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DNA in ancient bone – Where is it located and how should we extract it?

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SUMMARY

Despite the widespread use of bones in ancient DNA (aDNA) studies, relatively little concrete information exists in regard to how the DNA in mineralised collagen degrades, or where it survives in the material's architecture. While, at the macrostructural level, physical exclusion of microbes and other external contaminants may be an important feature, and, at the ultrastructural level, the adsorption of DNA to hydroxyapatite and/or binding of DNA to Type I collagen may stabilise the DNA, the relative contribution of each, and what other factors may be relevant, are unclear. There is considerable variation in the quality of DNA retrieved from bones and teeth. This is in part due to various environmental factors such as temperature, proximity to free water or oxygen, pH, salt content, and exposure to radiation, all of which increase the rate of DNA decay. For example, bone specimens from sites at high latitudes usually yield better quality DNA than samples from temperate regions, which in turn yield better results than samples from tropical regions. However, this is not always the case, and rates of success of DNA recovery from apparently similar sites are often strikingly different. The question arises as to whether this may be due to post-collection preservation or just an artefact of the extraction methods used in these different studies? In an attempt to resolve these questions, we examine the efficacy of DNA extraction methods, and the quality and quantity of DNA recovered from both artificially degraded, and genuinely ancient, but well preserved, bones. In doing so we offer hypotheses relevant to the DNA degradation process itself, and to where and how the DNA is actually preserved in ancient bone.

1. Introduction

The long-term survival of mineralised tissues such as bone (and to some degree, teeth) is normally dependent upon rapid burial in sediments, independently of whether terrestrial or marine. Subsequently, their chemical and physical properties undergo substantial change, in a manner determined by the environment. For example, in aerated soils fungi and bacteria colonise the pore spaces of bones and begin the breakdown of mineralised tissues within a few years (Bell et al., 1996; Jans et al., 2004). In contrast, cyanobacteria are principally responsible for initial microbial attack in freshwater and marine environments (Turner-Walker and Jans, 2008; Pesquero et al., 2010), which accelerates bone degradation by increasing its porosity (Nielsen-Marsh and Hedges, 1999). Despite an increasing body of knowledge about the degradation of the bone itself, much less is known about how the DNA in the bone degrades, and indeed, even how or where it is preserved. While some have argued that DNA in the mineralised collagen of bone and teeth hypothetically undergoes a retarded rate of decomposition because of its adsorption to hydroxyapatite (e.g. Collins et al., 1995; Hagelberg et al., 1989; Lindahl, 1993), and others have argued that the mummification of individual cells, and the physical exclusion of microbes and other external contaminants from the smallest pores of skeletal tissues may play a role in DNA survival (Hummel and Herrmann, 1994), we lack a comprehensive picture of the DNA–bone relationship.

The relationship is unlikely to be simple, as significant variation exists in the quality and quantity of DNA that has been recovered from old bone – even among samples collected from environments that appear to be similar. For example, DNA recovery success rate was high in several large-scale studies of permafrost-preserved samples, including bison (*Bison sp.*, success rate 352/442 [Shapiro et al., 2004]) and musk ox (*Ovibos moschatus*, success rate 207/446 [Campos et al., 2010b]). However, similar studies yielded much

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lower success rates, for example 27/122 saiga antelope (*Saiga tatarica* [Campos et al., 2010a]). Given that most of the samples were collected from places with similar environments (permafrozen soils) and were thus presumably exposed to similar diagenetic conditions (Hedges, 2002; Nielsen-Marsh et al., 2007; Smith et al., 2007), the question arises as to what caused the discrepancy? One potential answer could be differences in the types of degradation undergone during the samples' history, both taphonomic, and post-excavation (at least one study has argued that freshly excavated bones are better for ancient DNA analyses due to acceleration of degradation in museum storage resulting from elevated temperatures and greater access to oxygen [Pruvost et al., 2007]).

The degradation of DNA in archaeological bone is not a straightforward topic, as multiple chemical processes may act to both cross-link and fragment the molecule's chemical backbone, and nucleotide bases may be either removed or altered (e.g. Lindahl, 1993; Pääbo, 1989; Hansen et al., 2006). Regardless of the underlying chemical reasons, the end result of most of these processes is the same – the lengths of amplifiable DNA molecules decrease rapidly. Although several factors affect the rate of this decay, including environmental salt content, exposure to radiation, pH, and availability of oxygen and free water, it is temperature that is believed to play the key role in the longevity of aDNA molecules (Lindahl, 1993). In brief, an exponential relationship ensures that degradation rate rapidly increases with temperature (Lindahl and Nyberg, 1972). Thus for any given age, cold preserved samples are more likely to provide usable genetic material than those of a similar age that have been buried (or stored) at warmer temperatures (Smith et al., 2001, 2003).

While DNA degradation is obviously a key factor in determining whether aDNA can be recovered, an alternate explanation for variable success rates may be that the observed results do not reflect on the quality of the DNA *per se*, but where and how it is preserved in the bone, and the efficiency of the different extraction methods used. It is clear that a comprehensive understanding of bone composition and its diagenesis is crucial for determining the location of DNA in ancient bone, and hence for selecting appropriate samples for study and the extraction techniques to apply.

Macroscopically, bone is composed of two main architectures. At the jointed ends of long bones, and in flat sheet-like bones such as the sternum and skull vault, it comprises an outer layer of compact bone that surrounds a load-bearing network of intersecting planes and buttresses called trabeculae. These are termed cortical and trabecular bone respectively (the latter is also called cancellous or spongy bone). The mid-shafts of long bones are principally hollow tubes of cortical bone (Currey, 2002). Microscopically, bone consists of a hard, apparently homogeneous intercellular material, within or upon which can be found a number of characteristic cell types including osteoblasts and the osteoprogenitor cells that give rise to them; i.e. osteocytes, osteoclasts, and bone lining cells (that are essentially inactive osteoblasts) (Fig. 1). These cells cover all available bone surfaces, the exact type of cell depending upon the physiological status of the bone tissue; i.e. resorption, formation/mineralisation or quiescence (Ortner and Turner-Walker, 2003). Osteoblasts are mononucleate immature bone cells responsible for bone formation (Fig. 1). Located on the surface of osteoid seams, they secrete osteoid, a protein mixture that subsequently mineralises with a non-stoichiometric carbonated hydroxyapatite (HAP) to become the rigid, load-bearing solid that is bone mineral. Osteoblasts also produce hormones, such as prostaglandins or alkaline phosphatase, an enzyme that has a role in the mineralisation of bone (Ortner and Turner-Walker, 2003). Osteocytes are star shaped mature bone cells that originate when osteoblasts become trapped within the matrix they produce, occupying spaces in the bone known as lacunae (Fig. 1). Osteoclasts (Fig. 1) are large, multinucleated cells located on bone surfaces in what are called

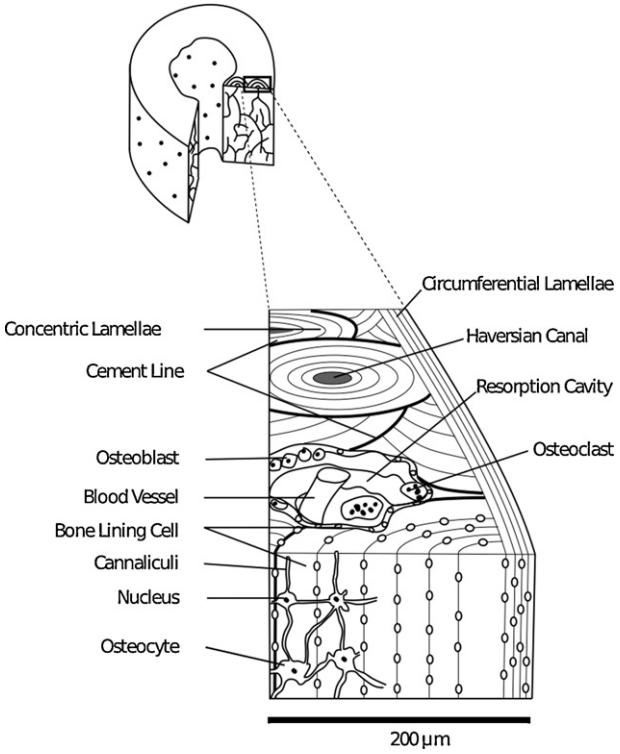

Fig. 1. Histological structure of compact bone. Modified with permission from Gilbert et al. (2005).

