Post-mortem environment and DNA quality: studies addressing the forensic utility of routine molecular analyses.

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Declaration

I declare that the research presented in this thesis, for the Master of Forensic Science at the University of Western Australia, is my own work. The results of the work have not been submitted for assessment, in full or part, within any other tertiary institute, except where due acknowledgement has been made in the text.

Lauren Swift
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ABSTRACT

Forensic anthropology involves the identification of human skeletal remains in the context of a medico-legal setting, achieved through the application of physical anthropological skills and methods (Byers 2011). Routine anthropological metric and non-metric assessments are performed and factors such as age, sex, ethnicity and stature can be relatively accurately estimated, which assists towards the positive identification of a victim (Franklin 2010). By performing such investigations, the pool of potential victims is reduced, allowing for more rapid identification through established biological processes (e.g. DNA sequencing). Many taphonomic processes (such as animal scavenging, climate variations and burial environment) have the potential to alter skeletal morphology and DNA and therefore the feasibility of subsequent molecular analysis. Disaster victim identification relies on both molecular analysis as well as anthropological assessment to achieve positive identification following skeletal exposure to extreme environmental conditions.

The present study examines the quality and quantity of skeletal DNA extracted from porcine long bones following prolonged exposure to varied taphonomic environments. Bones were subject to one of three test conditions; fortnightly cycles of wet/dry conditions, fortnightly cycles of freeze/thawing or submersion within 1% sulphuric acid. Medullary DNA extraction were subsequently performed on both intact and fragmented remains within each group at one, three and five month intervals. This was assessed against test control bones as well as fresh control bones at each time point. Preliminary quantification was achieved through nanodrop spectrophotometry; this was followed by gel electrophoresis. These results indicated that organic DNA extraction provided higher quantities of DNA compared to commercial extraction kits. DNA qualifications were measured following sequencing using three primers; two mitochondrial DNA primers and one nucleic (Pig growth hormone) primer. Trace scores (phred scores), QV20+ values and continuous read lengths were used to
gauge DNA quality. Each measure of DNA quality was assessed against five factors; treatment group, length of exposure to environment, method of extraction, bone fragmentation and primer selection, using one-way ANOVA.

Bones within acidic environments consistently elicited the lowest quality DNA, similarly the quality of DNA obtained through sequencing using Nucleic DNA was significantly lower than that obtained through MtDNA sequencing. While unsurprisingly the test control bones provided the highest quantities and quality of skeletal DNA. The result of the present study indicate that taphonomic processes not only negatively influence the morphology of skeletal remains, but also have the potential to promote DNA degradation following prolonged exposure. Primer selection proved to be the single greatest factor influencing the success of DNA amplification with mitochondrial primers providing higher quality DNA for every treatment group at all three extraction points. The conclusions of the present study have the potential to influence processes utilised for victim identification following natural or man made disasters. Despite bone diagenesis, skeletal molecular analysis has the potential to yield high quality sequences (in the case of freeze/thaw and wet/dry bones). With primer selection crucial to this success, the combined sequencing of nucleic and mitochondrial DNA would be recommended in order to achieve positive identification regardless of the taphonomy surrounding the remains.
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CHAPTER ONE

Introduction

1.1 Introduction

In a routine forensic investigation numerous avenues of analysis are employed in order to ascertain the identity of a decedent. In situations where skeletal material is the sole source of evidence, a forensic anthropologist is employed to establish (as accurately as possible) a biological profile (Kimmerle et al. 2008). Forensic anthropology involves the identification of human skeletal remains in the context of a medico-legal setting, achieved through the application of physical anthropological skills and methods (Cattaneo 2007). Routine anthropological metric (the acquisition of linear measurements between pre-determined points as per the method) and non-metric (a visual assessment based on a set of criteria specific to the bone and method used) assessments are performed, and factors such as age, sex, ethnicity and stature can be relatively accurately estimated, which assists towards achieving a positive identification (Franklin 2010). By performing such investigations, the pool of potential suspects (or victims) is reduced, allowing for more rapid identification through established biological processes such as DNA profiling. With regard to personal identification of skeletal remains, an important factor to consider during anthropological assessment is the availability of ante-mortem information on the decedent needed for comparison against the established biological profile; this might be lacking, making identification difficult. In order to achieve identification in these situations molecular investigations should be considered. DNA fingerprinting is one commonly used avenue of investigation; a genetic profile is created and matched to living relatives, samples from personal items, or previous biological samples that may exist.
1.2 Post-mortem skeletal disturbances

Relying on skeletal remains (as the medium through which identification is attempted) can be problematic when changes or alterations that can occur to bone via natural or deliberate interventions are considered. These post-mortem disturbances have the potential to influence the preservation of remains and thus adversely affect the accuracy of the biological profile, in addition to degrading the genetic material contained within. Taphonomic changes affecting bone can be subcategorised into those that have occurred naturally or those that are the result of deliberate intervention from a third party.

Naturally occurring taphonomic processes relate to the environment in which remains have been interned. Animal scavenging, temperature variations, the presence of water and soil composition are examples of naturally occurring taphonomic process that all have the potential to influence skeletal preservation (Sledzik 2002). Scenarios in which bone has been subjected to annual seasonal waterlogging and freezing provide examples of naturally occurring environments that have also been shown to alter the chemical composition and histology of bone. The latter increases bone degradation, which potentially impedes the ability of the anthropologist to create an accurate biological profile (Hedges 1995; Millard 1995). Naturally occurring post-mortem influences have been shown, (in an archaeological context) to alter bone morphology, however the extent to which the genetic material within the medullary cavity is affected is largely unqualified and thus forms the basis of the present study.

Furthermore increased acidity occurring as a result of either deliberate intervention or naturally occurring low pH environments also has the potential to influence not only the morphology of the bone but also the integrity of the genetic material within. Skeletal material subjected to low pH environments has previously been shown to cause osseous dissolution (Gordon and Buikstra 1981; Nicholson 1996), this loss of structural
integrity has the potential to then influence the utility of genetic material within the medullary cavity.

The final way in which skeletal assessments can be hindered relates to bones becoming highly fragmented as a consequence of the nature of death. The utility of fragmented remains for forensic investigation is of particular consequence when considering events such as mass disasters, mass burials and explosions, in which there is typically a high degree of fragmentation (Holland et al. 2003). Using September 11 as an example, some 250 fragments of remains were investigated each day during recovery and identification. Identification required the combined efforts of anthropologists, forensic dentists, in addition to the biotechnology group Bode. The vast majority of identifications (62%) were achieved using DNA fingerprinting (Holland et al. 2003) showing the importance placed on the necessity for the extraction of useable undamaged genetic material from fragmented and damaged skeletal remains.

1.3 Molecular identification

DNA sourced from bones and teeth is a commonly applied investigative tool (both forensically and archaeologically) to determine identification and lineage through profiling (Gill et al. 1994; Silva 2007). A small DNA sample can be extracted from bone or bone marrow, amplified using the polymerase chain reaction (PCR), and then matched to living family members in cases where anthropological assessment and identification through dental records is not possible. DNA from the medullary cavity can be used because the surrounding cortical bone is both thick and strong and thus acts as a protective barrier for the cells within. Post-mortem influences such as moisture, pH and temperature, therefore are believed to have little influence on the cells within (Soler et al. 2011b) (see Chapter Three for further discussion).

In contrast, the degree to which seasonal waterlogging, freeze thaw cycles, and immersion in acid affects the degradation of DNA within skeletal material has not been extensively investigated, and thus provides
the basis of the present project. Through controlled experimentation, the
degree to which DNA can be successfully extracted and amplified from
bones subjected to environmental stressors or deliberate damage will be
established; this has considerable importance for establishing investigative
standard operating procedures. Knowing under what conditions high
quality DNA (purity and concentration values of a sample) is likely to be
available will facilitate more efficient allocation of investigative resources,
saving both time and money. It also ensures that the integrity of evidence
is maintained by performing only destructive sampling that is likely to elicit
quantifiable DNA.

1.4 Aims

Knowledge of taphonomic impacts on skeletal evidence is beneficial to
forensic investigations. Situations that result in damage to skeletal tissue
have the potential to adversely impact the success of molecular analyses
(Alonso et al. 2005). The objective of the present project is to determine
under what circumstances bone can be sampled for molecular analysis
and to what degree burial environment affects DNA quality and quantity.
This will concurrently involve an assessment of commonly used DNA
extraction methods.

The specific aims of this project are as follows:

i. To establish the extent burial environment affects the quality and
quantity of DNA extracted from bone

Using laboratory simulations, porcine bones will be subjected to three
different taphonomic processes; two of the latter will mimic natural
seasonal variations within the burial environment, whereas the other will
relate to a deliberate attempt to damage remains. In a mass burial
environment bones often move through phases of becoming waterlogged
and dried (due to the depth of burial); deeper burials can reach the water
table, whereas shallow burials are subject to seasonal rainfall fluctuations.
Furthermore, liquid associated with decomposition can accumulate in
burial environments where drainage is insufficient (Wilson et al. 2007),
thus the latter will be one of the avenues investigated during this project. The freeze-thaw cycle can affect bones buried within shallow graves in environments where large fluctuations in temperature occur seasonally (or even daily) (Forbes et al. 2005); this comprises another avenue of investigation. Immersing bones in acid represents an attempt to deliberately conceal or discard remains and is therefore the final avenue of investigation being explored. In order to establish the effect that long-term exposure to the aforementioned environmental influences has on DNA quality and quantity. Sampling will be performed in duplicate at one month, three month and five month intervals. The effect of all aforementioned environmental influences will be assessed against controls at each stage.

