Investigation of the Effects of Heat on Bone Tissues to Inform Forensic Analysis

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Abstract

Following an incineration event bone is often the only surviving tissue, making the skeleton vital for obtaining a positive identification of the deceased. Discriminating features such as age, sex, height, and antemortem injuries can often be observed in skeletal remains and used as secondary identifiers in forensic examination. Understanding the effects of incineration on bone tissues and how this may impact skeletal markers is essential to improving standard methods of anthropological profiling for forensic identification. In addition to these secondary identifiers, bone provides a protective barrier for DNA which is highly individualising and hence is considered a primary scientific method of identification. Due to the structural degradation of bone, and the loss of organic material, obtaining a viable DNA sample from incinerated bone is problematic. There is a need for improved DNA extraction protocols for these incinerated samples, as well as methods of triaging multiple samples to facilitate an optimal outcome.

The primary aim of this research was to study heat-induced changes in bone microstructure to inform forensic evidence recovery and analysis. This was achieved by employing a range of techniques including x-ray powder diffraction (XRPD), micro computed tomography (microCT), and scanning electron microscopy (SEM) with energy dispersive x-ray (EDX). As previous research has suggested heat-induced changes in bone mineral resemble long term diagenetic changes, it was hypothesised that ancient DNA extraction methods may be appropriate for modern incinerated samples. This theory was tested by comparing demineralisation techniques designed for ancient DNA extraction to simpler techniques used for fresh samples. The quantity of DNA recovered was compared between methods to assess the optimal method for extraction from burned samples. As bone crystallinity has an impact on both DNA preservation and the ability to extract DNA from mineralised tissue, XRPD was used to assess heat induced changes in hydroxyapatite crystal size and structure across a range of temperature intervals. SEM and EDX were then used to visually assess these changes and provide basic elemental analysis. In the final part of this project, MicroCT was used to create 3D reconstructions of

incinerated bone samples to visually and quantitatively assess changes to bone porosity as a result of thermal insult.

The application of ancient bone demineralisation techniques was not shown to increase DNA yield from incinerated bones, with a shorter demineralisation period and retention of EDTA supernatant producing optimal results. XRPD analysis provided a possible explanation for this, showing that heat-induced changes to bone hydroxyapatite were far more impactful than the diagenetic changes seen in ancient bone, which could not be distinguished from unburned modern samples. Similarly marked heat-induced changes in bone structure were observed using MicroCT, where bone porosity was greatly decreased at high temperatures. This research highlights the variable nature of heat induced changes in bone which could prove valuable in prioritising sampling based on the likelihood of obtaining identifying information such as DNA.

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Signed: Date: 23.06.21

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Chapter 1: Introduction

The formal identification of a deceased person is a legal requirement in most jurisdictions. It also provides closure and comfort to the family of the deceased individual. Positive identification is not always an easy task – aside from logistical constraints such as decomposition, displacement and commingling of remains, death is a sensitive area for many individuals. Cultural and religious considerations must also be taken into account. Many belief systems do not approve autopsies, for example Jewish law Halacha requires remains to be buried immediately with some rare exceptions. Hindu practices require cremation of remains within 24 hours of death, and Islamic law requires remains be buried within this same timeframe to avoid loss of any parts of the body, which is considered sacrilegious. Repatriation of historical Aboriginal remains is another sensitive task faced by forensic anthropologists, who attempt to provide as much information as possible in order to identify the home country of the deceased individual so they may be repatriated. We as forensic professionals have a legal, ethical and humanitarian duty to ensure all human remains are granted identity and justice after death.

Forensic human identification, be it of victims of foul play, unexpected natural death, manmade disaster or simple misadventure relies at the most basic level on individual uniqueness. Without the inherent variation that exists in the anatomy, physiology and cultural aspects of every human, we would not be able to return identity to those who have lost it or had it taken from them. However, it is essential that we understand not only how individuals differ, but also how they do not. The skeleton is an anatomical feature that we all share; when all other traditional identifying features have been lost, the bones and teeth remain. Dental records, fingerprints and DNA are considered primary identifiers, meaning they are scientifically validated techniques (Schuliar & Knudsen, 2012). In the absence of primary identifiers, secondary traits such as tattoos, scars, or an anthropological profile may be used to support identification. In some cases, anthropological assessment of pathologies, abnormalities and injuries can provide highly individualising information (De Boer et al., 2020). However, if none of these features are present, analysis of the skeleton alone may not be sufficient for a positive identification. The mineralised nature of bone, however, protects and preserves one of the most discriminating aspects of human variation: DNA (Pinhasi et al., 2015).

1.1 DNA inheritance and analysis

DNA is a double helical compound consisting of an alternating sugar and phosphate backbone with attached nucleotide bases. Much like a binary coding system, the relatively simple alteration of base pairs (Guanine – Cytosine, Adenine – Thymine) provides an immensely complex blueprint instructing the development of an entirely unique organism (Lee et al., 1994). In the case of humans, these blueprints can be found within either the nuclei of cells or within cellular mitochondria. Nuclear DNA (nDNA) is tightly packaged within 46 chromosomes, 44 of which are autosomal non-sex chromosomes, whilst two are sex chromosomes. Autosomal chromosomes exist in pairs, with offspring inheriting one homologous chromosome maternally and the other paternally (Butler, 2009).

A specific physical location on a chromosome is known as a "locus", plural "loci", and variations of a specific locus are called "alleles". Determining which of the parents' homologs will be passed on to the offspring is a random process; this is primarily how a unique genetic profile is created. Meiotic crossing over is another aspect of genetic inheritance that increases genotypic variation. When chromosome homologs align during meiosis, DNA can be cut, shuffled between pairs and re-annealed to create a new, unique homologous pair. Although crossing over does not always occur in gamete production, the random allocation of parental chromosomes does. The combination of these processes results (with the exception of monozygotic twins) in a highly individualised genetic profile (Latham & Miller, 2018, Baranzini et al., 2010, Kaye, 2010). More recent studies have shown that examination of methylation patterns in DNA will even allow differentiation between identical twins (Stewart et al., 2015, Vidaki et al., 2013).

Although individual profiles are essential to establishing identity, there are some cases where forensic professionals are interested in establishing kinship between genetic samples. In these cases, mitochondrial DNA (mtDNA) can be used in place of, or in addition to nDNA (Fig. 1.1). As the name suggests, mtDNA is housed within the mitochondria of eukaryotic cells. It is theorised that the development of mitochondria and their unique genome is a result of endosymbiosis, whereby ancestral eukaryotic cells internalised energy producing bacterial prokaryotes (Zimorski et al., 2014). This resulted in formation of double-membraned organelle like mitochondria and chloroplasts, with the inner

membrane derived from the ancestral bacteria and the outer from the host-cell. As the mitochondria of all energy producing cells using aerobic processes were once isolated prokaryotic cells, they still possess self-contained, isolated genomes in the form of a single circular chromosome (Kodama & Fujishima, 2010). This means the mitochondrial genome now exists as multiple copies in each eukaryotic cell, unlike the single nDNA genome housed within the nucleus. MtDNA has a unique germline inheritance, with all genetic material required for organelle function (primarily production of adenosine triphosphate in the citric acid cycle) passed on predominantly by the mother (Thornton et al., 2014). This differs significantly from the recombinant nature of nDNA, which is inherited biparentally (Budowle et al., 2003). Although the mtDNA genome is not subject to the genetic randomisation of parental genes, it is far more susceptible to evolutionary alterations and mutations than nDNA. Base substitutions in mammalian mtDNA genomes have been calculated to occur at a rate of 0.02 substitutions per base pair per million years, which exceeds that of the nDNA genome tenfold (Brown et al., 1979). This makes the mitochondrial genome ideal for evolutionary genetic studies.

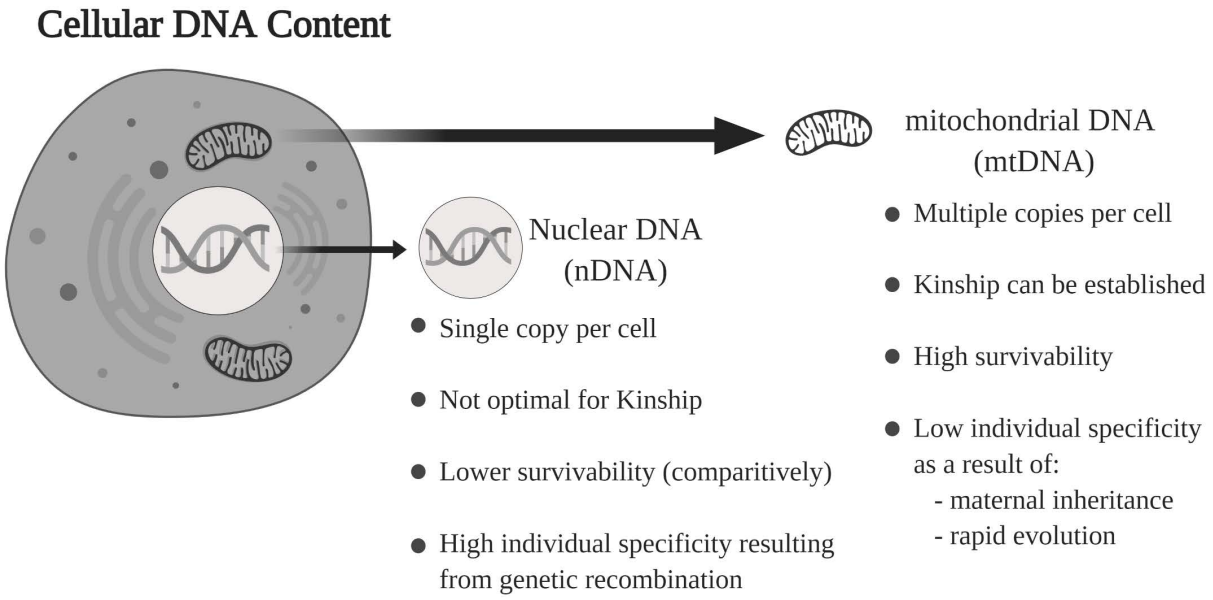

Fig 1.1. Comparison of structural and compositional differences between mitochondrial and nuclear DNA. Figure created with BioRender.com.

Despite the rapid rate of genomic evolution, this occurrence is slow enough that inter-generational genomes are unaffected. This means mtDNA is still very useful for establishing genealogy and kinship. In disaster victim identification (DVI), mtDNA can be used to identify maternally related family members following cases where related individuals are known to be amongst the deceased (vehicular crashes, house fires etc.) (Holland et al., 1993). However, the lack of individuality in the mitochondrial genome is a major caveat in many cases as the discriminatory power of mtDNA is much lower than nDNA, and profiles obtained can be matched to a larger pool of individuals (Bär et al., 2000, Giles et al, 1980). For this reason, in closed DVI situations where a majority of victims are documented (e.g., flight manifest) kinship is usually established using nDNA profiles voluntarily provided by families, as was used to aid in identifying many of the victims of Malaysian Airlines flight MH17 (Smith & Mann, 2015). However, in cases where remains are subjected to extreme degradation, mtDNA can have some advantage over nDNA due to the higher number of mitochondrial genomes and the circular molecular structure, which is more stable than nuclear helical DNA (Montelius & Lindblom, 2012, Graw et al., 2000). These factors result in higher survivability of mtDNA under challenging conditions, making it particularly useful for establishing kinship of degraded, skeletal remains (Coble et al. 2004). In Chapter 3 of this thesis, where alternate methods of DNA extraction from bone are assessed, I focused on nDNA, with the understanding that mtDNA has much greater survivability than nDNA, hence if nDNA can be retrieved mtDNA must also be available in equal or greater measures.

In order to compare and contrast two genetic profiles we must first identify regions of inter-individual variability, and DNA polymorphisms are ideal for this. These variations can be in the form of single bases, multiple bases, and repeated sequences. The insertions and deletions of individual bases are known as “sequence polymorphisms” whilst those that involve the insertion of multiple bases are known as “length polymorphisms”. Population genetic studies have mapped a large number of locations at which polymorphisms occur, which forensic professionals can then use to isolate and compare sequences from unknown and known samples. Initially, restriction fragment length polymorphisms (RFLP) served as the primary method of isolating genetic variation. This involves the

use of probes and simple restriction endonucleases to tag and digest the sample, after which the remaining fragments can be visualised using electrophoresis to provide a unique profile based on the varying length of fragments. Although still useful for genomic studies and general variation analysis, in forensic science it has largely been replaced by the use of polymerase chain reaction (PCR).

The advent of PCR had a massive impact on the field of genetics, particularly in forensic science. Unlike RFLP, PCR can be used to isolate and amplify miniscule quantities of DNA from extremely degraded samples in a fraction of the time (1-2 days vs 6-8 weeks) (Butler, 2009). PCR works by identifying a DNA sequence containing the chosen polymorphism, isolating it with the use of primers and exponentially replicating the target sequence multiple times creating enough of the target product for various forms of analyses (e.g., gel electrophoresis). Real-time quantitative PCR (qPCR) takes this process a step further and allows the number of target fragment copies to be measured in real time. This is achieved by use of fluorescent labelling (e.g SYBR® Green dye or TaqMan® probes) which binds to double-stranded DNA or primers. As the number of fragment copies increases with each amplification cycle, so too does the fluorescence. The point at which the fluorescence reaches a detectable level above background noise is known as the C_T (Cycle Threshold) or C_q (Cycle quantification) value (Karlen et al., 2007). In order to quantify the DNA concentration of an unknown sample, the C_q values of known samples are plotted against the log of DNA concentration to create a dosage-dependent standard curve. Unknown sample C_q values are then plotted against this curve to extrapolate the concentration of the sample. This is an example of absolute qPCR quantification, which is most commonly used in forensic analysis as opposed to relative quantification (used to measure gene expression).

Early methods of DNA profiling only allowed analysis of long repeat sequences, classified as “minisatellites”. The first minisatellites to be used were variable number tandem repeat (VNTR) markers, which were often analysed using RFLP in a process known as DNA “fingerprinting” (Butler, 2009). With increased genome mapping and the development of highly specific amplification techniques such as PCR, smaller markers soon replaced VNTRs. The term “microsatellite” is commonly used to describe this family of smaller markers, which have a much higher power of discrimination than the earlier minisatellites (Lee et al., 1994).

In addition to the highly polymorphic nature of microsatellites, these markers are also less affected by DNA strand breakage and are more readily retrieved from degraded samples due to their shorter length.

Today the most commonly used minisatellites are short tandem repeat (STR) markers, with STR-based human identification kits used in most forensic laboratories (Schichman et al., 2002). Traditional STR kits targeted amplicons of 100 to 450 base pairs in length, however for highly degraded DNA even fragments of this length may not be available. To overcome this "mini STRs" were developed which involved moving the primers targeting the amplicon as close as possible to the repeat sequence. While this improves the likelihood of recovering a useful DNA profile it can still be problematic for highly degraded samples. A possible alternative in these situations are single nucleotide polymorphisms (SNP), which as their name suggests, are a variation at a single base. SNPs which are largely bi-allelic are of interest to the forensic community for a multitude of reasons; the amplicons targeted for SNPs can be very short and within a population a SNP can be assigned a frequency of occurrence. Additionally, phenotypic predictions can be made based on varied allelic combinations. One of the shortcomings of SNPs is finding a multiplex assay that can amplify enough SNP targets for robust, discriminatory results (Butler, 2009). This, and the fact that extensive STR databases currently exist, means SNPs are unlikely to replace STRs in a forensic criminal investigation context in the foreseeable future.

The use of DNA for identification of human remains in a forensic setting most often occurs when a body is not visually identifiable. That is, the body is damaged or decomposed to an extent that it would not be reliable to confirm identity based on the opinion of a friend or relative viewing the facial appearance of the deceased. When taphonomic degradation is extensive, for example following advanced diagenesis, prolonged burial or incineration, mineralised tissue such as teeth and bones may be all that remains for genetic analysis. Teeth are particularly well suited for genetic identification as the DNA-rich pulp is protected by dense enamel (Balasse, 2002, Bryant et al., 1996). However, teeth are also easily displaced due to various factors such as disease, aging and diagenesis (Higgins & Austin, 2013), and in such cases bone may be the only substitute. DNA retrieval from these

mineralised tissues is often more complicated than from fresh material. Archaeologists and palaeobiologists have extensively researched DNA recovery from diagenetic skeletal remains, and some of this research can be applied to modern forensic casework (Latham and Miller, 2019). Substantially fewer studies have investigated the changes that occur in incinerated bone, and how this may affect DNA recovery. There is a particular need for this research in Australia where bushfires are frequent and severe, with climate change likely to aggravate the situation in future years (Lucas et al., 2007).

1.2 Bone microstructure and taphonomy

Successful forensic analysis of skeletal tissues that have been degraded by heat requires an understanding of what specific changes take place within the bone during the burning process. For DNA related investigations this knowledge is especially important to inform which bone to target and how best to liberate DNA from within these tissues. At the most basic level, bone can be divided into two main structural forms that can be observed macroscopically; trabecular and cortical. The trabecular (or cancellous) structure of bone tissue is produced by organic osteoids laid around spaces filled with red bone marrow. The osteoid is permeated by hydroxyapatite so that ultimately bone trabeculae are built of collagen fibres strengthened by hydroxyapatite (Trueman et al., 2008). By comparison cortical bone consists of Haversian systems that surround small blood vessels, and of osteocyte spaces (Cummaudo et al., 2019, Vaughan et al., 2012, Qiu et al., 2003). Some research has shown that at lower temperatures (<250 °C) the circularly organised lamellar layers of Haversian systems undergo significant changes, primarily the broadening of Haversian canals as observed with SEM (Chadefaux & Reiche, 2009). This reorganisation of bone internal structure is likely a primary driver of changing porosity. Bone is also often described as primary or secondary, which simply refers to the time and manner in which it is formed – primary bone mostly occurs during early development and repair, whereas secondary bone (also known as Haversian bone) is either deposited onto existing bone surfaces or onto sites where pre-existing bone has been resorbed (Hillier & Bell, 2007).

Both trabecular and cortical bone can be further divided into organisationally different microstructures that are particularly useful for species differentiation. Woven bone is a primary tissue

commonly found in fetal bones and repair sites. In early development, as well as fracture repair, woven bone is laid down in a rapid and disorderly fashion to provide a temporary scaffolding structure for mature bone to develop on (Mulhern & Ubelaker, 2012). Comparatively lamellar bone is a mature tissue that consists of consecutive layers of thin lamella deposited alongside parallel layers of collagen that can form either primary or secondary bone. Fast growing mammals including cows, pigs and sheep can also present a combination of woven and lamellar tissue known as fibrolamellar bone which is largely absent in humans and other primates (Martiniaková et al., 2007). This tissue consists of alternating layers of woven and lamellar structures which, although somewhat mechanically inferior to primary lamellar bone, can be laid down far more rapidly (Mulhern & Ubelaker, 2012). A subset of this tissue where lamellae are organised in a brick-like pattern is known as plexiform fibrolamellar bone, which is highly characteristic of large mammals (Burr & Martin, 1989). The presence of plexiform bone in animal models is likely an important structural difference that must be considered when making comparisons to humans.

Bone diagenesis and/or weathering results in chemical and mechanical destruction of bone. A consequence of this can be a severe decrease in bone density, which has been linked to the physical protection of endogenous DNA (Pinhasi et al., 2015, Collins et al., 2002). Additionally, decreased bone integrity dramatically increases the likelihood of microbial invasion, and contamination of target DNA. It has been suggested that ancient bones exposed to prolonged diagenetic processes may share similar structural composition to modern incinerated bones, however research supporting this is insubstantial (Piga et al., 2009, Stiner et al., 1995). Understanding these similarities (or lack thereof) is of particular importance to how DNA extraction from incinerated bone may be approached, especially with regards to the demineralisation step, which has been shown to be vital for ancient samples. As the name implies, DNA is slightly acidic due to the phosphates in the molecular backbone. This means nucleic acids project a slight negative charge which allows them to bind to ionised substances such as bone hydroxyapatite (HAp) (Busse et al., 2009, Paget et al., 1992). In addition to removal of calcium (which is a PCR inhibitor), demineralisation is essential for liberating small quantities of DNA bound to HAp crystallites. In archaic samples most cellular DNA has been lost, and a prolonged period of demineralisation is vital to release as much endogenous DNA as possible. The comparison of diagenetic ancient bone and modern incinerated bone is a common theme throughout this thesis.

Changes in bone integrity, and in turn the likelihood of obtaining viable DNA, can be assessed to some degree by microscopic and macroscopic investigation. Whilst it is often visually obvious if a sample is too degraded for analysis (i.e., calcined bone), caution must be taken as the depth of internal damage is not always visible from the exterior. This is where microscopic changes such as presence or absence of nucleated cells, osteon integrity and micro fracturing can be used to give some indication of DNA survival (Edson et al., 2013, Singh et al. 2013, Alers et al., 1999, Cattaneo et al., 1999). Collagen content has also been linked to DNA yield, with some studies suggesting an increased affinity for DNA binding as the collagen becomes mineralised (Campos et al., 2011, Götherström et al., 2002, Collins et al., 1995). Histological analysis using both demineralised/stained (i.e., haemotoxylin and eosin, masson's trichrome) and unstained (i.e., resin embedded slices) tissue can show these changes (Higgins et al., 2015, 2013), however decalcifying, cutting and mounting highly degraded or incinerated samples can be challenging.

As histological analysis requires a certain amount of sample destruction, it is not ideal for cases where target material is delicate or limited. In such cases, non-invasive alternatives such as Micro Computed Tomography (MicroCT) should be considered. MicroCT is a less traditional method for examination of structural integrity, however with some refinement it could prove a valuable screening tool for examination of forensic samples. This could be achieved by employing volumetric analysis (bone density, bone porosity etc.) of incinerated samples using three dimensional MicroCT reconstructions (Kuhn et al., 1990, Sandholzer et al., 2013). Utilising MicroCT in this way could provide both visual and quantitative insight into heat-induced changes in bone without the need for extensive sample preparation or destruction.

Research throughout this thesis attempts to clarify if changes to the microstructure of incinerated modern bones are comparable to those of ancient bone using a holistic approach to explore both large-scale and molecular taphonomy. Chapter 3 directly investigates the benefits of ancient DNA extraction demineralisation techniques on modern incinerated samples, whilst Chapter 4 investigates how HAp crystallites change during burning (compared to ancient Bison bone). Chapter 5 uses a combination of MicroCT and SEM to compare histological and microstructural changes in remains incinerated at various temperatures.

1.3 Ethical considerations

When conducting research on biological samples, a range of factors must be taken into consideration, especially with model selection. In some regards working on post-mortem tissues is less ethically constrained than research on live subjects however, as samples are often exposed to destructive processes, animal substitutes are generally preferable to human. As the outcome of this research is aimed at informing processes applicable to human tissues any substitute must represent humans as closely as possible whilst still taking various practical factors into consideration. In many cases, the need for similarity in bone macro/micro structure is overcome by factors such as developmental rate of an induced disease, or ease of handling. This is why small rodents such as domestic mice and rats are commonly used in osteoarthritis research – surgically induced osteoarthritis can be closely monitored in these models, and lesions develop much more rapidly than in larger species (Bendele et al., 2001). In the past, research on orthopaedic implants has used larger animals to varying degrees of success. The main structural difference between human bone tissue and that of large animals like sheep, cows and pigs is the presence of primary plexiform bone – this type of bone tissue is characteristic of these fast-growing animals and is mostly absent in human bones (Hillier & Bell, 2007). In immature animals, plexiform tissue can be located throughout entire long bones. For this reason, it is imperative that samples be collected only from mature individuals to increase the likelihood of obtaining secondary Haversian bone that is more comparable between species.

Domestic sheep (*Ovis aries*), have increased in popularity for orthopaedic implant studies as they have macroscopically similar bone structure to humans, are easily available for research and are easier to handle than some other large animals (for example cows and pigs) (Pearce et al., 2007). Although macroscopically similar, the microstructure of cortical sheep bone consists of irregularly shaped haversian canals as opposed to the dense, consistently shaped canals of human bone (De Kleer 2006, Mosekilde et al., 1987). Cows are also occasionally used in research; however their large size make them generally unfavourable for in vivo studies. Like sheep, they possess cortical bone consisting of irregularly shaped Haversian canals (Pearce et al., 2007). Aerssens et al., (1998) compared the structure and composition of various laboratory animals including sheep, cows, pigs and rats. The

findings suggest that although pigs (along with dogs) shared more biomechanical similarities with humans, cows were the intermediary, surpassing rats and sheep in terms of bone comparability.

Domestic pigs (*Sus scrofa*) are arguably the best substitute for human models due to the similarity of their body size and bone composition both macro and microscopically. Like humans, pigs possess densely organised, regularly shaped Haversian canals (although porcine trabecular bone is slightly denser) (Thorwarth et al., 2005). Furthermore, the genome of pigs is relatively similar to humans, which makes them similarly viable substitutes for DNA based research (Fadista et al. 2008, Hart et al., 2007). Although pigs are not ideal for in vivo studies due to their high growth rates, large size and noisy, aggressive natures, this issue is negated when using scavenged post-mortem tissues, making them ideal for forensic research.

No single species can perfectly replace human samples, and ultimately the animal models I used for my research were selected based on a combination of ethical and logistical considerations. Human samples would have been ideal, but due to the destructive nature of the research this was not possible. A porcine model was the obvious substitute for the DNA-based study covered in Chapter 3 due to the genomic similarities between humans and pigs. Additionally, pig carcases are readily available and easy to acquire, and as they are deceased none of the issues involved with housing and handling living animals apply here. Model selection was also based on availability, as was the case for the study presented in Chapter 4 where ancient bone was compared to modern samples. As this study was comparing and contrasting the effect of different taphonomic influences (diagenesis vs incineration) it was vital that samples were comparable. Ancient bone samples are much harder to obtain than fresh/modern material, and as I was only able to acquire ancient bovine samples (*Bison antiquus*), domestic cow (*Bos taurus*) was the only modern counterpart readily available. Despite some minor difference in density and organisation, mammalian bone microstructure is inherently similar. However, as different regions of the skeleton are afforded different levels of protection by the surrounding tissue thickness (Buikstra and Swegle, 1989), and the distribution of tissue differs between cows, pigs and humans, heat-induced changes to bone microstructure may not be directly comparable to humans. This is an unavoidable caveat of using animal models for forensic research.