Howship's lacunae (or resorption pits), and are responsible for bone resorption – the process of removing bone tissue by dissolving its mineralised matrix and breaking up the osteoid (Nijweide et al., 1986; Ortner and Turner-Walker, 2003). Compact bone is permeated by an interconnected network of pores represented by the Haversian canals and canals of Volkman, which carry blood vessels and nerves, and canaliculi and which contain the cytoplasmic processes that connect adjacent osteocytes (Fig. 1).

Structurally, the majority of bone is composed of bone matrix. This consists of both an inorganic fraction composed of cryptocrystalline carbonated hydroxyapatite (to which DNA may adsorb [Lindahl, 1993]), and an organic fraction composed principally of Type I collagen as well as various non-collagenous proteins and glycoproteins, such as glycosaminoglycans, osteocalcin, osteonectin, ostepontin, bone sialoprotein and cell attachment factor (Tuross, 2003). A simplified view of the relationships between collagen and hydroxyapatite is given in Fig. 2. Tropocollagen molecules (~300 nm in length and ~1.5 nm in diameter) self aggregate extra-cellularly into fibrils with mean diameters of around 50 nm (Tzaphlidou and Berillis, 2005). The fibril is stabilised by post-translational modifications and cross-links between adjacent collagen molecules. These intermolecular bonds are such that there is an offset in the alignment among the collagen molecules so that there are gaps between the end of one molecule and the beginning of the next. The collagen molecules interdigitate in such a way that there are gap zones (where there is high density of gaps) and overlap zones where the molecules are well aligned and more closely packed. The 40 nm gap zone together and the 27 nm overlap zone are responsible for the 67 nm banded appearance of collagen fibrils when seen in TEM images. The initial mineralisation of collagen takes place in the gap zone and progresses along the fibrils, small crystallites developing both within and on the surfaces of fibrils. Full mineralisation is accomplished by the replacement of water between fibrils by mineral and the bulk of the mineral load is deposited here.

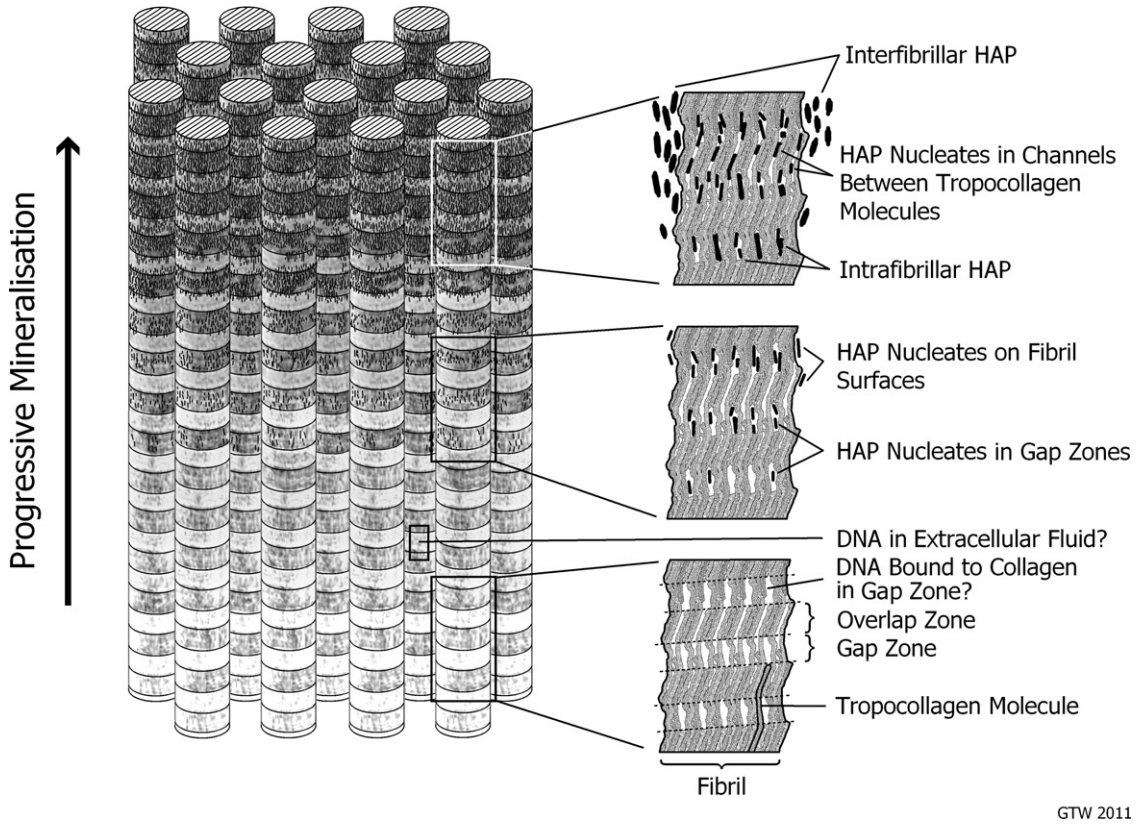

Fig. 2. A simplified view of the relationships between collagen and hydroxyapatite. The osteoid that forms the template for mineralised tissues forms by the extracellular self-aggregation of collagen tropocollagen molecules. These "collagen triplets" align and interdigitate to form rope-like fibrils with "gap zones" resulting from a quarter-staggered arrangement in the way the tropocollagen molecules align. The gap zones and overlap zones give the collagen fibrils a banded appearance that repeats every 67 nm. In the gap zones the fibrils are less closely aligned and more disordered than in the overlap zones. In bone the fibrils pack in a quasi-hexagonal arrangement with the banded zones in register and with diameters that range from 30 to 70 nm (mean diameter ~50 nm). The fibrils are fully hydrated with bound water and are surrounded by extracellular fluid containing non-collagenous proteins, proteoglycans, glycosaminoglycans and possibly cellular remnants – including DNA. Mineralisation is initiated in the gap zones and on the surfaces of fibrils. With progressive mineralisation, HAP platelets fill the gap zone and extend along channels within the fibrils between adjacent tropocollagen molecules. In the final stages of mineralisation, HAP crystals grow and fill the interfibrillar spaces, the mineral growing at the expense of the water content. Consequently the bulk of the mineral lies in the interfibrillar spaces. Dried, fully mature bone tissues comprise approximately 46% collagen, 46% HAP and 8% water by volume (Todoh et al., 2009).

While some evidence has been published that demonstrates that DNA can adsorb to hydroxyapatite and influence crystal growth (e.g. Lindahl, 1993; Okazaki et al., 2001), the nature of the collagen/DNA interactions are more obscure and have been rarely studied. However, theoretical models (Mrevlishvili and Svintradze, 2005) and in vitro experiments (Kitamura et al., 1997) strongly suggest that nuclear DNA not only binds to collagen but can act as a scaffold or matrix in the aggregation of collagen molecules into fibrils (fibrillogenesis). There is little evidence, however, that large strands of DNA are incorporated into mineralised collagen since this would distort the regular structure of the fibrils; something that has not been observed (Orgel et al., 2001). On the other hand, the possibility of short fragments of either nuclear DNA or mtDNA becoming trapped in aggregating or mineralising fibrils cannot be excluded. Furthermore, it is possible that the gap zones, which are more disordered than the overlap regions (Orgel et al., 2005) may also be sites where smaller DNA fragments may become bound to collagen molecules. These may then be encapsulated within HAP crystallites as mineralisation proceeds.