$i$. **Is DNA quality and quantity affected by bone fragmentation?**
Fragmented remains are often encountered in scenarios involving explosions, mass disasters and high impact deaths, such as aircraft and motor vehicle accidents (Clayton et al. 1995). It is therefore important to know the effect that fragmentation has on nuclear material within the medullary cavity. Bone as a solid unit acts to protect the DNA within its cavities and breaking this protective barrier exposes the molecular material within to potentially deleterious environmental conditions and thus has the potential to reduce DNA yield. The present project will investigate this effect by comparing the yield of DNA retrieved from intact and fragmented bones simultaneously at each of the aforementioned sampling periods investigated (see Aim 1). Knowing how fragmentation affects the utility of molecular analysis should facilitate the efficient allocation of resources during investigations to maximise potential for positive identification.

$ii$. **Establish the effectiveness of selected DNA extraction techniques**
The final aim of this project compares and contrasts the effectiveness of two extraction methods in relation to how they function to elicit DNA from the test bones. The purpose of this is to inform investigators of which method of DNA extraction can be successfully applied to bone affected by any of the modifiers tested.
The methods tested are:

- Organic extraction using phenol/chloroform
- Commercial extraction kit (Quiagen)

1.5 Sources of Data

Porcine (*Sus scrofa*) long bones are used as an analogue for human bone material. The pig material is ethically sourced from the domestic Linley Valley Abattoir (Linley Valley Rd Wooroloo, Western Australia) where they are humanely slaughtered for the purpose of food consumption (See Appendix 1). Porcine bones are used as the source material because human bone was not available, and also due to the similarities they share with human bone in terms of morphology (Pearce 2007). In addition to the widespread availability of pig bones, another factor contributing to this selection is the availability of the porcine specific molecular primers (Larkin et al. 2010). Once acquired from the Linley Valley Abattoir, the bones were manually defleshed and stored in a refrigerator at 4°C until they were required for experimentation (see Chapter Four for further details).

1.6 Thesis outline

This thesis comprises six chapters. Chapter Two provides insight into the background of the project: what is DNA? how is DNA useful in forensic investigations? how the environment affects bone degradation in general; in addition to exploring some issues faced during practical casework, such as disaster victim identification and mass burials. Chapter Three reviews literature relating to the effects of freeze/thaw and wet/dry cycles, as well as low pH environment, all have on bone morphology, histology and DNA quality. In addition, the chapter provides an overview of relevant previous work performed within this field, as well as a comparison of commonly used DNA extraction procedures. Chapter Four details the materials used and the methods applied, including all DNA extraction protocols and quantification and qualification protocols. Chapter Five comprises the results presented in relation to the aims of the thesis. Chapter Six discusses the results in the context of existing knowledge and provides as
assessment of how the present research has practical applications and benefits in modern forensic investigation.

1.7 Potential limitations

Studies involving decomposition in an Australian forensic context are hindered by the fact that animal analogues must be used in place of human remains. Accordingly, the present research is being conducted using domestic porcine (Sus Scrofa) bone, rather than human. While porcine bones resemble those of a human in terms of size, shape and strength, the fact that they originate from a foreign species should be noted when making direct comparisons and assumptions to a human context. Despite this, the use of porcine remains in place of human tissue has been proved effective in previous related studies (Haunshi et al. 2009; Sembon et al. 2008) that utilise pig specific markers for genetic analysis.

Laboratory simulations represent a highly controlled environment in which to conduct experiments. While they afford the opportunity to regulate factors such as temperature and humidity, and provide protection from predators and miscellaneous environmental stressors, it is obvious that they do not strictly mimic the reality of real-world environmental conditions. Variable temperatures, weather patterns, unknown predation and time periods are all factors that impact on real investigations, but are controlled in these experiments. It should, therefore, be taken into consideration that the outcomes of this experiment are scenarios in which confounding external factors are largely removed or controlled. The above external factors would (in reality) influence the outcome of any molecular investigation and should therefore be duly considered.

Further potential limitations include the possibility of molecular contamination from foreign parties. Steps taken to ensure that this does not occur include wearing protective equipment (such as gloves, lab coat, face mask and goggles) in addition to ensuring all equipment is autoclaved both prior and post use. The aforementioned protocols attempt to mitigate foreign DNA contamination, however this is a risk that is apparent in all
casework and should therefore be noted as a potential limiting factor for this research. Importantly, the use of pig specific primers prior to PCR should eliminate DNA sourced from foreign (human) origin being amplified and sequenced (Haunshi et al. 2009; Sembon et al. 2008).

Finally, this research will be conducted over a five-month period, which is a considerably shorter period than the post-mortem time frame associated with skeletonised remains. The latter is unavoidable within the construct of a twelve month Master by Research Thesis. The weathering processes tested is also an accelerated version of natural and deliberate post-mortem disturbances as a result of annual seasonal variations or intervention.

1.8 Summary

The outcome of this project has potential practical applications, not only for forensic casework, but also in an archaeological and historical context. The results will allow investigators to predict under what circumstances DNA profiling can be successfully utilised during an investigation, particularly those involving weathering, deliberate acid disturbance and high rates of fragmentation. Thus facilitating the correct and cost effective allocation of resources, as well as maintenance of skeletal evidence, for cases in which molecular intervention would otherwise be redundant.
CHAPTER TWO

Bone as an investigative tool

2.1 Introduction

This chapter explores the relevant background information on the use of bone and the genetic material within as a forensic investigative tool. The structure and function of DNA is first discussed along with a brief introduction into the development of PCR and sequencing technologies. This is followed by an explanation of the gross structure and function of bone, and its cellular components. How taphonomy facilitates the diagenesis of bone is then considered and the chapter concludes with a brief review of disaster victim identification and the holistic use of bone as an analytical tool. The purpose of this chapter is to establish a link between the breakdown of bone through burial taphonomy and the affect this has on its morphology, this facilitates inferences regarding the potential impact this may have on DNA integrity. Background information is also provided on the procedures and technologies that are employed in the present thesis.

2.2 Structure and function of DNA

Deoxyribonucleic Acid (DNA) is the basic building block for life; a cellular code that facilities the production of amino acids (and thus proteins) essential for normal cellular function. Structurally DNA is a double helix comprised of a backbone of alternating phosphate and sugar deoxyribose groups (covalently bound). To each sugar group one of four nitrogenous bases (adenine, cytosine, guanine or thymine) is covalently bound, forming what is known as a nucleotide (Brown 2011). This configuration is shown in Figure 2.1.
The four nitrogenous bases are classified into two groups according to their structure: purines (adenine and guanine); double ringed structures and pyrimidines (cytosine and thymine); single ringed structures (Figure 2.2). Due to their variation in structure, and therefore binding sites, purines and pyrimidines are complimentary units, that pair in a very specific manner according to the number of hydrogen bonds they share. Adenine pairs with thymine due to the availability of two hydrogen-binding sites, while cytosine pairs with guanine, as they have three binding sites each (Figure 2.2). This complimentary pairing rule gives DNA its double helix structure and causes each DNA strand to be a compliment to the strand opposing it (Moat 2002).

Nucleotides link together forming a polynucleotide chain that can be millions of base pairs in length. In order to fit vast amounts of genetic material into the nucleus of a cell, DNA helices are wound into chromosome structures. Humans have 23 pairs of chromosomes (22 autosomal and one pair of sex linked) per cell. Investigations involving genetic material typically focus on select regions of DNA within the chromosomes known as loci (Yunis 1977). Each DNA strand is given
directionality by numbering of the carbon atoms within the nucleotide sugar ring, the terminal end of the strand is known as the 5’ (prime) end while the opposing end is the 3’ (prime) end. Due to the antiparallel nature of DNA, each double helix has both a 5’ to 3’ strand as well as a 3’ to 5’ strand. This directionality is important, as synthesis can only occur in the 5’ to 3’ direction.

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Recommended IUPAC Name</th>
<th>Common Name</th>
<th>Synonyms</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Chemical Structure" /></td>
<td>1H-Purin-6-amine</td>
<td>Adenine</td>
<td>6-AminoPurine</td>
</tr>
<tr>
<td><img src="image2.png" alt="Chemical Structure" /></td>
<td>2-Amino-1,7-dihydro-6-H-purin-6-one</td>
<td>Guanine</td>
<td>2-Amino-6-oxopurine (2-amino-hypoxanthine)</td>
</tr>
<tr>
<td><img src="image3.png" alt="Chemical Structure" /></td>
<td>5-Methyl-2-4-(1H3H)-Pyrimidinedione</td>
<td>Thymine 5-Methyl 2,4-dioxopyrimidine (5-Methyl uracil)</td>
<td></td>
</tr>
<tr>
<td><img src="image4.png" alt="Chemical Structure" /></td>
<td>4-Amino-2(1H)-pyrimidinedione</td>
<td>Cytosine</td>
<td>4-Amino-2-oxopyrimidine</td>
</tr>
</tbody>
</table>

**Figure 2.2** Structural differences of each nitrogenous base, with particular attention being paid to the differences shown between pyrimidines and purines (Moat 2002).

### 2.3 Nuclear and Mitochondrial DNA

DNA is sourced from two locations within a cell; the nucleus and the mitochondria. The type of DNA contained within each differs in their location, mode of inheritance, function and forensic use. It is therefore important to understand and appreciate the differences between these variants in order to undertake the most effective molecular analysis.
2.3.1 Nuclear DNA (nDNA)

DNA is packed tightly within chromosomes contained in the nucleus; humans have their DNA arranged into 23 pairs of chromosomes (22 autosomal and one sex). nDNA consists of two antiparallel polymer strands that pair together (following base pairing rules); this structure is then wound into a double helix and further contorted until it is able to be arranged into each of the 46 individual chromosomes (Butler 2005). nDNA follows Mendalian inheritance rules, a diploid system whereby half of an individuals genetic make up is inherited from each parent; nDNA is, therefore, the targeted evidence in paternity disputes. nDNA is also predominantly used during DNA sequencing, with Short Tandem Repeats (STR) being the target regions mapped during analysis (See also Section 2.6.2).

