Mesolithic on the Move

Papers presented at the Sixth International Conference on the Mesolithic in Europe, Stockholm 2000

Edited by Lars Larsson

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Oxbow Books 2003

73. Mesolithic-Neolithic population relationships in Portugal: the evidence from ancient mitochondrial DNA

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Analyses of Mesolithic and Neolithic samples from Portugal have led to the formulation of an hypothesis that the population increased during and after a protracted period of shift in subsistence, during which there were biological responses to changing lifestyles. We report here on bioarchaeological analyses that support the hypothesis of change within a population which was not subject to marked gene flow, and provide preliminary information on the analysis of aDNA from Mesolithic and Neolithic skeletal samples.

Bioarchaeological background

The attempt to clarify relationships among Iberian populations using osteological data has a long history, particularly because of interest in Basque origins.

The initial attempts were based on craniometry M size and shape of skulls. While the method may be questioned, particularly with regard to environmental effects on skull size, it is not obsolete. For example, de la Rúa published "The craniofacial factors of the Basque skull, a comparative study" in 1992, and Lalueza Fox (1996; Lalueza Fox *et al.* 1996) has also recently published on Iberian craniometry. Our analyses have shown that, craniometrically, the Basques are northern Iberians, and that the Neolithic and Mesolithic Portuguese samples group together. We have, in fact, noted the near identity of the Neolithic Melides sample, which is craniometrically small, with the Portuguese Mesolithic (Jackes *et al.* 1997b).

Figure 73.1 suggests that size would indeed be a critical factor in discriminating among the geographical areas and chronological periods of Iberia based on these craniometric variables, and this was demonstrated using discriminant function analysis with the grouping variable indicated in figure 73.1. Although the craniometric variables are very well distributed in their correlations over the multiple extracted functions, generally interpreted as indicating that shape rather than size determines the clustering, size must remain a concern.

We have experimented with methods of reducing the effect of size on the discrimination, thus putting more weight on skull shape, using both Howells (1973) C scores and division by geometric means (DGM: see Jungers *et al.* 1995). The two methods of reducing the effect of size give similar results. However, there is a reduced spread over the major axis when using C scores, so that S.E. Spain groups with N. Spain. Because of this reduced discrimination, and following the lead of Collard and Wood (2000), we illustrate the DGM method of size-effect reduction in Figure 73.2.

Figure 73.2 shows the major functions resulting from a direct discriminant function analysis on the pooled sex means of a variety of adult skull samples (Figure 73.3)

	Mesolithic Portugal	**Neolithic Portugal**	**Basques**	**S. Spain**	**N. Spain**	**SE. Spain**
M1(GOL)	180.3	180.1	183.4	181.8	185.1	180.1
M5(BNL)	94.7	96.0	98.1	98.0	99.6	97.2
M8(XCB)	133.9	136.3	141.1	136.4	138.2	134.4
M9(WFB)	92.6	94.4	96.0	94.3	96.1	94.5
M45(ZYB)	122.7	121.4	125.7	124.6	126.9	120.8
M48(NPH)	69.5	65.1	69.9	68.3	68.9	65.3
M52(OBH)	29.2	31.3	33.8	32.1	32.9	31.3
M54(NLB)	23.9	23.4	22.7	23.9	23.7	22.5

Figure 73.1 Mean values (in mm.) of cranial measurements (Martin and Saller 1957–66; Howells 1973) by grouping variable.