It is quite feasible that, during bone resorption and formation, large amounts of mtDNA are released into the forming osteoid matrix following the apoptosis of osteoclasts or osteoblasts. Fragments of DNA would then be available to bind to the outer surfaces of collagen fibrils in the mineralising osteoid or to the surfaces of developing HAP crystallites. Of course in aDNA studies the picture is made even more complex by the potential release of tissue decomposition products, including DNA and collagen fragments released by chemical and/or microbial degradation of un-mineralised osteoid. The two proposed mechanisms for DNA preservation in bone, i.e. binding to: (a) mineral and (b) collagen have important implications for how DNA is most efficiently extracted, considering that most protocols involve the removal and discard of the mineral phase.

In this paper we synthesise new experimental evidence and previously published data, in order to present the current knowledge on DNA degradation in bone and the efficacy of various extraction methods in retrieving DNA from both artificially degraded and truly old bone. We offer hypotheses as to the DNA degradation process itself, and where and how the DNA is actually preserved long term in archaeological and fossil bones.

2. Materials and methods

We have undertaken two experiments in this study. The first investigates the degradation of mitochondrial DNA (mtDNA) and collagen in serial data sets of modern cow bone that had been left to degrade in different depositional environments: in two North European bogs, at Lejre (Denmark) and Rømyra (Norway). The third set of experimentally buried bones were interred on the sea bed at Marstrand Harbour (Sweden) as part of archaeological

experiments into bone and other cultural artefact diagenesis (Turner-Walker and Peacock, 2008). The second experiment consists of mtDNA and collagen degradation analyses on a dataset of genuinely ancient permafrost-preserved musk ox bone.

We investigated the relative amounts of DNA associated with the organic (principally collagen) and inorganic (principally hydroxyapatite) components of the bone using a DNA extraction protocol as outlined Campos et al. (2009) (protocol 19.5.2.2.). Briefly, bone samples were powdered, then demineralised through incubation in 0.5 M EDTA (pH 8.0) at room temperature (20 °C) for 24 h. The insoluble residue was centrifuged into a "collagen" pellet, and the supernatant was removed. We assume that any DNA present in this supernatant was liberated from the hydroxyapatite, thus to quantify this we purified it from the EDTA using a Qiaquick kit (Qiagen, Valencia, CA). DNA was recovered from the remaining pellet through digestion and purification using a DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). The DNA extractions, and subsequent quantitative real time PCR (qPCR) analyses were performed in a dedicated clean room facility following strict procedures to help limit the effect of contamination, including physical isolation from post-PCR laboratories, nightly UV irradiation of the laboratory and benches, use of full body suits and disposable latex gloves, and use of molecular biology grade reagents and consumables. DNA extractions, and subsequent qPCR analyses were performed on both the EDTA soluble and non-soluble fractions of the bone, that we postulate represent the DNA available in principally the inorganic, and organic, components of the bones, respectively (although see Section 4 for alternate possibilities). For convenience we henceforth refer to the two bone digestion fractions as the hydroxyapatite and collagen fractions.

2.1. qPCR analyses

As detailed below, each sample was extracted in duplicate or triplicate, so that subsequent data presented for each individual sample were based on an average of the two/three independent extractions. Furthermore, DNA was extracted from both the hydroxyapatite and collagen fraction independently, thus there were four to six extractions per sample. Subsequently quantitative PCR (qPCR) was used to generate relative quantitative data.

Each qPCR reaction was performed on a dilution series for each extract of 1×, 0.25× and 0.125×, using a Stratagene MXPro 3000P, under the following cycling parameters: enzyme activation 95 °C for 5 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, using primers as detailed in the relevant sections below. Following each qPCR run, a dissociation curve was implemented between 50° and 90 °C to monitor for amplification of primer–dimer or alternate non-specific amplifications. Each 25 μl reaction contained 1 μl DNA, 1× buffer, 2.5 mM $MgCl_2$, 100 nM each dNTP, 0.1 μl Amplitaq Gold (Roche, Basel, Switzerland), 400 nM of each primer, and 1 μl SYBR Green/Rox mix (Invitrogen, Carlsbad, CA). Following qPCR, the data (*Ct* values and dissociation curve melt temperatures) were assessed visually to ensure that inhibition or spurious amplification did not compromise the results. When compromised results were observed (principally due to inhibition or PCR set-up errors), such results were discarded and the relevant qPCRs were repeated.

The qPCR analyses were undertaken without use of a molecular standard. In contrast all analyses were undertaken using a relative internal control as follows. Firstly one sample in each experiment was chosen to represent the benchmark sample, either due to its virtue of being the undegraded control (sea and bog burial experiments), or the sample with the highest DNA concentration (ancient bone experiments). Once it was verified that the dilutions for the extracts on these samples did not show any sign of being affected by inhibition (demonstrated through appropriate relative PCR amplification curve shift), the particular dilution that yielded the lowest *Ct* value (hence had the highest DNA concentration) was furthermore specified as the standard. In order to calculate the relative content of DNA in the extractions, a standard curve was generated by arbitrarily setting the DNA value in the undiluted standard sample to 1,000,000, and for the 0.25× and 0.125× dilutions to 250,000 and 125,000, respectively. Ultimately the DNA content of all extractions could be expressed as a value, which was directly comparable to the standard extract. Finally, an average value could be calculated for the hydroxyapatite and pellet extractions from each sample, based on the average of the values for each of the replicate extractions. This final value was used to quantify the DNA in the various experiments detailed below.

2.2. Cow bone – seabed

The diaphyses of metapodials from freshly slaughtered cows were cleaned to remove marrow, washed and air-dried. Part of one diaphysis was kept in the museum store as an unburied control. Six sub-samples were then buried 0.5 m below the seabed in Marstrand Harbour (Sweden), over a period of one to three years. Half of the diaphyses were directly exposed to the sediment and half were isolated from the sediment by water-permeable geotextile. The salinity of the water was 25‰, with an annual temperature fluctuation between 5 and 12 °C (Björdal and Nilsson, 2008). The pH of the sediment varied between 7.2 and 7.5. Further details on the samples and the sampling location are described by Bergstrand et al. and Peacock. Small sections were sawn from each bone (including the unburied control) and stored frozen at −20 °C (with an unburied control) until they were processed in triplicate. DNA was analysed from the hydroxyapatite and collagen fractions of 0.01 g subsamples of bone powder drilled from the compact bone, with three replicates per time series per burial environment. The relative DNA content of the extracts was quantified using qPCR for an 82 bp fragment of the cow mtDNA control region, using primers QcowF (5′ GGGTCGCTATCCAATGAATTT 3′) and QcowR (5′ AGAGGAAAGAATGGACCGTTT 3′). Subsequent analyses of this dataset included (i) a comparison of the total DNA content in the sample through time (the sum of the average amount of the hydroxyapatite and collagen fractions per sample) and (ii) the percentage of the DNA in the hydroxyapatite and collagen fraction per sample.