2.3.2 Mitochondrial DNA (MtDNA)

MtDNA is contained solely within the mitochondrion of the cell and is comprised of a singular DNA ring of approximately 16,569 base pairs (Krawczak 1998). Each cell can contain over a thousand mitochondrion, making the amount of mtDNA per cell far greater than that of nDNA, which only contains 22 chromosomes. mtDNA (unlike nDNA) lacks an effective repair mechanism, which allows a high mutation rate. This facilitates more efficient forensic investigation because the mutations make the MtDNA more individualizing (Sigurðardóttir et al. 2000). Mitochondrial DNA is inherited in a haploid fashion, unlike nuclear DNA, which follows Mendalian inheritance. mtDNA is solely inherited from the maternal side, and is therefore effectively used to establish maternal lineage (Butler 2005). One famous example is that of the Romanov Family of Russia. Samples obtained from the skeletal remains of the royal family were effectively compared to maternal relatives of the Tsarina and used to establish identity (Gregory et al. 2006)
2.4 Central dogma

A fundamental principle of molecular biology is that of central dogma, which explains the flow of genetic information from DNA through RNA to proteins. This is achieved through the unwinding and separation of a DNA double helix into a replication fork, whereby two antiparallel polynucleotide strands act as templates for the formation of DNA/RNA. This function is facilitated by helicase, an enzyme responsible for unwinding and separating annealed DNA strands (Rodwell 1990). When DNA replication occurs, the DNA polymerase attaches to the template strands to read the sequence in a 3’ to 5’ direction. However, since the opposing strand runs antiparallel, the polymerase must function asymmetrically to simultaneously replicate both strands. The leading strand can be read and replicated easily, however the lagging strand (running 5’ to 3’) must be read and synthesized in small segments (known as Okazaki fragments) due to its orientation. RNA primers attach between Okazaki fragments during synthesis, but are later replaced by DNA once replication is complete (Murray 2009). In the case of gene expression, this complexity is not necessary, but rather an enzyme (RNA polymerase) reads the newly separated strand of DNA to produce an antiparallel strand of genetic material. If the section of DNA copied codes for a protein then messenger RNA (mRNA) is produced. This process is known as translation and is the first step in the process of central dogma (Rodwell 1990).

Once mRNA has been produced, the DNA template reforms and the mRNA migrate out of the nucleus and into the cytoplasm where it attaches to a ribosome. This attachment marks the start of the next stage of the central dogma process, transcription. The ribosome reads the template mRNA and attaches amino acids, as per base pairing rules to create a polymer of amino acids (Gilbert 1992). The polymer (or protein) produced is specific to the gene that was originally expressed and translated by the RNA polymerase. The principle of specific base pairing is paramount to the entire process of unwinding, reading and copying. It is this principle that is exploited during biomolecular investigations, with the aid of a
process known as polymerase chain reaction (PCR), which functions to exponentially increase any sample of DNA evidence.

2.5 Polymerase Chain Reaction (PCR)

Kary Mullis first developed PCR in 1983; this process increases the amount of genetic material from desired loci on the genome in order to facilitate sequencing. DNA Oligonucleotide Primers designed to specifically target and bind to these areas of the genome are added to a sample and repeated cycles of heating and cooling allow for DNA replication to be mimicked in a controlled environment. PCR is a cyclical process that involves three stages denaturation, annealing and extension. Movement through these stages increases the starting material by a factor of $2^n$, where “n” is equal to the number of cycles (Booth et al 2010).

2.5.1 Denaturing

The initial stage of PCR requires the double helix to unwind and denature, which is achieved by heating the sample to 94°C. At this temperature the weak inter-nucleotide hydrogen bonds are broken and the two DNA strands separate in the centre to produce a replication fork. This makes the two strands vulnerable to primer annealing (Voet 1999).

2.5.2 Annealing

The next stage of replication is that of primer annealing, whereby small sections of DNA (typically 20-30bp in length) known as primers bind specifically to their targeted regions of DNA (as per base pairing rules). A primer functions in pairs to anneal and replicate both antiparallel strands simultaneously, thus one primer is designed to target the 5’ and while the other targets the 3’. This is facilitated after cooling the mixture to between 45°C and 60°C, the temperature of this step is crucial; too hot and the primers cannot anneal sufficiently, and too cold has the potential to lead to non-specific binding. The temperature of this stage depends on the melting point of the primers used (Rychlik et al. 1990).
2.5.3 Extension

Extension of a newly formed strand of DNA occurs under the influence of Taq polymerase. Taq polymerase is a DNA polymerase originating from *T. aquaticus*, a bacterium that resides within hydrothermal vents. For this reason, Taq polymerase functions most efficiently within high temperatures, which makes it ideal for PCR where extension occurs under 72°C conditions. Taq uses free available nucleotides to synthesize an identical DNA sequence originating at the primer. Nucleotides are added at a rate of approximately 2,000 nucleotides per minute (Su et al. 1996). (Figure 2.3).

![Figure 2.3](image)

**Figure 2.3** Polymerase chain reaction, showing denaturation, and annealing and extension steps. The exponential nature of this analysis can be appreciated through this diagrammatic representation (Butler 2005).

For forensic analysis, 25-30 cycles are usually used to increase the amount of starting material by $2^{30}$, effectively a billion-time increase in DNA material. After this has been achieved, the amount of material is large enough for detailed analysis to occur without the issue of limited starting sample (Booth et al. 2010)
2.6 DNA profiling and the evolution of Short Tandem Repeat (STR) technology

Nuclear DNA is classified into coding and non-coding regions. Genes comprise the section of coding DNA that is responsible for the production of amino acid and proteins. Approximately 2% of the human genome is comprised of these coding regions; the remaining 98% is non-coding areas of unknown function. These sections are of forensic significance, as they are the target regions for DNA fingerprinting. Short tandem repeats (STR’s) (or microsatellites) are sections of (primarily) non-coding DNA comprised of 2-10bp repeats. Millions of STR’s are found within the human genome. Collectively they account to approximately 3% of its length (Mullis 1994). STR’s occur in consistent locations (or loci) throughout the human genome. The number of repetitions at any particular loci, however, varies amongst individuals. It is this variation that makes STR analysis the target of biomolecular investigations (Butler 2005). The evolution of DNA analysis can be separated into two developmental stages: Restriction Fragment Length Polymorphisms (RFLP) and STR analysis; these discussed in more detail below (Edwards et al. 1991).

2.6.1 RFLP: Minisatellite testing

On September 10 1984, while working in his Leicester University Laboratory, Dr Alec Jeffreys noticed the varied development in a piece of x-ray film containing the DNA of three individuals. The various differences and similarities between the three individuals allowed Jeffreys to deduce the potential use of the technology for paternity issues, suspect identification and sample matching (Jeffreys 1985). The basis of the technology relies on variations in the length of the minisatellite, which (like microsatellites) are areas of repeating nucleotide base pairings within nuclear DNA. During the process, minisatellites (that are larger in size than microsatellites) are targeted with restriction enzymes. The DNA sample is cleaved by these restriction enzymes along the site of minisatellites, resulting in variation of lengths at each site; these are then electrophoresed to allow separation based on size (Jeffreys 1985).
Multilocus probes are then introduced to the sample, which hybridize at the site of minisatellites to display a bar code like pattern; this pattern is believed to be highly unique between individuals and is known as Restriction Fragment Length Polymorphism. While this technology facilitated the identification and exoneration of criminals such as Colin Pitchfork, who in 1987 became the first individual incarcerated on the basis of DNA profiling evidence (Jobling and Gill 2004), the limitations were also considerable. Due to the multiple steps required for analysis, the process often took a number of weeks; it also required the use of a large amount of starting material, which was not always feasible in certain forensic cases where sample material was minute. Development of a more refined technology needed to continue in order for widespread use to be considered.

2.6.2 STR analysis

The basis of DNA sequencing is now based on STR analysis after amplification from PCR. The need for a large sample is eliminated through the use of PCR, which exponentially amplifies a particular loci of interest based on the primers used (Butler 2005). In essence STR analysis functions through the use of highly specific sequence primers, which bind to the desire microsatellite. These areas are then amplified via PCR, separated and detected through the use of either gel or capillary electrophoresis. By investigating multiple loci simultaneously, a highly specific pattern emerges that allows accurate identification (Butler 2005). The advantages of STR analysis over RFLP are as follow:

- PCR allows more sensitive reactions to occur allowing analyse to be performed on much smaller samples;
- PCR cycles occur rapidly and analysis can be complete in less than 24 hours as appose to multiple weeks, this make STR analysis both time and cost effective;
- Targeting STR, which are smaller loci than those targeted during RFLP, means that fragmented or degraded samples may still be
analysed, as the chances of survival is much higher for sections that are shorter (Gill 2005).

2.7 Gross structure and function of bone

Bone is a tough tensile composite of protein (collagen) and mineral (hydroxyapatite). Functionally bone acts as a biomechanical component of the musculoskeletal system, providing the framework and stability required for movement. Bone also provides structure and support for all of the soft and connective tissue of the body and is the site of tendon and ligament insertion that facilitates movement of the body (Martini 1998). The skeletal system is also where blood cells are produced, in addition to being a reservoir for minerals (such as calcium and phosphorus), growth factors and fat (White and Folkens 2005). Structurally bone can be separated into two categories, cortical and trabecular; these are discussed further below, including the microscopic functional units of each, its overall cellular structure, and the components and function of the bone marrow.

