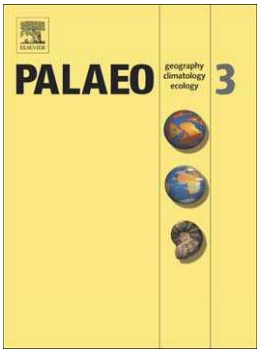

Article:
Rogers, K., Beckett, S., Chamberlain, A. et al. (2 more authors) (2010) Contrasting the crystallinity indicators of heated and diagenetically altered bone mineral. Palaeogeography, Palaeoclimatology, Palaeoecology, 296 (1-2). pp. 125-129. ISSN 0031-0182

https://doi.org/10.1016/j.palaeo.2010.06.021

Palaeogeography, Palaeoclimatology, Palaeoecology, Volume 296, Issues 1-2, 1 October 2010, Pages 125-129



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PII: S0031-0182(10)00370-6
DOI: doi: 10.1016/j.palaeo.2010.06.021
Reference: PALAEO 5457

To appear in: *Palaeogeography*

Received date: 23 November 2009
Revised date: 16 June 2010
Accepted date: 30 June 2010

Please cite this article as: Rogers, Keith, Beckett, Sophie, Kuhn, Samira, Chamberlain, Andrew, Clement, John, Contrasting the crystallinity indicators of heated and diagenetically altered bone mineral, *Palaeogeography* (2010), doi: 10.1016/j.palaeo.2010.06.021

Contrasting the crystallinity indicators of heated and diagenetically altered bone mineral.

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ABSTRACT

Modifications to bone mineral as a result of diagenesis or heating include a marked increase in crystallinity. Although these processes are not completely understood a number of simple, pragmatic approaches are in general use to quantify crystallinity and thus provide a relative metric for features such as preservation state. A preliminary investigation into the interpretation of crystallinity as measured by X-ray diffraction has been undertaken.

The microstructural changes associated with diagenetically altered (archaeological) and heated contemporary bone have been examined. A common analysis approach was adopted and thus direct comparison between the physical features of these material systems has been possible.

The data clearly demonstrate the pronounced anisotropic nature of the crystallite microstructure for both diagenetically altered and contemporary bone. The limitations of adopting simple crystallinity indices for characterising such materials are explored. Crystallite size and strain were shown to be dependent upon crystallographic direction. Overall, the diagenetically altered bone mineral possessed greater long range lattice order than that of contemporary heated bone. Further, significant differences between the directional nature of the microstructure of diagenetically altered and modern heated bone were observed.

This study has enabled a direct comparison of the effects of heating and diagenesis upon bone mineral. It has demonstrated the need to consider bone microstructure anisotropically.

Keywords: bone, crystallinity, X-ray diffraction, diagenesis.

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1. Introduction

The term `crystallinity` is used extensively throughout a number of disciplines to characterise a wide range of materials. It is an attribute that is related to the amount of long range structural order within a material that can be moderated by features such as grain boundaries and point/linear/planar defects. These are of interest as they can significantly modify a materials` physical and chemical properties. For archaeologists crystallinity measurement is regularly employed as an indicator of the degree of diagenetic change or thermal modification of bone mineral. These two processes have implications for assessing preservation state and determining the isotopic composition of bone but a technique that reliably distinguishes them remains elusive (Pijoan et al. 2007).

Crystallinity is frequently quantified by X-ray diffraction and infrared spectroscopy as these methods are sensitive to structural order. However, due to the complex physio-chemical nature of bioapatites a pragmatic approach is often adopted for crystallinity quantification and thus a number of indices that provide some relative measure of average crystallinity have been adopted. Within archaeological science, these indices derive from a number of practices including an infrared splitting factor (IRSF) which is based upon the degenerative splitting of a phosphate band (Surovell and Stiner, 2001; Weiner et al. 1993), a multiple peak IR summing method (Lebon et al. 2008), a simple measure of a single diffraction peak width (Hedges et al. 1995), or multiple diffraction peak widths (Person et al. 1995; Quattropani et al. 1999). The potential advantages of more sophisticated X-ray diffraction whole pattern fitting methods, although capable of providing direction dependent data (Stathopoulou et al. 2008; Piga et al. 2008), have not yet been fully realised in this context. Small angle X-ray scattering to characterise crystallite particle size has also been successfully applied to heated bone (Hiller et al. 2003). However the resultant values are difficult to compare directly to the more commonly used wide angle approach due to the confounding effect of microstrain.