1.4 Primer design and application

Opting to use a porcine model was not without challenges, primarily the lack of established primer sets. Primers are single-stranded DNA fragments designed to be complimentary to nucleotide regions on target DNA (Butler et al., 2009). During PCR, DNA strands are separated, allowing primers to bind and amplify the target material. Two primers, one forward and one reverse, are required to target both complementary DNA strands (Garibyan & Avashia, 2013). As no suitable published porcine nuclear primers were available, they had to be specifically developed in house and validated before I could apply them to my research. When designing primers various factors such as the number and composition of base pairs (GC to AT ratio), individual specificity of target fragment, annealing temperature and secondary structure of the molecule must be considered to optimise PCR efficacy. The optimal length of a standard primer is generally between 18 and 24 base pairs; any longer than this and hybridization times become excessive, whilst much shorter primers can result in amplification of non-target material (Wu et al., 1991). When extracting and amplifying DNA from highly degraded samples, short primers are preferable as they are more sensitive to fragmented DNA (Swango et al., 2006).

As GC bases contain more hydrogen bonds than adenine and thymine, increasing GC content can increase primer stability (Kämpke et al., 2001). It is generally suggested that a primer should consist of between 45% to 60% GC, and (if possible) end with either a G or C base "clamp" to promote binding at the 3' end of the strand (Dieffenbach et al., 1993, Sheffield et al., 1989). However, including too many G or C repeats near the 3' end should be avoided as this can lead to self-hybridisation, and subsequent primer-dimer (Abd-Elsalam, 2003). Hairpins are another example of a secondary primer structure that should be avoided. These structures form when two separate regions on the same primer are complementary, which allows the strand to fold back and anneal to itself forming a loop or "hairpin" (Fan et al., 2019). Online databases like primer-BLAST (Ye et al., 2012) can be used to ensure primer specificity, assess its potential for self-binding and identify temperatures at which problematic secondary structures may form (Thornton & Basu, 2011). Online databases and primer design tools can also be used to establish the melting point of a primer (T_m). Ideally, annealing

should occur at temperatures around 5 °C below T_m, and making sure this does not occur at particularly low temperatures is essential to prevent non-specific binding (Rychlik et al., 1990). Ideally there should be no more than 2 °C difference in T_m between primers. Although T_m can be calculated based on the GC content (higher temperatures needed for higher GC content), realistically this temperature may vary in the reaction itself (Bustin & Huggett, 2017). To achieve the best result, the primer reaction (e.g., thermocycling conditions) may require further optimisation.

Along with DNA quantity, DNA quality can also be assessed using qPCR by the amplification of two DNA target strands of different lengths (Swango et al., 2006, Niederstätteret al., 2007). qPCR primers are designed to select target areas of DNA that do not vary between individuals, only occur once in the genome, and are species specific. If the long target fragment can be successfully amplified, this indicates the DNA strands have not been severely degraded or broken (Nygard et al., 2007, Alonso et al., 2005). Common techniques of isolating desired DNA sequences involve the use of fluorescent dyes or probes. Fluorescent dyes such as SYBR® green bind to double-stranded DNA molecules, allowing the amount of DNA present after each thermocycle to be recorded (Higgins et al., 2015). Comparatively, fluorescent probes such as those used in TaqMan® assays bind to primer-specific sequences; this results in cumulative florescence of probes after each amplification cycle which is proportional to the quantity of DNA synthesised (Arya et al., 2005). Although TaqMan® assays are more product-specific, they are also more complex and costly (Tajadini et al., 2014). For this reason, I opted to use SYBR® green for my research.

1.5 Thesis scope

In the wake of destructive post-mortem processes, DNA and skeletal material are intrinsically linked, and the degradation of one often results in loss of the other. With this in mind, I have taken a multidisciplinary approach to investigate the following aspects of the bone-DNA relationship. The bulk of this thesis is presented in a series of published manuscripts with the exception of the introduction (Chapter 1), and discussion and conclusions (Chapter 6). The first manuscript (Chapter 2) is a literature review compiling established information on DNA extraction and amplification from incinerated samples. In addition to providing a general overview of some of the pitfalls and shortcomings of the currently available methods, I outline areas where knowledge is lacking, and the potential for further research.

The second manuscript (Chapter 3) explores direct DNA yield from burned bones using various methods of decalcification. Although using human samples for this study would have been ideal, ethical and logistical constraints made it impossible. Porcine primers were developed as an alternative, however this presented a new set of challenges. To address this, chapter 3 is separated into two parts; section 3a, which includes non-published but still relevant information relating to how porcine primers were developed and tested, and section 3b, which includes the published study exploring decalcification techniques using these primers. Initially it was believed that degraded modern bone samples should be exposed to prolonged decalcification prior to extraction in the same way that archaic bones are. With the previous study showing that bone calcite crystal structure only changes dramatically after 600 °C, I hypothesised that using extraction techniques designed for archaic bones may actually be too harsh for many burned samples, and a decreased EDTA incubation period could improve DNA yield. Additionally, research suggested that retaining digested supernatant could prevent the loss of vital DNA (with the increased potential for contamination) (Fondevila et al., 2008, Loreille et al., 2007), which I also decided to test. These altered extraction protocols were tested on a range of burned and unburned bone samples, following which absolute quantification using qPCR was used to assess the success of each method.

The third manuscript (Chapter 4) expands on the link between bone hydroxyapatite and DNA by investigating how heat-induced degradation can affect this link. A combination of x-ray powder diffraction (XRPD), scanning electron microscopy (SEM) and energy dispersive x-ray (EDX) were used to visualise and quantify changes to bone crystallite structure at various temperature intervals. Here I also discuss the potential use of the aforementioned techniques as screening tools to differentiate various bone types (e.g., ancient/fresh, porcine/bovine). The primary aim of this study was to assess if hydroxyapatite (HAp) crystallites would increase or decrease in size as a result of heating. As DNA extraction methods are designed to remove excess calcium from highly crystallised ancient samples, comparing the crystallite composition of ancient and modern incinerated bones could indicate if these methods are also appropriate for modern samples.

The fourth and final manuscript (Chapter 5) investigates the use of MicroCT as a method for assessing incinerated bone compared to standard SEM investigation. MicroCT provided information

on changes to bone porosity that could not be fully realised using SEM alone, additionally these changes could be both quantified and visually observed using 3D reconstruction. Ultimately the aimof this study was to assess what information MicroCT could provide on changes occurring in burned bone, and also to compare it to changes observed using SEM analysis. Histological analysis was also explored, however decalcification and subsequent cutting of incinerated samples was largely unsuccessful. The limited findings of this analysis have been included in appendix i.

1.6 References

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Chapter 2: A Review of the Current Understanding of Burned Bone as a Source of DNA for Human Identification

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By signing the Statement of Authorship, each author certifies that:

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Review

A review of the current understanding of burned bone as a source of DNA for human identification

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ABSTRACT

Identification of incinerated human remains may rely on genetic analysis of burned bone which can prove far more challenging than fresh tissues. Severe thermal insult results in the destruction or denaturation of DNA in soft tissues, however genetic material may be preserved in the skeletal tissues. Considerations for DNA retrieval from these samples include low levels of exogenous DNA, the dense, mineralised nature of bone, and the presence of contamination, and qPCR inhibitors. This review collates current knowledge in three areas relating to optimising DNA recovery from burned bone: 1) impact of burning on bone and subsequent effects on sample collection, 2) difficulties of preparing burned samples for DNA extraction, and 3) protocols for bone decalcification and DNA extraction. Bone decalcification and various DNA extraction protocols have been tested and optimised for ancient bone, suggesting that prolonged EDTA (Ethylenediaminetetraacetic acid) demineralisation followed by solid-phased silica-based extraction techniques provide the greatest DNA yield. However, there is significantly less literature exploring the optimal protocol for incinerated bones. Although burned bone, like ancient and diagenetic bone, can be considered "low-copy", the taphonomic processes occurring are likely different. As techniques developed for ancient samples are tailored to deal with bone that has been altered in a particular way, it is important to understand if burned bone undergoes similar or different changes. Currently the effects of burning on bone and the DNA within it is not fully understood. Future research should focus on increasing our understanding of the effects of heat on bone and on comparing the outcome of various DNA extraction protocols for these tissues.

1. Introduction

Previous research on DNA retrieval from thermally damaged tissues has been primarily palaeontology based, with a focus on establishing phylogeny of ancient remains. Far less attention has been given to retrieval of genetic information from incinerated tissues for forensic human identification of modern samples, for example identification of disaster victims. Twenty years ago, geneticists were not included in disaster victim identification mortuary teams, as dental records were considered a more effective means of establishing identity [1,2]. More recently the prevalence of DNA analysis in forensic identification has increased, although the process can still be costly and time consuming.

Even in contemporary remains, tissues may be compromised by factors such as fragmentation, burning, comingling of remains, and prolonged burial [3]. Some of these factors only damage the structural integrity of the tissue while viable DNA may still be attainable [4]. However, in the event of extreme tissue damage (eg. casualty events such as bush fires, structural fires, or bombings) even the DNA in skeletal elements can become contaminated or degraded. Following the September 11, 2001 World Trade Centre attack, DNA analysis was considered invaluable for identification as remains were fragmented, making identification from dentition alone almost impossible [5,6]. Similarly, during the 2009 Victoria bushfires forensic scientists took post-mortem DNA samples and compared them to ante-mortem samples from living individuals with missing relatives [7]. To obtain a usable DNA profile in cases like these there are many considerations involved in the process (Fig. 1) for example: which bones should be prioritised for maximum DNA yield, what DNA extraction process should be adopted to minimise contamination and inhibition, and what technique should be used for downstream analysis [8].

Over the past few decades, the methods used to obtain genetic profiles for identification of casualties of war and missing persons have

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improved dramatically [9–12]. With modern techniques providing more accurate results and becoming less labour intensive and less time consuming the ability to successfully investigate degraded and compromised remains genetically is becoming more likely. However, skeletal remains affected by incineration remain a challenge. An increased understanding of the changes that occur in burned bone and the potential implications to successful genetic analysis of these tissues will increase the potential to successfully identify individuals even when the remains are severely damaged. This paper aims to collate all currently available information with regards to the changes that occur in burned bone, the potential implications these changes have to successful genetic analysis, and current techniques and their limitations for this sample type.

2. Sample collection

2.1. Bone diagenesis

To improve the likelihood of success the practitioner must select which skeletal element to sample based on likely DNA yield. It has been suggested that bone density influences DNA yield, as denser tissues provide greater physical protection against damage [13,14]. For this reason, DNA is often sampled from teeth, which are protected by hard enamel [15–17], or dense, weight-bearing long bones (tibia or femur) [18,19]. Although some studies have suggested small skeletal elements of the distal skeleton (tarsal and carpal bones) can provide higher quantities of DNA than other skeletal elements [20–22], this is unlikely to be the case with incinerated remains. Bones of the hands and feet are often completely destroyed or lost during incineration due to their small size and the lack of soft tissues and density that affords significant protection to larger bones [23]. Rapid moisture loss during exposure to heat causes contraction of flexor muscles, which results in the characteristic pugilistic “boxers” pose seen in incineration victims [23]. This contraction also results in subsequent fracturing of the distal metacarpals and proximal phalanges, meaning these bones are rapidly affected by incineration and less than optimal for DNA sampling. The petrous (or petrosal) portion of the temporal bone has a greater density than many other skeletal sites and has been shown to yield much greater quantities of useable DNA, in some cases between four and 16 times more than teeth [24–28]. Use of the petrous bone as a source of DNA has largely been restricted to ancient remains, and potential uses in forensic cases and with incinerated remains have not yet been fully investigated. Research comparing DNA quality and quantity between different skeletal elements (e.g. long bone compared to petrous bone) under the influence of incineration would yield a better understanding of which bones should be prioritised for increased efficiency of DNA sampling in these cases.

Although this paper focuses on the effects of burning, it is important to note that in forensic case work skeletal samples are often exposed to multiple taphonomic effects, sometimes concurrently. Following (or prior to) incineration bones may also be subjected to various other forms of destruction and degradation, including animal predation, weathering and burial. DNA is preserved differentially across different skeletal elements- in fact differential DNA yield has even been found between different regions of the same bone (i.e. low yield femoral diaphysis vs. high yield epiphyses) [29], although the reasons behind these differences are not yet fully understood. Bones exposed to the elements can be subject to diagenesis which Collins et al. [30] break down into three pathways: 1) chemical degradation of organic bone material, 2) chemical deterioration of bone minerals and 3) invasion of microbes. These processes both increase the likelihood of contamination with exogenous DNA and environmental contaminants and decrease the organic content of the bone, resulting in a lower yield of viable DNA [30,31]. Weathering can be defined as chemical and mechanical deterioration and destruction occurring over time, the rate and severity of which is influenced by factors such as bone size, species and burial conditions [32]. Whether there is a direct correlation between bone appearance and likely success of genetic analysis remains debated. Misner et al. [33] investigated the correlation between the physical appearance of weathered bone and subsequent mitochondrial DNA quality and quantity. The authors concluded that the degree of visible bone weathering, although likely to reduce sample availability, had little effect on the DNA with no significant difference between differentially preserved samples.

Many studies have linked bone colouration and texture following fires to different burn durations and temperatures [34–42]. Walker et al. [43] used imaging software to generate average colour values for bone samples subjected to selected temperatures and durations of fire and concluded that collagen content could be estimated from bone colour. Fredericks et al. [44] compared bovine DNA yield following differential burning to bone colour using handheld digital colorimeters. This study confirmed that bone colouration could be linked to the successful (or unsuccessful) amplification of DNA. However, assessing bone colouration is a) somewhat subjective, and b) beholden to other variables besides heat. Additionally, uncontrolled burning, as occurs in disasters and industrial or domestic events does not occur at a constant temperature and hence will not necessarily mimic trial situations.

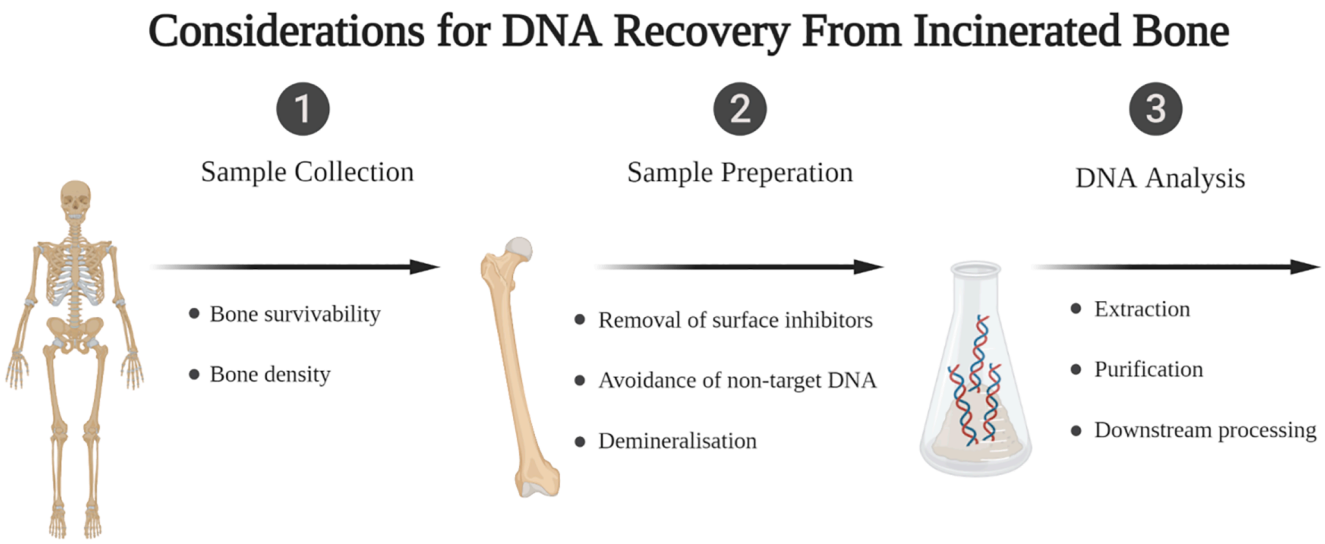

Fig. 1. Flowchart visualising the process of DNA recovery. Figure created with BioRender.com.

2.2. Microscopic and macroscopic bone analysis

Prior to subjecting a sample to DNA extraction, the likelihood of success may be able to be assessed to some degree using microscopic and macroscopic observations. As presence of nucleated cells and state of tissue osteons can be directly linked to DNA content quantifying these factors can give some indication of the likelihood of obtaining viable endogenous DNA [15,45–48]. Castillo et al. [49] assessed changes to the histological structure of bone following burning and found that at 300–400 °C all organic material disappeared, and crystalline structures began to form. Brain [50] described similar cellular changes in bone following differential burning. Although Brain did not investigate the presence of nucleated cells, the breakdown of other structures mentioned (carbon accumulation in lacunae, cracks appearing in the bone matrix, fusion of hydroxyapatite crystals) must also be considered, as they are likely indicators of DNA degradation.

In particular, the formation of hydroxyapatite crystals following burning may have implications for DNA extraction – if crystal aggregates in modern burned bones form in the same way as in diagenetically altered ancient bone, demineralisation techniques designed to extract endogenous DNA from these tightly-bound aggregates could be useful in forensic cases. This has been investigated to a degree using a combination of X-ray diffraction (XRD) and scanning electron microscopy (SEM) to compare average size of bone hydroxyapatite crystals between differentially preserved samples. The average size of hydroxyapatite crystals in human and bovine bone and teeth has been shown to increase exponentially at temperature increments above 500 °C [51].

Bone collagen content is another factor often considered directly linked to DNA yield, particularly in ancient remains [52]. Collagen diagenesis within ancient bone has been studied, however the mechanisms by which this process improves DNA survivability are somewhat unclear. One theory is that as collagen becomes mineralised, DNA binds to the positively charged hydroxyapatite calcium ions, which stabilises DNA and protects it from degradation [53,54]. Others have stated that mummification of individual osteocytes protected from contaminants by the small size of bone pores determines survivability of DNA in bone [55]. In regard to incinerated bone, studies have shown collagen is denatured at around 155 °C, and all traces of organic material are lost by approximately 400 °C [56,57]. However, the possible correlation between heat-induced collagen degradation and DNA loss has not been investigated. This is inherently difficult to inspect as DNA extraction techniques are not designed to target DNA within specific individual elements of the bone (e.g. hydroxyapatite crystals, mummified osteocytes), but rather all DNA within a sample. In forensic cases, it may be more beneficial to establish a means of assessing likely DNA content from gross morphology (e.g. bone colour, collagen content) to estimate the likelihood of collecting a usable sample. Theoretically, the same principle could be applied to hydroxyapatite crystal size using simple techniques such as SEM or XRD (X-Ray Diffraction). To do this the correlation between burn temperature, hydroxyapatite crystal size and DNA yield would have to be investigated.

3. Sample preparation

3.1. Tissue sampling

When working with incinerated remains samples cannot simply be cut from the bones' surface and used in the same way as an uncompromised soft tissue sample but must be processed in a manner which reduces contaminants whilst maintaining sufficient material for DNA extraction [58]. Finding viable sampling sites is especially difficult in burned remains as the loss of organic bone components at high temperatures can decrease bone size by 20% [59–61], rendering the remaining material warped and shrunken [34]. During extraction, improper handling and sample storage can lead to cross-contamination of target DNA with non-target DNA, which is often the case in mass disaster situations where inexperienced personnel are involved in sample recovery [62–64]. Additionally, environmental inhibitors such as bacteria, fulvic and humic acids, tannins, phenolic compounds, and ions can result in failure of downstream applications [65]. This can be avoided by thoroughly cleaning the bones' surface before extraction, after which all downstream processes should be performed in a dedicated laminar flow cabinet using UV-treated tools [66].

Cleaning commonly involves physical removal of the surface layer via sanding or drilling, followed by gentle rubbing with ethanol and/or sodium hypochlorite (NaOCl) to destroy DNA on the newly exposed bone surface [66,67]. Using this method, charring to the bones' exterior can sometimes be removed to expose less affected surfaces. However, it is often unclear how deep burning has penetrated the bone, and if the sample is entirely calcined the bone may be too fragile to survive this process. A clean sample must be pulverized to maximize surface area to volume ratio for successful release of DNA during extraction [68]. Collection of powdered bone may be achieved by drilling provided no heat is generated [69] as heat can further degrade any surviving DNA. The use of NaOCl, although effective for removal of DNA contamination, can also destroy targeted endogenous DNA. Korlević et al. [70] found that NaOCl treated samples contained significantly less DNA contamination, however 63% of the target DNA was also lost in the process. Furthermore, Dissing et al. [71] investigated the migration of ClO- ions from decontaminating agents into the tooth pulp cavity, and the subsequent effects on DNA yield using a 2% NaOCl solution to soak teeth for different durations (5 min and 30 min respectively). Results showed ClO- ions did indeed migrate into the tooth interior when applied to the enamel surface, but authentic DNA was still recoverable and exogenous DNA successfully removed if the duration of NaOCl exposure was limited to 5 min. Although not thoroughly tested using bone tissue, due to the increased porosity of its internal structure, migration of oxidizing ions into the bone matrix would likely be increased resulting in a greater loss of target DNA. Thus, submerging bone in NaOCl for prolonged durations may not be the best technique for cleaning these types of samples, and simply rubbing the surface of the bone with NaOCl followed by immediate drying may provide better results.

3.2. Sample demineralisation

Demineralisation involves removing the inorganic component of the bone to liberate DNA containing cells bound or trapped in the mineral matrix [15,72]. If demineralisation is not complete unreleased DNA may be discarded during filtering and if calcium ions are co-extracted with target material they may bind to target DNA and/or necessary reagents and inhibit polymerase chain reaction (PCR) [73]. Tissue is routinely decalcified using acidic agents such as hydrochloric acid (HCl), RDO Gold or Ethylenediaminetetraacetic acid (EDTA) [47,74]. Choi et al. [75] compared the outcome of demineralisation using the aforementioned agents for histomorphologic and DNA analyses, and found EDTA best preserved genetic material, a claim supported by many similar studies [46,47,76,77]. The method of demineralisation may require modification depending on the specific sample. In cases involving degraded material, total demineralisation has proven the most effective technique for achieving high DNA yields from bone and tooth samples [72,78–80]. Total pulverization (e.g. via cryogenic grinding) has shown to increase the rate of demineralisation and digestion of bone [81]. The advantage of total sample pulverization has been demonstrated in many studies; Rohland & Hofreiter [82] found the fine powder samples yielded significantly more DNA. Similarly, Sweet and Hildebrand [83] found cryogenically pulverising teeth to access the DNA-rich centre was faster and more effective than other methods of sampling and significantly reduced contamination.

Demineralisation is critical for ancient bone, as these tissue types are extremely petrified, and the breakdown of hydroxyapatite takes much longer than in fresh tissues [84] – it is not known if this is also the case for incinerated bones. Much like burned bones, ancient samples contain

little organic material to house DNA, and what remains is bound to dense crystal aggregates of hydroxyapatite [71]. Although liberating DNA from these aggregates can be challenging, the crystalline structure is extremely resistant to degradation and contamination, providing excellent protection to the DNA even from common oxidizing agents such as NaOCl and inhibitory exogenous material [85].

The process of total demineralisation involves submerging the skeletal sample in a chelating solution such as EDTA to remove the mineralised component before incubation in a lysis buffer. In some cases, multiple EDTA washes have been used to encourage complete dissolution of the bone. Discarding the wash solutions is considered to reduce contaminants [86,87]. However, Loreille et al. [72] hypothesised that in some circumstances discarding EDTA supernatant resulted in DNA loss. A solution to this was to integrate EDTA into the lysis buffer, eliminating the EDTA washing step and maintaining reagent volume and concentration, as well as providing 4.6 times more DNA on average than older protocols. Loreille's total demineralisation protocol is generally considered one of the most effective methods of DNA extraction from challenging bone samples. With regards to incinerated bone, however, a recent study [88] comparing the effectiveness of this protocol to the incomplete decalcification protocol described by Dabney [89], found that the later improved recovery of shorter DNA molecules (<185 bp).

Although most research agrees that ancient/diagenetic bones require prolonged, total demineralisation, notably less information exists regarding treatment of burned bones. A study by Ye et al. [58] indicated that as with ancient bones, an adapted EDTA extraction protocol could improve DNA extraction from charred bones. A major adaptation to the standard extraction protocol was the duration of EDTA demineralisation. The length of time required to completely demineralise a sample can be a trade-off between maximising the quantity of DNA extracted over the throughput of samples [90]. In cases where samples are few and precious (for example ancient bone), an over-conservative approach is justified. This is not always the case in forensic and disaster victim identification situations, where fast results are often required for a large number of samples [4]. In these cases, demineralisation protocols may require re-analysis. Remains subject to extreme trauma, for example burning, are likely compromised in a similar way to ancient bone, with decreased organic components although this has not been thoroughly investigated [34,91].

4. DNA extraction

Following demineralisation DNA extraction is required. A majority of research concerning burned bone DNA extraction is centred around collection and demineralisation of samples. Far less attention has been focused on subsequent extraction processes. All extraction techniques follow the same basic process: DNA is separated from cellular debris, isolated and purified. This can be achieved using phenol/chloroform, whereby the sample is suspended in a mixture containing Dextran Blue, acetate and EtOH, however due to the use of toxic chemicals (and incomplete inhibitor removal), this method has become obsolete for many samples in forensic casework [92]. A different class of extractions is solid-phase DNA extraction, most of which use silica-based binding that can double the amount of useable DNA recovered [93]. Unlike organic methods, silica-based techniques rely on a simple bind, wash, elute process [94]. The DNA is denatured, then binds to silica particles that can be separated from inhibitors and non-target material [95,96]. This method is quicker, easier, more cost efficient and better for removal of DNA inhibitors than other methods, therefore is especially useful in low-copy DNA contexts [68]. The advantages of silica-based methods has been thoroughly examined for ancient and modern degraded samples. A study by Pajnič et al. [97] presented an optimised silica-based protocol for extracting DNA from challenging ancient and contemporary remains. Although two burned femurs were included in this study, it could be expanded with the inclusion of a series of incinerated bones burned under comparable conditions. As it stands, there are no well-established, routine methods of extracting DNA from incinerated modern bone.