2.7.1 Cortical bone

Cortical (or compact) bone comprises the cortex (or outer shell) of most bones, which lies directly under the periosteum (White and Folkens 2005). Cortical bone is tougher, harder and stronger than cancellous bone. These qualities afford it the function of structural support and protection from external stressors. The physical properties of cortical bone arise from highly regulated functional units that run longitudinally within bone; these Haversian systems (or osteons) are comprised of concentric rings of mineralised collagen around a central canal where blood vessels and nerves run (Rho et al. 1998). Within the Haversian systems, microscopic osteocytes (bone cells; see below) reside within lacunae (or spaces); nutrients are delivered to the osteocytes through microscopic fluid-filled canals named canaliculi (White and Folkens 2005) (Figure 2.4).
2.7.2 Cancellous bone

Cancellous (spongy or trabecular) bone differs from cortical bone on a number of levels. Cancellous bone is far less dense than cortical bone (Figure 2.5). This is due to cancellous bone being comprised of bony spicules rather than concentric osteons, giving it a woven appearance (Rockoff et al. 1969). The primary functional unit is trabeculae, a microscopic projection of bony material that forms spongy lightweight and porous bone. Due to the lightweight composition of cancellous bone, it has a far greater surface area when compared to compact bone; this facilitates metabolic activity allowing cancellous bone to be easily remodelled (Rho et al. 1998). This lightweight and spongy bone is found within the vertebral bodies, between flat bones of the skull and at the epiphysis of long bones. These are the sites where blood cells are formed, (see also section 2.11).
2.8 Molecular structure and development of bone

Bone can be described in relation to its molecular and cellular constituents. At the molecular level bone is a composite material of organic and mineral elements. These function together to provide strength, robusticity, flexibility and elasticity (White and Folkens 2005). The principal organic and mineral components of bone are collagen and hydroxyapatite.
respectively; these components and the role they play in bone formation are explained below.

- **Collagen**: the organic element of bone is comprised of Type 1 collagen, which groups together extracellularly to form elongated fibrils. These fibrils are a large constituent of the extracellular matrix of bone, which provides structure, strength and elasticity. Within the fibrils there is, however, free space that is filled by the mineral element of bone (Buehler 2006).

- **Hydroxyapatite**: the mineral element of bone is comprised of calcium apatite, which includes phosphates. This element forms a crystal structure that infiltrates collagen fibrils to fill the free space left between them. Hydroxyapatite affords bone its flexibility and in combination with collagen overall robusticity (Cai et al. 2007).

At the cellular level, cortical and cancellous bone is comprised of the same three cells; osteoblasts, osteocytes and osteoclasts. These cells differentiate from osteoprogenitor cells during embryonic development in response to external signalling (Schwartz and Boyan 1994). The function of each and its location within bone is described below and illustrated in Figure 2.6;

- **Osteoblasts**: mono-nucleated cells responsible for the production of bone material and osteoid, a protein mixture that calcifies when infiltrated by hydroxyapatite. Once calcification occurs osteoblast become entrapped and mature to osteocytes. Large concentrations of osteoblasts can be found just beneath the periosteum (Olszta et al. 2007).

- **Osteocytes**: once osteoid calcifies the osteoblasts become entrapped and mineralise. The entrapped mature osteoblasts are then known as osteocytes, (or bone cells) which are responsible for the maintenance of bone tissue. Within compact bone the concentric rings of the Haversian system contains osteocytes within lacunae (Weiner and Wagner 1998).
- **Osteoclasts**: large multi-nucleated cells responsible for the resorption of bone that act in a phagocytic capacity to “eat away” bone, allowing for remodelling to occur. Osteoclasts are found primarily bordering the medullary cavity. (White and Folkens 2005).

![Visual representations of the cellular components of bone in their relative area(s) of origin](image)

**Figure 2.6** Visual representations of the cellular components of bone in their relative area(s) of origin (Martini 1998).

An appreciation of the molecular and cellular structure of bone is essential to understanding the mechanism behind bone breakdown during burial. Cell type and location within bone is of particular importance when considering its use for molecular analysis. Multinucleated cells are lysed for their genetic material during DNA analysis; these can originate in the medullary cavity or bone material itself (Terada et al. 2002).

### 2.9 Bone marrow and haematopoiesis

The bone marrow occupies the medullary cavity and is subcategorised into red and yellow types, with the former containing haematopoietic cells, and the latter largely comprises adipose tissue. The amount of each type of marrow within any bone varies according to age of the individual, as well as the metabolic environment. Generally speaking, red marrow occupies the majority of bone throughout infancy; this slowly becomes less moving into adulthood, until during later life the red marrow is confined to
the skull, vertebra, ribs, clavicle, sternum, pelvis and proximal ends of the femur and humerus (Vogler 1988). The primary biological function of the red marrow is haematopoiesis; the production of blood cells. This occurs when haematopoietic stem cells, within the intra trabecular tissue of the medullary cavity, differentiate and proliferate in response to external demand. They differentiate into one of three categories of blood cells (Fernandez and de Alarcon 2013):

- Erythroids; immature red cells that will eventually become circulating red blood cells;
- Lymphocytes; cells of the adaptive immune response, T-cells and B-cells;
- Myelocytes; cells of innate immunity, including macrophages, megakaryocytes and granulocytes.

While mature red blood cells do not contain DNA, the immature reticulocytes (a subcategory of erythroids), lymphocytes and myelocytes all contain genetic material (Davis 1994). The present research focused on the use of bone marrow as an investigative tool for molecular analysis, therefore knowledge of the cells within, as well as their genetic constituents, is essential. The manner in which those cells respond to external stressors is also essential and is further explored in Chapter Three.

2.10 Environmental influences (Diagenesis)

The primary aim of this thesis is to investigate the affect taphonomic disturbances have on the genetic material within bone. In order to understand the mechanism behind genetic disruption, an appreciation of the mechanism behind the breakdown (or diagenesis) of bone is required. Water content, temperature and acidity are all influencing factors that have been shown to affect bone preservation (Hedges 2002; Nielsen-Marsh 2000; Smith et al. 2007). If taphonomic factors have such an important role in the preservation of bone tissue, then it stands to reason the same
applies to the preservation of the molecular material contained within. Bone is broken down after burial through a process known as diagenesis (Zapata et al. 2006). Diagenesis has the potential to not only affect morphology, but also its molecular integrity. There is considerable debate over the parameters through which bone diagenesis can be assessed as well as the mechanism behind the process. Generally speaking, diagenesis can be assessed through four interrelated avenues that act specifically against both the mineral and organic aspects of bone; these are accordingly reviewed below.

2.10.1 Crystallinity

Hydroxyapatite is the crystalline composite of bone that provides flexibility and structure when combined with Type 1 collagen. Fresh bone is generally poorly crystalline due to this association with collagen (Surovell and Sitner 2001). The process of diagenesis, however, leads to a loss of collagen, as well as a potential intake of fluorine from the environment, which facilitates carbonate loss and results in an increase in apatite crystallinity (Surovell and Sitner 2001). The process behind increased crystallinity is debated, but it is generally appreciated that collagen acts as a stabilizing mineral, therefore once it is lost, the two elements disassociate and chemical changes in the hydroxyapatite occur more freely. The later results in apatite crystallization (Hedges 2002).

2.10.2 Porosity

This factor relates to how porous a bone is (or has become). In essence, it is a reflection of bone density, in relation to both micro- and macro-structures. Micro- and macro-porosity escalates as bone ages, but increasingly so in response to diagenesis. Bone porosity has been linked with overall water content, or fluctuation of water, within bone and the burial environment. A study by Nielsen-Marsh (2000) investigated eight archaeological burial sites across northwest Europe in an attempt to measure a number of digenetic parameters, one of which was bone
porosity. They concluded that porosity was directly related to protein loss, which occurred as a result of mineral leaching out of bone due to fluctuating water levels (or burials within a more acidic environment). These conclusions were further supported by Hedges (2002), who described porosity as a measure of the potential for water uptake and mineral loss as a result of the surrounding burial environment. Hedges (2002) investigated the theory that mineral loss via leaching is dependent on the pH of surrounding soils, as well as the mineral content. Leaching occurs when the amount of mineral in the soil is less than that within bone; this is associated with an acidic environment. Neutral soils already have high concentrations of calcium and phosphate, therefore equilibrium between the burial environment and bone is met much sooner and leaching is stopped (Gordon and Buikstra 1981).

2.10.3 Microbial attack

Microbial attack refers to the colonization and infiltration of microbes/bacteria into bone. This occurs when bacteria are prevalent within the burial environment and the newly decomposing remains provide a new area for potential colonization. Hedges (2002) strongly emphasize the importance of microbial attack during the initial stages of bone diagenesis as an avenue for collagen loss and therefore increased porosity, this leads to a loss of structural integrity within the bone. Bell et al (1996) reported evidence of microbial attack after only three months, to which they link an increased colonization of microbes with a loss of collagen and protein, as well as increased porosity. The mechanism behind how microbial attack results in a loss of collagen and protein is unclear, but it was shown by Jans et al. (2004) that microbial colonization is associated with an increase of crystallinity and porosity.

2.10.4 Collagen

As stated above, collagen acts as a stabilizing mineral within bone, therefore collagen level is often used as an indicating factor for organic
preservation. Smith et al. (2007) investigated collagen loss in archaeological bones dating to the European Holocene. They found that accelerated collagen hydrolysis was one of the most influential factors behind bone degradation. They were also able to show a correlation between microbial attack and collagen loss, which had previously been suspected but not empirically demonstrated (Smith et al. 2007). Similarly, collagen association with hydroxyapatite stops increased crystallinity from occurring within the mineral aspect of bone, but once collagen is lost, crystallinity increases, resulting in accelerated bone diagenesis (Rey et al. 2009).

2.11 Forensic applications

Bone as a physical entity is utilised through a number of avenues during forensic investigation. The context of the crime and the preservation of remains dictate how it may be used, but its physical properties often ensure a certain level of preservation far greater than soft tissue (Zehner 2007). The avenue of investigation taken with skeletal remains can be independent or incorporate multiple disciplines. When attempting to ascertain identification often the latter is the ideal approach.