The use of crystallinity indicators as reliable proxies for mineral alteration and bone integrity remains somewhat controversial with several studies questioning its reliability (Puceat et al. 2004; Trueman et al.

2008). Regardless of these issues, it is important to appreciate the limitations of material crystallinity determination and ensure that, when used, methods are appropriate for the materials being examined. For example, indices providing crystallinity indicator values averaged through a number of crystallographic directions (e.g. spectroscopy) may not provide an accurate indication of crystallinity changes in materials containing crystallites with a high morphological aspect ratio when this ratio becomes modified. Further, and particularly for diffraction methods, calculation of crystallinity indicators are confounded by crystallite preferred orientation, stoichiometry and inherent differences in instrument dispersion. These factors reflect the inherent anisotropic nature of bone minerals' mechanical properties that is essential for providing optimal weight bearing, e.g. bone mineral is formed in accordance with the principles of maximising resistance to stress.

Studying the problem of reliable crystallinity measurement to map microstructure in the context of diagenetically altered and heated bone which have previously been described as possessing similar crystallinity indicator values is of some value. Indeed both modification mechanisms have been extensively studied and are known to involve mineral recrystallisation. It has also been reported (Stiner et al. 1995) that weathering produces crystallinity changes similar to those associated with heat treatment when characterised by infrared spectroscopy and that boiling can mirror diagenetic effects (Roberts et al. 2002). Crystallite dimensions within diagenetically modified mineral (Bartsiokas and Middleton, 1992; Hiller et al. 2003) have been determined to be of similar value to those of contemporary bone heated to ~600 ^{o}C (Rogers and Daniels, 2002; Holden et al. 1995). Distinguishing between the processes of diagenesis and burning would therefore be valuable in studies of archaeological bone when both processes contribute to the recrystallisation state of the mineral. Thus when examining cremated bone, details of funerary practices may be inferred with more confidence.

This work focuses upon X-ray diffraction characterisation and extends the crystallinity indicator concept to identify separate crystallite size and microstrain contributions to structural disorder. The direction dependence of these features has also been examined. For archaeology, there is a desire to provide a more complete description of the diagenetic and thermal processes affecting bone mineral. Native bone mineral crystallites display significant anisotropy in many of their physical characteristics e.g.

morphology and lattice strain. It is therefore reasonable to suggest that any modifications to the mineral as a result of external factors should also display an anisotropic character which may be obscured using current crystallinity determinations.

2. Source of bone

The study compared bone mineral features from two principal groups, i.e. diagenetically altered and heat modified bone. For each group five species were considered and for each species three individuals were examined. For each principal group, the mineral was analysed in its native state and also following heat treatment.

Archaeological (diagenetically altered) bone samples were obtained from the excavation of an Anglo-Saxon settlement site at Fillingham, Lincolnshire (Chamberlain et al. 2000). The approximate stratigraphic period from which the bones were retrieved ranged from Anglo Saxon to the late 1700's A.D. and all were humeri except for three radii. Contemporary tissues were sourced from the Veterinary Laboratories Agency of DEFRA, local abattoirs and the North London Tissue Bank (human samples) and were all femurs. The archaeological material was identified anthropologically as deriving from the species of human, pig, cow and sheep/goat (undifferentiated). Corresponding contemporary tissues were used except that the sheep and goat specimens were distinguished. .

3. Methods

Samples of whole bone were cut transversely at the mid-shaft (approximately 5 mm in forming a ring, ~5 mm in height). Each ring was cut into equal segments to obtain, flat semi-lunar shaped fragments that possessed endosteum and periosteum surfaces. Our pilot studies indicated, that similar diffraction peak widths to those observed for archaeological material were attained from contemporary tissue heated to ~600 °C. Samples were thus heated in air to 600 °C using a carbolite tube furnace (CTF 16/75), at a rate of 10 °C per minute. This temperature was then maintained for two hours after which, the furnace was

allowed to cool naturally (over a period of ~ 12 hours) to room temperature. Prior to diffraction data collection, all fragments were independently pulversied using an agate pestle and mortar or a ball mill (Retsch MM2000). The powdered samples were then sieved through a 106 μm aperture stainless steel mesh.