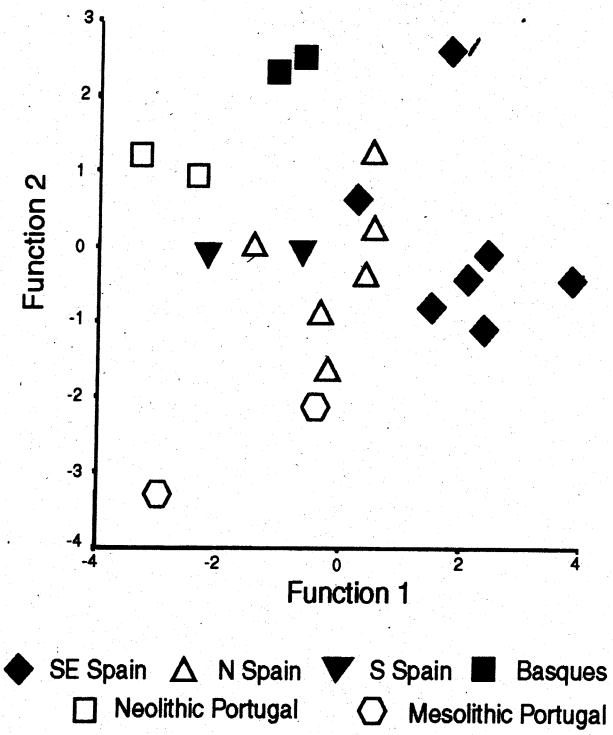

Figure 73.2 Direct discriminant function analysis of cranial measurements (size-effect adjusted): the grouping variable is as shown in the legend. The X axis illustrates the first canonical discriminant function which expresses 49.7% of the variation; the Y axis represents the second canonical discriminant function and 25.5% of the variation. Moita lies at the lower left.

from Mesolithic to modern times. As is common with archaeological data, the samples are varied, but we have used cranial measurements for which there are no missing data, and we have used only basic measurements that are standardised over two systems of measurement (Martin and Saller 1957–66; Howells 1973), and generally taken without error.

Figure 73.2 shows a clear differentiation between the two Portuguese Mesolithic sites, with Moita do Sebastião as an outlier, though separated from the Portuguese Neolithic sites only on the Y axis (with which orbital height is significantly correlated). The separation of Moita from Arruda is an unexpected result of size-effect reduction (compare with Jackes *et al.* 1997b, Fig. 1, male skulls only), but confirmation of a consistent finding in our research into other areas of Portuguese Mesolithic skeletal biology (Jackes and Lubell 1999a, 1999b).

Cranial size is at least partly determined by environmental factors: the separation of Moita and Arruda suggests that shape is also controlled by non-genetic factors, to some extent at least. On the other hand, we may assume that dental morphology, which is more genetically controlled, provides a more reliable method for determining population relationships.

In Jackes *et al.* (2001) Portuguese Mesolithic teeth, dated to around 7450 cal BP, are compared with teeth from several sites dated to the period 4500–5500 cal BP, and with a twentieth century Portuguese dental sample. [Calibrated dates are calculated at 1-sigma ranges. For the Mesolithic dates, we use $\Delta R = 250 \pm 35$ as suggested by Stuiver and Braziunas (1993:155), but see also Monge Soares (1993)]. The characteristics of premolar and first and second molar crowns are stable, and provide a method of examining genetic affinity, when outlier data provide scale, allowing for a more adequate interpretation of results. In Jackes *et al.* (2001) Canadian Iroquoian material provides the required outlier data, suitable because it is from an area in which unusually detailed archaeological work has been undertaken (e.g. Finlayson 1998). The conclusion, preliminary and based on a small number of traits, is that there is no evidence of any discontinuity in Portuguese dental morphology. The various Portuguese samples cluster closely, relative to the relationships between dental samples from the two Iroquoian sites.

Figure 73.4 shows the results of an analysis of the frequencies of 22 dental traits, representing aspects of the crowns of first, second and third mandibular and maxillary molars and distal mandibular premolars. The pooled left and right side incidences of the 22 traits are reduced to a matrix of Z values generated by an Anscombe transformation mean measure of distance (MMD) (the methods are outlined in Jackes *et al.* 1997a). Multidimensional scaling allows us to visualise the matrix, here in a three-dimensional form; the earlier (Mesolithic) samples are somewhat differentiated from the other later samples on the major (X) axis, but are themselves not identical.

In fact, analysis indicates that there is a statistically significant difference between the two Mesolithic sites, Moita do Sebastião and Cabeço da Arruda, despite their geographical contiguity and overlap in time. Two of the later samples, Paimogo I and São Paulo, are also significantly different from each other. All but one (Abrigo da Carrasca) of the post-Mesolithic Portuguese sites are significantly different from Arruda, but only Paimogo I is significantly different from Moita.

The dental trait analysis suggests that there is genetic heterogeneity within the Mesolithic of Portugal, which would be expected on the basis of small exogamous groups in which drift may occur. The dental trait analysis further suggests continuity across the Mesolithic/Neolithic boundary, with increased heterogeneity expected as a result of increasing population and greater sedentism. From the patterning of the significance of the MMD (mean measure of distance) statistics, Moita, especially, cannot be seen as representing a different population from that living in the same general area 2000 to 3000 years later. Analyses of more extensive data collected by Ana María Silva (pers. comm. 17/08/00) for the Late Neolithic and Chalcolithic (5500–4500 cal BP) sites indicate that even among these there are some differences which are marginally significant, and that finding is supported here.