2.11.1 Forensic anthropology

As previously stated in Chapter One, Forensic anthropological investigations reply entirely on evidence in skeletal remains. Estimations of age, sex, ancestry and stature are the basis of an osteobiography that can be effectively matched to suspected decedents (Kimmerle et al. 2008). The acquisition of this information is based upon assessments made in the skeletal remains. These assessments are either metrically attained from pre-determined measurements taken of select bones in accordance with a particular investigative method, or through non-metric visual analyses of bones as determined by a set of standards (Franklin 2010). In an ideal situation, a biological profile is used to find potential matches in an attempt to achieve positive identification. There are, however, numerous factors that compromise anthropological
assessments, a lack of ante-mortem information available for comparison, highly fragmented remains, co-mingled remains and taphonomy have the potential to alter bone morphology such that anthropological assessment is not sufficient enough to result in positive identification, in these instances DNA investigations become important.

2.11.2 Forensic biology

Due to the protection afforded by bone, molecular analysis of the medullary cavity has proven an effective tool for identification of decedents. DNA is contained within all nucleated cells of the body and can be extracted from the cells within the medullar cavity (marrow, osteocytes, osteoblast and osteoclasts) (Hochmeister et al. 1991). Soler et al. (2011a) successfully extracted DNA from the femoral diaphysis of 20 individuals buried between 1998-2001 across Brazil. The amount of DNA extracted was compared to the number of microscopy visible nuclei within the osteoclasts of the femur. The results of this investigation indicate that osteoclasts are highly nucleated cell that can provide the basis from which skeletal DNA analysis is attained.

Zehner (2007) demonstrated the extent to which foreign DNA can adhere to compact bone, leading to inaccurate DNA sequencing. Samples from two victims of the 2004 Indonesian Tsunami were assessed against ante-mortem references samples. It was shown that foreign DNA contaminated surface samples, including soft tissue and those taken from bone marrow of fragmented remains. The proposed mechanism of contamination occurred as a result of transfer when multiple victims were decomposing adjacent to (and even on top of) each other. Therefore, issues of DNA sample contamination are particularly prevalent for cases involving mass fatalities. In order to avoid potential contamination, the medullary cavity of intact remains is used as the source of individualizing DNA. Less contamination is expected due to the structural protection afforded to it by the outer cortex. Another potential factor limiting the utility of molecular analyses of bone is DNA degradation. This is a concern particularly in the
analysis of archaeological remains. The rate at which mtDNA and nDNA decay occurs exponentially after burial. nDNA decays at twice the rate of mtDNA, thus making analysis of ancient remains problematic (Allentoft et al. 2012). The present research specifically addresses the use of bone marrow as a source of DNA following prolonged burial; the degradation of DNA is accordingly assessed under varied environmental conditions.

2.12 Disaster Victim Identification (DVI)

The amalgamation of forensic anthropological assessment and DNA sequencing is utilised in a number of forensic investigations, but perhaps the most important (and difficult) are scenarios that involve identification of individuals following a mass disaster. When Disaster Victim Identification (DVI) is required, often-large quantities of biological material needs to be processed. The latter requires the combined efforts of an array of investigators, including anthropologists, pathologists and molecular biologists (Budowle et al. 2005; James 2005). The composition of any team of investigators relates to the context of death, as well as the post-mortem environment in which remains are recovered.

The Waco siege of 1993 is one such example where DNA analysis was paramount to identifying multiple remains. The siege occurred in a religious compound northeast of Waco, Texas. A fire broke out in the complex after prolonged gunfire between law enforcement and Branch Davidians. In total 72 members of the branch Davidians perished as a result of the fire. Thermal trauma resulted in highly fragmented and comingled remains. Molecular analysis was performed on 61 skeletal, deep tissue, organ and skin samples, and these compared to reference blood samples provided by known relatives. STR quadrupole and amelogenin analysis was performed on each sample, which resulted in the identification of 26 people. Quadrupole and amelogenin typing proved to be both accurate and reliable in this investigation, which reinforced the robustness of DNA sequencing in mass disaster and forensic scenarios (Clayton et al. 1995).
Another example of holistic forensic approaches to disaster identification is that of the September 11 (2001) World Trade Centre (WTC) attack. After the hijacking and subsequent collision of American Airlines Flight 11, and United Airlines Flight 175 into the North and South WTC towers, 15,000 fragmented skeletal remains were submitted for molecular analysis in order to identify 2,792 individuals (Biesecker et al. 2005; Brenner and Weir 2003; Holland et al. 2003). As of 2005, 850 of 1,594 individuals were identified using DNA profiling alone, and over 1,000 when including anthropological assessment (predominately dental record matching from odontologists).

The 2004, South East Asian tsunami provides yet another example of collaborative forensic efforts for victim identification following a natural disaster. Schuller-Gotzburg and Suchanek (2007) provide an overview of the identification process for some 4,000 victims. They concluded that 80% of the non-Thai victims were positively identified with the aid of forensic odontologists using dental records, but issues arose when a lack of ante-mortem information existed for certain individuals (particularly younger individuals who had not experienced regular dental visits, and those people of lower socio-economic status with no dental record/missing teeth) in these instances DNA identification and fingerprint analysis was utilized. When DNA analysis was utilized accurate positive identification was achieved when extraction occurred from bones samples or from within the medullary cavity as these areas provided protection from external influences (Zehner 2007).

The combined effort of forensic teams provides an increased probability that identification will be achieved, providing some degree of closure solace to the families of those affected by disaster. The success of these avenues of investigation is dependent upon many factors including, access to remains, speed of recovery and availability of ante-mortem information and familial genetics (Budowle et al. 2005). The present study aims to contribute to this particular forensic discipline by assessing the
effect that prolong exposure to taphonomic elements has on skeletal remains both morphologically and genetically.
CHAPTER THREE

Review of relevant literature

3.1 Introduction

This chapter reviews previous research into the effect that seasonal waterlogging and drying, periods of freezing and thawing, as well as environments of low pH, have on the integrity of bone and the DNA contained within. The literature explored will focus primarily on archaeological cases, but contemporary examples are also explored in relation to DNA degradation. This chapter establishes the present study within the context of relevant literature, explores links between published research, and demonstrates how the present study has the potential to fill knowledge gaps within the field of victim identification, particularly in cases involving severely weathered and/or damaged skeletal remains. Identification based on DNA analysis from weathered bone material is a relatively under-investigated field, thus this study aims to provide relevant data to help guide forensic practice and stimulate further research.

3.2 Waterlogging

The following section will explore relevant literature pertaining to the effect of water and water movement (hydrology) on skeletal material. Alternatives to skeletal morphology are explored in relation to the effect that is observable or quantifiable through histology, altered mineral content, and porosity. Following this, the extent to which DNA quality and quantity can likewise be affected by this process is explored.

3.2.1 The effect of waterlogging on bone

The degree to which water (both stagnate and fluctuating) affects bone diagenesis has been extensively studied (e.g. (Bocherens et al. 1997; Hedges 2002; Hedges 1995; Nielsen-Marsh 2000; Stojanowski et al. 2002; Von Endt 1984). Within a burial environment, hydrology (the
process of moving water) controls the saturation level of the surrounding groundwater with respect to the mineral portion of bone (calcium and phosphate). Continually moving water prevents the surrounding groundwater from becoming saturated, and thus promotes the dissolution of hydroxyapatite (Cox 2000) leading to bone diagenesis.

This extremely complex process is described by Hedges (2002) who states that the loss of minerals and increase in porosity is attributed to the movement of water within the burial environment. This is amplified within low pH, semi aquatic environments, when a larger surface area is exposed to the environment (e.g. when bone has become highly fragmented) and when recharge of the surrounding water occurs readily. Hedges (2002) also adds that diagenesis occurs due to the uptake of environmental solutes that displace the naturally occurring skeletal solute, which in turn leads to the slow breakdown of the bone.

Within the scientific literature, research in relation to this topic is largely archaeological in nature (e.g. (Nielsen-Marsh 2000) and the conclusions drawn from each study are thus broad, with the bones having been subjected to a plethora of varied environmental factors prior to testing. Some common inferences, however, can be drawn from these studies, which are accordingly reported below.

iv. Bocherens (1997)
Bocherens investigated differential preservation (with respect to chemical composition) of skeletal remains excavated from a French Neolithic paleochannel. Skeletal remains were excavated from an upper bank (emerged area) where water flow was subject to tidal variation, and also from the base of the channel (immersed area) where water flow was constant. Two locations (Bercy and Louviers) were studied to facilitate cross-site comparisons. Histological and chemical analyse were conducted on all excavated remains; 70 µm thin slices were prepared and an assessment of histological integrity performed. The visual assessment categorised the preservation state on a scale from “very bad” (microscopic structure has been lost with Haversian systems indiscernible) to “good”
(structural integrity remains with Haversian systems and osteons clearly distinguishable). Chemically, carbon and nitrogen content was assessed from 5 mg powder samples of each specimen.

The level of nitrogen present in the sample mimics the level of collagen and therefore gives an indication of structural integrity and diagenesis. Levels ranged from 0.1% N (indicating no collagen was preserved) to 4%, which is the level of collagen present in fresh bone samples. The results of this study show that at both sites the bone in the immersed (or upper bank) had the lowest levels of nitrogen (averaging 0.7% N in Louviers and <2% in Bercy) and therefore poorer collagen preservation. These remains were found to exhibit “very bad” histological preservation meaning they had lost microscopic structural integrity. Interestingly, those bones were also prone to a higher incidence of microbial attack compared to the remains within the lower bank (or immersion zone). Conversely, the remains from the lower bank had comparatively “good” histological preservation (i.e. bone was intact) and had averaged nitrogen contents of 2.7% in Louviers and 2.5% in Bercy. Their results thus show that bones from the immersed area had less degradation compared to those from the fluctuating areas of the upper bank.

This was a pioneering study because it introduced the concept that nitrogen content of bone has a positive correlation with collagen content, and can thus reflect the level of diagenesis exhibited. This concept has been continually used as a means of reporting bone degeneration. The results of this study were further confirmed by the findings of subsequent research (e.g. (Nielsen-Marsh 2000; Stojanowski et al. 2002) that are explored further below. Those studies suggest that moving water increases the dissolution of hydroxyapatite, as the bone and surrounding environment cannot reach equilibrium with each other.