X-ray diffraction data was collected using a PANalytical X'Pert PRO powder diffractometer with CuKα radiation. The diffraction range was 10-80°/2θ, with a step size of 0.013°/2θ and an equivalent count time per step of 150 seconds.

4. Data analysis

Diffractogram parameterisation was performed using Topas (v4.1, Bruker-AXS). A whole pattern fitting approach was employed although the unit cell contents were not refined. Usually when employing such an approach, a smooth function is used to describe how peak widths vary with scattering angle (Piga et al. 2008; Stathopoulou at al, 2008). We removed this constraint so that each peak was treated independently. This then enabled the determination of accurate and reliable values of diffraction peak shape parameters. The structural model for apatite was based upon a hexagonal lattice (space group, $P6_3/m$) as this is the most appropriate model for biological apatites that contain no long range order along the OH^- channels. Crystallinity indicators based upon a single peak width (e.g. as used by Koch at al, 1997; Hedges et al. 1995) and the Person method (Person et al. 1995) were derived for each sample. Also for comparison with previous literature, coherence lengths were calculated from the Scherrer equation (Klug and Alexander, 1954) after correcting the peak widths for instrumental broadening.

A silicon diffraction standard (NBS640c) was used to correct for instrumental broadening. Peak shape analysis was performed with some circumspection. Peaks were excluded from subsequent analysis when overlapping, caused by broadening, resulted in equivocal fitting. This was especially the case for data from contemporary unheated tissue in the 2θ region 30-35^o.

Simple crystallinity indicators were calculated for each diffractogram using the full width at half maximum (fwhm) of the apatite 002 peak and, following Person et al (1995), the resolution of the 202, 300, 211, and 112 diffraction maxima. As an alternative to the simple crystallinity indicators and to characterise the apatite`s direction dependent features, we also constructed Williamson-Hall (W-H) plots (Hall and Williamson, 1951; Langford et al. 1993; Rogers and Daniels, 2002) from the corrected peak widths. Each point within a W-H plot represents the broadening of a particular diffraction peak (plotted on the abscissa as fwhm.cos(θ)) and thus represents the structural disorder along the corresponding crystallographic direction. This data was also used to calculate size and strain estimates for particular crystallographic directions. Crystallite sizes were determined from W-H intercepts (size=λ/intercept) and microstrains determined from W-H gradients.

Reproducibility for the determination of each parameter was assessed by examining intra-individual variation. Extra material (human) from archaeological and contemporary groups was prepared, treated and analysed multiple times to determine the experimental repeatability.

5. Results

The reproducibility measurements showed that the difference in repeated parameter determination was <1% and thus the variability observed between groups could not be ascribed to measurement error. Differences between parameter values below are reported to be `significant` if, following a normality test, a students t-test p-value was found to be $p<0.05$.

3.1 Conventional crystallinity indicators

Visual inspection of the diffractograms (e.g. Fig. 1) indicated that both archaeological and heated sample groups possessed peak widths that were demonstrably narrower than those of the contemporary,

native bone and broader than those of a crystalline calcium hydroxyapatite standard (NIST SRM2910). Fig. 1 illustrates this by presenting the diffractograms for heated and unheated samples from one bovine individual and the highly crystalline, apatite standard. Single (abscissa) and multiple peak crystallinity indicators (ordinate) for unheated archaeological and heated contemporary bone are presented for all samples within Fig. 2. The single peak approach was based upon the width of the 002 maxima only and the multiple peak method that of Person et al (1995). As a benchmark, the corresponding indices for the apatite standard were determined to be 0.11 (single peak) and 1.23 (multiple peak). For each sample group there is a clear, but quantitatively different, correlation between the indices.

3.2 Direction dependence approach

Williamson-Hall plots illustrating the typical behaviour of diffraction peak widths as contemporary bone was heated to 600 °C is shown in Fig. 3. Data from all species have been averaged (error bars are standard errors, number of samples for each point = 15). There is a widely scattered distribution of peak widths for unheated bone indicating the marked direction dependence of the mineral crystallites' habit and microstrain. Heating to 600 °C significantly reduces all the peak widths. This behaviour is typical of contemporary bone and may be used to characterise the lattice direction dependence of any such recrystallisation process.