There are two general approaches to silica-based extraction; suspension in a silica solution and kit-based binding to silica beads or membranes. Silica suspension is a common alternative to silica binding that allows for processing of substantially larger sample volumes than commercial kits [82]. This is a necessity for aDNA extraction where large sample volumes containing small quantities of DNA are processed in batches. However, Dabney et al [89] states that the efficiency of this method is dependent on fragment length, with a strong bias towards longer fragments. In situations where only short fragments are available (as is often the case in forensic casework) using commercially available kits in place of silica suspension may provide better results. These kit-based methods are also preferred by forensic professionals as they are simple to set up, easily automated, and validated for use. Two of the main silica-binding methods are described in Qiagen kit user manuals [98,99] which are presented in Fig. 2.

Rothe and Nagy [65] compared these two techniques and outlined the advantages and disadvantages of both. Although DNA yield was significantly greater using the membrane method compared to the beads, co-extraction of inhibitors was also a much greater problem. Wen et al. [100] suggests that capillary-based monoliths may be more effective again than commercial spin column techniques with an efficiency of 86% compared to 49% respectively. However, this was not the case for larger sample volumes, and the lack of device stability and reproducibility suggest that commercial technologies such as spin columns are the best option at present [100,101]. When working with ancient DNA demineralised EDTA supernatant is generally discarded prior to extraction due to the high likelihood of co-extracting contamination and inhibitors [72,86,87,97]. However, it is not unlikely that this could vary in burned samples. Ancient bone contains virtually no exogenous DNA and there is very little risk of discarding targeted material in the supernatant. Comparatively, burned samples may still contain exogenous DNA in addition to crystallite-bound endogenous material, therefore co-extracting demineralised supernatant may actually improve DNA yield. Although this could increase the likelihood of contamination, it is a potential trade-off that should be more thoroughly investigated in future research.

5. Areas for expansion

Extracting DNA from (relatively) fresh modern bone tissue is a well-established process; large companies supplying DNA equipment and consumables (e.g. Qiagen) will generally provide clear protocols for using these sample types. Furthermore, the protocol for extracting DNA from ancient samples is well standardised (i.e. prolonged demineralisation to liberate bound DNA). Interestingly no such protocol exists for modern burned samples, and most decisions on DNA analysis in these situations are made ad-hoc on a case-by-case basis. There is a growing need for identification based on these sample types; for example, many of the World Trade Centre remains were never identified due to DNA contamination from commingling of remains [4]. Similarly, authorities fear that many victims of the recent Grenfell tower fire in London may never be identified as remains were almost completely incinerated and DNA destroyed. As many residents were illegal immigrants, dental records were not available, and DNA was considered the only option for identification [102].

Knowing that certain regions of the skeleton provide more protection to DNA than others, research into differential DNA yield from non-standard sampling sites following extreme tissue damage is essential. If these non-standard sites (e.g. petrous bone, metacarpals) provide higher quality and quantities of DNA, standard procedures for DNA sampling during disaster victim identification and other mass casualty events could be re-examined and adjusted if needed. A study by Kulstein et al. [103] assessed different sites on the skeleton by their DNA content, however this same analysis has not been conducted on incinerated

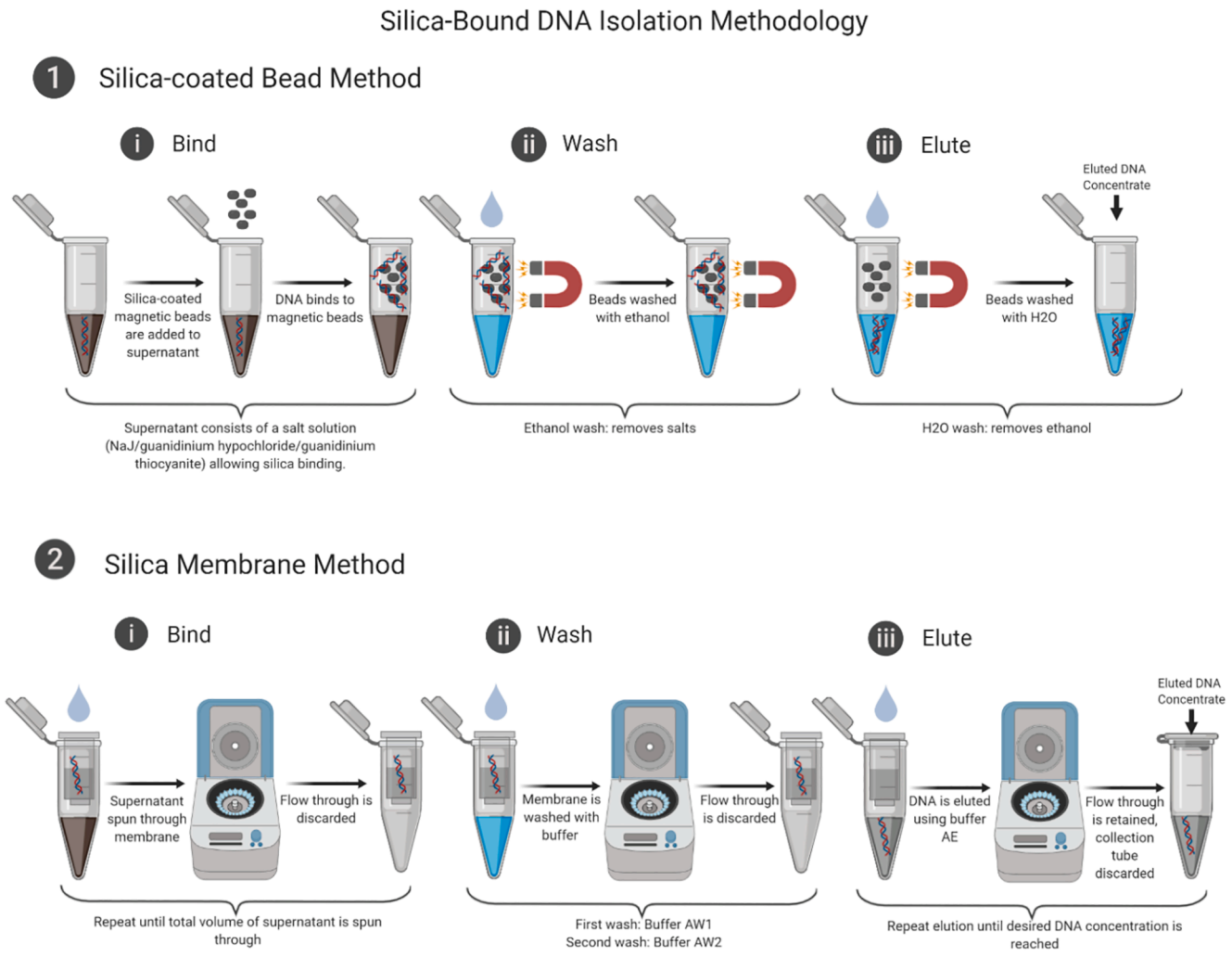

Fig. 2. Flowchart comparing silica-based methods of DNA extraction. Figure created with BioRender.com.

remains. If a graded system for DNA yield from incinerated bone was established, this system could be used to prioritise sampling procedures based on likely presence of DNA. Additionally, downstream DNA processes could be refined to maximize DNA yield, minimize time used and optimise accuracy of identifications. DNA analysis in human identification has greatly advanced and is often considered the gold standard in forensic science. Many studies have already presented strategies for improving DNA sampling and extraction from degraded skeletal material [104–107], however due to the differences between diagenetic and incinerated bones, these techniques may not be applicable. This suggests a need for further study applying some of these conservative extraction techniques to incinerated samples.

Many conclusions made in the existing literature are formulated from "best case scenario" research, using pristine bone samples prepared in the laboratory under controlled conditions. However, this somewhat defeats the purpose of such research – in a realistic situation, if bones are well preserved and uncontaminated, there is usually equally well preserved soft tissue, which is much easier to sample for DNA analysis. In this case, what is needed is a better understanding of DNA retrieval under less than ideal circumstances, so the effect of these circumstances (e.g. exposure to extreme heat) can be studied. A possible objective for future work would be the establishment of standards to estimate likely DNA yield based on simple tests such as ATR-FTIR spectroscopy, x-ray diffraction and scanning electron microscopy [108]. This could also be addressed by investigating whether the general macroscopic appearance (e.g. colour, texture, size) of incinerated bones can be indicative of DNA survivability, and whether various skeletal sites provide different quantities of DNA. It is apparent that a multi-dimensional, multi-disciplinary approach is required to understand DNA on different levels – not just DNA extraction and amplification, or histological analysis or gross anatomy, but rather a combination of these techniques to establish the most efficient and reliable way of sampling DNA from burned remains.

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CRediT authorship contribution statement

Meghan Mckinnon: Conceptualization, Formal analysis, Investigation, Writing - original draft. **Maciej Henneberg:** Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Denice Higgins:** Conceptualization, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 3: DNA Extraction from Incinerated Bone

3a. Validation of porcine primers

3a.i Introduction

Human DNA extraction and amplification assays are well developed and readily available, however accessing human tissue for forensic research can be difficult. By comparison, animal tissue is easy to source but there is significantly less published research and few commercially available DNA kits. Before DNA extraction and amplification can be conducted species-specific primers must be developed, their thermocycling conditions determined, and their general effectiveness tested. As the use of porcine DNA is most common in agricultural research and the food industry, pre-existing primers for domestic pigs are all mitochondrial due to the multi copy nature and increased robustness of mtDNA. For this reason, prior to the commencement of candidacy, two porcine nuclear primer sets (Table 3.1) were developed by my primary supervisor (DH). Upon starting my candidacy, I tested these primers, created standards and developed standard curves to allow me to accurately quantify DNA extracted from porcine bone. I then used these primers to test DNA extraction methods as documented in the study presented in part b of this chapter.

Table 3.1. Summary of porcine primer sets developed for use in this study.

	Sequence (5'-3')	Length	Tm	GC%	Self complementarity	Product Length
Primer pair 1 (long)						
Forward	CTCTGACCTGAGTCTCCTTT	20	55.89	50.00	5	74
Reverse	CAAACACGAGAAAGACTCCA	20	55.64	45.00	3	
Primer pair 2 (short)						
Forward	CTCTGACCTGAGTCTCCTTT	20	55.89	50.00	5	150
Reverse	CGGCTTTGTCACACGAG	17	55.94	58.82	5	
Details:	Sus scrofa isolate TJ Tabasco breed Duroc chromosome 3, Sscrofa 11.1, products associated with cytoplasmic actin 1					

3a.ii Methods

Quality control testing of the developed primers began with extraction and standard PCR amplification of DNA from fresh porcine tissue. A porcine long bone still encased in flesh was obtained from a local butcher. The surface dermal layer was removed, and a small section of tissue was collected using sterile forceps and a scalpel. Unlike later extractions involving bone tissue, no decalcification step was required. A standard Qiagen DNeasy Blood and Tissue kit was used to extract DNA with the following protocol:

1. Add 40 µl of proteinase K and 160 µl of Buffer ATL to 2 ml tube containing sample
2. Incubate tube at 55 °C overnight using a thermal shaker
3. Centrifuge at 10,000 rpm for 5 min to pellet any remaining undigested material
4. Remove 1 ml of supernatant to new 5 ml tube, add 1000 µl AL buffer
5. Vortex for 15 s and add 1000 µl Ethanol (100%)
6. Vortex for 15 s (critical to avoid formation of precipitate)
7. Pipette 650 µl of solution into DNeasy spin column in 2 ml collection tube
8. Centrifuge at 8000 rpm for 1 min, discard flow-through (hazardous) and reuse collection tube
9. Repeat steps 7-8 until entire sample spun through
10. Place DNeasy spin column in new 2 ml collection tube, add 500 µl Buffer AW1 and centrifuge for 1 min at 8000 rpm. Discard flow-through and collection tube.
11. Place DNeasy spin column in new 2 ml collection tube, add 500 µl Buffer AW2 and centrifuge for 3 min at 14,000 rpm to dry DNeasy membrane. Discard flow-through and collection tube.
12. Place DNeasy mini-spin column in clean 1.5 ml microcentrifuge tube and pipette 100 µl Buffer AE directly onto DNeasy membrane.
13. Incubate at room temperature for 10 min, then centrifuge for 1 min at 8000 rpm to elute.
14. Transfer DNA extract to clean tube, make a 30 µl working aliquot.

Extractions were performed in a dedicated low-copy laboratory and tools and equipment were regularly sterilised in a UV cabinet.

A standard PCR was performed on the fresh DNA extracts using an Eppendorf® thermal cycler; contents of the PCR master mix and thermocycling conditions are presented in table 3.2. Two master mixes were created, one using the short amplicon reverse primer and another using the long amplicon reverse primer.

Table 3.2. Summary of Plat Taq HiFi Master Mix components and PCR thermocycling conditions.

Plat Taq HiFi Master Mix		**Thermocycling Conditions**	
Setup:	Volume (µl):	Step	Duration/temp
ddH2O	14.275	**Denaturation:**	94 °C hold 2 min
HiFi buffer 10 x	2.5	**x30 Cycles:**	
RSA (10 mg/ml)	2.5	1. Denaturation	94 °C hold 15 s
MgSO4 (50 mM)	1.0	2. Annealing	56 °C hold 15 s
Primer L	1.0	3. Extension	68 °C hold 45 s
Primer H	1.0	**Extension:**	68 °C hold 10 min
dNTPs (10 mM each)	0.625		
Plat Taq HiFi (5 U/µl)	0.1		
Extract	2.01		
TOTAL VOLUME (per sample):	25.0		

A high sensitivity DNA assay was then used to test for specificity of the amplified product and presence of primer dimer. This was achieved using an Agilent 2100 Bioanalyzer (2021 Agilent Technologies, Inc.), which uses capillary electrophoresis in a chip-based format allowing for largely automated analysis of DNA. The Bioanalyzer is ideal for high resolution analysis of small sample volumes, although the limited sample throughput (12 samples per chip/cartilage) and high cost can be a major drawback. This technology was ideal for my pilot study as only two primer products (four replicates for each) were investigated for downstream applications. The resulting electrophoresis assay was well resolved, with bands matching in each replicate and no evidence of non-target amplification (Fig. 3.1). Similarly, the electropherogram showed fragments were of the expected length and non-target peaks were negligible (Fig. 3.2).

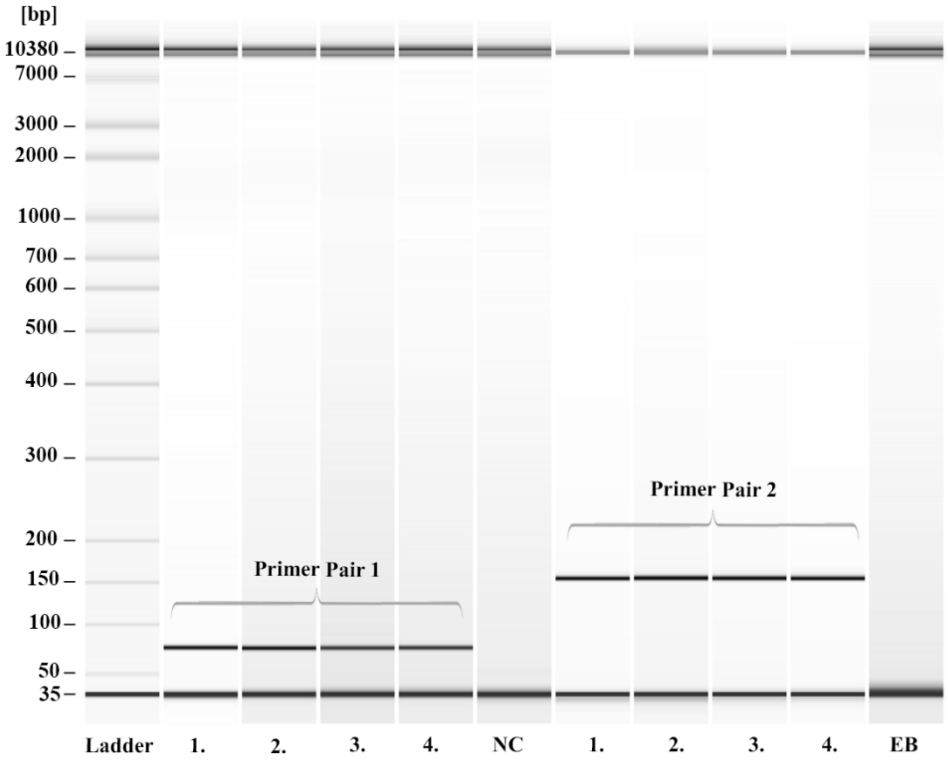

Fig 3.1. High sensitivity DNA electrophoresis assay. In addition to sample replicates (n=4 per primer set), negative controls (NC) and extraction blanks (EB) were included.

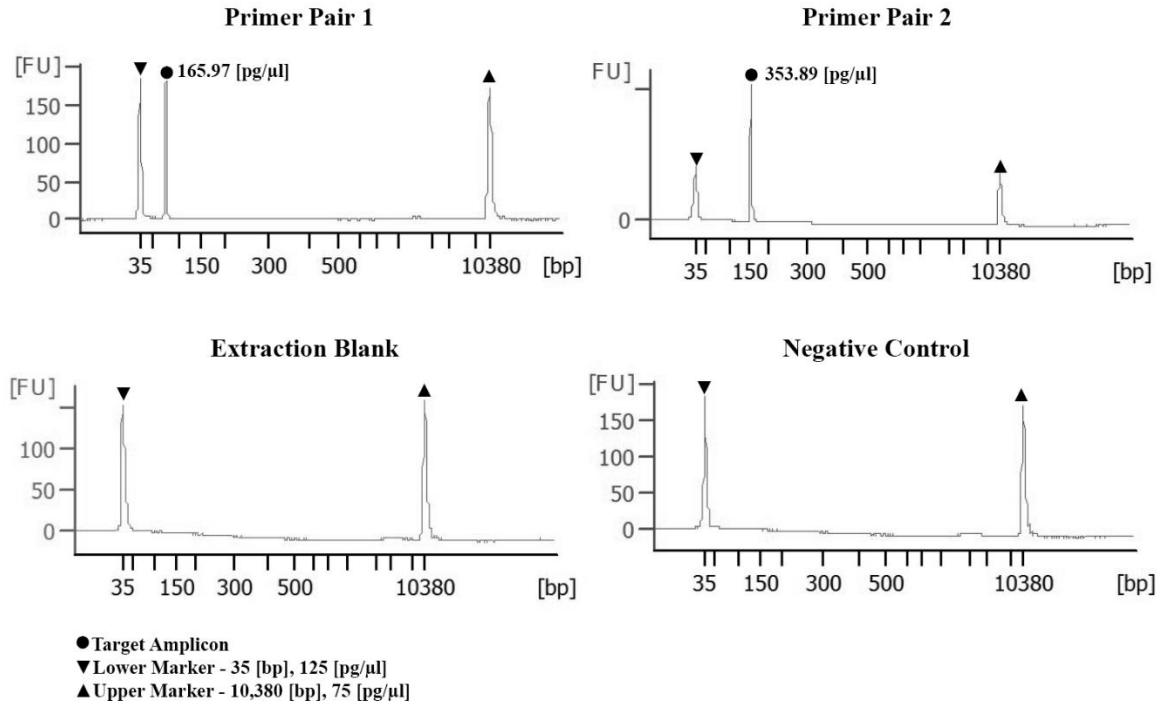

Fig 3.2. High sensitivity DNA electropherograms for both long (primer pair 1) and short (primer pair 2) amplicons.

Post-PCR product was then purified using AMPure paramagnetic beads (Argencourt Bioscience Corporation), which selectively bind to post-PCR DNA fragments, allowing for non-target material such as nucleotides, enzymes and excess primers to be removed using simple washing. A Qubit 2.0 fluorometer was subsequently used to determine DNA concentration of the stock solution. Melt-curve analysis was conducted on the purified product using a Rotor-Gene 3000^{TM} real time thermal cycler (Corbett Research) to determine optimal thermocycling conditions. Melt curves for each primer pair were well resolved, and gradient qPCR between 50-60 °C showed 56 °C was the optimal amplification temperature (Fig 3.3). Peaks corresponded to 80.3 °C for amplicon 2 (primer pair 2) and 83.0 °C for amplicon 1 (primer pair 1).

Step	Duration/temp
Denaturation:	94 °C hold 2 min
x35 Cycles: **1. Denaturation** **2. Annealing** **3. Extension**	 95 °C hold 30 s 56 °C hold 30 s 68 °C hold 45 s
Extension:	60 °C hold 10 min
MCA:	66-96 °C, 3 °C/s hold 5 s

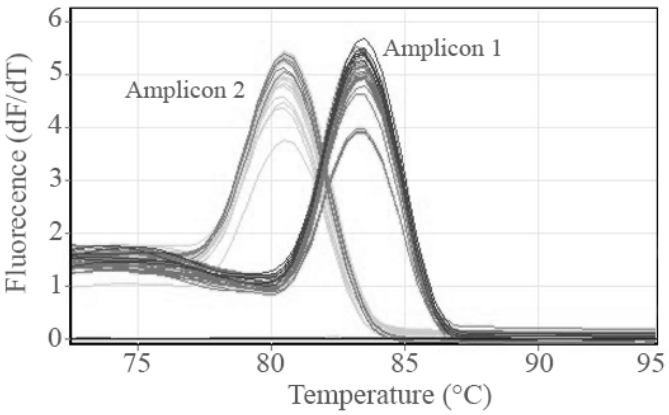

Fig 3.3. Optimal thermocycling conditions with gradient melt curve analysis step. Subsequent figure shows cumulative melt peaks for both amplicons.

After establishing concentration and thermocycling conditions, a standard curve was created using purified extract, which was diluted in series (Fig. 3.4). The standard curve was then used to compare amplification profiles of unknown samples to those of known concentration, along with extraction blanks and negative controls.

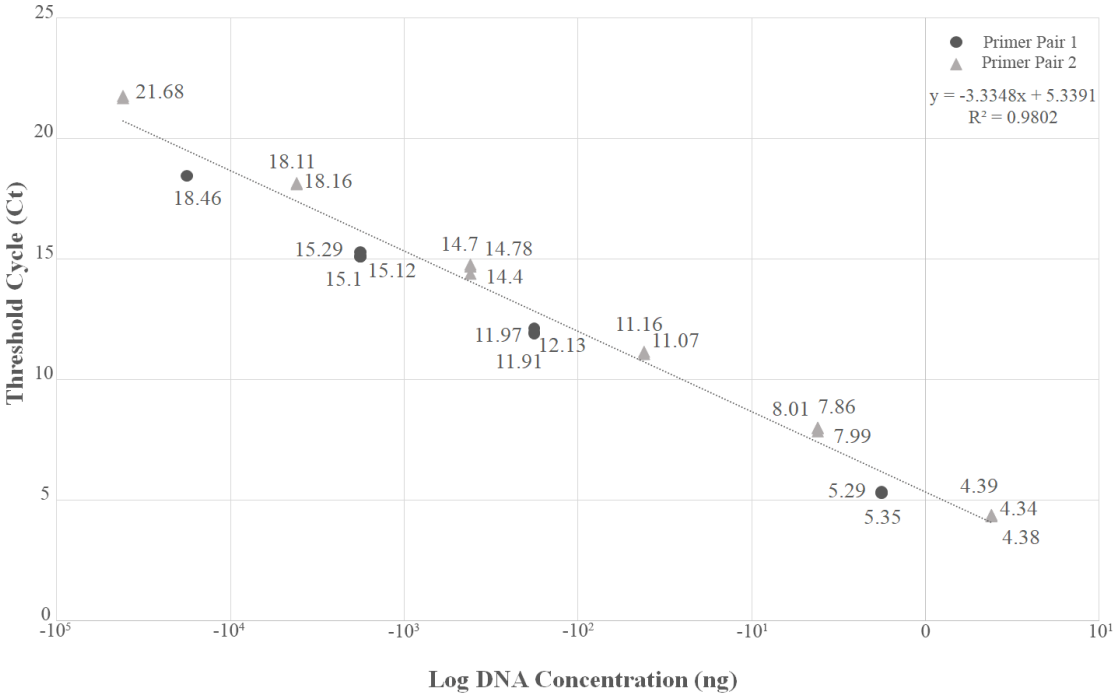

Fig 3.4. Line of best fit representing logarithmic decrease in DNA concentrations at increasing dilution. Each standard dilution was run in triplicate however, some data were excluded from the standard curve as not all dilutions were included in the final run.

3a.iii Concluding statement

From this analysis the developed primers were deemed fit for purpose and implemented in the next study comparing demineralisation procedures for DNA extraction.

3b: Comparison of Bone Demineralisation Procedures for DNA Recovery from Burned Remains

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Overall percentage (%): 90%

Certification: This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

Signature & Date: __ _____

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Co-Author: Denice Higgins (secondary author)

***Contribution to the paper**:* Conceptualization, methodology, investigation, resources, writing – review & editing, supervision, project administration, funding acquisition.