2.3. Cow bone – bogs

A similar experiment to the above was performed on cow metapodials buried in two wetland bogs, environments that are common in cold and temperate climates of the northern hemisphere and from which well-preserved bones are sometimes recovered. These are formed by the accumulation of dead plant material, mainly mosses and lichens, and are a common burial environment for archaeological material in NW Europe. The samples were buried at depths of 1 m for between one and four years, at Lejre (Denmark) and Rømyra (Norway). The pH of the sites and average annual soil temperatures at the burial depth were as follows: Lejre pH 5.6, 8.6 °C, Rømyra pH 5.0, 4.2 °C. For full details on these samples and the physical and chemical characterisation of the two sites see Turner-Walker and Peacock (2008). DNA was subsequently extracted in duplicate from each sample from both the hydroxyapatite and the collagen fractions, and analysed by qPCR, as above. Furthermore, an acid insoluble collagen fraction was prepared from the experimentally degraded bones following Smith et al. (2007). Briefly, sub-samples of the bone were demineralised in excess HCl (0.6 M, 4 °C, 3 days); the supernatant removed and the remaining acid insoluble fraction was rinsed three times with ddH_2O, lyophilised and weighed. The results are reported as weight percent of original bone powder. To prepare a 'purer' collagen fraction, the acid insoluble fractions were gelatinised in weak HCl (pH

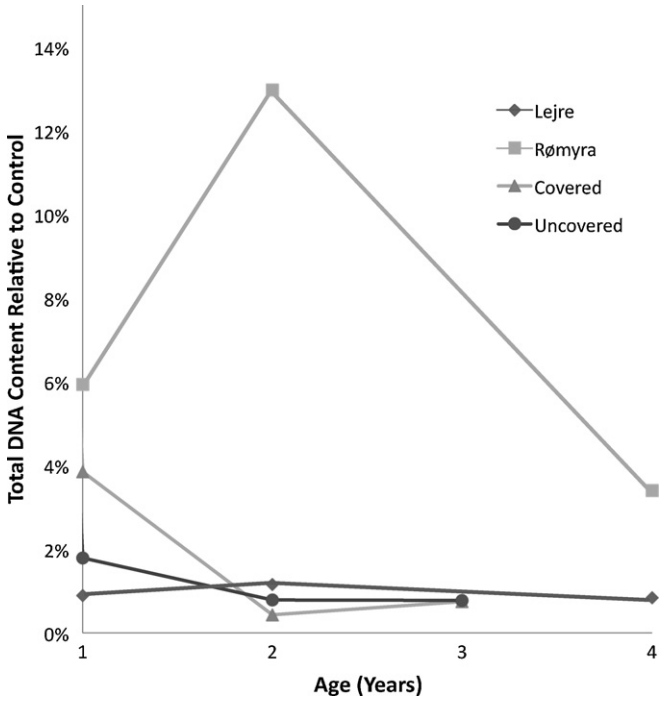

Fig. 3. Relative percentage of PCR amplifiable mtDNA present in the cow bone datasets in comparison to unburied control sample.

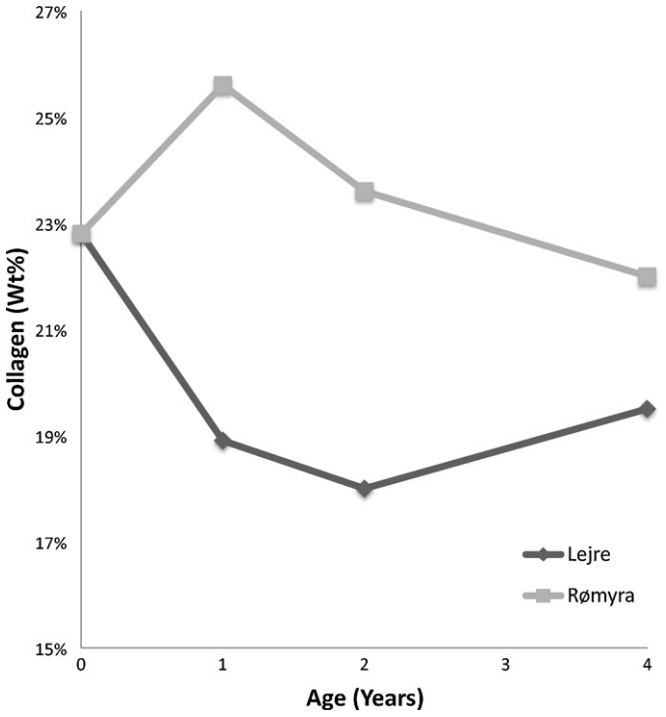

Fig. 4. Percentage collagen content (by weight) of cow bones from the experimental bog-burial sites.

3.0; 80 °C for 24 h, the centrifuged at 2000 × *g*) and the supernatants were lyophilised and weighed. The 'gelatinised' fractions are reported as weight percent of original bone powder.

2.4. DNA and collagen survival in ancient musk ox bone

Nine permafrost preserved musk ox (*O. moschatus*) bone samples (also studied in Campos et al., 2010b) were used in this study. DNA was extracted from triplicate samples of 0.01 g bone, from both the hydroxyapatite and collagen fractions of the decalcified bone, as above. The mtDNA content of the extracts was quantified using qPCR with primers 59F (5′ ATCAGCCATGCTCACACATAACTG) and 149R (5′ GGGCCTTTGACTGGCCATAG) that amplify 90 bp of the mtDNA control region. Final quantities of DNA per sample were calculated relative to the highest value in the total musk ox dataset, and analysed for the relative content in the hydroxyapatite and collagen fractions for each sample as detailed above. The percentage by weight of the acid insoluble collagen fraction was determined for each of the fossil bones, as above, modern bovine bone powder was used as a control. Radiocarbon dates have been published for six of the samples, and range from 14,000 ± 80 to infinite, radiocarbon years before present (^{14}C BP) (Campos et al., 2010b).

3. Results

3.1. mtDNA retrieval in the different burial environments

Results from the burial experiments show a rapid decrease in the amount of total mtDNA recovered in comparison to the unburied control. In both bog and sea burial data sets the total amount of amplifiable mtDNA in the cow bone drops to <10% of the total mtDNA recovered from control bone, by the first year after burial (Fig. 3). After this very rapid decrease, this amount appears to stabilise, at least during the three–four years encompassed by this study. With regards to the samples placed on the seabed, there appears to be little difference between the bones that were exposed to the sediment and those isolated by geotextile (Fig. 3). Examining the organic preservation in the bog-buried bones (Fig. 4) those from Lejre appear to lose collagen over the first two years and then stabilise. In contrast, the collagen in the Rømyra bones is better conserved, and even seems to increase in the one-year sample. This is consistent with a slight surface demineralisation of the bones at Rømyra identified previously (Turner-Walker and Peacock, 2008).

The question of where the mtDNA is preserved in the bones is more complicated. For both data sets, the relative level of the mtDNA in the collagen versus hydroxyapatite fraction rapidly decreases after death (Fig. 5). For the bog data, where this relationship can be compared to total bone collagen content, we observe that in the control (unburied) bones, around 75% of the amplifiable mtDNA is obtained from the collagen, whereas about 25% comes from the hydroxyapatite extract (Figs. 5 and 6). After only one year of burial the relative proportion of the collagen and hydroxyapatite extracts has been reversed (Figs. 5 and 6). Whether the total amount of mtDNA recovered (and also the amount in the hydroxyapatite and collagen fractions, which depends on the total mtDNA content) is linearly, exponentially or otherwise correlated with the total amount of collagen remaining in the bone is unclear. While our data suggests a possible linear relationship, more data would be required, in particular from bones containing lower levels of collagen, to resolve this question (Fig. 6, inset).

3.2. mtDNA content in ancient musk ox bones

Do the results for real ancient bones bear any similarities to the experimental burials? The majority of ancient samples exhibited good collagen survival, with several specimens having lost only a couple of percent of their original collagen. Although the total relative mtDNA content of the ancient samples showed no correlation with sample age (Table 1), as with the buried bones there is a correlation between bone collagen and mtDNA content (Fig. 7). Assuming that the sample with the highest mtDNA content is an outlier where some different mechanism has influenced preservation, if this is excluded from the dataset then the remaining