Fig. 4 shows Williamson-Hall plots that compare archaeological bone with heated (600 °C) contemporary material. There is a significant amount of dispersion within the broadening from both groups indicating lattice direction anisotropy in coherence length (long range lattice order). Peak widths from the archaeological unheated tissue are significantly less than the corresponding contemporary bone widths (shown within Fig. 3). This peak narrowing characterises the diagenetic recrystallisation, previously also characterised with the infrared 'splitting factor' (Surovell and Stiner, 2001). Fig. 4 also serves to indicate that the recrystallisation is significantly different for the heated and diagenetically altered mineral. Comparing the populations within Fig. 4, it is apparent that there are differences in the microstructural

lattice dependence of these tissues. Diffraction peaks arising from (hk0) planes are significantly narrower for the heated than the diagenetically altered mineral whereas the 00ℓ peaks are broader for the heated bone.

3.3 Quantification of crystallite size and strain

Differences between diagenetically altered and heated mineral may be quantified by comparing coherence length ratios determined for specific crystallographic directions i.e. <00ℓ> and <hk0>. For the archaeological and heated contemporary tissues these ratios (broadening of <00ℓ>/broadening of <hk0>) are 3.1± 0.3 and 2.0 ±0.2 respectively, indicating a significant difference ($p<0.05$) between the lattice dependent broadening of these groups. To examine the broadening in more detail, contributions to peak broadening from size and microstrain were determined from the 00ℓ peak widths. Table 1 provides these semi-empirical values for each of the principal bone groups averaged for all species. The estimated errors become greater as the crystallite size increases and/or the strain reduces as these situations both correspond to diffraction peaks becoming more similar in width to the instrument function. Not surprisingly the unheated contemporary tissue group has the smallest crystallites possessing the largest microstrain (at least along <00ℓ>). Crystallites of the unheated archaeological group are significantly greater in size than those of the contemporary group and the heat treatment of both groups results in significant increases in crystallite size. Thus within the archaeological material, crystallite size increases associated with diagenetic change are further compounded by heating. However, the processes are unlikely to be simply additive given the anisotropic nature of the changes that occur and the somewhat different recrystallisation mechanisms. A further interesting observation is that, although heating the contemporary tissue results in a significant reduction in strain, heating the archaeological bone produces a significant increase in strain.

3.4 Species dependence

Inter-species differences in microstructure were also examined. Fig. 5 shows Williamson-Hall plots that compare archaeological bone for various species. This illustrates the interspecies magnitude of variation and clearly demonstrates that the mineral of human bone possesses less disorder through all crystallographic directions when compared to other species. In particular, significant differences in hk0 coherence lengths were found between the human samples and those of all other species for both unheated archaeological and heated (600 °C) contemporary bone. Further significant differences could be demonstrated when comparing diffraction peak width pairs (paired by crystallographic plane) between groups of species. For example, for all the archaeological tissues, each species could be discriminated on the basis of peak broadening, with the exception of pig and sheep/goat.

6. Discussion and Conclusions

X-ray diffraction data have been used to contrast the crystallinity of diagenetically altered and heated bone mineral. The two direction averaging methods examined were shown to be correlated but produce equivocal values when comparing bone mineral samples that possess significantly different direction dependencies. For example, Fig. 2 illustrates that diagenetically altered and heated samples with similar Person crystallinity indicator values can simultaneously possess significantly different 002 peak widths. The converse of this is also shown to be the case. This is a direct consequence of microstructural differences between the sample groups that are obscured by the single and multiple peak approaches to crystallinity determination. This ambiguity is likely to occur if crystallinity is determined with spectroscopy as this also provides direction average values. It is therefore conceivable that previous work using crystallinity as a marker for preservation state (e.g. Trueman et al. 2008) may have been confounded by the lack of direction dependence within the analyses e.g. microstructrual changes may be occurring undetected.

Using a method that embraces direction dependent characteristics, it has been shown that the recrystallisation processes associated with heating and diagenesis produce apatite crystallites with

significantly different physical microstructures. In general, the diagenetically modified mineral has, on average, significantly larger coherent scattering domains.