In summary, then, we see little evidence for population

Samples	Source	Grouping variable
Moita do Sebastião	Meiklejohn	Mesolithic Portugal
Cabeço da Arruda	Meiklejohn	Mesolithic Portugal
Melides	Meiklejohn	Neolithic Portugal
Casa da Moura	Jackes	Neolithic Portugal
Guipuzcoan Basques (Aranzadi study)	Morant 1929	Basques
Basques (19th century)	de la Rúa 1992	Basques
Mediaeval Granada Muslims	Fox *et al.* 1996	S. Spain
Bronze Age Granada (mainly)	Fox *et al.* 1996	S. Spain
Visigothic North Meseta	Fox *et al.* 1996	N. Spain
Mediaeval Christian Cantabria	Fox *et al.* 1996	N. Spain
Bronze Age Central Catalonia	Fox *et al.* 1996	N. Spain
Mediaeval Barcelona Jews	Fox *et al.* 1996	N. Spain
Illot des Poros (Talayotic Majorca)	Fox *et al.* 1996	N. Spain
Mediaeval Christian Central Catalonia	Fox *et al.* 1996	N. Spain
La Bastida (Totana, Murcia)	Walker 1985	S.E. Spain
Cova del Palanqués (Novarrés, Valencia)	Walker 1985	S.E. Spain
Cueva de las Lechuzas (Villena, Alicante)	Walker 1985	S.E. Spain
Cova de les Llometes (Alcoy, Alicante)	Walker 1985	S.E. Spain
Cova del Morro de la Barsella (Torremanzanas, Alicante)	Walker 1985	S.E. Spain
Cova de la Pastora (Alcoy, Alicante)	Walker 1985	S.E. Spain
Cova de Beni Sid (Vall d'Ebro, Alicante)	Walker 1985	S.E. Spain

Figure 73.3 Samples used for craniometric analysis.

discontinuity between the Mesolithic and the Neolithic in western Iberia. In fact, we have also argued for a certain degree of continuity in broader terms of biological change M that is, in terms of trends through time rather than abrupt alterations with some sort of "revolution" in diet and lifestyle.

While our published stable isotope data appear to provide a clear differentiation in terms of diet, we draw attention to the heterogeneity of the Mesolithic data despite the minimal sample representation.

Figure 73.5, illustrates samples analysed by Schwarcz for our project (Lubell *et al.* 1994), for Cunha (H. Schwarcz, pers. comm. 20/04/99), and for the Los Canes Mesolithic material (P. Arias Cabal pers. comm. 23/12/99; Arias Cabal and Garralda 1996). It indicates that evidence for clear differentiation of Mesolithic and Neolithic may well be eroded as more data appear.

Our analyses over the last 16 years started from an hypothesis of a change in health with the introduction of domesticates, but with no prejudgement on the question of whether immigration would complicate our research on the Portuguese Mesolithic-Neolithic transition. We have found little evidence for a decline in health (Jackes *et al.* 1997a), and what there is contradicts previous ideas (Lubell *et al.* 1994). Our attempts to understand the transition based on studies of skeletal and dental morphology are limited by the geographical concentration of the Mesolithic material, and by taphonomic factors resulting from Neolithic ossuary burial, but we see a repeated pattern of gradual change beginning in the Mesolithic, with no sign of immigrant genes. While dental morphology may well provide some basis for clarifying genetic affinities, dental traits are not inherited in a simple fashion, and may not provide definitive answers. Nevertheless, there is now interest in the inclusion of anthropological data within an increasingly sophisticated archaeological approach to questions of migration and culture change (Sutton 1996; Burmeister 2000; Shennan 2000).

While proliferating studies of modern European Y chromosome and mtDNA can suggest the shape of the past (Richards *et al.* 2000; Rosser *et al.* 2000; Semino *et al.* 2000) there is no adequate control of the time-scale, and thus it is not possible to rely on the accuracy of reconstructions of past population affiliations based on the present-day gene frequencies.

In the light of all this, it is obvious that the analysis of aDNA is essential to the clarification of relationships among Iberian archaeological samples.