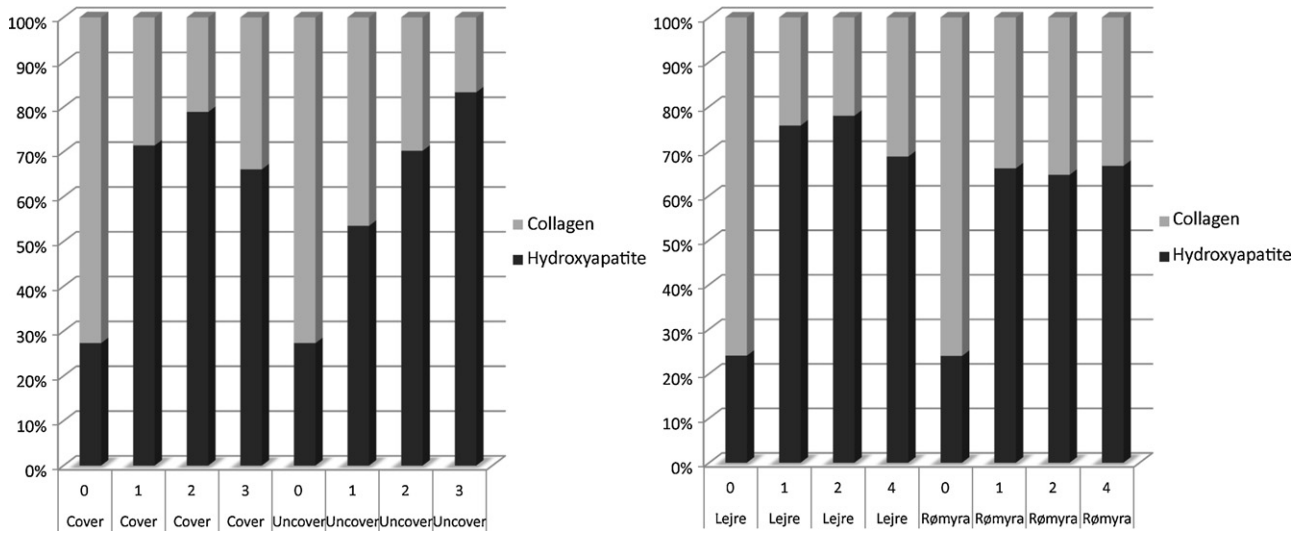

Fig. 5. Relative percentage of DNA in the hydroxyapatite versus collagen fractions of the cow bone datasets. Fig. 4A: bone samples buried at sea in Sweden (Marstrand), left either uncovered or covered. Fig. 4B: bone samples from Danish (Lejre) and Norwegian (Rømyra) bogs. Age of sample (years) is indicated along *X* axis.

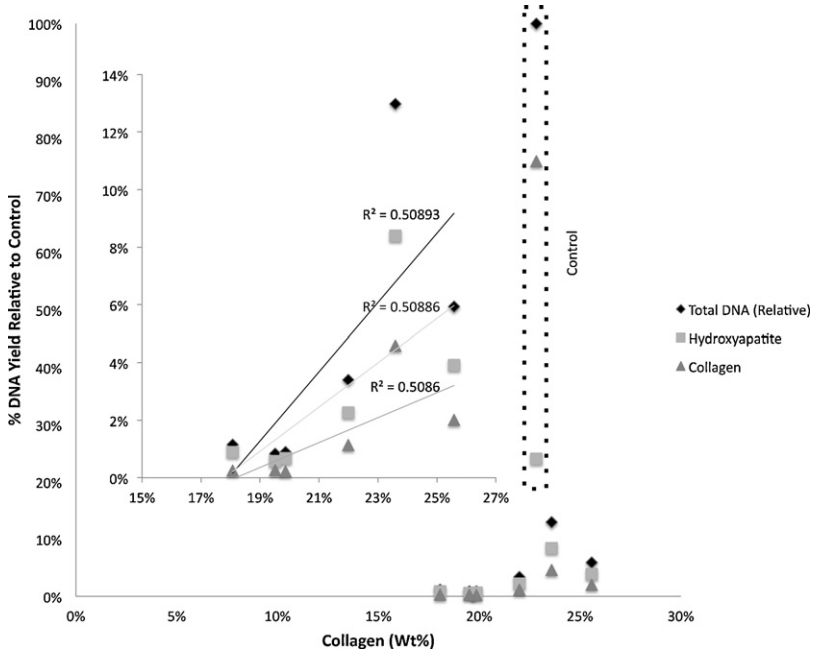

Fig. 6. Total DNA yield of the bog burial samples, and those in the hydroxyapatite and collagen fractions, relative to unburied control, compared to bone collagen content (by weight). Inset: Same with control sample removed.

Table 1
Collagen content and radiocarbon dates, where available, of the samples used in this study. The results are reported as weight percent of original bone powder.

Musk Ox	Relative DNA[a]	Ratio[b]	Collagen	^{14}C years BP[c]	^{14}C Lab no[d]
993	92%	506%	8.7%	Infinite	AAR11727
927	4%	428%	17.5%	N/A	
995	4%	300%	13.8%	N/A	
955	10%	32%	20.8%	24,150 ± 210	AAR12059
950	34%	7%	20.7%	14,730 ± 90	AAR11746
971	100%	−15%	19.8%	14,000 ± 80	AAR11754
924	2%	−32%	14.1%	20,350 ± 160	AAR12055
962	3%	−114%	15.2%	N/A	
954	5%	−182%	17.2%	10,755 ± 65	AAR12058

[a] Total mtDNA relative to sample with highest level (971).
[b] Ratio of DNA in the collagen versus hydroxyapatite fraction. Positive value indicative in more DNA in the collagen fraction, negative indicative of more DNA in the hydroxyapatite fraction.
[c] N/A = no data available.
[d] From Campos et al. (2010b).

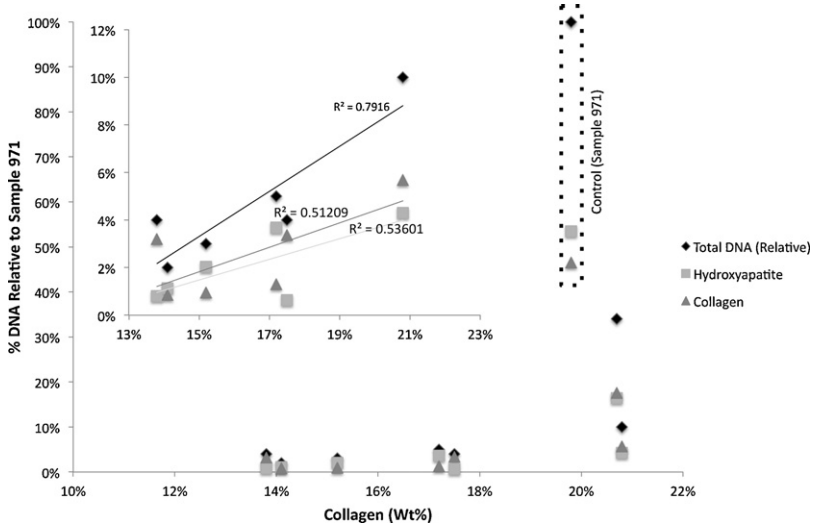

Fig. 7. Total DNA yield of the ancient musk ox samples, and those in the hydroxyapatite and collagen fractions, relative to the sample containing highest level of DNA (Sample 971), compared to bone collagen content (by weight). Inset: Same with highest DNA containing samples removed.

specimens appear to show a linear relationship between collagen survival and mtDNA preservation (Fig. 7, inset) – although as discussed above, more data from less well-preserved bones is needed to verify this relationship. We furthermore observe that although the relative mtDNA content of the hydroxyapatite versus collagen fractions of each sample varies, there are similar proportions of mtDNA in the hydroxyapatite and collagen fractions (Fig. 7, inset).

4. Discussion

Under the (possibly controversial–see Schwarz et al., 2009) assumption that our mtDNA assay reflects approximately the total DNA content of a bone, and that the DNA extracted from the hydroxyapatite and collagen extractions accurately represent DNA preservation in those two components of the bone, our data allows us to develop a number of hypotheses based on both the degradation of DNA in bone and how it is preserved. With regard to the validity of the latter point, we caution that the relationship may not be as straightforward as we postulate. Although currently there is no data to test the hypothesis, it is conceivable that during bone drilling some of the DNA that was originally present in the collagen matrix may be solubilised/disrupted and thus be released in EDTA digestion. If so, this would lead to an overestimate in the DNA component in the mineral phase of the bone. A further potential unknown is the extent to which collagen is fully demineralised during EDTA treatment. We postulate that the mineral hiding in the gap zone (Fig. 2) is the last to be sequestered, and may remain in the collagen after EDTA treatment. Since DNA is implicated in the self-aggregation of collagen molecules into fibrils, this gap zone may also be a location for some DNA preservation. To fully resolve this issue, future experiments will need to be performed. In this paper however, we discuss the implications of the data, under the simplest assumption, that the DNA liberated in the two fractions is a direct representation of its presence in the hydroxyapatite and collagen components, respectively.