Bone mineral crystallites have been reported previously to have rod and plate like morphologies. In most tissues there is probably a mix of both, for example reflecting local variation in carbonate composition. Coherence lengths derived from diffraction data are a spatial average over a large population of crystallites, in contrast to TEM estimates that are derived from direct observation of relatively few crystallites. The data presented here shows that the diagenetically altered mineral has rod shaped coherent domains with the long dimension significantly greater and the short dimension significantly less than that of contemporary bone heated to 600 °C. This is in agreement with previous work where diffraction was used with some direction dependence to study human remains (Prieto-Castello et al. 2007). The increase in strain observed when the archaeological bone is heated may possibly be due to an increased number of different foreign ions within the surrounding milieu becoming improperly incorporated within the lattice of the recrystallised apatite. These results serve to reinforce the thesis that, at least up to 600 °C for contemporary and archeological material, the crystallite physical properties should not be direction averaged, but reported with lattice dependence. Further, there are significant differences between coherence length and corresponding crystallite size values for all archaeological and contemporary mineral. This is due to the lattice microstrain which is clearly an important contributor to diffraction peak broadening.

Previous work examining a range of modern mammalian bone and archaic human tissue did not reveal any significant difference between species when a single peak approach was employed (Koch et al. 1997). However, by examining groups of paired microstructural data, we have demonstrated significant differences between species that are apparent for both the archaeological and contemporary tissues. In particular the human material is significantly different to all other species. An exception to the species discriminating ability is that peak widths of the archaeological pig group were not significantly different to that of the corresponding sheep/goat group. This may be due to the increased diversity in this parameter arising as a result of combining data from sheep and goat populations. Where goat and sheep could be

treated separately (e.g. contemporary tissues), data associated with pig were significantly different to that from both sheep and goat.

Acknowledgements

We wish to acknowledge the Cranfield Forensic Institute for provision of the analytical equipments used to produce the diffraction data presented here. We are also grateful to the U.K.'s Department of Environment, Food and Rural Affairs for supplying the animal tissues and the North London Tissue Bank for the human tissues.

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Figure & Table Captions

Fig. 1. Unprocessed diffractograms of (a) unheated contemporary human bone, (b) contemporary human bone heated to 600 ^{o}C, (c) archaeological human bone, (d) archaeological human bone heated to 600 ^{o}C (e) a calcium hydroxyapatite, highly crystalline standard (NIST SRM2910). The diffractograms have been offset for clarity.