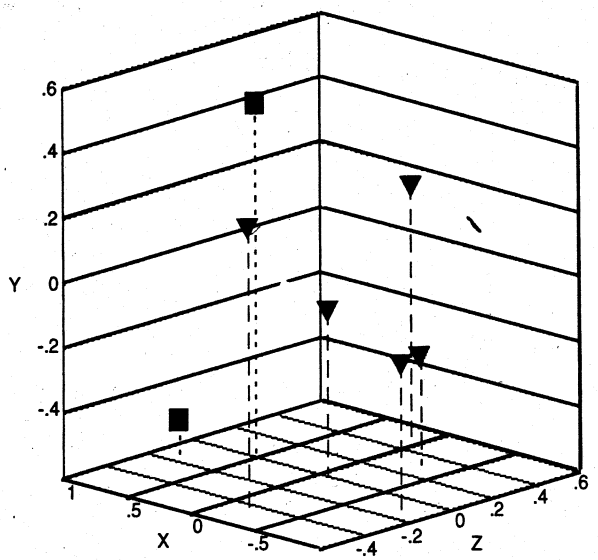

Figure 73.4 Multidimensional scaling of dental trait incidences: Mesolithic (squares) are somewhat differentiated from the Late Neolithic/Chalcolithic (triangles) samples on the first dimension (X). Note that the Late Neolithic/ Chalcolithic samples are broadly spread along the X axis, which accounts for 47% of the displayed variation. The second dimension (Y) expresses 28% of the variation, and separates Moita (above) from Arruda (below). The third dimension (Z) covers 25% of the variation, which occurs among the Late Neolithic/Chalcolithic samples.

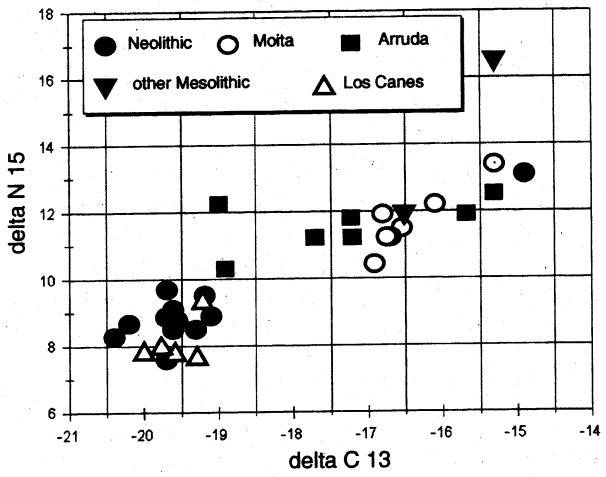

Figure 73.5 Scatter of stable isotope values for Portuguese Mesolithic and Neolithic individuals, and for five individuals from the Spanish site of Los Canes. In general the Neolithic diet is based on terrestrial components (bottom left), while Portuguese Mesolithic people have a more marine-based diet. Two individuals from Arruda are tending towards a more terrestrial diet, while the Spanish Mesolithic individuals have a diet indistinguishable on stable isotopes from the Portuguese Neolithic.

DNA analysis

The rapid development of new technologies for analysing DNA has led to increasing interest in DNA as a tool for examining population origins. There are two approaches – examination of DNA from large numbers of modern individuals from diverse populations with statistical analysis to predict population origins and secondly, examination of DNA extracted from ancient remains. In this paper we attempt to use DNA analysis to examine the degree of genetic continuity between the Mesolithic and Neolithic populations in Portugal.

Cells contain both nuclear and mitochondrial DNA (mtDNA) and both are valuable for examining prehistoric populations. With the exception of most of the Y-chromosome, nuclear DNA undergoes recombination, and nuclear encoded genes may be subject to positive or negative selection in a population because the proteins they code for may confer an advantage or disadvantage for survival. DNA which does not undergo recombination and which does not code for proteins is therefore of particular interest in this respect. Regions of the mitochondrial genome and Y-chromosome DNA have these characteristics and both may undergo mutations resulting in sequence changes that accumulate along the paternal lineage (Y-chromosome) and maternal lineage (mitochondrial DNA) (Lell and Wallace 2000). As these sequence changes accumulate they give rise to population specific sequence variations or haplotypes. Sequence variants sharing a common ancestor are designated as haplogroups.

Despite the common feature of non-recombination, there are important differences between mtDNA and Y-chromosomal DNA. In addition to the difference in parent of transmission, mtDNA has a higher mutation rate than nuclear DNA and permits fine distinction between populations. The Y-chromosome has a lower mutation rate and there are fewer informative sequence changes. However, the presence of both slow and fast evolving markers (de Knijff 2000) has facilitated the designation of ancestral haplogroups and haplotypes within Europe (Rosser *et al.* 2000; Semino *et al.* 2000) based on the examination of Y-chromosome material from a large number of modern Europeans.