Nielsen-Marsh investigated the relationship between site environment and bone diagenesis, with a specific focus on the role of water and water movement. Degradation of bone was measured based on altered histology, collagen content and porosity. A histological index (0-5) was used to assess the level of microscopic damage that had occurred to the bones over time, with a score of 0 indicating poor preservation (or a loss of histological integrity), whereas 5 indicated fresh (or unaltered) bone. Following Bocherens et al. (1997), collagen content was determined based on nitrogen levels taken from powdered samples. As noted in the previous study, the amount of nitrogen present in bone is indicative of collagen, and therefore gives a relative measure of the level of degradation. Porosity was measured based on water content per gram of the sample. The study examines long bones of both humans and animals from four archaeological burial sites across northwest Europe, the oldest of which dates to 12,000 years BP.

Histologically it was shown that bones within fluctuating water were poorly preserved compared to those in dry or saturated conditions. Similarly, the water-covered bones were also found to have increased porosity and lower collagen content. These findings support the theory that fluctuating water causes increased degradation of bone, and thus serves as a strong grounding from which the present research thesis seeks to develop and explore this concept. The study concludes by inferring that the level of water movement within a particular area is likely critically involved in the preservation (or destruction) of buried skeletal remains. The main limitation of this paper is that the mechanism(s) explaining how hydrology affects bone preservation are not explored in enough depth. It is highly likely that a number of other contributing factors assist in the degradation of bone, with hydrology being an important, but not the sole, determinant.
vi. **Summary**

The general consensus in the literature investigated in this section, regarding the effect that hydrology has on bone diagenesis, is that fluctuating water causes high levels of diagenesis (Pike et al. 2001). How the latter has the potential to affect the DNA within the bone was not considered in any of those studies. In fact, the manner in which this process affects the potential use of these bones as tools for identification was not explored in any of the papers reviewed above, thus highlighting the requirement for empirical research of this nature.

### 3.2.2 The effect of waterlogging on DNA integrity

The effect that hydrology and waterlogging has on the integrity of human DNA has scarcely been investigated, with associated research into environmental effects on skeletal DNA being even less researched. Therefore, analogues for human DNA (such as bacterial DNA) are reviewed in the following section and inferences between the two are made where possible. Bacterial DNA is primarily stored in plasmids (circular chromosome) within the nucleoid of the cell (Wearing 2010). While bacterial DNA is smaller in size compared to human DNA, the ability to effectively extract it from waterlogged environments shows how this particular post-mortem stressor affects the integrity of DNA in general (Hollar 2012). This section will also explore studies that address the ability to correctly identify persons involved in natural disasters (e.g. SEA Tsunami) based on DNA extracted from various sources in the body after it has been subjected to extreme waterlogging.

i. **Palla et al. (2013)**

This study investigated the likelihood of identifying bacteria based on the extraction of DNA in ancient waterlogged wood. Fragments of wood from wrecks within the Mediterranean Sea (off the coast of Sicily) were removed and samples analysed for the presence of bacterial DNA. Swabs taken from the wrecks were inoculated on agar plates and bacteria colonised. PCR was then used to amplify the DNA to facilitate sequencing
and species identification. Five different species of bacteria were identified, showing that the survival of intact high quality DNA was possible within prolonged waterlogged conditions. Further, amplification was achieved for each sample, which demonstrates that waterlogged environments can still yield viable DNA suitable for identification analyses. This study thus established that DNA can survive in both waterlogged and marine environments. It is important to note, however, that this study analysed the DNA of bacteria and the likelihood of skeletal DNA experiencing the same level of preservation under similar conditions has not yet been investigated.

ii. Zehner (2007)

Disaster Victim Identification (DVI) often requires the analysis of DNA extracted from victims that have been exposed to extreme environmental stressors. Zehner investigated the likelihood of obtaining high quality (able to be sequenced) authentic DNA profiles from victims of the Boxing Day Asian Tsunami. Samples were taken from two victims across varied locations in the body. A tissue (muscle) sample, intact skeletal sample (femur), and a bone marrow sample, were all taken from damaged remains and amplified and sequenced for the presence of foreign adhered DNA, as well as authentic DNA belonging to the victim. This investigation resulted in the successful amplification of all DNA samples, however material obtained from the soft tissue and medullary cavity was found to represent foreign DNA (i.e. someone who had handled the remains following recovery) due to the divergent sexes.

The results of this investigation indicate that authentic DNA had degraded beyond amplification potential, due in part to the prolonged exposure to the very hot, humid and waterlogged environment. Authentic amplifiable DNA was, however, obtained and sequenced from a femoral bone sample, suggesting that despite the environmental stressors, the DNA was protected. This investigation indicates that the DNA within intact skeletal elements were unaffected by the environment, unlike that of the soft tissue
and medullary cavity, which had been exposed to the environment due to the fragmentation of the human remains.

**iii. Summary**
The ability to extract high quality DNA from skeletal remains subject to waterlogging is possible. The result of the research above show that DNA can be unaffected by prolonged exposure to stagnate and fluctuating water.

### 3.3 Freeze/thaw cycle

The following section evaluates literature that explores the concept of freezing and the freeze/thaw cycle; specifically how this process affects skeletal morphology, encourages bone diagenesis, and lastly how it affects the quality and quantity of DNA within those biological samples.

#### 3.3.1 The effect of freezing and freeze thaw cycles on bone preservation

A considerable amount of research exists relating to how freezing and the natural freeze/thaw cycle can affect bone morphology. Pioneering studies (Linde and Sorensen 1993) addressed this issue by focusing on how prolonged freezing affects the mechanical properties of bone, such as strength under torsion and compression. More contemporary investigations have considered issues such as fracture morphology and microstructure (Andrade et al. 2008; Karr 2012a; Karr 2012b).

* *Pelker (1983)*
The aim of this study was to investigate the effect freezing had on the strength and elasticity of long bones and vertebrae. This was tested using 90 femora and 324 vertebrae (of rats) subjected to torsion (femur) and compression (vertebra) mechanical loading following prolonged exposure to varied freezing temperatures. Five test groups were used: group one was freeze dried at -70°C with less than 3% moisture; three frozen groups at -20°C, -70°C and -196°C, and finally a control group. This study demonstrated no change in the mechanical properties of the vertebra or
long bones exposed to any of the three frozen groups. However, a significant reduction in the torque value and torsional stiffness (values of 30 for torque value and 25 for torsional stiffness compared to the control bones at 100) was observed in the torsion strength of the long bones that were freeze dried; similarly these bones experienced micro fractures along the periosteal surface of the bone.

The results of this study are supported by Panjabi et al. (1985) who analysed frozen human vertebra (-18°C) and demonstrated that despite prolonged exposure and thawing (some samples were frozen for 232 days), the mechanical properties of the vertebra were not adversely effected. While this study does establish the concept of freezing potentially affecting various mechanical and physical aspects of bone, the use of rats as a proxy for humans is not as appropriate as other animal analogues.

ii. Linde and Sorensen (1993)
The aim of this study was to investigate the affect freezing and a freeze/thaw period has on the mechanical properties of trabecular bone. The proximal tibiae of two males were transected into 74 sections, those sections were then divided into three groups. The first group were stored in ethanol, the second frozen at -20°C for periods of 1 and 100 days and the final group frozen also at -20°C for either 1 or 100 days, thawed for a day, and then refrozen under the same conditions. The freeze thaw cycles were repeated up to five times in some instances. Following freezing, the samples were then tested using unconfined non-destructive compression exerted by an INSTRON universal test machine. The result of their experiments demonstrated little change occurring with regard to the mechanical properties of the bone, with only a slight increase in stiffness with prolonged freezing. Minor changes in the viscoelasticity of bone after prolonged freezing was also observed, however this was minimal.

While this investigation analysed human trabecular bone, they were highly fragmented portions of long bones. In order to understand the overall effect freezing and freeze/ thaw cycle has on the mechanical and physical properties of bone, whole bone need to be analysed concurrent with
fragmented remains. Despite this, it can be generally seen from the latter results that the effect prolonged freezing has on bone is minimal. Following this trend of investigations into the effect freezing has on the mechanical properties of bone, studies began to address how microstructure and fracture morphology were likewise affected under freezing conditions.

iii. Tersigni (2007)
This study investigated how bone microstructure is affected following exposure to freezing conditions. Human tibiae and femora were frozen at 0°C for a period of 21 days; these were then analysed using a Scanning Electron Microscope (SEM) to determine histological affects, specifically how Haversian systems and osteons were affected. The results of this investigation showed that at just 0°C, microcracks formed around the centre of the Haversian systems, thus compromising the structural integrity of the bone. Similarly, macroscopic cracks were visible along the periosteal surface of the bones following 21 days of exposure. This study indicates that freezing has a fundamental effect on the structural integrity of bone at both microscopic and macroscopic levels. While this study is effective in communicating the ways in which freezing can affect skeletal structure, the mechanisms behind how this occurs are not explored. Furthermore, using just one constant temperature to freeze the remains means that comments about the patterns of damage observed are only applicable at this temperature. By using a variety of temperatures to freeze the remains, a more comprehensive understanding of the effect of freezing could be elucidated.