Signature & Date: __ _____

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Research paper

Comparison of bone demineralisation procedures for DNA recovery from burned remains

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ABSTRACT

Recovering DNA from modern incinerated bones can be challenging and may require alteration of routine DNA extraction protocols. It has been postulated that incinerated bones share some similarities with ancient bones, including fragmented DNA, surface contamination and highly mineralised structure, all of which can inhibit the successful recovery of genetic material. For this reason, ancient DNA extraction protocols are often used for incinerated modern samples; however, their effectiveness is still somewhat unclear. Much of this uncertainty exists around the demineralisation step of extraction, specifically the length of incubation and retention or removal of supernatant. As obtaining human samples for forensic research can be challenging, porcine models (*Sus scrofa domesticus*) are often used as substitutes. This study developed real time PCR assays for porcine nuclear DNA in order to investigate the effects of modified demineralisation protocols on DNA yield from femurs exposed to either short (60 min) or prolonged (120 min) burning. Gradient PCR results indicated 56 °C was the ideal amplification temperature for targeted amplicons, with melt curve analysis showing short and long amplicons corresponded to 80.3 °C and 83 °C peaks respectively. Results of altered extraction protocol showed a trend towards higher DNA yields from longer demineralisation periods however this was not significant. By comparison, retaining supernatant post-demineralisation resulted in significantly greater DNA yields compared to discarding it ($P < 0.009$). Although DNA content yield decreased with burn duration, the demineralisation treatment variations appeared to have the same effect for all burn lengths. These results suggest that for incinerated modern bone retaining the supernatant following demineralisation can dramatically increase DNA yield.

1. Introduction

Post-mortem human remains may be subjected to a wide variety of physical and environmental influences resulting in differential preservation, particularly in large-scale disasters [1]. These influences result in fragmentation, burning, extreme weathering, contamination and comingling of remains [2], with variable impact on DNA content. For victims of forest, structural and vehicular fires sampling of hard tissue may be the only way of obtaining viable genetic material for identification, as soft tissues are usually compromised or unavailable [3,4]. As DNA in bone is intimately associated with the mineral structure demineralisation has been shown to be a vital step in liberating DNA from highly degraded samples [5–8]. Demineralisation involves decreasing the activity of inorganic bone calcium (Ca^{2+}), breaking down the mineral structure to liberate DNA trapped within the crystal bone matrix [9]. As calcium can inhibit polymerase chain reaction (PCR) its removal will also enhance downstream processes [10]. Choi et al. [11] compared the outcome of demineralisation using various acidic and chelating agents for DNA recovery, concluding that EDTA best preserved genetic material. This finding has been supported by many similar studies [12–15].

For ancient bone samples, total demineralisation utilising prolonged incubation periods (>12 h) has been shown to be the most effective technique for maximising DNA yields [5–8]. However, this may not be ideal for all samples, and demineralisation procedures may require modification depending on sample preservation. Rohland & Hofreighter [16] suggest that prolonging demineralisation for ancient samples does not always increase DNA typing success, but state that it can decrease the amount of undigested bone which may improve DNA yield.

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Prolonged demineralisation has been shown to be critical for ancient tissues that are highly mineralised, and contain virtually no DNA outside the protective hydroxyapatite crystalline lattice [17]. By comparison, modern samples may not require demineralisation as there is usually sufficient cellular material to provide DNA that is not bound to hydroxyapatite. For this reason, modern hard tissue DNA protocols (e.g. QIAamp DNA Mini Kit by Qiagen) do not include a demineralisation step. Modern burned bone, however, is believed to present many of the same structural and compositional changes as diagenetically altered bone, therefore DNA contained within this tissue may behave in a similar manner to ancient samples [18]. Not only is there debate over demineralisation duration, but whether or not the supernatant should be retained has also been questioned [19]. For ancient samples the supernatant is often discarded to ensure removal of inhibitory material and exogenous DNA contamination that may be present [20,21]. However, for modern bone samples where DNA is also contained within cellular components, a large amount of target endogenous DNA may also be discarded with the supernatant [8]. Due to the current lack of established protocols for modern burned remains, sampling and demineralisation is often performed ad-hoc, which can be particularly problematic in high-throughput situations where samples are limited in size and number and must be processed quickly (i.e. mass disasters).

Due to ethical restrictions on the use of human samples, animal models are often used as an alternative. Domestic pigs (Sus scrofa domesticus) are a common substitute for humans due to the similarity in body size and bone composition [22–24]. Furthermore, the genome of pigs is relatively similar to humans, which makes them viable substitutes for DNA based research [22]. In this study we developed PCR assays targeting porcine specific DNA and investigated demineralisation duration and supernatant retention or disposal on nuclear DNA (nDNA) yield from porcine bones exposed to temperatures >100 °C. We compared DNA yield from samples demineralised using a long incubation period (12 h), common in ancient DNA protocols, to a shorter (1 h) period. We also examined a third treatment group where, in addition to a shorter demineralisation period, the supernatant was retained.

2. Methods

2.1. Sample preparation

Three porcine long bones (encased in flesh) were exposed to an open fire, for either a short (60 min) or long (120 min) duration. No additional accelerants were used besides naturally occurring bush material. Bone powder was then obtained:

- Soft tissues removed using scalpels and forceps.
- Surface cleaned of any remaining tissue by alternate gentle rubbing with Isopropanol (IsoWipes) and 3% sodium hypochlorite solution.
- Bone sampled using a hand-held drill (pre-cleaned with 3 % sodium hypochlorite) fitted with a new 3.5 mm drill bit for each sample. Shavings collected on aluminium foil in a clean petri dish.
- Drill speed <100 RPM to reduce heat generation that could denature the DNA [25].
- 180 mg of bone shavings was obtained from each compact bone then divided into 9 × 2 ml tubes (Fig. 1).
- Samples stored at −20 °C immediately after drilling.

2.2. Silica column extraction

Extractions were performed in a dedicated low-copy pre-PCR lab in a fume hood. All surfaces and equipment pre cleaned with sodium hypochlorite. Extractions performed in triplicate (as was amplification and quantification). A negative control was included in each group.

For creation of qPCR standards DNA was extracted from fresh porcine soft tissue using QIAamp DNA Mini Kit following the manufacturer's instructions (Qiagen).

For bone samples, the same extraction process was used with the addition of a demineralisation step prior to digestion following Loreille et al. [8] with slight modification as described below:

- In a 2 ml tube 950 µl of 0.5 M EDTA was added to 20 mg of ground bone sample.

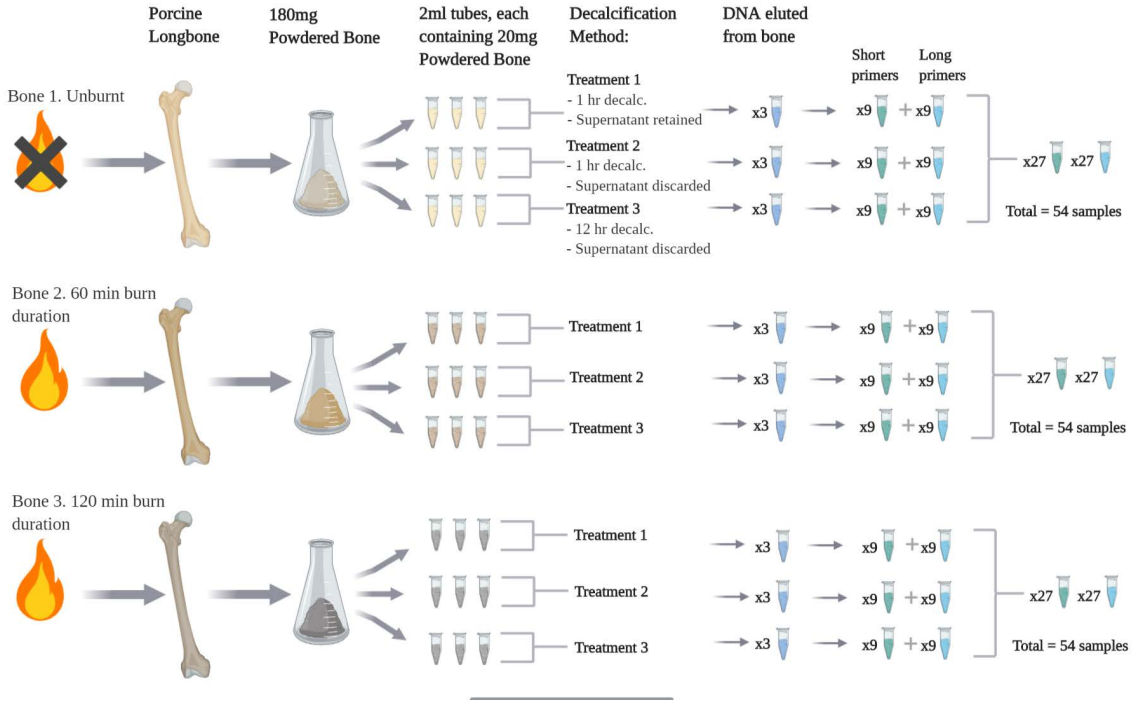

Fig. 1. Flowchart depicting the allocation of samples into treatment groups. Figure created with BioRender.com.

- Samples placed on a thermal shaker, incubated at 55 °C and continually mixed for;
  - 1 h (Qiagen standard incubation, treatment groups 1 & 2), or
  - 12 h overnight (prolonged incubation treatment group 3).
- EDTA supernatant was subsequently either:
  - Retained (treatment group 1), or
  - Discarded (treatment groups 2 & 3)
- Samples were digested using EDTA (950 µl), Proteinase K (40 µl), and Buffer ATL (160 µl).
- cRNA was added with Buffer AL (3 µl cRNA per 1000 µl Buffer AL).
- Samples were filtered through spin columns in multiple fractions of 650 µl.

2.3. Primer design and efficacy

A single forward and two reverse porcine specific primers were designed to target two amplicons (one short and one long) in a non-repeating area of the porcine nuclear genome (Table 1). Two amplicons were selected for the purpose of quality control. Along with effectively doubling the sample size, using two amplicons of different lengths mean that in cases of severe degradation, shorter fragments may still be detectable [26]. PCR primers were checked for specificity using the NCBI basic local alignment search tool (https://www-ncbi-nlm-nih-gov.proxy.library.adelaide.edu.au/tools/primer-blast/).

To avoid complications of multiplexing primers independent assays were developed for short and long amplicons. The optimal annealing temperature for each PCR assay was determined using gradient PCR. All reactions were performed with the Rotor-Gene 3000™ real time thermal cycler (Corbett Research). PCR cycling conditions were: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, primer extension at 68 °C for 45 s, with a final extension step at 60 °C for 10 min, followed by Melt Curve Analysis [MCA] (66 °C–96 °C, 3 °C/s). The specificity of primers to a single binding site was assessed using the post qPCR melt curve to visualize the dissociation kinetics. The assays were conducted in a 15 µl total reaction volume containing 0.5 µl of both the forward and reverse primer (10 mM), 0.5 µl Rabbit Serum Albumin [RSA](10 mg/mL) and 1 µl porcine genomic DNA extract. PowerUp™ SYBR™ Green Master Mix (Invitrogen™) made up the final 7.5 µl of the total reaction volume.

2.4. Creation of qPCR standards

Standards for each primer pair were created by amplifying DNA extracted from porcine soft tissue using an Eppendorf® thermocycler. A reaction volume of 25 µl was used, consisting of 14.2 µl H2O, 2.5 µl HiFi buffer, 2.5 µl RSA (10 mg/mL), 1.0 µl MgSO4 (50 mM), 1.0 µl forward/reverse primers (10µM), 0.6 µl dNTPs (10 mM), 0.1 µl Platinum® Taq HiFi (5U/µl) (Invitrogen™) and 2.0 µl extract. Post-PCR product was diluted to 1:10 concentration and run on a bioanalyser (Agilent High Sensitivity DNA kit, 2100 expert software). The high sensitivity DNA assay showed amplified sequences were identical in length to target amplicons. Most samples tested were free of primer dimer, and the few that were not, contained only negligible amounts which were removed using AMPure paramagnetic beads (Argencourt Bioscience Corporation, Beverly, Massachusetts). Neat control concentrations were quantified using a Qubit 2.0 fluorometer (InvitrogenTM), then diluted in series into six 45 µl standard aliquots (Table 2).

Table 1
Primer sequences used for amplification of target DNA in qPCR.

Primer Name:	Sequence (5'-3')	Primer length	Amplicon Length
Forward	CTCTGACCTGAGTCTCCTTT	20	
Reverse short (fragment 1)	CGGCTTTGTCACACGAG	17	74
Reverse long (fragment 2)	CAAACACGAGAAAGACTCCA	20	150

2.5. Quantification of samples

Sample extracts were run alongside both negative and positive (PCR standards) controls using both primer pair assays (short reverse primer and long reverse primer). For each assay 27 samples of unknown concentration were analysed, resulting in a total of 54 samples for each of the three burn groups (Fig. 1). Thermocycling was conducted using the optimised protocol described earlier, and Rotor-Gene software was used to apply the standard curve to the unknown samples. Absolute quantification values were collated and analysed in IMB SPSS statistics [27].

3. Results & discussion

The premise of this study centres around the comparability of archaic bone (defined here as deposited >200 years) to thermally altered bone, the assumption being that in both cases exogenous DNA is released during demineralisation whilst minuscule quantities of endogenous material will be bound to tight crystal aggregates of HAp [28]. Ancient DNA extraction techniques have been designed with this in mind and are optimised to remove external contaminants (by discarding initial supernatant) whilst liberating bound material (by prolonged demineralisation) [8,9]. Similar to ancient material, we expected a great deal of external contamination to be present in thermally altered bone. We predicted that like archaic bones, prolonged demineralisation would be required to liberate DNA, as bone becomes increasingly crystalline when heated making DNA less accessible [17,29]. The reality behind this is unclear, and previous studies have produced mixed results [19,30]. Although demineralisation is vital for dense, degraded and contaminated samples, there is some uncertainty on how this process should be conducted. The primary objective of this study was to optimize demineralisation techniques for maximum yield of nuclear DNA from thermally altered porcine bone – specifically EDTA incubation period length and impact of retaining demineralised supernatant.

3.1. Development of qPCR assays

Gradient PCR identified 56 °C as best temperature for targeted amplicons, amplification at this temperature resulted in highly specific, identical peaks for all triplicates. The short and the long amplicons corresponded to peaks at 80.3 °C and 83 °C, respectively, which were well resolved in the Melt Curve Analysis profile. The standard curves comprised a six-point, dilution series for each primer set, with each dilution level performed in three replicates. Reliable quantification was established for the range of input DNA shown in Table 2 per reaction, with an acceptable linear range (R2 = 0.0999). For both DNA targets the amplification was repeatable and reproducible over the three replicates.

Table 2
DNA fragment copies within neat standard (assessed using Qubit 2.0 fluorometery) and copies in subsequent serial dilutions.

Serial Dilution of Standards	Short fragment conc. (ng/µl)	Short Copies/µl	Long fragment conc. (ng/µl)	Long Copies/µl
Standard 6 (Neat)	2.4	60,100,000,000	5.6	69,200,000,000
Standard 5	0.24	6010,000,000	0.56	6920,000,000
Standard 4	0.024	601,000,000	0.056	692,000,000
Standard 3	0.0024	60,100,000	0.0056	69,200,000
Standard 2	0.00024	6,010,000	0.00056	6,920,000
Standard 1	0.000024	601,000	0.000056	692,000

3.2. Effect of DNA extraction protocol

In all burn states retaining the supernatant and demineralising for 1 h was the most effective technique, resulting in the highest number of fragment copies recovered by both assays (Figs. 2). Discarding the supernatant had the greatest impact on results, and substantially reduced the quantity of DNA recovered in both burned and unburned samples. Comparatively, demineralisation duration had little effect on the quantity of DNA recovered except in unburned samples, where a longer demineralisation resulted in a slightly improved DNA yield.

Our data was not normally distributed; hence a non-parametric approach was used. Analysis of variance shows significant differences ($P < 0.05$) in DNA yield between EDTA treatments in all burn groups with the exception of long fragments in the 60-minute burn group ($\chi^2 = 1.055$, $P = 0.59$). Post-hoc Mann-Whitney tests (Table 3) showed that within all burn groups there was no significant effect of incubation time on DNA yield when supernatant was discarded. In nearly all cases retaining the supernatant significantly increased DNA copies. Although not significant, a general increase of short (+16 %) and long (+45 %) fragment DNA was apparent where incubation was increased from 1 to 12 h (supernatant discarded). Comparatively, incubating for 1 h and retaining the supernatant significantly increased DNA yield when compared to the prolonged 12 h incubation and discard of supernatant (short = +91 %, long = +127 %) and when compared to 1 h incubation and discard of supernatant (short = +64 %, long = +56 %).

Overall, increased demineralisation duration (12 h) showed no improvement over the shorter duration (1 h). Whilst a longer demineralisation period appeared to perform better in unburned samples, this was not significant. In regard to disposal of demineralised supernatant, our results clearly show a significant improvement in DNA fragment yield from samples where supernatant is retained. Prado et al., 2002 explains that cellular lysis EDTA treatment causes defragmentation of the bone matrix and subsequent release of DNA to extracellular medium, which is then lost during subsequent washes. Prado et al. found that co-extracting supernatant with the undigested pellet ensured retention of usually discarded DNA. This aligns with the current study, where in some cases retaining supernatant more than doubled DNA fragment copies. In some situations, the bone pellet may be digested in its entirety (total demineralisation) to yield more DNA [8]. This method ensures all DNA within a sample is extracted, however with particularly degraded/dense samples this can be time consuming, and often results in co-extraction of surface contamination [5]. In such cases, disposal of undigested material may result in an unavoidable loss of DNA [31,16].

Table 3
Mann-Whitney test multiple comparisons showing specific relationships between each EDTA treatment within each burn group. Also included is the average percentage difference in DNA yield from one treatment to the other.

	12 h + discard vs. 1 h + discard		12 h + discard vs. 1 h + retained		1 h + discard vs. 1 h + retained	
Fragment Length:	Short	Long	Short	Long	Short	Long
Unburned	0.730	0.077	0.000*	0.000*	0.094	0.005*
60 min. burn	0.796	0.546	0.000*	0.321	0.000*	0.743
120 min. burn	0.387	0.190	0.000*	0.000*	0.000*	0.000*
Grouped	0.727	0.701	0.000*	0.000*	0.000*	0.009*
Concentration diff	±16 %	±45 %	**±91 %**	**±127 %**	**±64 %**	**±56 %**

* Significant ($P > 0.05$).

3.3. Effect of burn duration

In this study although DNA yield decreased as expected with longer durations of burning this was not always significant (Table 4). In all EDTA treatments unburned samples contained significantly more long and short DNA fragment copies than burned samples ($p < 0.01$). Comparatively, DNA yield from 60 min and 120 min were not significantly different ($p > 0.29$) with the exception of EDTA treatment 1 (1 h decalcification, supernatant retained) where short fragment yield was significantly greater in the 60 min burn group than the 120 min.

DNA has a strong affinity for inorganic bone due to ionic binding between positively charged calcium cations (Ca^{2+}) in hydroxyapatite and negatively charged phosphate groups in the DNA backbone [32,33]. As bone crystallisation changes dramatically when exposed to high and/or prolonged temperatures, it is logical to assume this process would affect bound DNA. Studies show that when exposed to temperatures of 500−600 °C bone enters a highly crystalline phase, with hydroxyapatite crystal size increasing dramatically [18,34–36]. It is likely

Table 4
Mann-Whitney test multiple comparisons showing specific relationships between each duration of burning within each EDTA treatment group.

	Unburned vs. 60 min		Unburned vs. 120 min		60 min vs. 120 min	
Fragment Length:	Short	Long	Short	Long	Short	Long
EDTA Treatment 1	0.000*	0.006*	0.000*	0.000*	0.387	0.666
EDTA Treatment 2	0.001*	0.014*	0.001*	0.000*	0.931	0.297
EDTA Treatment 3	0.004*	0.001*	0.000*	0.000*	0.031*	0.423

* Significant ($P > 0.05$).

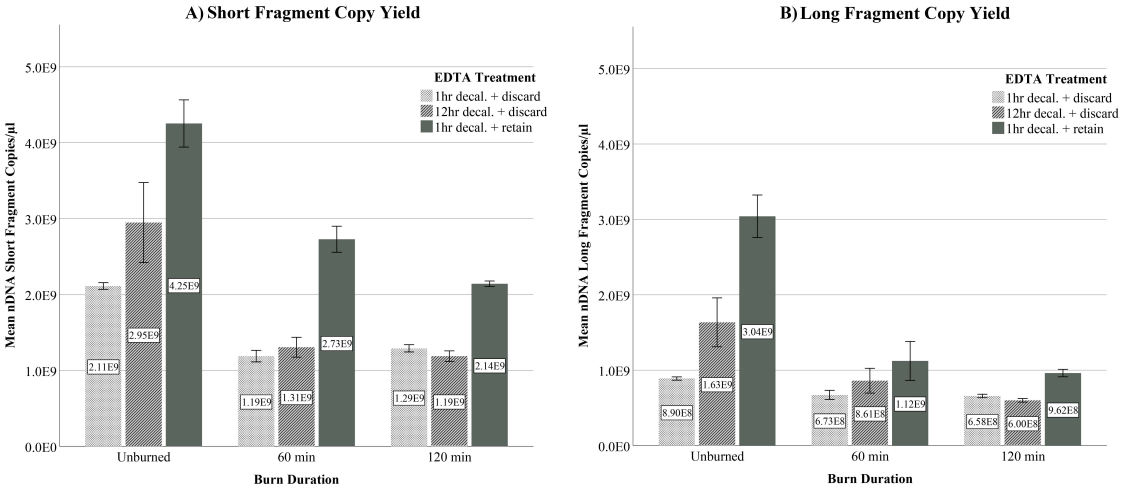

Fig. 2. Comparison of mean endogenous A) short and B) long nDNA fragment copies between EDTA treatment groups with standard error bars.

that the samples in the current study did not reach the 600 °C point of recrystallization, meaning that despite incineration of the outer soft tissue the interior was still relatively well protected even at the longer duration. This is where limitations of the current study must be considered. The very nature of attempting to recreate a realistic model of burning meant that many variables could not be controlled. Although consistency was maintained between samples (i.e. same accelerants used, same weather conditions) the exact temperature that samples were exposed to could not be measured. Whole porcine hocks were used to closely mimic the general dimensions of human bones and tissue thickness, the logistics of which meant an electric muffle furnace could not be used – additionally using a homogeneous heat source such as a furnace would not accurately represent a disaster event. Although less than ideal, the duration of burning presented an opportunity to assess diversity that may occur amongst burned samples in identification cases.

Based on our results, we can make some general recommendations regarding demineralisation methodology for samples where bone has not yet reached calcining temperatures (>600 °C). During demineralisation EDTA supernatant should be retained to prevent accidental disposal of genetic material. Where total demineralisation is not possible, undigested bone pellets should be retained for later analysis. If standard demineralisation protocol does not produce acceptable results, the undigested pellet can be processed as a last resort. Although this study strongly supports retention of supernatant during demineralisation, there are limitations that must be counteracted. Fore mostly the retention of surface contaminants, which is the main reason for disposal of supernatant for archaic samples [20]. The use of albumin such as BSA (in our case RSA) can significantly reduce inhibitor interference of polymerases and is commonly used for archaic samples [37]. Decreased inhibition also removes the need for excessive post-PCR purification, which can be time consuming [38]. In addition to RSA, we opted to include carrier RNA (cRNA). Although not directly linked to inhibition and contamination of samples, using carrier molecules in silica-based extraction can significantly increase yield where target DNA is expected to exist in low quantities (e.g. archaic/degraded material). The use of cRNA is still somewhat debatable, with some studies showing no improvement in DNA yield with its addition [39]. However, it has also been suggested that other constituents (such as EDTA) could affect the interaction of cRNA with silica columns, decreasing its effectiveness [40]. These processes are not necessary for all sample types, however in cases where samples are highly degraded those with the time and resources may benefit greatly.

4. Conclusion

This study developed a real time PCR assay for porcine nuclear DNA and demonstrated that modern burnt porcine bone did not respond to extraction techniques in the manner of archaic bone, but more appeared to mimic fresh bone. An increase in the duration of demineralisation was shown to have little effect on the yield of DNA recovered. Under the specific conditions investigated, the efficiency of DNA recovery from burned remains was improved by co-extraction of the demineralised supernatant and a brief (1 h) EDTA incubation period. Although similar changes in bone crystallinity may occur in both burned and archaic samples, the interaction between mineral and DNA may vary between these sample types. Hence, these sample types will need differing extraction techniques to optimise DNA yield. In future work, prolonging burn duration to reach calcining temperatures, along with the use of accurate temperature sensors could significantly improve our understanding.

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Availability of data and material

The raw dataset generated for the purpose of this study is available on reasonable request via the corresponding author.

CRediT authorship contribution statement

Meghan Mckinnon: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Denice Higgins:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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Chapter 4: A Comparison of Crystal Structure in Fresh, Burned and Archaic Bone – Implications for Forensic Sampling

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Certification: This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

Signature & Date: _____ 23.06.21

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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A comparison of crystal structure in fresh, burned and archaic bone – Implications for forensic sampling

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ABSTRACT

Standard protocols for extracting DNA from bone are variable and are largely dependent on the state of preservation. In archaic samples, endogenous DNA is believed to be tightly bound to crystal aggregates in the Hydroxyapatite (HAp) matrix requiring prolonged demineralisation to allow its release. By comparison, fresh bone contains abundant cellular material, discounting the need for demineralisation. Recommendations for incinerated bone, specifically how viable sampling sites should be selected and the ideal techniques for DNA recovery are unclear, and the protocol used is often selected based on macroscopic sample appearance.

It has been postulated that like archaic bone, burned bone is 'highly degraded' and therefore aDNA techniques may present better results for DNA recovery than using fresh protocols. However, little research has been undertaken comparing the crystal structure of burnt, fresh and archaic bone. This study uses a combination of XRPD and SEM analysis to compare the crystalline profile and microscopic appearance of burned bone subjected to temperatures ranging from 100–1000 °C, with archaic and fresh samples. Although macroscopically visually different, fresh samples and samples heated up to 500 °C showed no microscopic differences or significant changes in crystallinity. By comparison, samples heated above 500 °C became significantly more crystalline, with HAp crystal size increasing dramatically. Archaic samples were different again, more closely resembling the amorphous fresh samples than the highly crystalline incinerated samples. These results suggests that, potentially, samples burned at 500 °C or lower can be treated as fresh samples, whilst samples exposed to higher temperatures may require adapted protocols. Whether or not these highly burned samples require demineralisation needs to be investigated.