Mitochondria are unique organelles because they have their own DNA (about 16,000 bases) coding for a few of the mitochondrial proteins and the machinery required to make those proteins. The entire mtDNA genome has been sequenced (Anderson *et al.* 1981). Each mitochondrion has 2–6 copies of its DNA, so there are many copies of mtDNA per cell whereas there is only one copy of the nuclear DNA. This provides an advantage when working with prehistoric samples because the mtDNA yield per sample is much higher than nuclear DNA. European mtDNAs fall into a number of broad haplogroups determined by sequence changes specific to a population.. Many of these sequence changes occur in the control region of mtDNA. Each haplogroup can be further subdivided into haplotypes. Based on examination of

mtDNA from large numbers of modern Europeans, the major European haplogroups have been designated H, I, J, K, T, U3, U4, U5, V, W and X (Torroni *et al.* 1994; Richards *et al.* 1996, 2000; Simoni *et al.* 2000).

In this study, our approach was to examine Mesolithic and Neolithic remains from Portugal to determine whether there was evidence of genetic continuity between the two populations.

Haplogroups can be analysed by restriction fragment length polymorphisms (RFLPs), a technique where the sequence change causes either a loss or gain of recognition site for an enzyme that will cut DNA at a specified sequence of bases. Some workers have taken an alternative approach and sequenced the control region, a sequence of about a thousand bases where many of the population specific sequences occur. This analysis broadly correlates with the RFLP analysis.

There are several challenges in ancient DNA analysis. The material may be poorly preserved, but there is an approximate correlation between collagen content and success of DNA extraction. The yield of DNA is poor, and it is highly degraded. There may be mutations in the DNA caused by contact with chemicals, heat and ultra-violet irradiation that have accumulated with time. A major problem is that of contamination by extraneous DNA (Handt *et al.* 1996) which may occur at any time from initial excavation to laboratory analysis. Obsessive attention to laboratory technique is required to both avoid contamination, and to detect it should it occur.

Hypothesis

Our hypothesis was that evidence of continuity of the Neolithic and Mesolithic in Portugal would be demonstrated if the same distribution of mtDNA haplogroups were present in the Mesolithic and Neolithic samples examined. The appearance of new haplogroups or a difference in distribution of haplogroups in the Neolithic samples might be evidence migration from outside.

Samples available for analysis were from Neolithic and Mesolithic sites in central Portugal. The Mesolithic sites are Moita do Sebastião, Cabeço da Arruda and Cabeço da Amoreira, three shell middens located very close to each other near Muge on a small tributary of the Tagus. The Neolithic sites represent a larger geographic area and include the caves of Casa da Moura, Feteira, Furninha, Fontainhas (all located in the Estremadura northwest of Lisbon), and Melides (to the south of the Tagus near Sines). In total, we analysed 13 Neolithic samples (10 teeth, 3 bone) and 15 Mesolithic samples (all bone), ranging in date from 7200–8000 cal BP (see Lubell *et al.* 1994). We were also able to analyse a sample of Late Pleistocene wolf bone from Casa da Moura, which proved to be a valuable control.

Methods

The remains were visually well preserved. Great care was taken to avoid contamination by extraneous DNA at all stages of the analysis. All samples were extracted and analysed in duplicate. The second extraction was done on a separate occasion from the first. The sample was cleaned and the outer layer was scraped away. It was then soaked in bleach to destroy any remaining surface DNA (contaminating DNA is most likely on the surface), then immersed in liquid nitrogen, which allows the bone or tooth to be easily reduced to powder using a pestle and mortar. DNA was extracted from 500 mg aliquots of bone powder using guanidine thiocyanate to release DNA which was then adsorbed to a silica resin, then purified and eluted (Boom *et al.* 1990).