iv. Andrade et al. (2008)
A more in depth study (in relation to varied temperature and length of exposure) was undertaken by freezing portions (n = 48) of the ilium of rabbits at -20°C and -70°C for periods of 30, 60, 90 and 120 days. Following this, a histological analysis was performed to investigate the affect that prolonged and varied freezing temperature had on the microstructure of bone, and therefore, how this may affect structural
integrity. Results indicated that at -70°C, 100% of the tissue tested had been modified after prolonged (90 and 120 days) exposure. Modification was present in the form of shrinking nuclear areas of all cells (osteoblasts, osteoclast and osteocytes) within the cortical bone, and the expansion of the space occupied by cells within the medullary cavity. Andrade also found that collagen denaturation occurred proportionally with a decrease in temperature, leading to the eventual degradation of bone. The highest degree of modification occurred at -70°C after prolonged exposure, with the length of exposure appearing to have a greater affect on microstructure than temperature. This study was able to show that at extremely low temperatures the diagenesis of bone is accelerated, however, the temperatures used during these experiments are extreme (outside of glacial areas and environments subject to permafrost) and thus this should be taken into account when comparing the degradation of bone in a forensic casework context.

v.  (Karr 2012a)
The aim of the initial study (Karr 2012a) was to assess how bone degradation is influenced by extreme climates, such as freezing conditions at -20°C. Horse and cattle bones (humerii, radii-ulnae, metacarpals, femora, tibiae and metatarsals) were analysed; the bones were frozen over a period of 1, 10, 20, 40 or 60 weeks and then fractured using an anvil and hammer. Following this, each bone was given a freshness fracture index (FFI) corresponding to a combination of the helical fracture outline, the angle of the fracture surface, and the texture of the fracture area. Each of the latter criteria were scored between 0 and 2, and summed for a total score out of 6. Higher scores indicate greater ease of fracturing, increased fracture morphology and therefore increased bone degradation. Morphology was assessed against fresh control samples that were likewise fractured using the above process. Initial results indicate that the force required to fracture frozen bones was less than the force required to fracture both fresh bone, and the bones subjected to other climatic conditions (e.g. extreme heat). The FFI scores of frozen bones slowly rise, indicating that over time the bones degrade when subject to
very cold conditions. The latter process is, however, relatively slow. In order for the results of this investigation to be usable, further research into the affect freeze/thaw cycle needs to be conducted; in doing so, natural seasonal climates can be mimicked making the research more applicable to real life scenarios.

A follow up study (Karr 2012b) was performed to determine the effect a freeze/thaw period, as well as prolonged freezing under more realistic conditions (2°C), had on bone degradation (as assessed using the Freshness Fracture Index). This investigation utilised the bones of sheep and cattle (femora, tibiae, humerii and radii–ulnae). These were separated into five groups according to different environments; hot dry conditions (40°C); room temperature (22°C); mild freezing conditions (-2°C); freeze/thaw conditions (-20°C followed by 22°C); and constant freezing (22°C). Their results accord with their initial study, with the fracture freshness index indicating diagenesis across all five groups. The slowest degradation occurred to frozen bones; followed by bones subject to the freeze/thaw cycle while those refrigerated experienced the fastest rate of deterioration, (Figure 3.2).
While this study reiterates the results of their initial study it does go further by attempting to explain the mechanism behind the diagenesis, stating that freezing leads to a loss of moisture, which in turn affects the collagen content of bone. Likewise, freezing has the potential to affect chemical decay, as well as leading to overall bone loss over time.

vi. Summary
It has been demonstrated that freezing and a freeze/thaw cycle have adverse effects on the structural integrity of bone, with diagenesis appearing to be aided by prolonged exposure to freezing temperatures. While these studies positively indicate that freezing does lead to bone diagenesis and morphological change, it is stated that for bone to be degraded beyond being of evidentiary value required prolonged freezing (years) extending past the parameters explored in the studies reviewed above.

3.3.2 The effect of freezing and freeze thaw cycles on DNA integrity
Limited literature exists on the effect prolonged burial within sub-zero climates has on the integrity of contemporary skeletal DNA; there is also restricted research on the effect of repeated freeze-thaw cycles and the likelihood of positive extraction and sequencing. Examples of DNA sequencing of individuals exposed to extreme cold for centuries/ millennia do however exist, for example Ermini et al. (2008) in which MtDNA was sequenced from the Tyroline Iceman. These example have however not be discussed in depth during this section as they do not provide examples of the freeze/thaw cycle and they focus on ancient DNA sequencing. In order to assess the prospect of extracting usable DNA from contemporary frozen climates, studies focusing on the effect of freezing and freeze-thaw cycles have on the integrity of viral DNA, and DNA related to fertility treatment practises (e.g. spermatozoa DNA, blastocysts), have been used. Inferences from these studies can be made between their outcome and
the likely success of DNA extraction from skeletal material obtained within varied temperature climates.

i. Krajden et al. (1999)
The aim of this paper was to assess the effect repeated freeze-thaw cycles have on the integrity of viral DNA. Common virological examinations often require samples to be frozen and thawed a number of times and the impact this has on the yield of DNA was unknown. A total of 42 specimens (21 hepatitis B positive and 21 hepatitis C positive) were frozen at -70 °C, followed by thawing within a 25 °C bath. This cycle was repeated up to eight times to establish if (or when) DNA would be affected. The outcome of this study demonstrated stability of the viral DNA after the maximum amount of cycles (eight). There was no significant loss of viral information, and even a trend toward a slight increase in the concentration of DNA after each cycle. This study therefore concluded that freeze-thaw cycles have little affect on the integrity of DNA within viruses. However, the use of viral DNA is not directly comparable to human skeletal DNA due to differences in structure and number.

ii. Kader et al. (2009)
The aim of this study was to investigate the affect that freezing has on the DNA of blastocysts (small cell masses formed during early mammalian development). This was investigated based on cryopreservation techniques using glycerol or propanediol. A total of 124 blastocysts were slowly frozen to -140 °C over two hours. Following this, samples were stained to visualise any DNA damage. The outcome of this investigation showed an increase in DNA fragmentation and cell apoptosis after periods of extreme freezing. This study, designed to identify the affect cryopreservation has on blastocysts, was able to show how cellular damage occurred in the form of apoptosis and DNA fragmentation. DNA preservation was favoured when blastocysts were gradually frozen over 35 mins to a temperature of -40 °C. Despite this research not investigating the freeze/thaw cycle, it is still useful in adding to the expectation of DNA retrieval from material subject to extreme and prolonged freezing. This
research is, however, limited in that it deals with DNA from fragile blastocysts DNA, and not DNA extracted from protected skeletal remains.

iii. Summary
The results of the research reviewed above show that there have been conflicting finding with regards to the degradation of frozen DNA. Since no study exists detailing how freezing affects the integrity of skeletal DNA, inferences need to be made from studies that have investigated analogues of human DNA. There is potential for the freezing process to affect DNA if the temperatures are severe enough (below -140°C), and the exposure prolonged, however to unequivocally say this further research is needed.

3.4 Low pH
The final section of this chapter investigates literature relating to the effect that low pH environments have on the structural integrity and morphology of bone, as well as the effect that they have on DNA quality and quantity.

3.4.1 The effect of low pH on bone preservation
The effect that pH has on bone morphology has been investigated extensively in the literature, with relatively consistent results (Gordon and Buikstra 1981; Nicholson 1996; Nielsen-Marsh et al. 2007). The general trends throughout these studies demonstrate favourable skeletal preservation within alkaline or basic environments (pH < 7) with a more acidic environment (pH >7) causing osseous dissolution.

i. Gordon and Buikstra (1981)
The aim of this study was to investigate the affect soil pH has on the preservation of human skeletal remains. Two burial sites in Illinois (USA) were investigated, with a total of seven burial mounds excavated. A total of 63 adult and 32 children were excavated and visually assessed to determine the level of fragmentation.

In addition to the visual assessment of the skeletal remains, soil samples were taken from each burial to measure pH. This study separated adult
and child remains when considering the effect pH had on preservation. There was an observable difference between the preservation of adult and juvenile remains. Within the adult remains, 84% of skeletal damage or (poor preservation) was attributed to decreasing pH. Within the juvenile group this factor only accounted for 23% of the variation, however it is noted that this could increase if further age sub-categorisation was to occur prior to testing. The concept that acidic environments promote skeletal diagenesis is considered throughout this study. The study however only provides a broad overview of the concept of soil pH effecting bone preservation. Soil pH for each area is not divulged therefore the parameters of what constitutes low pH environment is not made clear, nor is the underlying mechanisms causing bone degradation explored. Similarly, the ages of these specimens would have been a compounding factor for preservation that is seemingly ignored throughout the study. The method of bone assessment is also largely subjective and ignores other mitigating factors that could contribute to the degradation of bone such as scavenging and root infiltration.


Nicholson (1996) investigated the affect of varied soil pH (acidic pH < 7 v’s basic pH > 7) on the preservation of fish, bird and mammal bones that were buried for a seven-year period. Unlike the study performed by Gordon and Buikstra (1981) (see above) exact pH measurements were taken for each of the four sites investigated. Two sites were found to be acidic (pH 3.2-4.5) while the remaining two sites were comprised of neutral (pH 6.5-7.0) and basic soils (pH 7.5–8) respectively. A mix of bird, fish, rat, cattle and sheep bones were buried within each of the above-mentioned sites and excavated after seven years. The skeletal remains were assessed on a scale between 1 and 5, with 1 indicating whole/intact remains and 5 indicating highly fragmented friable remains. Soil samples were also collected from each burial.

The study demonstrated preferential preservation of those remains within the basic and neutral soil (pH values between 6.5 and 8.0). Those remains
that were interred within acidic soils experienced marked degradation. Site 10 (pH 3.2-4.5) experienced the poorest preservation between the four sites, with no fish bones remaining and an average score of 3 being attributed to the highly degraded bird and mammal bones that remained. It is interesting to note, however, that the second acidic environment (site 15 pH 3.3-4.5) had a higher degree of skeletal preservation with an average score of 3.5 to 4 (intact whole bones were excavated from all of the test animals) attributed to the remains.

