta^{13}C$ of collagen samples from the proximal end of the bone differed systematically with distance from the extremity, so that sampling from the proximal portion of the bone does not provide material suitable for comparison between individuals. Samples from different individuals drawn from randomly distributed places near the proximal end of the bone will yield a higher sample variance and will be biased toward material laid down early in life.

In contrast, carbon and nitrogen isotopic ratios from the caudal distal surface did not vary systematically with distance from the bone extremity. A homogeneity test using duplicates showed that this part of the bone gave consistent $\delta^{13}C$ values, but less satisfactory consistency in $\delta^{15}N$. Because both random and systematic errors will be minimised, sampling from this region of the tibiotarsus should maximise the ability to detect subtle differences between sympatric taxa and the respective sexes and ages. This is likely to be of considerable importance for studies of moa ecology, given that multiple taxa were often sympatric (Holdaway & Worthy 1997) and were highly sexually dimorphic (Bunce *et al.* 2003).

Our study demonstrated that the positions of samples of moa bones taken for isotopic analysis must be standardised. Some previous studies have examined the isotopic ecology of moa, albeit with small sample sizes (Worthy & Holdaway 2002). Bone samples for these studies were typically drawn from the proximal caudal surface of the tibiotarsus so that the sampling hole would be less conspicuous mid-shaft. Consequently, the results can be regarded as only indicative. Although it is probably valid to infer from these earlier studies that moa lived in tall vegetation, detailed comparisons between taxa or between sexes will be compromised.

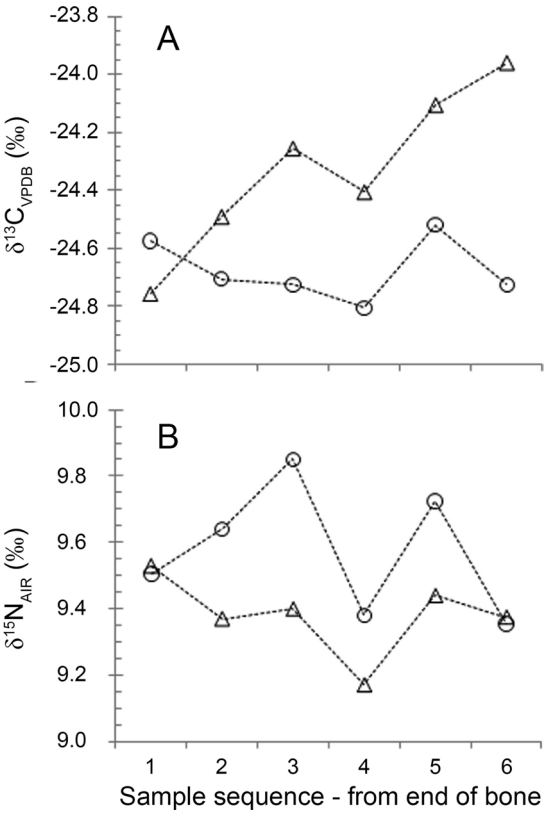

Fig. 2. Bone collagen $\delta^{13}C$ (A) and $\delta^{15}N$ (B) values for distal (○) and proximal (Δ) ends of the *Euryapteryx curtus* tibiotarsus. Note the steady increase in $\delta^{13}C$ with distance from the proximal end. Sample positions shown in Fig. 1.

The progressive increase in $\delta^{13}C$ with distance from the proximal extremity of the tibiotarsus, the decrease with increasing depth (corresponding to earlier stages of the maturation process), and apparent constancy at the distal end are all consistent with the similarity of growth patterns between moa and their near relatives, the theropod dinosaurs (Horner & Padian 2004; Padian *et al*. 2004; Turvey & Holdaway 2005; Turvey *et al*. 2005). These patterns will be explored in future research, but for the purposes of the present study can be accounted for as follows. The distal tibiotarsus is part of the ankle joint (colloquially, "knee" in birds) and its articular surfaces would be ossified relatively rapidly during development, while the individual was in a relatively constant isotopic environment; continually growing bone could cope with the axial loading at the proximal end.

If the individual changed its isotopic environment as it grew and matured, the developing bone would have recorded the transition. For example,

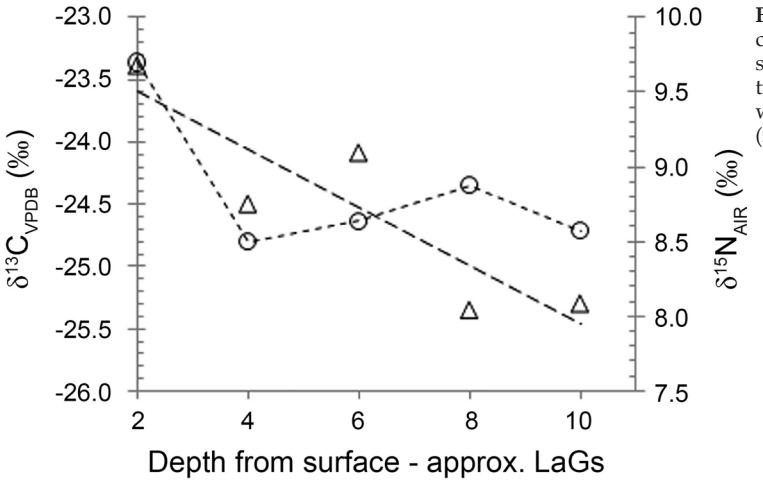

Fig. 3. Depth dependence of bone collagen $\delta^{13}C$ and $\delta^{15}N$ for mid-shaft sample of the *Pachyornis elephantopus* tibiotarsus. The regression with depth was statistically significant for $\delta^{13}C$ (Δ) (regression line shown) but not $\delta^{15}N$ (○).

an increase in $\delta^{13}C$ could reflect movement from closed canopy forest to more open edge habitats. However, the data indicate that $\delta^{15}N$ remained constant throughout this developmental process.

Conclusions

Choice of samples of fossil material for stable isotopic analysis depends on many factors, including quality of post-mortem preservation, a need to maximise sample size from collections that include damaged specimens, ease of sampling, and curatorial considerations, such as minimising damage to archive material and preservation of morphological features. Samples from different parts of the same individual may or may not yield comparable results, and the possible presence and magnitude of such differences are not well known (Todd *et al*. 2010). To minimise spatial and temporal variation when sampling collagen of slow-growing, long-lived birds for palaeoecological studies, the proximal end of long bones should be avoided because the collagen there will be subject to growth-related inhomogeneity in $\delta^{13}C$. Ideally samples should be extracted from the entire depth of cortical bone at the distal end of the moa tibiotarsus. However, other considerations, such as damage to or loss of the ends of bones in an assemblage that would significantly reduce the sample size, or the need to limit sampling damage to morphologically non-informative sections of the bone, can force a compromise position to be chosen.

Sampling from the mid-shaft allows all identifiable bones, including those with damaged or missing distal ends to be sampled, and avoids damaging or destroying important morphological features. Although this region exhibits ^{13}C depletion with depth, the effect can be minimised by using only a slice of solid surface bone from the core, and this position has the advantage of ensuring that a significant mass of cortical bone will be available for ^{14}C dating and ancient DNA analyses.

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