We opted to look at all Caucasian haplogroups so that the haplogroup of each sample could be identified. A limiting factor to this approach may be the amount of DNA available from each sample. Overall, H, I, J, K are the most common Caucasian haplogroups encompassing more than 60% of the population. The remainder are more recently described and tend to be the more minor. Based on analyses of modern Portuguese populations (Corte-Real *et al.* 1996; Simoni *et al.* 2000; Torroni *et al.* 1998), we expected the predominant haplogroups to be H, K, T U4 and V. We used the polymerase chain reaction to amplify about 100 bp of mtDNA which is about the largest fragment size that can be analysed for ancient DNA. DNA haplogroups were assigned after restriction enzyme digestion and electrophoresis. Only one key sequence which identifies each haplogroup was analysed. All analyses were validated by including a negative control for the amplification reaction, and a negative control for the extraction procedure, in this case, wolf bone. These controls are a check for DNA contamination during these procedures.

Results and conclusions

Haplogroup I was excluded from all the Neolithic samples and six of seven Mesolithic samples. One sample failed to amplify. None of 21 samples, both Neolithic and Mesolithic, belonged to haplogroup U6. For the haplogroup V, four samples failed to amplify, and none of the remainder belonged to this haplogroup. Thirteen samples have been analysed to date for the haplogroup K. Eleven (seven Neolithic and four Mesolithic) were not haplogroup K. To our surprise, we found two individuals who, using the K specific primers, have a larger than expected amplified product. Although we have not excluded an artifact, the result is reproducible for one sample. The second sample has not yet been re-analysed. The fragment is not cleaved by restriction enzymes used to detect the haplogroup K. We plan to sequence this DNA fragment to gain more information. One sample is from Arruda (Mesolithic) and the other from the Feteira (Neolithic),

and the only sample we have so far analysed from this site.

This is interesting because these sites are separated by about 2,500 years, and represent different palaeoeconomic patterns and distinct burial practices. Feteira is a Neolithic ossuary cave in karstic limestone containing disarticulated human skeletal remains (Zilhão 1984). Arruda is an open-air Mesolithic shell midden occupation site on the terrace of a tributary to the Tagus estuary, and the human remains there are predominantly individual, and sometimes multiple, burials of complete bodies (Roche 1967, 1989). Thus, in view of the current discussion about the ways in which the Neolithic was introduced to western Iberia, this preliminary evidence is most intriguing because it suggests biological continuity along the female line (at least) between Mesolithic and Neolithic populations (for contrasting interpretations of what this might imply for the archaeological record (see Jackes *et al.* 1997a, 1997b; Zilhão 1998).

The absence of haplogroup V is notable as it is prevalent in Southwest Europe, particularly in the Basque population. However, a study of 121 Neolithic and Bronze Age Basque samples by Izagirre and de la Rúa (1999) has also shown absence of haplogroup V individuals. Haplogroup V is a derivative of haplogroup H, and has been calculated from data on modern samples to have arisen in Southwest Europe about 12,500 years ago. Izagirre's conclusions are that the Iberian population might have been heterogeneous and subject to a founder effect, or that the polymorphism arose at a later date than calculated. The absence of haplogroup I and U6 in our samples is not surprising. Haplogroup I is predominantly found in North and East Europe (Simoni 2000) and U6 is mainly North African (Macaulay *et al.* 1999).

In conclusion, our results to date demonstrate the feasibility of DNA analysis from Iberian skeletal remains. However, sample size is critical in this kind of analysis, and we would probably need 50 samples from each period to draw valid conclusions about this population. The polymorphism amplified using the haplogroup K primers is of interest, particularly as the samples come from two distinct sites. We will continue our analysis, but results at present suggest genetic continuity between the Neolithic and Mesolithic populations of Portugal.

Acknowledgements

The research reported here was funded by a Social Sciences Research Grant from the University of Alberta (to DL). Continuing research is now funded by a grant from the Social Sciences and Humanities Research Council of Canada (to DL, MJ, FB and others). Our samples come from previous work by DL and MJ in Portugal (funded by SSHRC), from Eugénia Cunha (Departamento de Antropologia, Universidade de Coimbra; new samples from Moita and Arruda), and from João Zilhão (Faculdade de Letras de Lisboa; Feteira samples). We thank Christopher Meiklejohn (University of Winnipeg) for data collected during our joint work in Portugal; Pablo Arias (Departamento de Ciencas Históricas, Universidade de Cantabria) for permission to use the stable isotope data for Los Canes; and Dr. M.M. Ramalho (Serviços Geológicos de Portugal, Lisbon) for permission to work on materials under his care. Sample preparation and processing was done by Ann O'Neill and Kerri Albertson in Bamforth's laboratory. Participation at MESO2000 was made possible by our SSHRC grant (for DL) and the Department of Laboratory Medicine and Pathology, University of Alberta (for FB).

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