1. Introduction

The successful recovery of DNA from degraded remains is a challenge faced in various fields of study including palaeobiology, anthropology and forensic science. In many cases, bones and teeth are the only surviving materials available for analysis. Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is a highly crystalline substance consisting primarily of calcium and phosphate that is the basis of all forms of biological apatite [1]. The term "bioapatite" is often used to refer to the less crystalline, carbonate-containing form of hydroxyapatite that can be found in living bone. Whilst hydroxyapatite (HAp) is the main inorganic component of bone, collagen makes up the primary organic component [2]. The ratio of these components can influence the likelihood of recovering useable genetic information from the sample [3–5]. Under different taphonomic conditions the structure of bone can be radically altered hence the techniques used to extract DNA may need varying dependant on preservation. In some instances skeletal remains may have been subjected to burning or thermal damage which can change the structure of bone at both the primary (histological) and secondary (macroscopic) levels, and severely decrease the availability of organic material. Under extreme temperatures (>500 °C) HAp crystals have been shown to increase in size, whilst organic content decreases [6]. It has been suggested that the mechanisms by which HAp crystals enlarge under higher temperatures may be similar to those in bones that have undergone prolonged natural diagenesis - for simplicity's sake from here on in we will be using the term "archaic" to refer to bones deposited for at least 200 years that show evidence of digenetic changes (eg. Mineral loss and replacement) [7]. Harbeck et al. demonstrated complete loss of

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the organic content of bone at approximately 400 °C [8], however this threshold is subjective as in most forensic cases the length of burning is unknown. Tissue thickness, which is a highly variable, can delay the degradational effects of burning [9], and some studies have found protein components still present in bone burned at 600 °C and 700 °C [10,11]. Additionally, variables such as bone density, duration of burning and fuel source could also impact the exact temperature at which organic material is lost. As the temperature interval at which organic material is present could overlap bone recrystallization temperatures (~500 °C), potentially DNA could still be found within the HAp matrix at these higher temperatures.

The assumption that modern degraded bone is similar in crystalline structure to archaic bone has led to the use of conservative ancient DNA extraction techniques in some forensic casework. Decalcification is vital in DNA extraction from archaic bone, due to the potential binding of DNA to mineral [12–15]. It is believed that virtually all endogenous DNA in archaic remains is contained within dense HAp crystal aggregates which must be disrupted to obtain a usable genetic profile [16]. Decalcification requires prolonged incubation in an acidic agent (commonly ethylenediaminetetraacetic acid), increasing the time needed for the DNA extraction process. Decalcification is generally not performed on modern/fresh bone as DNA is readily available within the organic components, and decalcification can actually result in a loss of vital genetic material [17,18,11]. It is possible that the destructive processes associated with high temperatures may alter the crystalline structure of modern bone in a way that resembles archaic bone indicating that ancient DNA decalcification protocols would be more appropriate for these samples than fresh sample protocols. However, if this is *not* the case a prolonged demineralisation period may be inappropriate and could actually reduce the effectiveness of DNA recovery. To investigate this, the crystalline profiles of modern and ancient bones need to be compared. If significant changes in bone crystalline structure are detected following burning, knowing the point at which these changes occur and the temperature at which the crystalline structure in burned bone resembles that in fresh bone could be essential in determining which protocol is appropriate for a specific sample.

The rate and extent of bone degradation following death is influenced by environmental factors. For cases of prolonged soil exposure, the activity of ions within soil can be considered a driving force behind the destruction or preservation of both inorganic and organic components. White and Hannus [19] state that calcium (Ca^{2+}) and phosphorous monoxide (PO) ions exist in a state of equilibrium with HAp and disturbing this balance can result in differing degrees of degradation. Microorganisms decompose collagen in the presence of water and oxygen resulting in increased carbon dioxide (CO_2), bicarbonate (HCO_3^-) and hydrogen (H^+) ions in the soil [20]. In calcium-rich soils the rate of bone degradation will be decreased as Ca^{2+} ions can be absorbed into the bone, thereby replacing ions leeched out into the soil and stabilising the HAp structure; by this same mechanism, the rate of degradation of bones deposited in water would be different again [19]. This has implications for DNA preservation through HAp Ca^{2+} ionic binding [21,22].

In some ways heat-induced degradation is similar to prolonged diagenesis, as both result in the initial loss of organic collagen whilst the inorganic component survives until later stages. Bone heating eliminates hydroxide (OH-) groups through dehydration, and in cases where a greater concentration of calcium is present Calcium Oxide (CaO) can be produced as a secondary by-product at higher temperatures [23]. Although heat does not radically change the elemental composition of HAp the interatomic arrangement can be altered resulting in a change in "phase". This phase change can be quantified using X-Ray Powder Diffraction (XRPD) analysis [24] which examines the scatter patterns of monochromatic X-ray beams created by the lattice planes of a sample. The process produces a peak profile unique to the angle of scattering. The intensities (heights) of the peaks reflect the positioning of atoms within the crystal lattice [25]. The profiles produced by XRPD can appear as smooth, wave-like hills, sharp peaks or a combination. Materials that produce a diffraction pattern of continuous sharp peaks are crystalline in nature, whereas those with single wavelike patterns contain amorphous materials [26,27]. It is not uncommon to see a combination of these peak types, particularly in samples containing both inorganic and organic material [24]. Once a diffraction pattern has been produced for the sample of interest, published reference patterns from specific compounds can be aligned with the target profile peaks to identify elemental composition [28]. Once the elements are identified, their crystal size can be calculated using the X-ray wavelength, peak position (2θ) and the width of a target peak at half its full height [Full Width at Half Maximum (FWHM)] (Fig. 1).

XRPD analysis can be supplemented by semi-quantitative analysis of the crystalline composition using techniques such as Scanning Electron Microscopy (SEM). Unlike XRPD analysis which provides information on the full volume of a powdered sample, SEM only allows visual and elemental analysis to be studied at specific regions. Secondary Electron (SE) images are produced when the electron beam scans the sample and is reflected off to produce a topographical image of the samples' surface. Additionally, Energy-Dispersive X-ray spectroscopy (EDX) can provide elemental and chemical analysis. In EDX the electron beam hits the sample causing its atoms to become energised. The reaction of the atoms to this energy depends on the charge of their shells. The transition of energy produces an X-ray unique to the atomic number and properties of a specific element [29]. Both XRPD and SEM can be considered minimally destructive, as only a tiny portion of powdered bone is required for acceptable results. It should be noted that although SEM provides a useful tool for visual assessment of a sample, the EDX feature provides a highly localised and somewhat superficial analysis of sample chemistry. For this reason, EDX elemental analysis is best used as a supplementary tool to more advanced techniques such as XRPD.

Here we use XRPD, SEM and EDX analysis to examine and compare the crystalline profiles of modern fresh bone, modern burned bone and bone exposed to prolonged burial.

2. Methods

2.1. Sample selection and preparation

Thirty long bone samples were collected, including 21 from three mature domesticated cow bones (*Bos taurus*), two from archaic permafrost (~20,000 years before present) bison bones (*Bison antiquus*) and seven samples from domestic pigs (*Sus scrofa)* (1 control sample, 1 sample per each of the 6 temperature treatments). The three fresh bovine long bones and one fresh porcine long bone were each cut into seven 1 cm thick cross-sections. One section from each bone was retained as a control sample and the remaining sections were burned in groups of four for each of six temperature intervals (100 °C, 300 °C, 500 °C, 700 °C, 900 °C, 1000 °C). An electric kiln with in-built thermocouple (Woodrow Hobby Fire Kiln Mini, © 2019 Keane Ceramics, NSW, Australia) was programmed to maintain a consistent temperature ramp-up speed of 400 °C/h per run. Samples were introduced to the kiln once the desired hold temperature was reached, burned for 60 min, and then removed once the kiln completed the cooldown cycle. Each sample was photographed and weighed using a calibrated electronic scale before and after burning. Two

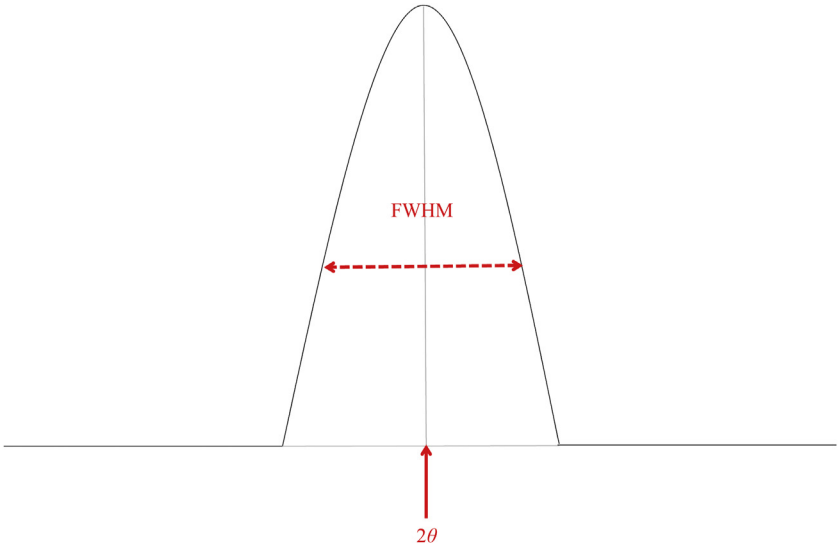

Fig. 1. Illustration showing two key measurements used to establish characteristics (i.e. crystal size) of a target X-Ray Powder Diffraction (XRPD) peak: Full Width at Half Maximum (FWHM) and 2-theta position (2θ).

grams of powdered bone was obtained from each sample via manual grinding with a mortar and pestle. Samples unable to be easily pulverized using a mortar and pestle were subjected to an additional slow speed drilling step (<100 revolutions per minute, 3.5 mm drill bit).

2.2. XRPD and SEM analysis

XRPD analysis was performed using a MiniFlex 600 X-Ray Diffractometer 9 (©2019 Rigaku Corporation, Tokyo, Japan) with CuK_{α} radiation (λ = 0.15418 Å) and a working voltage of 20 kV/2 mA. Slit size was 1.25°θ and wavelength was measured at 10° per minute with a 0.02 step size. Peaks are only identified in XRPD if they are larger than 3 nm and the crystallite in question makes up at least 3% of the whole sample [16]. Once peak profiles were obtained, phase analysis was performed using PDXL: Integrated X-ray powder diffraction software (© 2019 Rigaku Corporation) which uses the Crystallography Open Database (COD) as a source of crystal structures and compositions. As apatite crystallites have a hexagonal structure [6,30], peaks corresponding to the *a*- and *b*-axis overlap substantially. As a result, average crystallite size can only be obtained along planes perpendicular to the *c*-axis [31]. Based on published data, crystallographic planes (*hkl*/Miller indices) found in HAp include 002, 211, 300, 202, 310, 222 and 213 [31,32], however some peaks may overlap at lower temperatures where samples are less crystalline. For this reason, only planes clearly identified across all samples were used to establish average crystallite size. The average crystallite size was calculated for each plane using the Scherrer formula [33]:

$$D = K\lambda/(\beta cos\theta)$$

Where D is the average crystallite size (in nm), K is the Debye-Scherrer constant (0.89), λ is the x-ray wavelength specific to the machine used (0.15418 Å), θ is Bragg diffraction angle and β is line broadening in radians (peak full width at half maximum divided by 2). Additionally, SEM analysis was performed using a FEI Quanta 450 FEG Environmental Scanning Electron Microscope (©2019 Thermo Fisher Scientific, Massachusetts, USA) with 15 kV and 3.0 spot size working conditions. Bone powder was applied to sticky carbon buttons and sputter coated with platinum (10 nm) to reduce interference and static discharge. This was performed by Adelaide Microscopy staff using a Cressington 208 HR sputter coater (©2019 Cressington Scientific Instruments, England, UK). Captures were taken from three sites on each sample, at 20,000× and 50,000× magnification. At 50,000× magnification, images were also assessed using EDX AZtec software (©2019 Oxford Instruments plc, Nanoanalysis). In this software elemental identification was performed for each sample, with platinum excluded from analysis as sample coating would likely alter results. Three point analyses and one area analysis were performed for each sample site.

2.3. Statistical analysis

IBM SPSS [34] was used to test data distribution for normality (Shapiro-Wilk) and the appropriate method for ANOVA and multiple comparison testing (parametric or non-parametric). Additionally, Spearman's Rho correlation was used to explore relationships between variables.

3. Results

3.1. Impact of sample size

The Shapiro-Wilk test showed the data was not normally distributed. Given that with an appropriately large sample size biological data almost always follows a normal distribution, it is likely that our sample size caused this result rather than abnormalities in the data itself. To adjust for this we applied non-parametric analyses which are more robust than parametric tests as they do not rely on distribution. This means that any statistically significant results obtained in this way are made under the most conservative conditions possible.

3.2. Post-burning sample changes

All samples exposed to sintering temperatures (>500 °C) were extremely brittle and easy to powder, however the fresh, low temperature-treated and archaic samples required drilling. The range of colour changes observed in post-burn samples can be seen in Fig. 2. Sample weight was seen to decrease at all temperatures but was more evident above 300 °C. Porcine bones

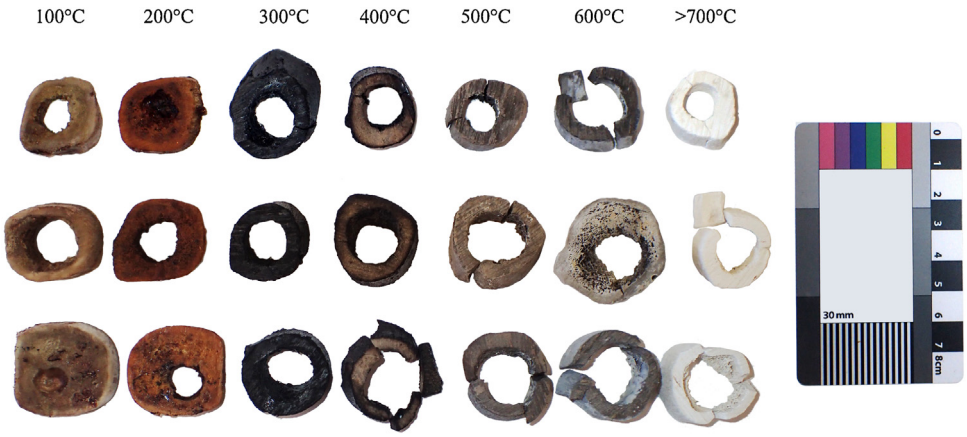

Fig. 2. Selection of bovine samples from three different individuals exposed to various temperatures. Above 700 °C there was no further visible colour change.

showed a slightly greater weight loss than bovine samples (Fig. 3).

3.3. XRPD profile analysis

The primary peaks evident in all x-ray diffraction patterns were a positive match for archived phase data of HAp crystals and were seen in all samples (Fig. 4.1-4.4). However, the high intensity peak (~32° 2θ) showed a substantial overlap between *211*, *300* and *202* planes of reflection at lower temperatures, and individual peaks were not apparent until ~700 °C. Only three planes presented clearly defined peaks in all samples analysed. These planes had Miller Indices (*hkl*) of *002*, *310* and *213* and were selected for further investigation. In samples burned at temperatures of 700 °C and higher, a secondary phase was identified at theta (2θ) positions 37° (*200*) and 53° (*220*) as CaO [35,36] (Fig. 4.3, 4.4). Average HAp crystallite size values indicate that for all crystallographic planes the greatest crystallite growth occurs between the 500 °C and 700 °C (Table 1). Average crystallite size

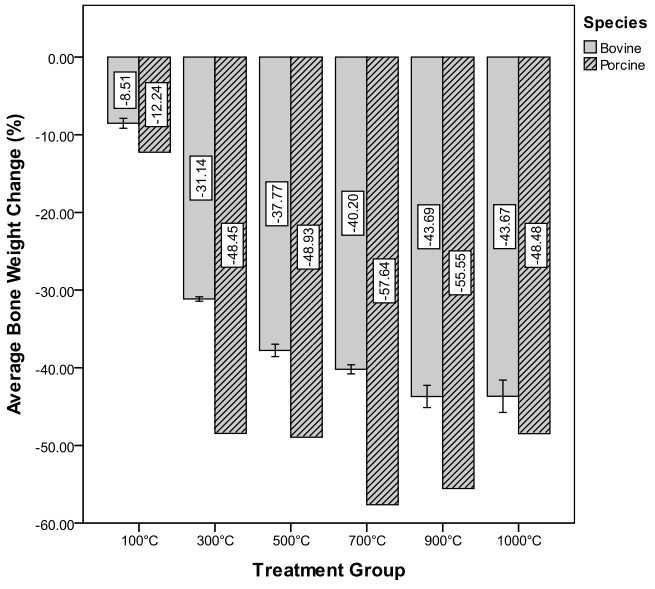

Fig. 3. Histogram showing average percentage of bone weight loss for modern bovine and porcine samples analysed within each temperature/treatment group with standard error bars.

increased by a total of 191% (*002*), 805% (*310*) and 1260% (*213*) from the lowest (unburned) to the highest (1000 °C) temperature groups.

3.4. XRPD ANOVAs and correlation analysis

Kruskal-Wallis H test (supplementary S1) showed significant differences between temperatures for both HAp crystal size ($P < 0.013$) and bone weight loss ($P = 0.004$). No significant difference was found in crystal size ($P > 0.083$) between unburned archaic and modern bovine samples. Mann-Whitney tests showed in the case of the *002* crystallite plane, the only significant change was seen between 500 and 700 °C where crystallite size increased dramatically (36.9 nm). By comparison the *310* plane presented significant changes at two intervals; a slight increase (1.46 nm) in crystallite size between 100 and 300 °C and a dramatic increase (55.75 nm) between 500 and 700 °C. Lastly, the *213* plane showed the most change, with a small decrease (-4.38 nm) occurring from 100 to 300 °C followed by increases from 300 to 500 °C (6.33 nm), 500–700 °C (54.76 nm) and 900–1000 °C (8.95 nm). Spearman's rho non-parametric correlation showed a significant ($p < 0.001$) positive relationship between temperature and HAp crystallite size for all three planes, as well as temperature and weight loss (Table 2).

3.5. SEM energy-dispersive x-ray spectroscopy descriptive statistics

Basic statistics (Table 3) show a trend towards increased carbon and oxygen at higher temperatures, whilst calcium appears to decrease. Other elements show peaks and drops at certain temperature intervals, but as there is substantial overlap of standard errors for most elements, no distinct pattern can be discerned.

3.6. SEM ANOVAs and correlation analysis

Kruskal-Wallis H test (supplementary material S2) showed that calcium was the only element that differed significantly between temperature groups ($P = 0.007$), however no significant differences in elemental composition were found between modern and archaic samples ($P > 0.237$). Further post-hoc analysis showed the weight percentage of calcium decreased significantly ($p = 0.016$) from the unburned group to the highly burned (1000 °C) group. Spearman's rho analysis (Table 4) showed a relatively weak but still significant ($p = 0.042$) positive correlation between temperature and carbon weight percentage. Correlation

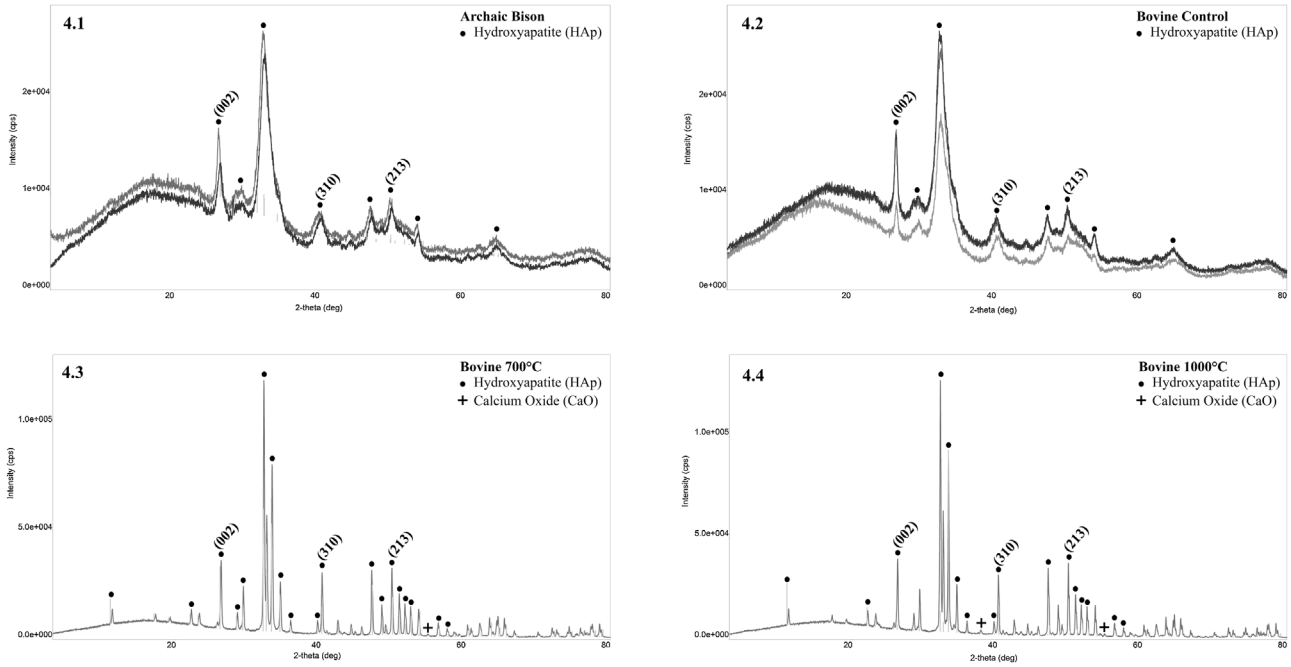

Fig. 4. X-ray diffraction phase patterns for archaic bison bone (4.1), modern untreated bovine bone (control) (4.2), and the same modern bone treated at 700 °C (4.3) and 1000 °C (4.4) temperature intervals.

Table 1
Average size (nm) of three primary HAp crystallographic planes in archaic samples and in modern samples before and after burning at controlled temperature intervals.

Temperature (°C)	Avg. HAp Crystallite Size (nm) ± SD			Avg. HAp Crystallite Change (nm)		
	(002)	(310)	(213)	(002)	(310)	(213)
Archaic:	15.18 ± 4.72	7.44 ± 0.53	2.98 ± 0.79	–	–	–
Unburned:	19.69 ± 1.11	7.16 ± 0.52	5.26 ± 0.44	–	–	–
100	17.46 ± 2.51	6.11 ± 1.10	8.06 ± 1.14	−2.23	−1.05	2.8
300	18.67 ± 3.33	7.57 ± 0.73	3.68 ± 2.45	1.21	***1.46**	***−4.38**
500	17.81 ± 0.41	7.04 ± 0.40	10.01 ± 1.03	−0.86	−0.53	***6.33**
700	54.71 ± 2.40	62.79 ± 0.84	64.77 ± 1.15	***36.9**	***55.75**	***54.76**
900	54.62 ± 6.22	59.48 ± 4.88	62.60 ± 2.78	−0.09	−3.31	−2.17
1000	57.35 ± 1.54	64.79 ± 4.43	71.55 ± 6.31	2.73	5.31	***8.95**
* Significant ($P < 0.05$) Total Crystallite Increase 0-1000°C:				191%	805%	1260%

Table 2
Spearman's rho correlation showing the strength of relationships between variables.

		Crystallite Size (nm)/Bone Loss			Crystallite Size/Temp			Bone Loss/Temp
		(002)	(310)	(213)	(002)	(310)	(213)	
Spearman's rho	*Correlation (ρ)*	**0.679	**0.710	**0.807	**0.718	**0.721	**0.845	**0.975
	Sig. (2-tailed)	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	23	23	23	23	23	23	23

** Correlation is significant at the 0.01 level (2-tailed).

Table 3
Average elemental weight percentage for samples within all treatment groups.

		Carbon %	Oxygen %	Sodium %	Magnesium %	Calcium %	Trace Elements %
Treatment Group:	*A.Bison*	18.77	39.63	0.95	–	41.26	2.65
	Unburned	13.00	36.51	1.02	0.72	52.36	–
	100 °C	18.76	44.07	1.19	0.66	36.38	2.21
	300 °C	26.92	45.70	1.33	0.74	32.79	–
	500 °C	24.12	41.23	0.99	0.76	35.93	–
	700 °C	19.03	42.55	0.90	0.96	35.72	1.49
	900 °C	20.07	48.43	1.16	0.82	30.62	–
	1000 °C	30.28	46.44	3.03	0.94	29.57	0.46

Table 4
Spearman's rho correlation showing the strength of relationships between elemental weight percentage and temperature increase.

		Temp/Carbon	Temp/Oxygen	Temp/Sodium	Temp/Phosphorous	Temp/Calcium
Spearman's rho	*Correlation* (ρ)	*0.418	0.191	−0.113	0.170	*−0.367
	Sig. (2-tailed)	0.042	0.066	0.495	0.099	0.000
	N	24	93	39	95	95

* Correlation is significant at the 0.01 level (2-tailed).

between temperature and calcium weight percentage was similarly weak but significant ($P = 0.000$), however this relationship was inverse.

3.7. Semi-quantitative analysis of SEM images

Fig. 5 shows a sample of SEM images comparing archaic bison bone to modern bovine samples burned at various temperatures. A notable similarity is apparent between the archaic bison samples (Fig. 5a) and modern bovine samples burned at low temperatures (Fig. 5c), with both presenting striated surfaces. At 500 °C, burned bone begins to restructure, with many samples developing small pits (~0.5 μm) on the surface (Fig. 5e). Samples burned at ≥ 700 °C showed the most dramatic change, with apatite crystals becoming less dense and more granulated in appearance (Fig. 5f). From 600–1000 °C these crystalline granules become visibly larger and appear to "melt" into each other at the highest temperature increment (Fig. 5h). Barring some minor individual sample variation, analysis of the porcine SEM images showed a similar pattern of crystallization from low to high temperatures (supplementary material S3). The only exception to this was the samples burned at 700 °C, where granulation (though similar) presented more 'rod-shaped' crystal aggregates compared to the relatively spherical shaped aggregates of the bovine samples.