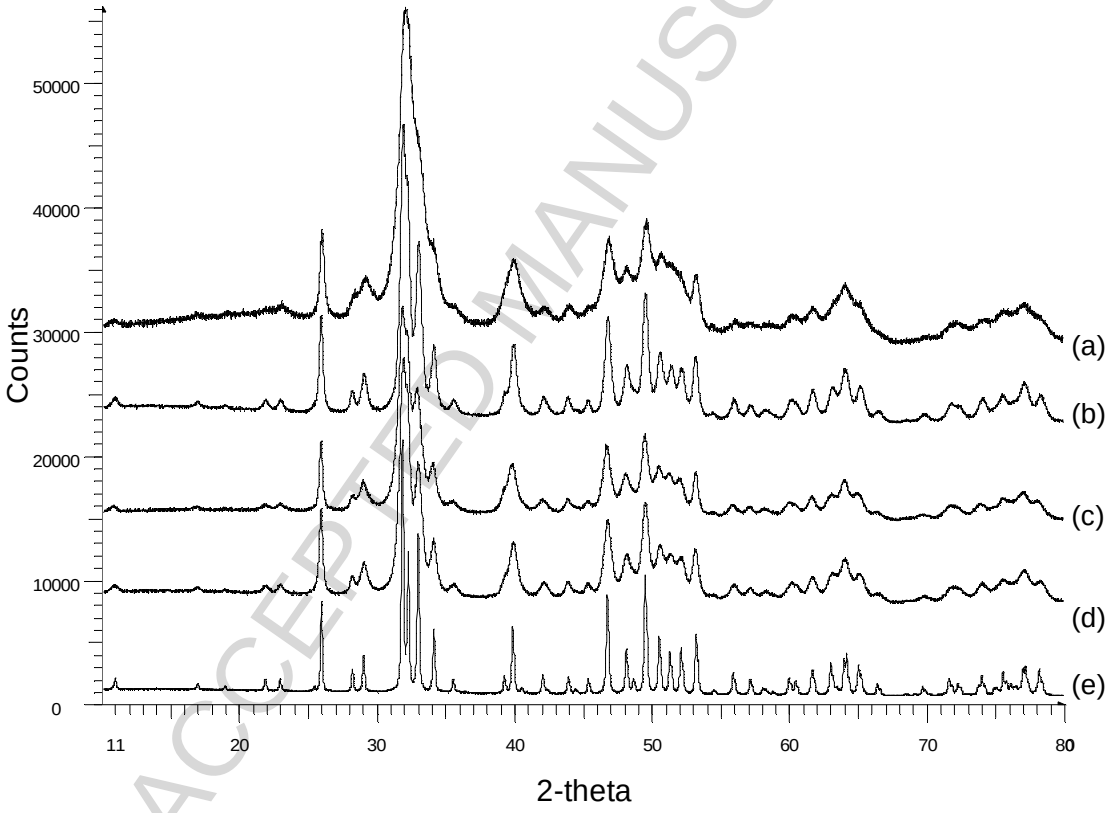

Fig. 2. Crystallinity indicators calculated from Person (1995) and the 002 full width at half maximum for all archaeological bone samples (◊) and contemporary bone heated to 600 ^{o}C (■).

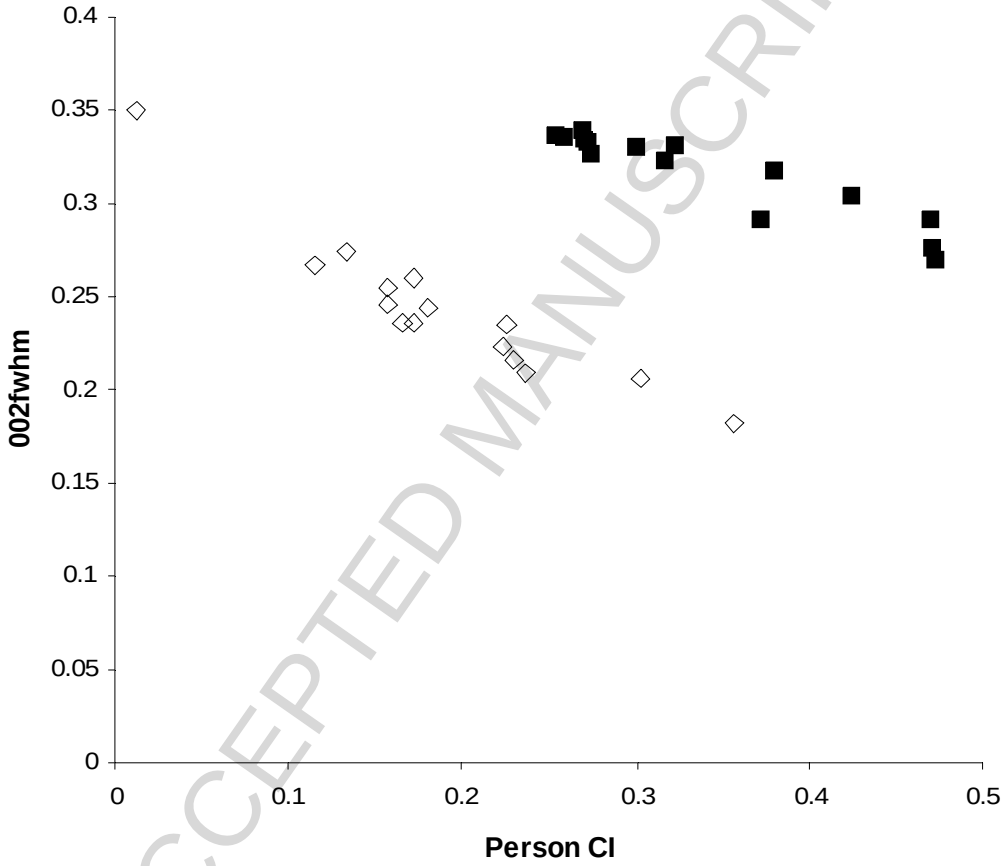

Fig. 3. Williamson–Hall plots indicating instrument corrected diffraction peak widths plotted for each unequivocal peak within the diffraction data. Data presented are contemporary bone (X) unheated, and (■) heated to 600 ^{o}C. Error bars are standard errors, (n = 15).