4.1. Rapid DNA loss in bone over the first year post death

The analyses of the mtDNA content in buried cow bone during the first few years of burial, whether in bogs or on the seabed, indicate that, independent of the physical and chemical characteristics of the burial environment, there is large decrease in the DNA content within the first year after death. It is known that microbial attack on un-butchered bone occurs early in the diagenetic process (Jans et al., 2004; Trueman and Martill, 2002; Yoshino et al., 1991). One plausible explanation for the observed decrease in mtDNA is the rapid putrefaction of soft tissue (e.g. osteoblasts, osteoclasts, bone-lining cells and blood cells) in the bone, as opposed to the degradation of the presumably more refractory DNA trapped within osteocyte lacunae or the mineralised osteoid. This is consistent with the observation of relatively little change in the collagen content, but large loss of total DNA, of the buried bones.

4.2. The role of the organic and inorganic matrix for long-term DNA survival

An additional explanation for the above may relate to rapid initial loss of the ca. 2% of collagen that constitutes the un-mineralised osteoid that lies directly under the bone-lining cells of all living bone. In particular, should a proportion of the DNA be bound to this, then one would expect it to be lost as this exposed collagen degraded in the early post-mortem period. Given the magnitude of the mtDNA losses we observed in our cow bones over the first year of burial (>90%), it seems most likely that this is a complementary, rather than alternate, explanation to the above.

An additional question relates to what underlies the observations that a significant proportion of the mtDNA in the non-control cow, and all ancient musk ox bones, is found in the hydroxyapatite fraction. A commonly accepted theory of DNA preservation in bone is that it is somehow trapped in the organic matrix, either forming a complex with collagen, entombed within protein matrix, or cross-linked to proteins (Mrevlishvili and Svintradze, 2005; Kitamura et al., 1997). Of course the internal porosity of bone during post-mortem degradation may also harbour fragments of collagen and other proteins released by the hydrolysis or microbial degradation of un-mineralised osteoid. This potentially provides an additional route for the adsorption and complexing of DNA in ancient bones. This 'working hypothesis' underlies why many methods of DNA extraction from bone involve discarding the hydroxyapatite fraction, that represents the dissolved inorganic component (e.g. Leonard et al., 2000), instead focusing on recovering DNA from the collagen remainder. Furthermore, there is some evidence that collagen promotes the stabilisation of the hydration shell and double helix of DNA through hydrogen bond formation (Mrevlishvili and Svintradze, 2005), which might prolong DNA survival. However, should the findings discussed above

be accurate, it would imply that the inorganic component of bone is at least as important in the long-term survival of DNA. It has previously been speculated that DNA may be stabilised by hydroxyapatite (Lindahl, 1993; Okazaki et al., 2001), and can survive within bioapatite crystals in bone (Salamon et al., 2005). It is presumed that this adsorption or encapsulation takes place in vivo during growth and remodelling of bone. Another possible mechanism is that post-mortem degradation of the cellular components of bone tissues releases fragments of DNA of various lengths into the microscopic pore spaces of bone where they mingle with a solution saturated in calcium and phosphate ions, and thus may be adsorbed by or encapsulated in re-precipitating HAP. Further support for this mechanism is provided by the sizes of aDNA fragments usually recovered from ancient bone which are typically in the range 60–150 bp (Prüfer et al., 2010) equivalent to 22–54 nm. These lengths compare favourably with the typical sizes of HAP crystallites found in bone at 2–5 nm thick, 15–55 nm long and 5–25 nm wide (Nudelman et al., 2010).

Thus we postulate four possible locations for the post-mortem preservation of DNA fragments in ancient bones; (a) bound to the collagen fibrils and subsequently overlain by HAP as the fibril mineralises, (b) bound to and/or encapsulated by HAP crystallites growing in the interfibrillar spaces as the osteoid mineralises, (c) adsorbed onto collagen fibrils released into the macroporosity of bone as un-mineralised osteoid undergoes chemical or microbial degradation, and (d) bound to and/or encapsulated by HAP crystallites as bone mineral re-crystallises or re-precipitates. Of these mechanisms (a) and (b) may take place in vivo, while (c) and (d) would be post-mortem or diagenetic phenomena.

Our data clearly support the hypothesis that aDNA may survive closely bound to mineral phases, suggesting that the hydroxyapatite component is at least, or even more, important than the organic component. We note that it is unlikely that the pattern of DNA yields in the collagen and hydroxyapatite extractions is due to an effect of the EDTA extraction protocols because there were no observable differences in results from the ancient musk ox when temperatures and EDTA volumes were modified (PFC and MTPG, unpublished data).

Our observations are not unique, in a similar experiment on ancient mammoth bone Schwarz et al. (2009) demonstrate that approximately 40% of the 84 bp fragments of the mtDNA recovered were similarly present in the hydroxyapatite fraction, while Ottoni et al. (2009) also observe that substantial amounts of DNA can be recovered from the hydroxyapatite fraction of 9th to 10th century cattle bones from Coppergate, York, UK.

For the majority of our ancient musk ox samples (6/7 samples), the proportion of mtDNA in each of the extraction fractions is sufficiently high (ratios of between 1:4 and 1:1) that the discarding of either one of the fractions would lead to a loss of a considerable proportion of the total mtDNA. In addition to obvious ramifications for studies that aim to recover as much endogenous DNA as possible from an ancient sample, the observations enable us to hypothesise further on the relationship between the DNA, collagen and hydroxyapatite.

Firstly there appears to be a 'threshold' effect with regards to collagen content – above a certain level the DNA is predominantly in the collagen fraction (in this data set ca. 20%, although we caution that this value is based on a relatively limited number of observations, so future analyses will be required to substantiate this). We speculate that if our above mentioned hypothesis about the role of collagen in un-mineralised osteoid is correct, it may be that the 'best preserved' musk ox simply have some un-mineralised osteoid remaining. This is certainly plausible given their permafrost origin. Secondly, should DNA be bound to collagen and hydroxyapatite separately, one would expect a loss of total bone collagen to correlate with a DNA loss in the collagen fraction, but not in the hydroxyapatite fraction. However, as is clear from both the musk ox and buried bone data sets, this is not the case (insets for Figs. 6 and 7). Specifically, the mtDNA content of both fractions decreases in a similar manner. This suggests that either (i), the DNA is simultaneously bound to the hydroxyapatite and the collagen, and loss of one leads to loss of the other, or that (ii), the loss of collagen by hydrolysis both leads to the loss of the collagen fraction of the DNA, but also exposes the hydroxyapatite to dissolution/re-precipitation, thus losing any DNA bound exclusively to the hydroxyapatite.

One possible explanation for the relatively large proportions of DNA found in the collagen and EDTA extracts is that the DNA becomes trapped in the osteoid as it forms. It is thought that once the osteoid is formed by osteoblasts it is slowly mineralised by the progressive replacement of water by mineral (Lees, 2003). Any extracellular DNA that lay in the osteoid would become encapsulated by bone apatite and thus shielded from degradation, the mineral in turn being protected by the collagen – a sort of mutual protection (Collins et al., 2002). Although mature bone contains approximately 23% collagen by weight, collagen makes up approximately 50% by volume. Thus if a buried bone suffers partial collagen loss via chemical degradation, with the resulting opening up of the microporosity, the exposed bioapatite may be subject to partial dissolution and reprecipitation (Collins et al., 2002; Hedges and Millard, 1995). Any DNA encapsulated in the bioapatite would thus also be lost. Assuming that DNA is initially evenly distributed throughout the osteoid, then, when the bone is subsequently sampled and demineralised, half the surviving DNA will be released into the EDTA solution, leaving half remaining bound to the collagen.