4. Discussion

In forensic research there is always a trade-off between conducting studies realistically or accurately, and in this case logistical restrictions led us to select the later. Electric furnaces capable of reaching and holding controlled temperatures above 1000 °C are extremely difficult to access as there is rarely a need for them; finding one large enough to burn entire fleshed bones was not possible. Herein lies one of the primary limitations of this study; to suit the dimensions of the furnace we were forced to pre-section bones prior to burning. This means we were not able to investigate the likely impact of soft tissue shielding, and the effect of increased bone size on heat penetration. Though these factors definitely require consideration, measuring them was beyond the scope of this study. The relatively small sample size must also be considered - though significant results were obtained through reliable and consistent analyses, increasing the sample size would only strengthen these results and allow for further more in-depth statistical models. Finally, the use of permafrost bones as representatives of "archaic" samples should be discussed. Increased age and severe diagenesis are not intrinsically linked, meaning that a variety of factors besides age determine the degree of preservation. Permafrost bones are a prime example of this, as they are far better preserved than other samples originating from the same period. Again this was an issue of logistics; due to the destructiveness of the sampling process and the inherently precious nature of archaic samples, it was not possible to access bones of non-permafrost origin. Subject to increased availability, conducting similar analysis on other types of archaic samples could prove highly beneficial in supplementing the results presented here.

One of the primary directives of this study was to compare and contrast changes to the mineralised structure of bone samples exposed to either high temperatures or natural diagenesis. Given the assumption that there is an intimate relationship between bone hydroxyapatite and DNA, variation in mineral structure may indicate that alterations to DNA extraction protocols are required dependant on the taphonomic conditions to which the sample

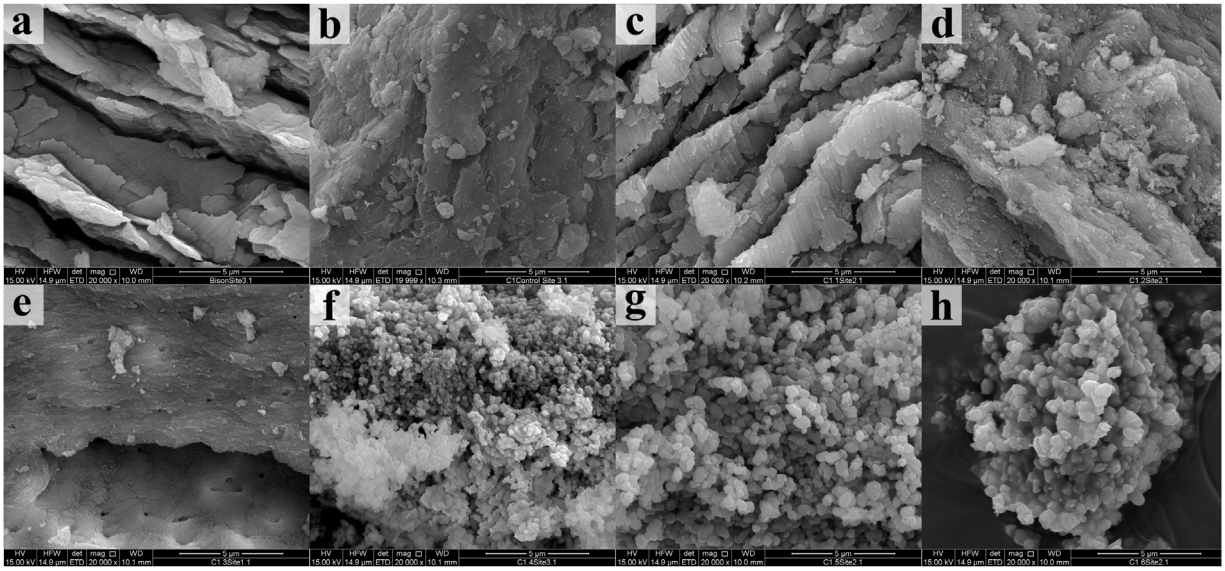

Fig. 5. SEM images taken at ×20000 magnification (scale bar = 5 microns) – a. untreated archaic bison bone, b. untreated modern bovine bone and subsequent images show the same modern bone treated at 100 °C (c.), 300 °C (d.), 500 °C (e.), 700 °C (f.), 900 °C (g.) and 1000 °C (h.).

has been exposed. The link between DNA binding to HAp and DNA preservation is not a new concept; in the past, HAp columns were used for protein chromatography due to their affinity for DNA binding [37]. Similarly, many studies have found DNA yield from HAp containing structures such as bone or teeth is significantly greater than that from other degraded tissues [38–40]. However, it is only in the past decade or so that the relationship between mineral structures and DNA preservation has been described as more than simply "physical protection" of genetic material. This is likely because the exact mechanisms of the relationship are poorly understood, and its importance is often overlooked. It is commonly stated that bone structure is the driving factor behind DNA preservation, and denser cortical bone provides greater physical protection for DNA than trabecular bone, where a greater surface area is available for microbial invasion. This relationship is multidirectional as increased microbial activity results in decreased collagen and therefore increased porosity [5,20]. However, as far back as 1965, Bernardi suggested that the interaction between nucleic acid and HAp calcium was the main factor in DNA/HAp absorption [41]. Götherström et al. states that HAp has a strong affinity for DNA and that ancient DNA degradation can be linked to mineral resorption and crystallinity loss [4]. Brundin et al. established that DNA from *Fusobacterium nucleatum* incubated in a HAp medium produced significantly greater quantities of nuclear material than samples incubated in other media – this was attributed to DNA's affinity for HAp binding [42]. This affinity is believed to be a direct result of ionic binding between positively charged calcium cations (Ca^{2+}) in HAp, and the negatively charged phosphate groups that form the sugar-phosphate backbone of the DNA double helix [43,44]. With this in mind, it is logical that dense bone would contain greater quantities of DNA, not only as a result of a lack of porosity, but also due to increased calcium content [41]. Therefore, any process affecting the ratio and abundance of HAp cations and anions (Ca^{2+}/PO^{-}) can be expected to affect the quantity of DNA retained within mineralised tissues. Exposure to high temperatures, as well as natural weathering and diagenesis are destructive processes that very likely alter these ionic ratios.

A mixture of amorphous material (likely collagen) and crystalline HAp was seen in the XRPD phase patterns of the fresh (and low temperature) modern bones examined in the current study. This is consistent with Bartsiokas & Middleton's statement that "modern bone mineral can be regarded as poorly crystalline, contaminated hydroxyapatite" [45]. Comparatively the archaic samples examined in this study presented peak profiles and HAp crystallites virtually indistinguishable from those of modern unburned and low temperature (100 °C) samples. This was not consistent with the findings from other research demonstrating that archaic samples produce sharper diffraction patterns indicative of purer crystalline structures, as well as larger crystallites than modern samples [46–48]. Visual assessment of SEM images showed no evidence of the "rod-shaped crystals" Rogers et al. described as being present in archaic bone and absent in modern unburned samples [48]. Based on this study alone, archaic bison bone could not be separated from fresh samples simply by using XRPD, SEM and EDX. This suggests that diagenetic processes did not greatly alter the mineral structure of the bone. However, when cutting the archaic bone, we found it considerably harder to section than the fresh and burned samples, indicating a difference exists between the bone types. Although we were not able to detect significant differences in the crystallinity of the permafrost samples, there are likely other processes affecting the bone that were not examined in this study. It is worth noting that although permafrost samples are frequently used in ancient DNA studies, they are far less degraded than bones of similar age found in other regions. Heat treatment had a dramatic effect on the crystallinity of samples. In most cases, the temperature that samples were exposed to could be approximated based on crystal size and appearance using SEM. In all samples exposed to 700–1000 °C, HAp crystals became significantly larger and CaO appeared as a secondary phase bi-product. Concurrently, a significant decrease in calcium content was observed with increased temperature. Bones more abundant in Ca^{2+} ions have presented an increased affinity for DNA absorption [37,49]. Knowing this, temperatures high enough to break down and/or convert bone calcium to CaO would likely result in a loss of any DNA bound to the calcium. Alternately, lower temperatures likely have less of an effect on HAp-bound DNA. Endogenous DNA bound to HAp potentially remains present up until 500–700 °C where significant recrystallization is observed. Although the sample size in this study is small, the colour of the burned samples was clearly correlated with the temperature to which they were burned [31]. If this is representative in all burned bones and is indicative of DNA content it would provide an excellent screening tool for triaging of samples to determine which are likely to provide genetic information.

With regard to inter-species differentiation; although HAp crystal size was not deemed significantly different between porcine and bovine samples, visual assessment of the SEM images showed crystals of porcine samples heated to 700 °C were more rod-shaped than the plate-like crystals formed at the same temperature within bovine samples. This suggests some morphological differences may exist – or it could simply be that the rod-shaped crystals are precursors to the next crystalline form, and also occur in bovine samples at temperature ranges that were not recorded (i.e. during the 600 °C range). Another explanation is simple topography; Rogers et al. described bone mineral crystallites as having both rod and plate like morphologies in most tissues reflective of local variation [48]. The only major difference seen between species was the percentage of weight lost during burning, where porcine samples lost significantly more weight than bovine samples. As bovine bones are considerably larger than porcine bones this could indicate that the deeper tissues in large bones are afforded more protection than those of smaller bones, however due to the limited number of porcine samples included in the current study this could also be a random aberration. Although our results largely point towards a lack of variation between the two species investigated, we cannot automatically assume no difference will exist between non-human (bovine and porcine) and human bones.

This work could be expanded on by including a broader range of sample types - for example fresh and degraded modern human (especially for forensic contexts), and severely degraded archaic bone. Preservation of DNA depends on a range of variables, and only changes in bone crystallinity and elemental composition were analysed here. In addition to the techniques used in the presented study, a variety of other techniques could be used to expand the current understanding of how bone is altered by various forms of trauma, and how this affects DNA recovery. This knowledge would not only improve DNA recovery from challenging samples but assist in the differentiation and possibly even identification of fragmented remains.

Key points

1. Archaic bison bones and unburned modern bovine bones could not be distinguished based on crystalline profiles and elemental composition.
2. The crystalline profile of both bovine and porcine bone subjected to heat remained unchanged until temperatures exceeded 500 °C.
3. The crystalline profiles of burned bone suggests decalcification prior to DNA extraction should not be performed on samples that have not undergone recrystallization (~600 °C).

4. To maximise success, different DNA extraction protocols are required for differentially burned bone samples.

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Availability of data and material

The raw dataset generated for the purpose of this study is available on reasonable request via the corresponding author.

CRediT authorship contribution statement

Meghan Mckinnon: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Maciej Henneberg:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Ellie Simpson:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration. **Denice Higgins:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None of the listed authors have conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.forsciint.2020.110328.

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Chapter 5: Effects of Thermal Insult on Bone Tissue as Observed by Micro Computed Tomography

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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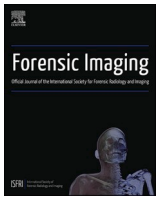

Invited article

Effects of thermal insult on bone tissue as observed by micro computed tomography

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ABSTRACT

A thorough understanding of the effects of heat on bone morphology is required for reliable analysis of burnt skeletal remains in forensic anthropology and bioarchaeology. However, thermal damage often prevents application of the usual analytical techniques in both modern and archaeological contexts. This study uses Micro Computed Tomography (MicroCT) to assess heat-induced changes in incinerated bovine (*Bos taurus*) long bones at temperatures between 100 and 700 °C. Scans provided data on bone porosity and surface to volume ratio, which were compared between samples. Macroscopic comparisons were also undertaken using 3D reconstructions of samples. Results showed a decrease in porosity between unburned samples and those burned at the highest temperature (700 °C). This change was not unidirectional, with an unexpected increase at 500 °C. The lack of uniform exponential change suggests that more than one mechanism influences bone morphology in response to thermal insult. The initial significant decrease in porosity may be attributed to the loss of organic material (100–200 °C) which had the most dramatic impact on bone morphology, whereas the increase at 500°C coincides with hydroxyapatite re-crystallisation which is known to occur around this temperature. This study demonstrates that MicroCT can be readily applied to burned bone which is often fragile and difficult to examine. Additionally, unlike most microscopic techniques MicroCT is three dimensional, allowing the internal structure of the entire bone to be investigated without destruction of the sample. The changes observed in bone morphology due to heating provide valuable insight which can inform subsequent investigation and analysis of these samples.

Introduction

Skeletal tissues persist long after soft tissues have been lost and can provide insight into the identity of an individual, their lifestyle and even how they died. This is valuable in many contexts including bioarchaeological, anthropological and forensic investigations. Thermal insult, however, can significantly affect the structural integrity of bone, obscuring signs of antemortem changes and limiting the analytical techniques available to obtain data. For example, radiocarbon dating, and stable isotope analysis of ancient bone are routinely used to reconstruct past diets, environments, and migration patterns [1, 2]. However, following a) loss of collagen, b) bone recrystallization (i.e. after burning or prolonged natural diagenesis), or c) exchange/fixation of structural carbonate, these analyses are not always possible [3].

In addition to chemical and microstructural alterations, bone can severely warp under intense heat. This can prove challenging for anthropologists who rely primarily on gross morphology to determine biological characteristics such as origin (i.e. human or non-human), sex, height and age [4]. Evidence of trauma (e.g. blunt force, knife wounds) may also be lost due to heat induced changes, depending on the severity and duration of heating. In addition to fracturing and shrinkage, a variety of features (Fig. 8) such as colour, water content, collagen structure, hydroxyapatite crystallization and the appearance of calcium oxide are known to be affected when bone is exposed to high temperatures [5, 7]. The exact temperature that these changes occur at varies depending on factors such as bone density, burn duration, and calcium phosphate ratio [6, 7]. Blackened bone indicates a loss of the majority of organic material, that changes to elemental carbon. Whitened bone indicates complete replacement of organic carbon with hydroxyl groups, and conversion from an amorphous to crystalline mineral [8]. It is

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notoriously difficult to identify at precisely what temperatures these changes occur due to the large number of variables; bone type and density (e.g. cortical bone vs. trabecular) being a primary factor.

Microscopic analysis is often used to examine bone to reveal identifying characteristics and antemortem and postmortem changes. However, traditional microscopy techniques are destructive and only provide a localised view of a small area, hence, variation in other regions of the bone may be overlooked. Additionally, both diagenesis and thermal insult can severely degrade bone tissue, limiting histomorphology and microscopic analysis [9]. Decalcifying samples for slicing and staining becomes nearly impossible for incinerated bone due to the loss of organic matter and collagen framework during burning. A study by Cattaneo et al. [10] found that despite significant shrinkage of osteons, microscopy was highly accurate in identifying the origin (human or non-human) of burned bone samples embedded in resin. However, scanning electron microscopy of resin embedded bone still only provides a localised view of a single surface of the sample, requires specialised processing and causes significant destruction of the bone. The difficulties associated with investigation of burned samples indicate a need for a visual triaging tool that would allow non-destructive assessment of bone for targeted analysis. MicroCT could potentially fill this niche, as it allows three-dimensional inspection of an entire sample with the ability to slice through at various levels without physically slicing the bone.

Computed Tomography (CT) is an imaging tool common in medical and bioarchaeological fields that is now also used in forensic investigations [11–13]. Being non-invasive, CT is ideal for this type of work, where practical and/or ethical boundaries prevent the destruction of delicate samples. In some cases, CT (along with Magnetic Resonance Imaging) is beginning to take the place of full autopsies, with cause of death established from in-depth examination of full-body scans [14, 15]. By comparison, Microcomputed Tomography (MicroCT) is still in its infancy when it comes to industry applications and is primarily used for research. Although much higher resolution than ordinary CT, MicroCT is substantially more expensive, and might be considered inutile as currently only small samples can be analysed. However, where a microscopic approach is required e.g. fragmented/small samples, MicroCT could well augment histological analyses and/or be used as a screening tool. Kuhn et al. [16] demonstrated the usefulness of this technique on fresh human bone by comparing trabecular bone cube micro-CT scans to corresponding histological sections, finding that micro-CT measures of bone volume fraction (P_P) and trabecular plate density (P_L) were not significantly different from those measured from histological sections. To date Sandholzer and collaborators [17, 18] appear to be the only researchers consistently using MicroCT to investigate heat induced changes in biological material. Thermal insult can severely hinder the process of identification, in both forensic and anthropological contexts [19–21]. In order to establish an accurate biological profile, anthropologists need a thorough understanding of the changes occurring in bone during the processes of burning. This current study aims to examine these changes in bone burned at various temperatures as observed by MicroCT.

Methods

Samples

Eight 10mm thick cross-sections were collected from the cortical bone shaft of three individual domestic cow (*Bos taurus*) femurs, with each allocated to a different temperature treatment (three sections (one from each bone) per temperature group, N=24). An electric kiln with digital temperature control and in-built thermocouple (Woodrow Hobby Fire Kiln Mini, © 2019 Keane Ceramics, NSW, Australia) was used to burn samples at 100 °C temperature intervals (unburned, 100 °C, 200 °C, 300 °C, 400 °C, 500 °C, 600 °C, 700 °C). For consistency a ramp-up (heat-up) speed of 400 °C/h per run was maintained, and upon reaching the desired temperature samples were inserted and burned for 60 min durations. After 60 min elapsed, a cooldown cycle would begin and samples could be removed.

Scanning & reconstruction

In order to develop the correct CT protocols for the samples, a pilot study of three samples burned at low (unburned) intermediate (400 °C) and high (700 °C) temperatures was conducted. A Bruker Skyscan™ 1276 apparatus and associated software was used. By this process the following settings for the study were determined:

- Filter: aluminium-copper (Al-Cu)
- Voxel resolution: 8.04 μm
- Exposures: 820 ms
- Voltage: 100 kV
- Current: 200 MA
- Rotation step (degrees): 0.200

Again, we used test samples to determine the optimal settings for reconstruction, using NRecon (Skyscan, Kontich, Belgium). Optimal settings were determined as:

- Smoothing: 2
- Ring artefact reduction: 8
- Beam hardening correction: 30 %
- Output log: 0.029

Using the Skyscanner's native software CTAn (Skyscan, Kontich, Belgium) we conducted both 2D and 3D analysis on a consistent volume of interest (VOI) containing 600 slices for each sample. At this point one of the most vital factors of MicroCT analysis had to be considered: thresholding. All morphometric variable measurements can only be performed on segmented or binarised images, which is achieved via "thresholding". The threshold is a number corresponding to a greyscale value above which all pixels are considered bone, and below which are considered non-bone [22, 23]. One method of obtaining these values is "local" thresholding, where values are determined only by the grey levels of surrounding voxels. This provides a uniform threshold that is appropriate for samples that possess the same mineral structure and density but may not suit samples presenting local variation i.e. cortical and trabecular bone. An alternative method used to overcome the issue of local variation is "adaptive" thresholding, whereby multiple local threshold values are collected, and the average of the maximum and minimum values are used to binarise the data [22, 16]. Due to the extreme likelihood of morphological variation in burned bone, we opted to use the latter method. Adaptive pre-thresholding within the 2D space was trialled on various samples until 50-140 (with a radius of 6 voxels) was determined the most universally appropriate threshold setting.

Following thresholding, scans were despeckled (pixels <250 removed from 2D space), and a shrink-wrap function applied to restrict further analysis to the targeted area. Finally, 3D analysis was used to generate quantitative data on the following variables: bone surface to bone volume ratio (BS/BV), mean pore volume (Po.V) and mean pore surface area (Po.S), bone (BV) and porosity (Po) percentage, and surface to volume ratio of pores (PS/PV). Additionally, 3D modelling was used to visually display sample changes, and inverse thresholding was applied to highlight regions of non-bone (porosity) within the bounds of each sample. This was used to create a visual reconstruction of porosity. A summary of variables and their abbreviations is provided in Table 1. These variables are commonly used for measuring changes in bone morphology in clinical studies, and are likely to be severely altered by thermal insult. The information in the table was based off the Bruker MicroCT handbook [24].

In simplified terms, bone surface to volume ratio (BS/BV) measures the relationship between the whole exterior surface of the bone compared to the volume of solid material within the bounds of the bone.

Table 1
Description, definition and abbreviation of all MicroCT measured variables. Variable names follow Bone ASBMR nomenclature [25].

Variable	Definition	Abbreviation	Unit
Bone surface / bone volume ratio	The ratio of solid surface to volume measured in 3D within the VOI (Volume Of Interest).	BS/BV	mm^{-1}
Total pore space volume	The total volume of all pores within the VOI.	Po.V	mm^3
Total surface area of pores	The total surface area of all pores within the VOI.	Po.S	mm^2
Percent bone volume (bone volume/total volume)	The proportion of the VOI occupied by binarised solid objects.	BV	%
Percent pore volume (pore volume/total volume)	Total porosity is the volume of all pores as a percent of the total VOI volume.	Po	%
Pore surface area to volume ratio (pore surface/pore volume)	Average ratio of pore surface area to pore volume within the total VOI.	PS/PV	mm^{-1}

This can be indicative of the surface complexity of the sample, for example a less uniform surface would present a higher surface area to volume ratio, compared to a flatter, more uniform surface. This also applies to the complexity of pores (PS/PV). Total pore surface (Po.S) and volume (Po.V) both measure pores contained within the internal bounds of the solid object. These may be defined circular pores, cavities or fractures, the consistency of which influences the average surface to volume ratio of pores. Again if this value is high it can indicate the presence of multifaceted and/or varied porosity, whereas low values indicate more uniform porosity and/or decreased surfaces e.g. rounded pores.

Scanning electron microscopy

Following CT scanning, a selection of samples were mounted on carbon tabs and coated by Adelaide Microscopy staff for Scanning Electron Microscopy (SEM). A 10nm thick platinum coating was applied using a Cressington 208 HR sputter coater (©2019 Cressington Scientific Instruments, England, UK), samples were then examined at various magnifications ranging from <200x (mm) - 20000x (μm). A FEI Quanta 450 FEG Environmental Scanning Electron Microscope (©2019 Thermo Fisher Scientific, Massachusetts, USA) with a working voltage of 15 kV and 3.0 spot size was used to collect captures of bone topography for visual analysis.

Statistical analysis

Data collected in this study followed a normal distribution, therefore parametric analyses were justified. One-way ANOVAs were conducted to determine if significant differences exist between temperature treatments, and Tukey's post-hoc tests were then performed to establish variation between specific groups. Pearson's correlation was applied to show the relationship (or lack thereof) between bone morphometry variables and temperature change, and if said relationships were present, raw data were plotted and displayed using a logarithmic regression model.

Results

Although not always significant, an overall trend was apparent for all variables investigated, with the possible exception of BV and PS/PV. From initially high surface and porosity values (Table 2) of unburned bone, there was a dramatic decrease until 300 °C then a linear increase to a peak at 500 °C degrees, before decreasing once more. This is emphasised in Fig. 1, where BS/BV of the unburned and 500 °C sample groups clearly overlap. This trend is again apparent in Fig. 2a and b,

Table 2
Average values obtained for each variable assessed at each temperature interval.

	BS/BV (mm^{-1})	Po.V (mm^3)	Po.S (mm^2)	BV (%)	Po (%)	PS/PV	*N*
Unburned	18.84	55.48	2,355	73	27	52.33	3
100°C	14.71	28.04	972	76	24	45.33	3
200°C	10.11	14.46	511	80	20	41.33	3
300°C	6.00	5.13	325	94	6	88.33	3
400°C	12.17	10.50	624	86	14	75.00	3
500°C	17.31	15.48	881	80	20	63.33	3
600°C	12.40	10.12	613	86	14	76.00	3
700°C	7.79	5.46	303	90	10	76.67	3

however, the values for unburned samples are significantly greater than for all temperature treatments, despite the increase at 500 °C (Table 2). Comparatively, change in the percentage of bone and porosity (BV and Po) only somewhat follows this trend, and the extremely low porosity at 300 °C is more prominent than the subsequent increase at 500 °C (Table 2). Pore surface to pore volume ratio (PS/PV) showed the greatest deviation from this general trend, presenting lower values in unburned, 100 °C 200 °C samples before increasing at 300 °C (Fig. 3).

Data shown in Table 3 indicate significant differences only exist in Po.V (P=0.006), Po.S (P=0.003) and PS/PV (P=0.001) at different temperature intervals. Furthermore, post-hoc analysis showed that Po.V was *not* significantly different between unburned and 100 °C samples (P=0.281), however, unburned samples were significantly more porous than samples burned over 200 °C (P<0.034). This was also the case for Po.S, where unburned samples were not significantly different from 100 °C samples (P=0.059), but pore surface area significantly decreased over 200°C (P<0.007). Comparatively, PS:PV multiple comparisons showed that samples burned at 200 °C degrees had significantly lower ratio of pore surface to pore volume than those of higher temperatures (with the exception of 500 °C samples). Samples burned at 300 °C presented ratios that were significantly higher than those of lower temperatures (<200 °C).

Pearson's correlation showed that all variables with the exception of BS/BV were significantly, but weakly correlated (P<0.01, R<0.52) with temperature (Table 4). Of these significant correlations, only BV and PS/PV was positive (bone volume and pore surface to volume ratio increased with increasing temperatures), whilst the other variables were all negatively correlated (decreased with increasing temperatures). The strongest correlation exists between increasing temperature and decreasing Po.V (R=0.72). Logarithmic curves fitted to the data (Figs. 4 and 5) showed the same significance (or lack of) for each variable, with increasing temperature and decreasing Po.V showing the best fit for this regression.