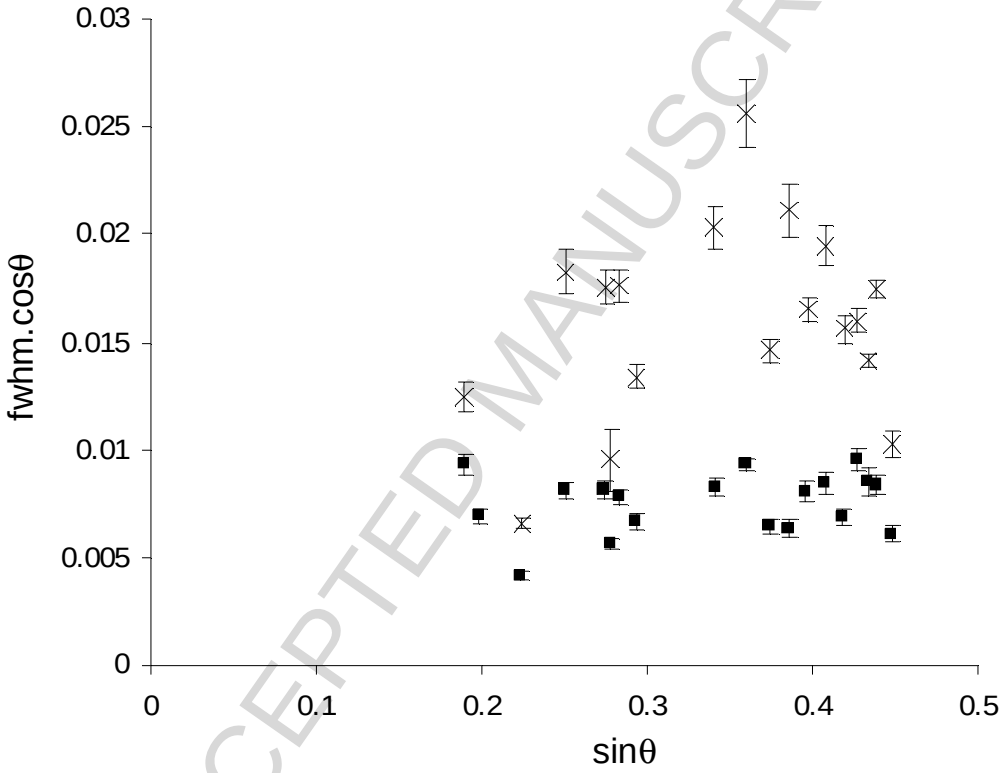

Fig. 4. Williamson–Hall plots indicating instrument corrected diffraction peak widths plotted for each unequivocal peak within the diffraction data. Data presented are unheated archaeological bone (◊), and contemporary bone heated to 600 ^{o}C (■). Further, for the archaeological bone, widths corresponding to hk0 (⟠) and 00ℓ reflections (♦) are indicated. Error bars are standard errors, (n = 15).