Despite these hypotheses, however, it is clear that the relationship of DNA and the inorganic matrix is not straightforward. Firstly, we are unable to explain why our buried cow bone data set contained much larger proportions of mtDNA in the hydroxyapatite than collagen fractions. Does this represent a very early stage of diagenesis in which poorly mineralised osteoid decays and releases calcium and phosphate ions that subsequently reprecipitate elsewhere, trapping DNA fragments arising from soft tissue decay? Through a series of experiments targeting different size DNA molecules, Schwarz et al. (2009) have observed that the hydroxyapatite fraction is enriched in smaller DNA fragments (both mtDNA and nuclear DNA – nuDNA) when compared to the collagen fraction. They postulate two different explanations for this, (i) that the DNA associated to the mineral matrix is more prone to post-mortem degradation, and/or (ii) there is a filtration effect, where small DNA fragments present in the collagen fraction are either released or retained in the matrix depending on their size. It is clear that future studies will be required to clarify the relationship between DNA, collagen and hydroxyapatite further.

4.3. The relationship of DNA and collagen preservation

Several methods, such as collagen content, crystallinity index analyses, amino acid racemisation, thermal age calculations, and cytosine to uracil deamination patterns (observed as cytosine to thymine, C → T, or guanine to adenine, G → A, changes in ancient sequences) have been suggested as proxies for survival of DNA in bone samples (e.g. Götherström et al., 2002; Poinar et al., 1996; Schwarz et al., 2009; Smith et al., 2003). If correct, these would serve as valuable tools to predict DNA presence in ancient samples prior to the undertaking of a genetic study – something that would be especially useful when resources are scarce and a large number of samples are available to choose between. The validity of such proxies requires their correlation with the amount of DNA present in the bone. In this regard, our data is supportive of studies that have argued for a correlation between DNA yield and protein content in bones (e.g. Poinar et al., 1996; Poinar and Stankiewicz,

1999), and contradictory to several other studies on older samples that have failed to note such a relationship. For example, Schwarz et al. (2009) do not observe a clear correlation between collagen and DNA content in a series of permafrost preserved bones. Given that our ancient musk ox data only shows a relationship of collagen versus total mtDNA content for the samples that contain relatively low levels of mtDNA, it may be that the samples studied by Schwarz et al. (2009) were of sufficiently high quality to not show the relationship. In another study, Ottoni et al. (2009) observed that DNA preservation in archaeological bone is not related to the presence of intact collagen fibrils, and that even bones with severely thermally damaged collagen contained high quantities of DNA. This discrepancy may relate to the fact that damaged collagen fibres may not necessarily mean that the collagen has been removed from the bone. A similar explanation may underlie why Collins et al. (2009) found that DNA amplification success is not related to the degree of aspartic acid racemisation (a function of collagen degradation) – racemisation does not necessarily relate to collagen loss.

5. Conclusion

Although much remains to be learnt about the relationship between DNA and the organic and inorganic components of bone it is clear from the results presented both here and published previously, that any extraction method that involves discarding the hydroxyapatite-containing EDTA supernatant following demineralisation (e.g. Leonard et al., 2000) is less efficient in terms of DNA recovery than methods that retain it. Thus, to maximise DNA recovery both fractions should be considered. This likely underpins the reason why aDNA extraction methods that rely only on the use of EDTA to liberate DNA, or that simultaneously couple EDTA digestion with proteinase K (and possibly with a detergent such as SDS) are reported to be so efficient (e.g. Rohland and Hofreiter, 2007; Yang et al., 1998; Żołędziewska et al., 2002).