Visual assessment

3D cross-sections of samples showing significant or remarkable variation in all variables measured are presented in Fig. 6. Other samples are shown in supplementary material 1. The presence of microfractures was observed in high temperature samples, however, these were mostly surface level, and did not show up clearly on the 3D models. The unburned and 700 °C samples showed the greatest difference, particularly in porosity. Before burning, bone presented large circular pores, which decreased in size during burning. Narrow porous "channels" appeared at higher temperatures. Some temperatures (e.g. 300 °C) showed the presence of more uniform porosity, which may represent fractures along consistent planes. At 700 °C nearly no porous space was present. This finding was mirrored in the SEM images collected (Fig. 7). Again only samples showing remarkable variation are included here, more can be found in supplementary material 2. As with the 3D reconstruction and analysis, 300 °C, 500 °C and 700 °C samples presented substantially different surface appearances. Micro fracturing appears at 300 °C (Fig. 7 A and D) with very few small/circular pores, whilst at 500 °C (Fig. 7 B

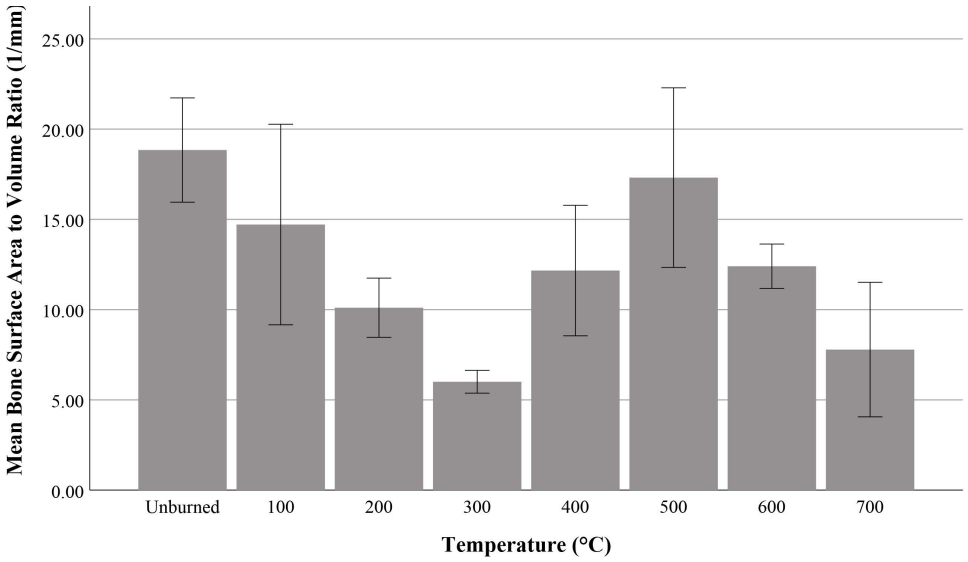

Fig. 1. Histogram of mean bone surface to bone volume ratio (BS/BV) across increasing temperature intervals.

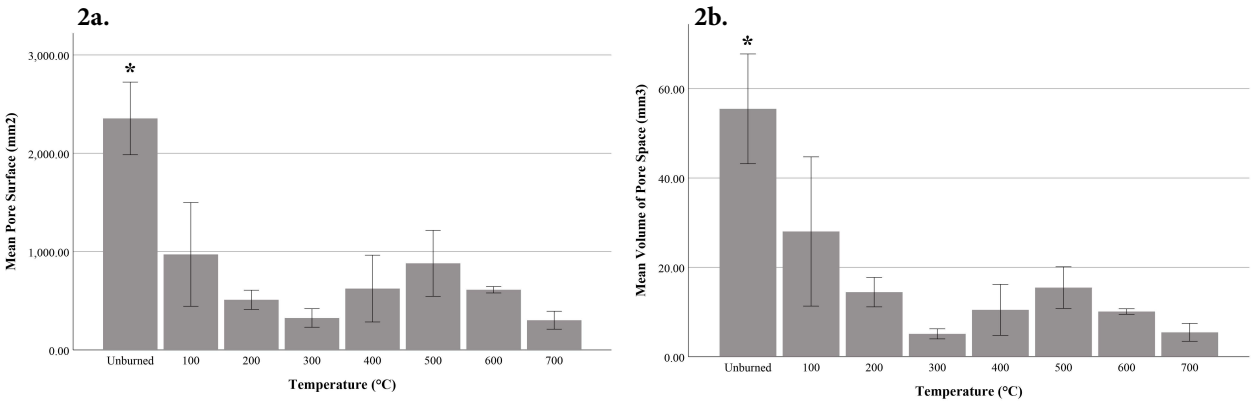

Fig. 2. Histograms showing a) mean pore surface (Po.S), and b) mean volume of pores (Po.V) across increasing temperature intervals.

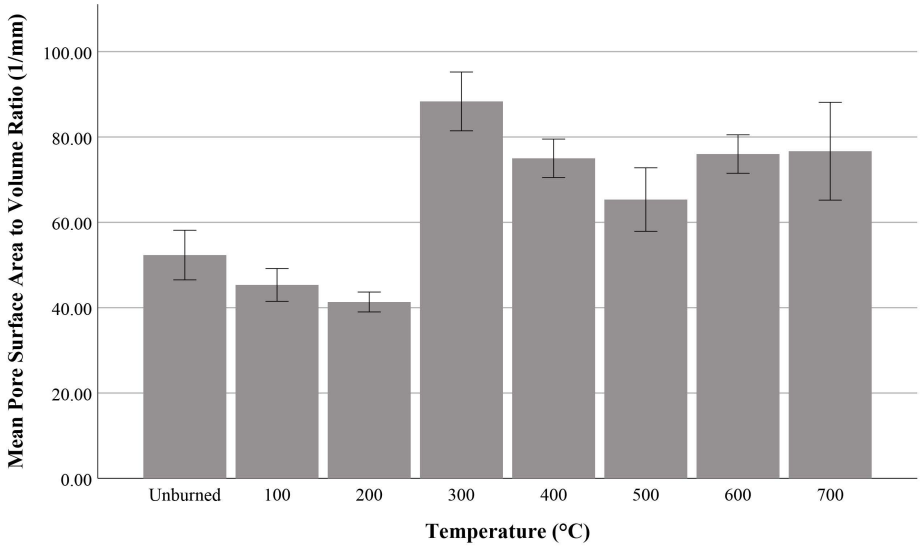

Fig. 3. Histogram showing the ratio of pore surface to pore volume (PS/PV).

Table 3
One-way ANOVA F-test table showing variation within each morphometric variable across all temperature treatments.

Variable x Temperature	F	Sig.
BS/BV (mm^{-1})	1.657	0.190
Po.V (mm^3)	4.487	**0.006***
Po.S (mm^2)	5.236	**0.003***
BV	2.311	0.078
Po (%)	2.311	0.078
PS/PV	6.979	**0.001***

* =*P* is significant

Table 4
Pearson's correlation (one-tailed) and curve fitting (logarithmic regression) between temperature and morphological variables.

	Pearson's Correlation		Logarithmic Regression		
Variable x Temperature	***N***	**Sig. (1-tailed)**	**R**	**R^2**	**Sig.**
BS/BV (mm^{-1})	24	0.127	0.32	0.10	0.118
Po.V (mm^3)	24	**0.001***	0.72	0.51	**0.000***
Po.S (mm^2)	24	**0.006***	0.65	0.43	**0.000***
BV	24	**0.014***	0.52	0.27	**0.009***
Po (%)	24	**0.014***	0.52	0.27	**0.009***
PS/PV	24	**0.002***	0.58	0.34	**0.002***

*=Correlation is significant

and E) samples resembled those of lower temperatures (unburned-100 °C). The most dramatic change was seen in the 700 °C sample (Fig. 7 C and F), where small, circular pores appear consistently alongside larger, narrow crescent-like pores.

Discussion

The purpose of this study was twofold; to provide insight into the structural changes occurring in bone during burning, and to explore MicroCT as an investigative tool for burned bone compared to other techniques (i.e. SEM). As a non-destructive method capable of three dimensional analysis, MicroCT is ideal for fragile samples such as incinerated bone. We aimed to provide a better understanding of what specific changes could be observed using this method, and highlight the benefits it may have over other techniques. A major advantage of MicroCT is the ability to supplement quantitative data with visualised 3D reconstructions. Bone porosity can be assessed numerically as well as visually, providing a more cohesive understanding of how pore complexity changes under thermal insult. The primary finding was that bone porosity significantly decreased after 100 °C, whereas the ratio of pore surface to pore volume was significantly higher at temperatures above 300 °C. The increased ratios suggest that pores are changing in shape and complexity at higher temperatures, which was supported by visual assessment. Using both MicroCT reconstruction software and SEM topographical microscopy, dramatic changes in general bone structure from unburned to incinerated (700 °C) samples were observed. Focusing specifically on porosity, only the 3D MicroCT reconstructions clearly show the change in pore dimensions at 300 °C, as well as the overall loss of porosity at high temperatures.

It is difficult to directly compare results obtained in the current study to other research, as we could find no examples of MicroCT being used to measure porosity of incinerated bone. However, the observed overall decrease in pore space was unsurprising, based on a broader understanding of the taphonomy of burned bones. Many studies using visual assessment (e.g. SEM, radiography, histology) attribute loss of porosity to a) major degradation of organic material (primarily water and collagen), and b) recrystallization of bone mineral. Firstly organic material is lost at temperatures above 100 °C and below 200 °C [26], with collagen denaturation occurring at around 155 °C [8]. This process results in decreased porosity as the bone loses elasticity and shrinks, and cavities previously containing organic material are filled with carbonised debris [8, 27]. Secondly, carbon is replaced by hydroxyl groups which, in the case of dense cortical bone, fill in the spaces left by organic material resulting in a purer, crystalline form of hydroxyapatite [28–30]. The greatest decrease in porosity observed was between unburned samples compared to 100 °C samples. Although this could be a result of the aforementioned loss of organic material, it is surprising that this decrease was greater than in high temperature samples (i.e. 500–700 °C) where significant structural changes were also seen. One possible explanation for this is the trace amounts of trabecular bone seen in some 3D reconstructions (particularly unburned samples), which could have resulted in higher porosity values. Despite being collected from the same section of femoral bone, it was impossible to generate identical sample slices. Although we did not visually observe pre-treatment differences from one sample to the next, it is not unlikely that some degree of microscopic/elemental disparity existed prior to burning. As samples were collected from femoral shafts and no trabecular material was apparent macroscopically, it was surprising to see it on the MicroCT images. Less trabecular bone was apparent in samples burned at temperatures over 100 °C. The fragile lattice structure of trabecular bone is less likely to survive carbonisation, meaning it is often

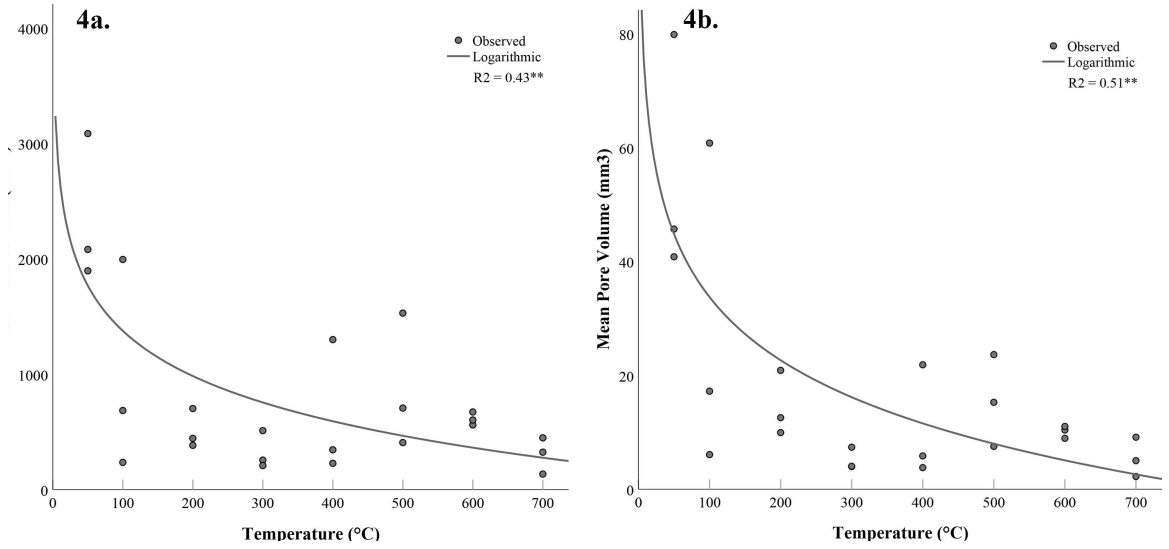

Fig. 4. Logarithmic regression model showing a) mean surface of pores (Po.S) and b) mean volume of pores (Po.V) with increasing temperature.

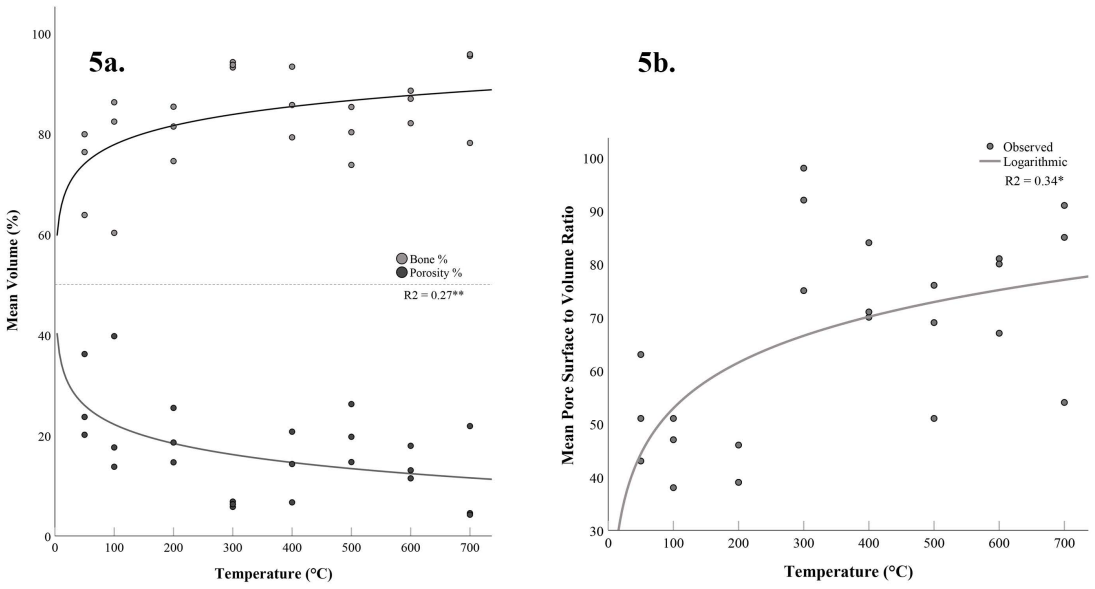

Fig. 5. Logarithmic regression model showing a) the change in percentage of bone (BV) and porosity (Po) and b) ratio of pore surface to volume (PS/PV) with increasing temperature.

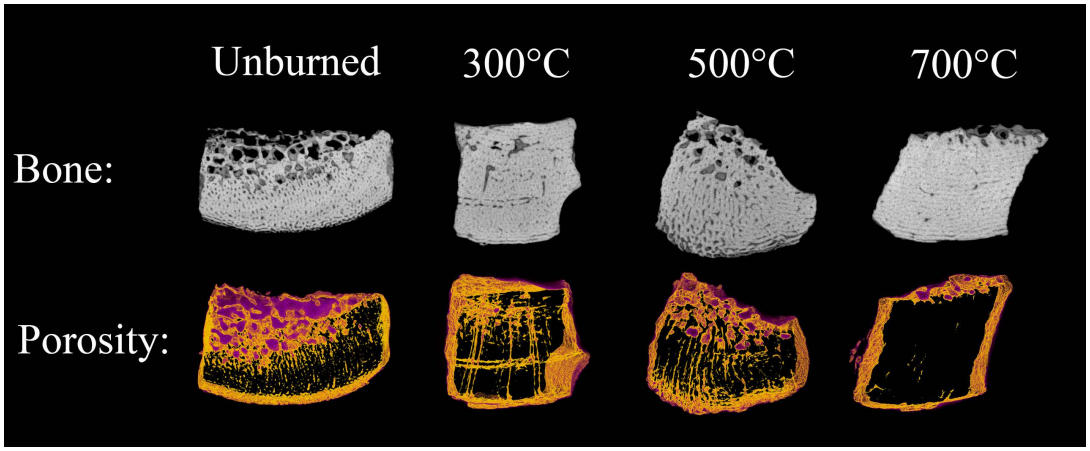

Fig. 6. MicroCT 3D cross sections of samples at selected temperature intervals. Bone material is shown in greyscale. The second row shows an "inverse" representation of bone, with non-bone areas in the region of interest selected to represent porosity.

lost before recrystallization occurs – this is consistent with our findings [31–33]. Some research has shown that at lower temperatures (< 250 °C) the circularly organised lamellar layers of Haversian systems undergo substantial changes, primarily the broadening of Haversian canals as observed with SEM [34]. This reorganisation of bone internal structure, along with the dramatic recrystallization of hydroxyapatite at between 500 and 600 °C [35–37] are likely a primary drivers of changing porosity.

It is apparent from this work that MicroCT may have potential applications in a much greater range of areas than it is currently used in. Thompson [38] noted that heat-induced changes in porosity and fracturing adversely affected standard anthropological techniques such as morphological and metric assessment of bone. MicroCT could provide a means of quantifying how much the internal structure of bone may have been altered during burning, and digital measurements can be taken from CT scans that account for bone shrinkage and degradation. Another potential application is non-invasive investigation of bone pathologies, for example porous lesions on the orbital roof (*cribra orbitalia*), growth arrest lines of varying bone densities (Harris lines), and thickening of the frontal bone resulting from hormonal changes (hyperostosis frontalis) [39–41]. While these morphological changes may be clearly identified on unburned skeletal remains, incineration can make macroscopic diagnosis impossible from examination of bone exterior alone [42]. It has been postulated that heat-induced changes in bone structure and composition may be similar to that of diagenetically altered ancient bone [43]. This has largely been assessed using x-ray powder diffraction (XRPD) analysis, which calculates changes in mineral crystallite size though measurement of diffraction peaks [44, 45]. Although a useful technique, XRPD requires some destruction of the sample, and does not allow visualisation of the interior microstructure of bone. MicroCT could augment this process and provide some contextual information on the similarities (or lack thereof) between modern incinerated and ancient bones. Aside from a lack of research, the major issue with implementing MicroCT in many of the aforementioned contexts comes down to logistical and financial constraints. Like all novel technologies MicroCT will likely became more affordable with time. Most current MicroCT

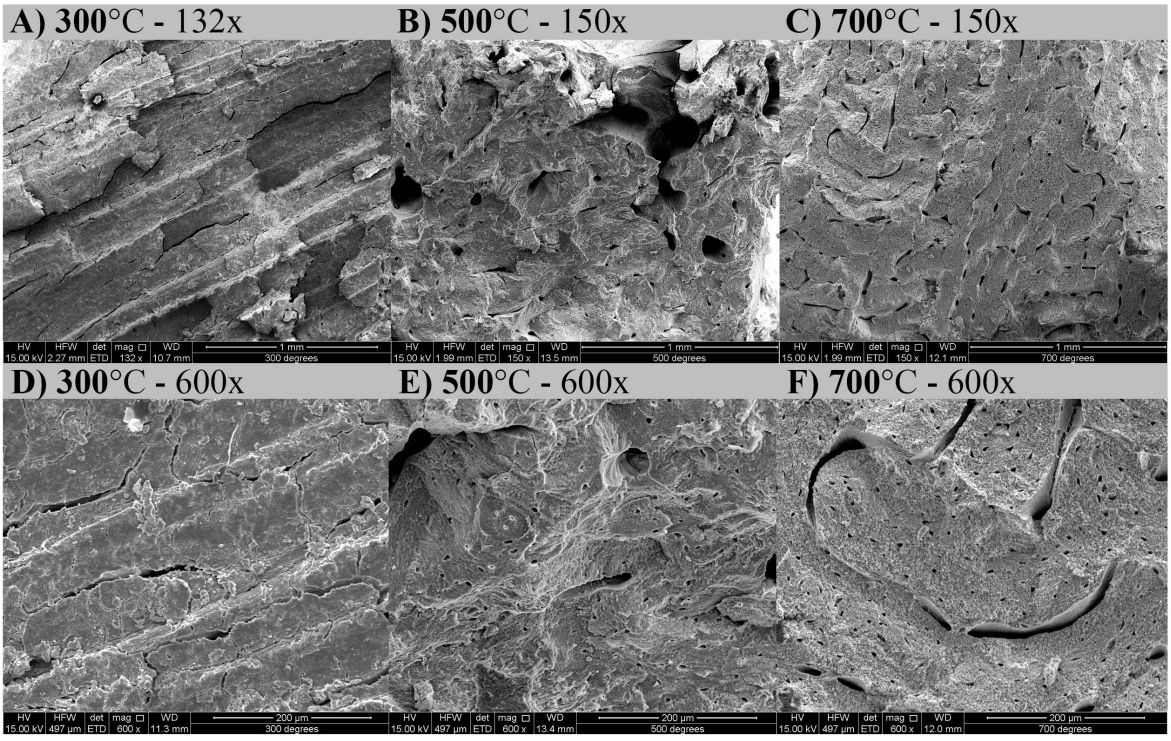

Fig. 7. SEM images of samples from three different temperature ranges (300, 500 and 700). These are presented at lower magnification (< 150x) to show the uniform structure of the bone (A-C), as well as higher magnification (600x) for a more localised view (D-F).

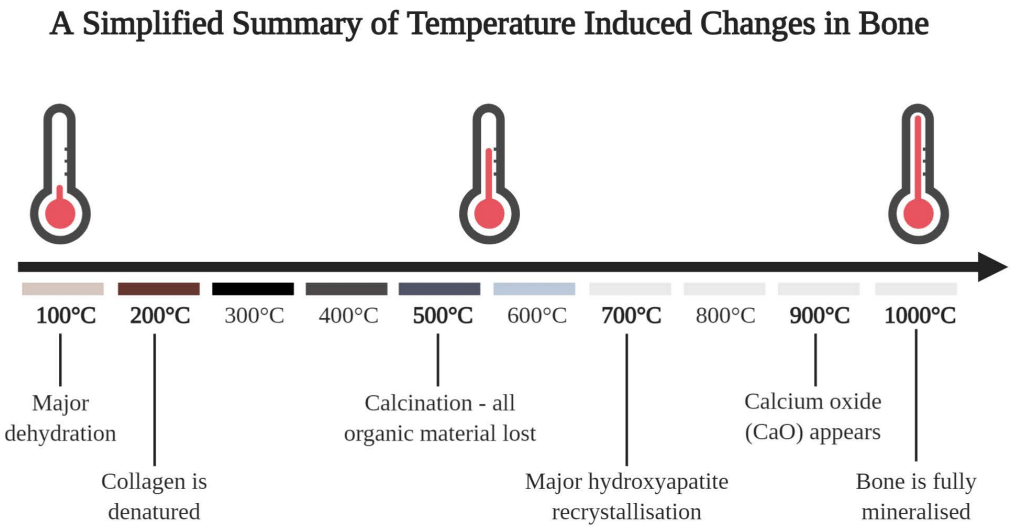

Fig. 8. Simplified diagram showing changes known to occur in bone at increasing temperatures. Changes in bone colouration are represented above each temperature interval, however it must be noted that this (and other factors shown) is variable. Created with BioRender.com.

systems are excellent for investigating small, localised sections of bone. However, a major issue is sample housing volume, meaning larger intact samples (i.e. an entire skull) can only be digitised using ordinary CT instead of the high resolutions afforded by MicroCT. Large volume MicroCT scanners do exist, for example the Nikon XT H 225ST CT cabinet system, which can scan large and heavy objects at much higher resolutions than medical CT systems. However, these are uncommon (at the time of writing there is only one in Australia), and the standard resolution is still less than that of a small volume MicroCT system such as the Bruker SkyscanTM 1276. If large volume MicroCT scanning could be applied to a range of incinerated intact bones, in addition to intact bones affected by various pathologies, a more complete understanding of the similarities (or differences) in porosity could be assessed.

Conclusion

This study showed that changes to bone microstructure, specifically porosity, could be assessed using MicroCT in a more efficient and possibly more effective manner than SEM. Although it is highly unlikely that MicroCT will entirely replace the need for other analytical

techniques, its value as a potential triaging tool should be considered. Due to the three dimensional nature of CT scanning, the internal structure of precious or delicate samples can be investigated without destruction. This could allow for identification of artefacts or regions of interest that could subsequently be avoided or targeted for further investigation. The application of MicroCT in casework certainly warrants further study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fri.2021.200437.

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Chapter 6: General Discussion and Conclusions

6.1 Discussion

Calcified tissues such as bone and teeth are inherently resilient, meaning they have great potential for providing antemortem information and postmortem information, even long after death has occurred. The skeleton has become a staple of forensic identification as it can provide a wealth of anthropological evidence on factors such as age, sex and stature of unidentified remains. Additionally, DNA profiles can often be obtained from skeletal material even after other tissues have been lost. Although significant research has focused on determining identification from skeletal remains, the impact of extreme trauma on these tissues remains relatively understudied. Incineration of remains is a common occurrence in DVI situations, motor vehicle accidents, and household and industrial fires and understanding how this might affect typical procedures such as DNA analysis and anthropological profiling is vital to achieving a successful outcome. The comprehensive review presented in the second chapter of this thesis highlights how little this is understood and suggests some areas of focus for future research. While compiling this review it became apparent that no established protocols exist for extracting DNA from incinerated bones, likely due to the fact that the chemical and compositional differences between burned and unburned bone, beyond a decrease in organic content, is not fully understood (Harbeck et al., 2011, Kalsbeek & Richter, 2006, Collins et al., 2002). Some studies have suggested that incinerated bone is structurally similar to archaic bone, implying that ancient DNA (aDNA) extraction techniques could be beneficial for forensic investigation of burned samples (Piga et al., 2009, Stiner et al., 1995). As DNA is considered a primary method of scientific identification of unknown human remains the main focus of my first study, chapter 3b, was to trial aDNA extraction techniques on burned bone.