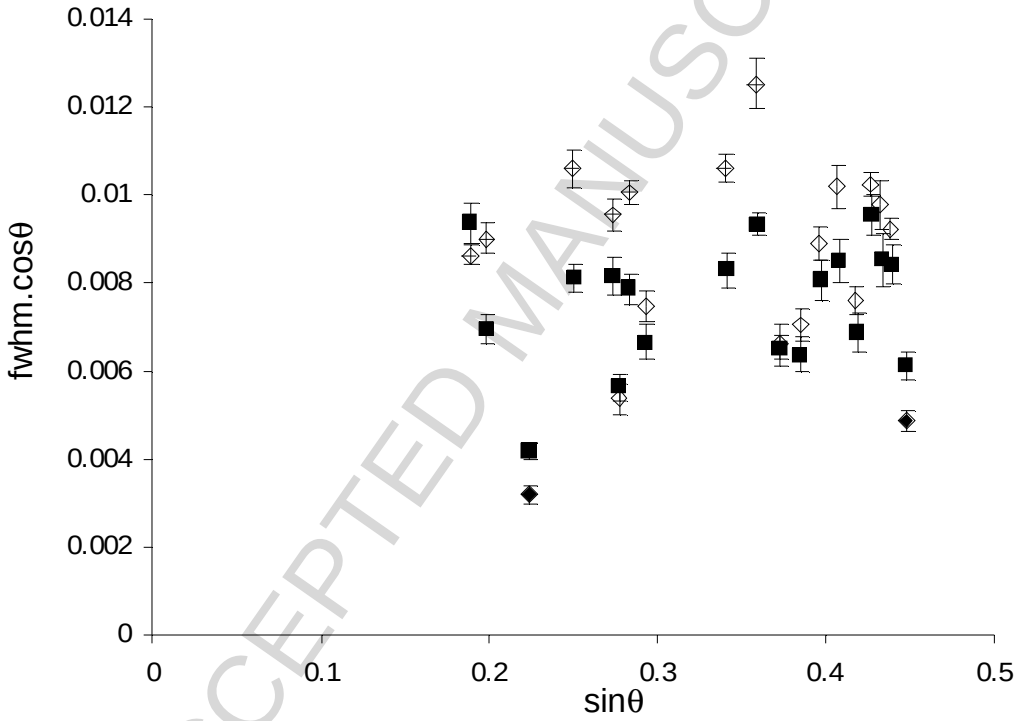

Fig. 5. Williamson–Hall plots indicating the instrument corrected diffraction peak widths plotted for each species with the archaeological series. Data presented are (◊) cow, (■) human, (Δ) pig, and undifferentiated (●) sheep/goat.

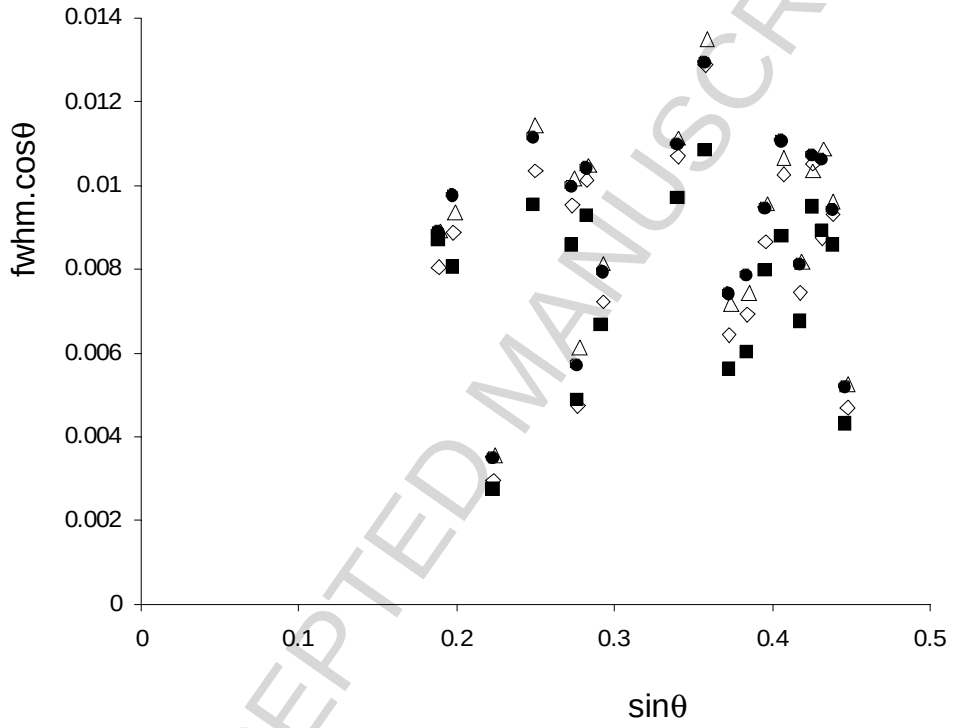

Table 1. Size and strain values measured along the <00ℓ> crystallographic direction for archaeological and contemporary bone before and after heating.

	Size / nm	**strain x 1000**
a-uht	96 ± 12	7.46 ± 0.17
a-600	141 ± 17	11.98 ± 0.20
c-uht	48 ± 3	16.38 ± 1.7
c-600	62 ± 2	8.65 ± 0.65