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References

Bell, L.S., Skinner, M.F., Jones, S.J., 1996. The speed of post-mortem change to the human skeleton and its taphonomic significance. Forensic Sci. Int. 82, 129–149.
Björdal, C.G., Nilsson, T., 2008. Reburial of shipwrecks in marine sediments: a long-term study on wood degradation. J. Archaeol. Sci. 35, 862–872.
Campos, P.F., Willerslev, E., Gilbert, M.T.P., 2009. Isolation of DNA from ancient samples. In: Liu, D. (Ed.), Handbook of Nucleic Acid Purification. Taylor & Francis, Boca Raton, pp. 441–461.
Campos, P.F., Kristensen, T., Orlando, L., Sher, A., Kholodova, M.V., Götherström, A., Hofreiter, M., Drucker, D.G., Kosintsev, P., Tikhonov, A., Baryshnikov, G.F., Willerslev, E., Gilbert, M.T.P., 2010a. Ancient DNA sequences point to a large loss of mitochondrial genetic diversity in the saiga antelope (*Saiga tatarica*) since the Pleistocene. Mol. Ecol. 19, 4863–4875.
Campos, P.F., Willerslev, E., Sher, A., Orlando, L., Axelsson, E., Tikhonov, A., Aaris-Sørensen, K., Greenwood, A.D., Kahlke, R.-D., Kosintsev, P., Krakhmalnaya, T., Kuznetsova, T., Lemey, P., MacPhee, R., Norris, C.A., Shepherd, K., Suchard, M.A., Zazula, G.D., Shapiro, B., Gilbert, M.T.P., 2010b. Ancient DNA analyses exclude humans as the driving force behind late Pleistocene musk ox (*Ovibos moschatus*) population dynamics. Proc. Natl. Acad. Sci. U. S. A. 107, 5675–5680.
Collins, M., Riley, M., Child, A., Turner-Walker, G., 1995. A basic mathematical simulation of the chemical degradation of ancient collagen. J. Archaeol. Sci. 22, 175–183.
Collins, M.J., Nielsen-Marsh, C.M., Hiller, J., Smith, C.I., Roberts, J.P., Prigodich, R.V., Wess, T.J., Csapo, J., Millard, A., Turner-Walker, G., 2002. The survival of organic matter in bone: a review. Archaeometry 44, 383–394.
Collins, M.J., Penkman, K.E.H., Rohland, N., Shapiro, B., Dobberstein, R.C., Ritz-Timme, S., Hofreiter, M., 2009. Is amino acid racemization a useful tool for screening for ancient DNA in bone? Proc. R. Soc. B-Biol. Sci. 276, 2971–2977.
Currey, J.D., 2002. Bones: Structure and Mechanics. Princeton University Press, Princeton.
Gilbert, M.T.P., Rudbeck, L., Willerslev, E., Hansen, A.J., Smith, C., Penkman, K.E.H., Prangenberg, K., Nielsen-Marsh, C.M., Jans, M.E., Arthur, P., 2005. Biochemical and physical correlates of DNA contamination in archaeological human bones and teeth excavated at Matera, Italy. J. Archaeol. Sci. 32, 785–793.
Götherström, A., Collins, M., Angerbjörn, A., Lidén, K., 2002. Bone preservation and DNA amplification. Archaeometry 44, 395–404.
Hagelberg, E., Sykes, B., Hedges, R., 1989. Ancient bone DNA amplified. Nature 342, 485.
Hansen, A.J., Mitchell, D.L., Wiuf, C., Paniker, L., Brand, T.B., Binladen, J., Gilichinsky, D.A., Ronn, R., Willerslev, E., 2006. Crosslinks rather than strand breads determine access to ancient DNA sequences from sediments. Genetics 173, 1175–1179.
Hedges, R.E.M., 2002. Bone diagenesis: an overview of processes. Archaeometry 44, 319–328.
Hedges, R.E.M., Millard, A.R., 1995. Bones and groundwater – towards the modeling of diagenetic processes. J. Archaeol. Sci. 22, 155–164.
Hummel, S., Herrmann, B., 1994. Y-Chromosomal DNA from Ancient Bones Ancient DNA. Springer-Verlag, New York, pp. 205–210.
Jans, M., Nielsen-Marsh, C., Smith, C., Collins, M., Kars, H., 2004. Characterisation of microbial attack on archaeological bone. J. Archaeol. Sci. 31, 87–95.
Kitamura, E., Iwamoto, C., Sakairi, N., Tokura, S., Nishi, N., 1997. Marked effect of DNA on collagen fibrillogenesis in vitro. Int. J. Biol. Macromol. 20, 241–244.
Lees, S., 2003. Mineralization of type I collagen. Biophys. J. 85, 204–207.
Leonard, J.A., Wayne, R.K., Cooper, A., 2000. Population genetics of ice age brown bears. Proc. Natl. Acad. Sci. U. S. A. 97, 1651–1654.
Lindahl, T., 1993. Instability and decay of the primary structure of DNA. Nature 362, 709–715.
Lindahl, T., Nyberg, B., 1972. Rate of depurination of native deoxyribonucleic acid. Biochemistry 12, 5151–5154.
Mrevlishvili, G., Svintradze, D., 2005. DNA as a matrix of collagen fibrils. Int. J. Biol. Macromol. 36, 324–326.
Nielsen-Marsh, C.M., Hedges, R.E.M., 1999. Bone porosity and the use of mercury intrusion porosimetry in bone diagenesis studies. Archaeometry 41, 165–174.
Nielsen-Marsh, C.M., Smith, C.I., Jans, M.M.E., Nord, A., Kars, H., Collins, M.J., 2007. Bone diagenesis in the European Holocene II: taphonomic and environmental considerations. J. Archaeol. Sci. 34, 1523–1531.
Nijweide, P.J., Burger, E.H., Feyen, J.H.M., 1986. Cells of bone: proliferation, differentiation, and hormonal regulation. Physiol. Rev. 66, 855–886.
Nudelman, F., Pieterse, K., George, A., Bomans, P.H.H., Friedrich, H., Brylka, L.J., Hilbers, P.A.J., de With, G., Sommerdijk, N.A.J.M., 2010. The role of collagen in bone apatite formation in the presence of hydroxyapatite nucleation inhibitors. Nat. Mater. 7, 1004–1009.
Okazaki, M., Yoshida, Y., Yamaguchi, S., Kaneno, M., Elliott, J.C., 2001. Affinity binding phenomena of DNA onto apatite crystals. Biomaterials 22, 2459–2464.
Orgel, J.P.R.O., Miller, A., Irving, T.C., Fischetti, R.F., Hammersley, A.P., Wess, T.J., 2001. The in situ supermolecular structure of type I collagen. Structure 9, 1061–1069.
Orgel, J.P.R.O., Irving, T.C., Miller, A., Wess, T.J., 2005. Microfibrillar structure of type I collagen *in situ*. Proc. Natl. Acad. Sci. U. S. A. 103 (24), 9001–9005.
Ortner, D., Turner-Walker, G., 2003. The Biology of Skeletal Tissues, in Identification of Pathological Conditions in Human Skeletal Remains, second ed. Academic Press, 11-35.
Ottoni, C., Koon, H., Collins, M., Penkman, K., Rickards, O., Craig, O., 2009. Preservation of ancient DNA in thermally damaged archaeological bone. Naturwissenschaften 96, 267–278.
Pääbo, S., 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc. Natl. Acad. Sci. U. S. A. 86, 1939.
Pesquero, M.D., Ascaso, C., Alcalá, L., Fernández-Jalvo, Y., 2010. A new taphonomic bioerosion in a Miocene lakeshore environment. Palaeogeogr. Palaeoclimatol. Palaeoecol. 295, 192–198.
Poinar, H., Hoss, M., Bada, J., Paabo, S., 1996. Amino acid racemization and the preservation of ancient DNA. Science 272, 864.
Poinar, H.N., Stankiewicz, B.A., 1999. Protein preservation and DNA retrieval from ancient tissues. Proc. Natl. Acad. Sci. U. S. A. 96, 8426–8431.
Prüfer, K., Stenzel1, U., Hofreiter, M., Pääbo, S., Kelso, J., Green, R.E., 2010. Computational challenges in the analysis of ancient DNA. Genome Biol. 11 (5), R47, doi:10.1186/gb-2010-11-5-r47.
Pruvost, M., Schwarz, R., Correia, V.B., Champlot, S., Braguier, S., Morel, N., Fernandez-Jalvo, Y., Grange, T., Geigl, E.M., 2007. Freshly excavated fossil bones are best for amplification of ancient DNA. Proc. Natl. Acad. Sci. U. S. A. 104, 739.
Rohland, N., Hofreiter, M., 2007. Comparison and optimization of ancient DNA extraction. Biotechniques 42, 343–352.
Salamon, M., Tuross, N., Arensburg, B., Weiner, S., 2005. Relatively well preserved DNA is present in the crystal aggregates of fossil bones. Proc. Natl. Acad. Sci. U. S. A. 102, 13783–13788.

Schwarz, C., Debruyne, R., Kuch, M., McNally, E., Schwarcz, H., Aubrey, A., Bada, J., Poinar, H., 2009. New insights from old bones: DNA preservation and degradation in permafrost preserved mammoth remains. Nucleic Acids Res. 37, 3215–3229.

Shapiro, B., Drummond, A.J., Rambaut, A., Wilson, M.C., Matheus, P.E., Sher, A.V., Pybus, O.G., Gilbert, M.T.P., Barnes, I., Binladen, J., Willerslev, E., Hansen, A.J., Baryshnikov, G.F., Burns, J.A., Davydov, S., Driver, J.C., Froese, D.G., Harington, C.R., Keddie, G., Kosintsev, P., Kunz, M.L., Martin, L.D., Stephenson, R.O., Storer, J., Tedford, R., Zimov, S., Cooper, A., 2004. Rise and fall of the Beringian steppe bison. Science 306, 1561–1565.

Smith, C.I., Chamberlain, A.T., Riley, M.S., Cooper, A., Stringer, C.B., Collins, M.J., 2001. Neanderthal DNA: not just old but old and cold? Nature 410, 772–773.

Smith, C.I., Chamberlain, A.T., Riley, M.S., Stringer, C., Collins, M.J., 2003. The thermal history of human fossils and the likelihood of successful DNA amplification. J. Hum. Evol. 45, 203–217.

Smith, C.I., Nielsen-Marsh, C.M., Jans, M.M.E., Collins, M.J., 2007. Bone diagenesis in the European Holocene I: patterns and mechanisms. J. Archaeol. Sci. 34, 1485–1493.

Todoh, M., Tadano, S., Giri, B., Nishimoto, M., 2009. Effect of gradual demineralization on the mineral fraction and mechanical properties of cortical bone. J. Biomech. Sci. Eng. 4, 230–238.

Trueman, C., Martill, D., 2002. The long term survival of bone: the role of bioerosion. Archaeometry. 44, 371–382.

Turner-Walker, G., Peacock, E.E., 2008. Preliminary results of bone diagenesis in Scandinavian bogs. Palaeogeogr. Palaeoclimatol. Palaeoecol. 266, 151–159.

Turner-Walker, G., Jans, M., 2008. Reconstructing taphonomic histories using histological analyses. Palaeogeogr. Palaeoclimatol. Palaeoecol. 266, 227–235.

Tuross, N., 2003. Recent Advances in Bone, Dentin and Enamel Biochemistry, in Identification of Pathological Conditions in Human Skeletal Remains, second ed. Academic Press, pp. 65–72.

Tzaphlidou, M., Berillis, P., 2005. Collagen fibril diameter in relation to bone site. A quantitative ultrastructural study. Micron 36, 703–705.

Yang, D.Y., Eng, B., Waye, J.S., Dudar, J.C., Saunders, S.R., 1998. Technical note: improved DNA extraction from ancient bones using silica-based spin columns. Am. J. Phys. Anthropol. 105, 539–543.

Yoshino, M., Kimijima, T., Miyasaka, S., Sato, H., Seta, S., 1991. Microscopical study on estimation of time since death in skeletal remains. Forensic Sci. Int. 49, 143–158.

Żołędziewska, M., Gronkiewicz, S., Dobosz, T., 2002. Comparison of various decalcificators in preparation of DNA from human rib bones. Anthropol. Rev. 65, 75–80.