Before DNA extraction methods could be compared some issues with creating a “realistic” incineration scenario were faced. I struggled to gain access to a suitable furnace that was both of sufficient size for intact bones and capable of reaching and holding different temperatures at a controlled ramp-up speed. As an alternative I opted for an open fire scenario, using only naturally occurring bush material as fuel. At first, not knowing the temperature of the fire which the samples were burned in seemed like a major limitation. However, on further consideration I decided that this

was a more realistic representation of a true incineration scenario. Ultimately, I decided to trade-off an unrealistic but controlled experiment for a realistic environment where some variables might not be controlled for. Another reason controlling temperature was not vital was that the primary focus of this pilot study was to test different demineralisation methods on bones that were either fresh or incinerated, not the effects of burning at different temperatures. I also wanted to test demineralisation methods on bone that had undergone differing severities of burning as I expected this to have an impact on bone integrity. For this reason, I included different durations of burning as an alternative to controlled temperature. This made the study more robust, allowing me to investigate if burn severity would impact the success of different demineralisation methods, a step advocated in aDNA methods.

Although, as expected, I discovered DNA yield decreased with increased time of exposure even after 120 minutes of burning in an open fire DNA could still be recovered. Although much of the pre-existing research in this field focuses on the effects of burn temperature rather than durations, a notable study by Grela et al., (2021) investigated multiple burn durations at a fixed temperature (400 °C) and achieved similar results. The primary finding of my study, however, was not the differences between burn durations but the effectiveness of modified extraction techniques. Burned bone did not respond especially well to ancient bone extraction protocols, in fact using less conservative methods resulted in greater DNA yield so the ideal extraction method was still to be determined. Grela et al., 2021 compared the effectiveness of four commercial extraction kits on both mitochondrial and nuclear DNA recovery from bone and teeth. They showed that whilst bone provided more DNA than teeth, heat negatively impacted the stability of mitochondrial DNA in both of these hard tissues. This study (and others) also highlighted that both bone and teeth are demanding tissues to work with. Other techniques to overcome the demands of incinerated tissues should be considered. These could include free silica suspension, which is particularly useful for large sample volumes possessing low quantities of DNA (Rohland et al., 2010), and direct PCR where samples are not isolated or purified to prevent the loss of small quantities of DNA (Mercier et al., 1990).

The use of aDNA extraction methods on incinerated modern bone was suggested based on the premise that their crystallite structures were similar. My extraction study, presented in chapter 3b, did not support this, suggesting that the differences between ancient and burned modern bone are more complex than just organic content. Hence, I decided to investigate further what actually changes within the bone through the use of XRPD and SEM analysis. Whilst the focus of chapter 3 was to investigate the impact of incineration on the outcome of DNA analysis, chapter 4 investigated the possible mechanisms behind this outcome. The overall directive was twofold; could I use XRPD and SEM to observe structural/compositional changes at different temperature intervals and if so, would these heat-induced changes bear resemblance to ancient bones (as suggested by the literature) or are they more like fresh modern bones (as suggested by results shown in chapter 3). Unlike the previous study I was able to access an electric furnace with digital temperature controls, although a similar issue with fitting intact samples was faced. The overall conclusion of this investigation was that not all burned bones are equal; incineration at different temperatures results in a spectrum of changes with little differences observed between samples burned below 600 °C, after which recrystallisation is dramatic. This suggests that simply applying separate techniques for “burned” versus “unburned” bones will likely be ineffectual for many samples. This could also indicate that whilst aDNA extraction techniques investigated in the previous study may have been ineffectual, they may still benefit some burned samples exposed to higher temperatures. Comparing the success of extraction techniques in bones burned above and below this 600 °C threshold would be an interesting subject for future research.

In regards to comparing archaic and incinerated samples, no ascertainable difference in crystalline profile or appearance was observed. Despite these results, there were obvious differences between these samples, for example the dense consistency of archaic bones made them significantly harder to cut than fresh bones. It is more likely that although archaic bone and modern bone may have similar crystalline profiles and microscopic appearance at low temperatures, there are many other differences that cannot be observed using XRPD or SEM imaging. This could simply be differences in porosity, collagen content or water content that could result in radically different responses to DNA extraction

methods. To expand on this further I considered other technologies that could be used to clarify the relationship between incineration and tissue destruction, and histology seemed like the next logical step. Moving forward, the results of chapter 4 were particularly useful in narrowing the focus of this next study by eliminating some temperature intervals where little or no change was observed in bone structure. As a PhD project is heavily time constrained, this was a very useful finding.

For my final study I first tried examining histological variation between unburned and burned bone samples. In addition to examining heat driven compositional changes in bone that could affect DNA recovery, I was also interested in linking these changes to observable effects of burning. However, histology proved to be impossible on samples burned at higher temperatures, and no useful results were obtained (Appendix i.). This ended up being a positive experience as it highlighted a need for non-destructive methods of viewing changes in the whole bone. In some forensic contexts samples may be so degraded that attempting to analyse them is a waste of resources (as I experienced first-hand), however this is not always clear by simple visual appraisal. Having a method of evaluating bone integrity without destruction of precious samples would be invaluable. Although much was learned from XRPD and SEM, both these methods were limited by the need for some destruction of the sample and inability to examine the whole bone. Methods such as synchrotron high resolution imaging (or synchrotron-computed micro-CT SAXS/WAXD spectra (Ma et al., 2016)) can be used to conduct 3D ex-vivo reconstruction of complex internal bone structure down to the molecular level. Although this would be an ideal method of investigating structural changes of incinerated bones, the current astronomical cost and logistical constraints make it unlikely that this avenue of research will be pursued in the foreseeable future. More affordable and accessible technology such as Fourier transform infrared and Raman spectroscopy have been used to provide quantitative assessment of incinerated bone, however this provides very little in the way of visual assessment (Thompson et al., 2011, Morris & Mandair, 2011). A more realistic and comprehensive approach for my research was MicroCT scanning, which I used for my final study.

Chapter 5 demonstrated that differences in bone microstructure between burned and unburned samples can clearly be observed using MicroCT without the need for any sample destruction.

Interestingly, although the overall decrease in bone porosity roughly correlates with the increased crystallinity shown in chapter 4 there were additional fluctuations in porosity (primarily at 500 °C) only observed using MicroCT. This is likely due to the much larger scale of MicroCT, which emphasises the need for multiple analytical techniques to assess incinerated bone on different levels. Ultimately, assessing burn temperature and duration is somewhat redundant in a practical context as these variables are not known in a real event. By comparison, linking compositional changes to observable effects of incineration could be vital for development of sample triaging. By consolidating the changes in crystallite size observed in chapter 4 with visual changes observed using MicroCT and SEM imaging, I was able to form a more comprehensive overview of the impact of incineration. Although standard medical CT scanning is routinely used in many forensic laboratories, the higher resolution MicroCT scanners needed to examine tissue integrity are largely limited by sample housing capacity and cost. However, this knowledge is still valuable from a research perspective, and may be particularly useful in the future as these technologies become more accessible and affordable.

Bone is an excellent source of discriminating information, however its behaviour under nonoptimal conditions requires a greater understanding. The results of this thesis showed that not all burned bones are equal and should be treated as such. Incineration of bone tissue is a complex process and therefore requires multiple approaches for a comprehensive understanding. All of the techniques explored in this thesis had advantages and disadvantages (Fig 6.1.), with some offering more information than others at the expense of accessibility or cost. Knowing what each of these techniques can offer when investigating incinerated remains is one of the primary findings of this study. There is a close relationship between research and application, as each informs the other. For this reason, although not all methods explored are applicable in a practical context, it is vital that they are understood for further development. Even as the usage of MicroCT is limited now by factors such as sample housing volume and cost, technological advancements could see these issues overcome in the near future. When this happens, knowing exactly how this can be used for analysis is essential. Additionally, knowing not only how these techniques can be used in cases of incineration, but also understanding the impact of burning itself is vital. The other primary finding of this study demonstrated how burning

at different severities affects DNA recovery and bone integrity. Developing cohesive triaging tools that could use this information to increase the likelihood of successfully recovering information from burned bone would be an excellent future direction.

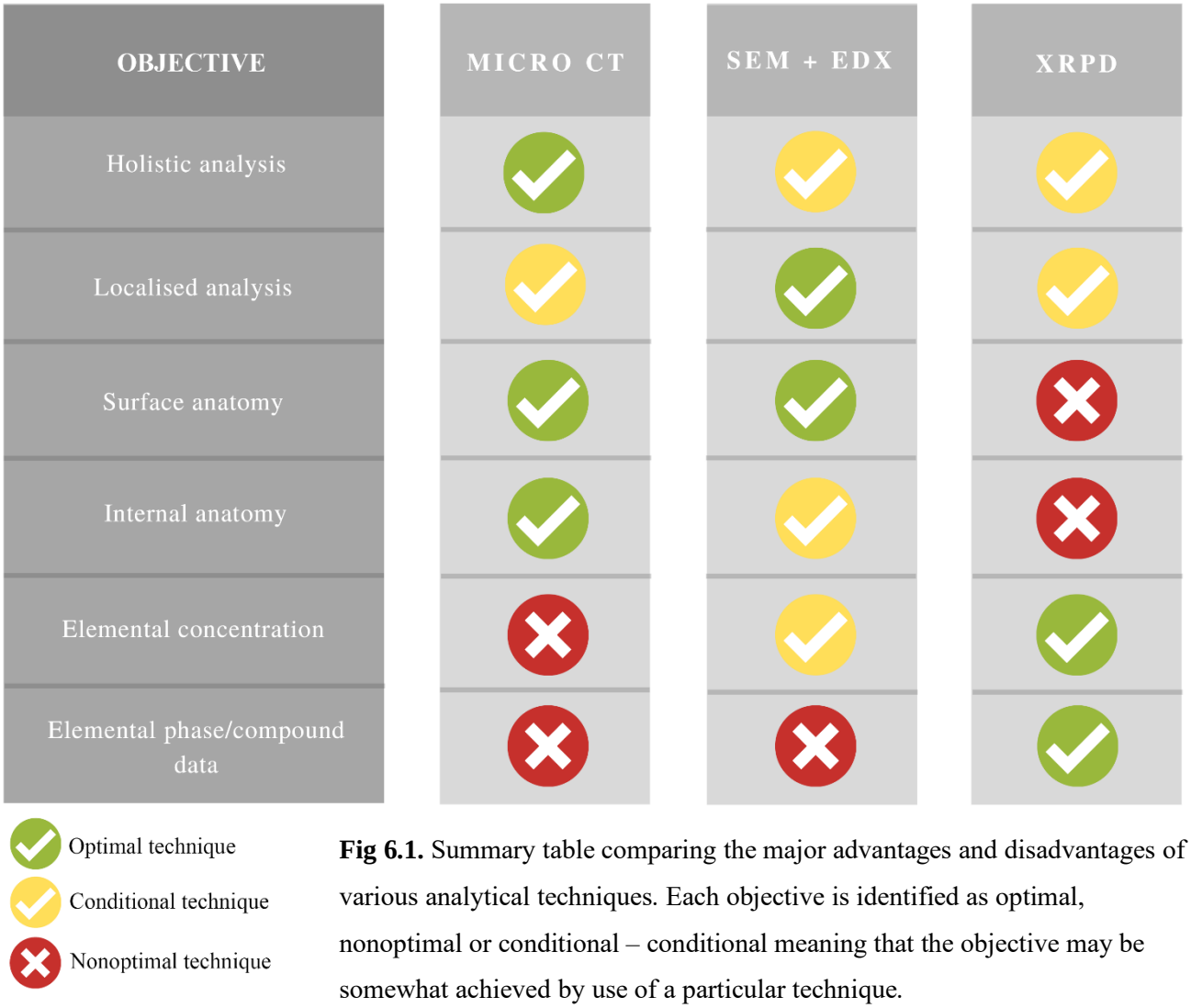

Fig 6.1. Summary table comparing the major advantages and disadvantages of various analytical techniques. Each objective is identified as optimal, nonoptimal or conditional – conditional meaning that the objective may be somewhat achieved by use of a particular technique.

6.2 References

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Appendix

Appendix i. Histological analysis of incinerated bone

The histology of incinerated bone compared to archaic samples was initially going to form another chapter of this thesis. To achieve this, decalcification was performed on a range of burned and archaic samples using the following method:

1. Small rectangular bone samples (~5x10 mm) were collected from larger sections.
2. 50 g of ethylenediaminetetracetic acid (EDTA) was dissolved in 4525 ml distilled water.
3. Sodium hydroxide (~25 g) added to EDTA solution until neutral pH 7.0 achieved.
4. Samples placed in individual test tubes and EDTA solution added.
5. Tubes placed on shaker plate set to low speed to gently agitate samples overnight
6. Each day-old solution was pipetted off, and fresh solution added.
7. Samples regularly x-rayed for signs of decalcification, frequency of x-rays was dependent on the current speed of decalcification.

Following a month of EDTA incubation the incinerated samples would not decalcify, and subsequent cutting, mounting and staining were unsuccessful. Even after prolonged decalcification, incinerated samples were still so dense that attempts at cutting resulted in destruction of the sample and significant damage to microtome blades. Comparatively partial success was achieved with some archaic remains, specifically those taken from permafrost bison bone (Gold Run dig site, ~10-20 Ka), and human burial sites at St Mary's Cemetery (>100 years), Metaponto (~2.2-2.6 Ka) and Pompeii (1.9 Ka). Even these archaic samples did not completely decalcify and after 28 days x-rays indicated that decalcification would likely not progress further (Fig i.1.). The presence of calcified regions, as well as the already degraded nature of the tissue resulted in severe tearing of most tissue samples (Fig. i.2).

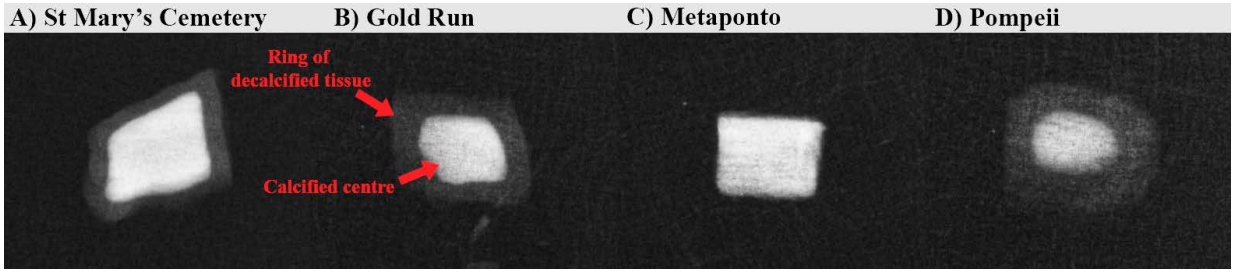

Fig i.1. X-rays of archaic bone samples decalcified in EDTA for a period of 28 days. At approximately 19 days samples developed a ring of decalcified tissue around the calcified centre. By 28 days no further changes of the calcified centre were observed, so decalcification was halted.

Waiting for samples to decalcify only to find most were not viable was disappointing, particularly as the focus on incinerated bone could not be further examined using this method. However, this did provide some useful information about archaic samples and direction for future work. Although too few samples are presented here to provide any statistical significance, it appears that the age of the samples impacted the success of decalcification and tissue preparation (fig i.2). Although the much more recent St Mary's sample presented the most intact osteons, the permafrost bison sample from the Gold Run site was very well preserved considering its advanced age. This supports the X-ray diffraction analysis presented in Chapter 4, which concludes that the permafrost bison bone is not significantly different in structure to modern bovine samples. It is also interesting to note that the histological appearance of intact human osteons is not dramatically different from bovine osteons.

Following unsuccessful decalcification and histological analysis of incinerated bones, vacuum-based resin infiltration was considered as a possible alternative to mounting. Resin embedding removes the need for decalcification by infiltrating bone pores and reinforcing stability, which then allows extremely dense and/or brittle incinerated material to be polished down, exposing the interior without loss of structural integrity (Nielsen & Maiboe, 2000, Erben, R 1997). Preparing bone in this way allows for in situ examination of the interior morphology in an unaltered state. This technique is often used on dense archaic bone (Turner-Walker & Jans, 2008, Yang et al., 2003), and it was theorised that this may be equally effective for incinerated material. Test samples burned at 100 °C and 700 °C were prepared using EpoFix cold resin kit (© 2021 Electron Microscopy Sciences). Once embedded in epoxy resin, samples were polished down to expose the interior. Embedded samples were then

examined using SEM with semi-satisfactory results (fig 6.4). Although this is a useful technique to explore then interior structure of bone, higher quality images were obtained from the powdered samples presented in Chapter 4.

The histomorphology of incinerated bone is something that could be further explored – I only trialled one method of embedding, and this did not present significantly better results than un-embedded samples. Although Methylmethacrylate (MMA) is the most commonly used medium for resin embedding of undecalcified samples (Sterchi & Eurell, 1995, Buijs & Dogterom, 1983), other commercially available resin kits may actually provide more reliable results without use of hazardous chemicals (Mohsin et al., 2006). None of these resin embedding techniques have been properly optimised for burned samples, and exploring this further could be of significant value for forensic analysis.

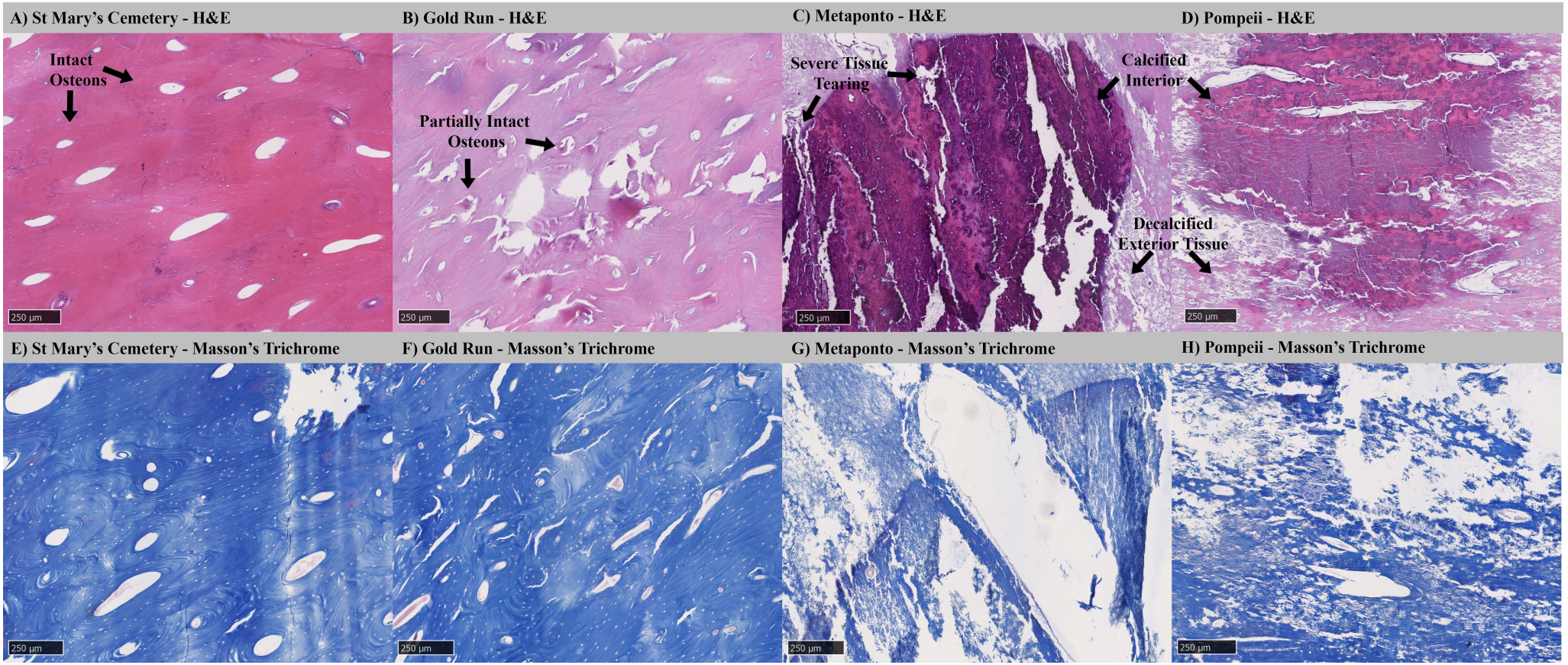

Fig i.2. Histological sections of archaic bone stained with H&E (A-D) and Masson's Trichrome (E-H). Although the H&E staining appeared to work slightly better than Masson's Trichrome on degraded tissue, the two stains were not dramatically different. The St. Mary's samples (A & E) presented the most intact tissue and osteons, whilst samples from Metaponto (C & G) and Pompeii (D & H) did not completely decalcify, resulting in severe tearing of the tissue and a lack of intact osteons. The permafrost Gold Run samples (B & F) were the intermediary, showing some tearing and partially intact osteons.

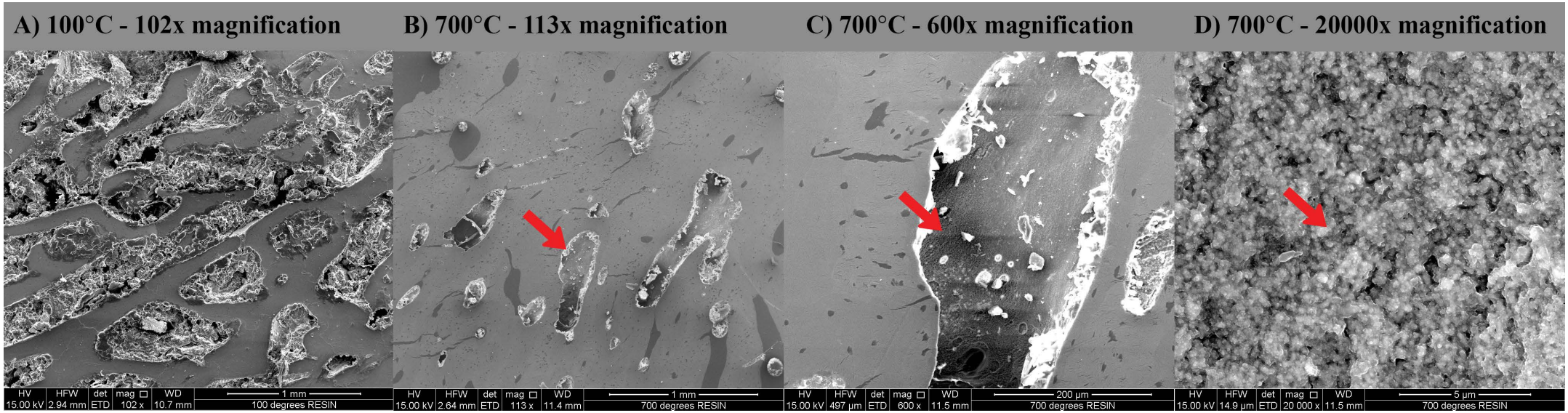

Fig 6.4. SEM captures of resin embedded sample at low to high magnification. Figure A) shows a comparison sample burned at 100 °C, which is significantly more porous than the incinerated samples presented in B) - D). The red arrow represents a region of interest on the same sample examined at higher magnification in figure C) & D). The granulated appearance of bone at higher magnification C) is comparable to SEM captures presented in Chapter 4, however this was only observed within small pockets of tissue.

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Appendix ii. Awards and other achievements

Presentations

- 2021 ***"Recovering DNA from burned bone"*** ANZFSS SA branch award presentations, oral presentation via zoom.
- 2020 ***"Comparing crystal structure in burned remains – implications for forensic sampling"*** Florey postgraduate research conference. Poster presentation via zoom.
- 2018 ***"Can DNA be extracted from burned bones: analysis of various sampling sites in pig and human models"*** Research presentation given at Forensic Science South Australia. Oral presentation.
- 2018 ***"Growth patterns and individual variation in mid-sagittal facial soft tissue depth from childhood to adulthood"*** 87th Annual Meeting of the American Association of Physical Anthropologists. Austin, Texas, USA. Poster Presentation.

Teaching/lectures

- As a teaching assistant in Anthropological Anatomy II, I demonstrations during practical exercises, help grade student assessments and provided the following lectures:
    - 2018 ***"Skeletal Identification"*** Oral presentation.
    - 2018 ***"Ecosensitivity of Human Biological Characters"*** Oral presentation.
    - 2018 ***"DNA extraction from severely damaged bones: an analysis of various sampling sites and extraction methods"*** Oral presentation.

Collaborations/professional acknowledgements

- Forensic Science South Australia (FSSA) – In addition to allowing me to present my proposed research at the beginning of 2018, FSSA allowed me to complete a three month contract working as mortuary technician whilst COVID-19 prevented me from working on my thesis.

- Advanced DNA, Identification & Forensic Facility (ADIFF) – Staff at the ADIFF provided technical expertise, some consumables and access to low-copy DNA laboratory space.
- Faculty of Engineering, Computer and Mathematical Sciences, Adelaide University – Staff provided training, technical expertise and use of x-ray diffractometer.
- Adelaide Microscopy – Staff at Adelaide Microscopy provided training and access for use of MicroCT, SEM & EDX equipment, also assisted with interpretation of results and technical expertise.
- Australian New Zealand Forensic Science Society (ANZFSS) – ANZFSS provides a lecture series dedicated to developments in forensic science, which is directly related to my area of research. Becoming a committee member has allowed me to network with experts in the field.
- SA Museum – Anna Russo of SA museum allowed me to assist Ellie Simpson in auditing the museum's stored skeletal collection, which was invaluable to the development of my anthropological profiling skills.

Other achievements

- Publication of honors work: Mckinnon, M, Simpson, E & Henneberg, M 2018, 'Growth Patterns and Individual Variation in Mid-sagittal Facial Soft Tissue Depth from Childhood to Adulthood', *Journal of Forensic Sciences*, vol. 63, no. 6, pp. 1641-1651.
- Ross Vining Memorial Student Scholarship – I was awarded this scholarship for the abstract I submitted for the 22nd Triennial Meeting of the International Association of Forensic Sciences. The conference was to be held in Sydney, 2021, and the scholarship was to assist with the cost of registration, airfares and/or accommodation. However, the event was cancelled due to COVID-19.