The study concludes that pH alone is insufficient for predicting skeletal preservation. This study, as well as being thorough in its approach by using a diverse range of soil profiles, also attempts to explain the mechanisms behind preferential preservation. It is hypothesised that lower pH soils are subject to attack from proteases that degrade collagen and thus bone material. It also noted the presence of heavy fungal growth across all bones within the low pH environments, and attempts to correlate this to a higher degree of damage from infiltrating hyphae (filaments produced by bacteria). Despite these factors, the inclusion of fish and bird remains does not allow for direct comparison to occur with humans skeletal remains; the purpose of investigating the affect of pH on skeletal remains has the most impact when morphologically similar mammals are used as study subjects.

iii. Nielsen-Marsh et al. (2007)
The aim of this study was to investigate how soil chemistry (pH) affects the preservation of skeletal remains. A total of 219 archaeological burials and accompanying soil samples were analysed. A thorough assessment of diagenetic parameters was performed on all remains excavated, including: percentage of collagen lost; porosity; skeletal density; cracking index; and an assessment of histology based on the Oxford Histological Index (Smith et al. 2007). In this study both articulated and disarticulated remains were investigated. Bones within highly acidic environments (pH 4.5) experienced poor preservation (Figure 3.3).
The conditions of the skeletal remains were affected by soil pH, only 10% of skeletons were present following burial within acidic soils (pH 4.0-4.5), 90% had not survived within the environment. The study concluded that in the event of remains being buried within acidic environments, in the interest of skeletal preservation, immediate excavation should be undertaken to minimise further diagenesis.

iv. Summary

The mechanism of bone diagenesis within acidic soil has been the subject of much research. The consensus is that acidic environments promote the dissolution of the mineral constituent of bone (Nafte 2000). During this process, hydroxyapatite disassociates into its constituents calcium and phosphorus, thus causing bone to effectively dissolve (Surabian 2012). It is thus well established that acidic environments have a detrimental effect on bone preservation. The affect that this has on the likelihood of obtaining
skeletal DNA from interned remains is also of importance when considering identification during mass disasters, upon the discovery of clandestine burials, or as part of missing person’s investigations.

### 3.4.2 The effect of low pH on DNA integrity

The ability to obtain high quality DNA from remains found within an acidic environment has not been extensively investigated. A small number of studies have attempted to investigate this issue and address the impact pH has on the structural integrity of DNA. Like that of bone, DNA responds similarly under different pH environments, notably it is degraded under the influence of low pH environments (Lindahl 1993).

#### i. Hagelberg et al. (1991)

The aim of this study was to address the effectiveness of extracting mitochondrial DNA from ancient remains excavated from soils of varied chemistries. The remains examined were exhumed from Abingdon cemetery, which was utilised initially during the medieval period (5th to 13th century) and primarily throughout the English Civil War (1642-1651). Over 100 human skeletons were excavated from the alkaline soils (exact pH values not given) of the cemetery; sections of the femur were cut and DNA extraction was performed from the bone sections. With the exception of one individual, amplification was achieved for all the remains. This study demonstrated preservation of skeletal material (and thus DNA), within an alkaline environment. This project, however, did not investigate any remains exhumed from an acidic environment thus the notion that DNA is adversely effected by low pH is only an untested hypothesis.

#### ii. Burger et al. (1999)

The aim of this study was to assess the ability of extracting sequenceable DNA from ancient teeth from varied burial environments (e.g. arid, limestone, surface burials) across the United Arab Emirates (UAE), Nepal and Lichtenstein. A total of 38 teeth from individuals buried between the first and second millennia BC were analysed. Each site represented unique burial environments; the site within the UAE was comprised of wadi
(valley) sediment in a semi arid climate, with the soil being largely a composite of limestone. The second site in Nepal was also semi arid but within an alpine region where cool dry winds had mummified a large portion of the remains, furthermore the site experiences large microbial infestations year round. The final test site in Lichtenstein was comprised of loamy clay soil mixture. Humic acid measurements were taken for all environments with medium to high reading occurring within Lichtenstein and medium to low measurements for Nepal and UAE.

It was found that the highest percentage of PCR products and allele reproducibility was from the surface burials at Lichtenstein (90% and 92% respectively). The site in Nepal had the lowest PCR product amplification (20%) and none of the samples could be successfully amplified during sequencing. This study was able to show that within varied pH environments differential levels of DNA extraction was achieved, due to varied levels of skeletal preservation. The chemical structure of bioapatite (calcium phosphate that resembles hydroxyapatite found within the dentition) is altered steadily under the influence of an acidic environment (pH between 6.0 and 4.5 modifies hydroxyapatite to brushite, pH lower than 4.5 completely dissolve hydroxyapatite leaving only soil silhouettes), which leads to degradation and a loss of structural integrity that in turn facilitates DNA degradation.

The author states that oxidation and hydrolysis are the primary mechanisms behind DNA degradation. Oxidation and hydrolysis cause the denaturation and loss of bases, in addition to causing depurination (breaking purine bonds) and depyrimidination (breaking pyrimidine bonds) of the individual DNA strand. This has the potential to culminate in the fragmentation of DNA and thus affect potential amplification. All results where amplification was unsuccessful (or hindered) occurred in remains shown to be high in levels of humic and fulvic acids occurring as a result of autolysis and decay of remains within humid environments.
iii. Summary

The affect that pH has on skeletal remains is well established. The degree to which DNA within these remains can be extracted and amplified has also been investigated however in a limited capacity. In order to establish clear links between low pH environments and DNA degradation/preservation further research needs to be conducted.
CHAPTER FOUR

Materials and Methods

4.1 Introduction

The present study addresses the concept of the degradation of DNA in bone marrow after prolonged periods of both natural and deliberate weathering. The purpose of this chapter is to detail the source of DNA material analysed in this study, including the specimens sampled and preparation protocols. The methods employed to simulate natural seasonal weather fluctuations and deliberate taphonomic alterations are also defined. Finally, the chemical methods used to test DNA degradation are examined; these include DNA extraction, amplification and qualitative and quantitative procedures.

4.2 Materials

4.2.1 Sus Scrofa specimens

This study uses adult long bones (femur) from the domestic pig (Sus Scrofa) as an analogue for human skeletal remains. Porcine bones were selected as the experimental subject due to their morphological similarities with human skeletal remains (Pearce 2007) in addition to the ready availability of the porcine specific molecular primers (Larkin et al. 2010) required for the DNA analyses.

The articulated hind legs of 14 pigs were acquired from the Linley Valley Abattoir, Perth, WA. These legs included the articulated femur, tibia, fibula and trotter (tarsals, metatarsals and digits). The specimens were randomly selected from animals that were humanly slaughtered for the purpose of food consumption. Ethics approval for the material procured from the abattoir was granted on the 11th of March 2014 (see Appendix 1). In addition, three porcine femora were purchased from the local butcher (supplied by Linley Valley Abattoir) during each sampling period to be
used as fresh controls (see section 4.3.1). The legs were manually disarticulated and defleshed to expose the femur (see section 4.2.2). The femur was the target bone for DNA extraction due to the fact that the long bones are the site of haematopoiesis throughout infancy and adulthood. Thus the bone marrow within the femur should (in its natural state) be rich in nucleated cells from which DNA can be extracted.

4.2.2 Sample preparation

The femur, tibia and fibula were disarticulated and adherent flesh was carefully removed from the bones manually using forceps, scalpels and scissors. This process revealed the fresh bone lined in periosteum. The flesh that was removed was disposed in biohazard bins for subsequent incinerated. Each bone was photographed to document its initial appearance; an example of a pig femur is shown in Figure 4.1

![Figure 4.1 Sus Scrofa left femur (A) after disarticulation, defleshing and cleaning. An anatomical illustration of an articulated hind leg is provided for reference (Theobald 1913)]

Following sample preparation the bones were subdivided into defined experimental categories (see section 4.3 and Table 4.1 below) and stored
within a laboratory fume hood. As the second aim of this study concerns the difference in DNA preservation between intact and fragmented long bones, half of the experimental subjects required further preparation. Six femora were randomly selected and sectioned in the sagittal plane, in order to simulate exposure of the medullary cavity. Long bones were cut across the mid-shaft to simulate fragmentation. This was performed using a METABO Bas 260 Swift band saw. Once the samples had been prepared they were then subdivided into the various post-mortem alterations.

### 4.3 Post-mortem alterations

In order to assess the effect natural and deliberate taphonomic processes have on the integrity of medullary DNA, laboratory simulations were designed to mimic these environments. Two of the environments simulate natural seasonal variations experienced in a prolonged burial in tropical, flood prone and seasonally frozen environments. Another experiment modelled deliberate taphonomic intervention using low pH (Sulphuric acid). Each test group contained a complete and a fragmented femur, per time point; for example each group contained eight bones in total (see Table 4.1 below).

#### 4.3.1 Test groups

4.3.1.1 *Bones subjected to cycles of freeze and thaw*

These bones were subjected to alternating cycles within a freezer (constant -20°C) and then thawing at room temperature (20-24°C) under a fume hood. Each phase of the cycle lasted two weeks, thus one cycle was complete after one month (28 days).
Table 4.1 Schematic of samples according to treatment group and extraction time.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bone No</th>
<th>1 month</th>
<th>3 months</th>
<th>5 months</th>
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<tbody>
<tr>
<td><strong>Fresh control (FC)</strong></td>
<td>01I-FC1</td>
<td>01I-FC1P 01I-FC1Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>02I-FC3</td>
<td></td>
<td>02I-FC3P 02I-FC3Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>03I-FC5</td>
<td></td>
<td></td>
<td>03I-FC5P 03I-FC5Q</td>
</tr>
<tr>
<td><strong>Test Control (TC)</strong></td>
<td>04I-TC1</td>
<td>04I-TC1P 04I-TC1Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>05I-TC3</td>
<td></td>
<td>05I-TC3P 05I-TC3Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>06I-TC5</td>
<td></td>
<td></td>
<td>06I-TC5P 06I-TC5Q</td>
</tr>
<tr>
<td></td>
<td>07F-TC</td>
<td>07F-TC1P 07F-TC1Q</td>
<td>07F-TC3P 07F-TC3Q</td>
<td>07F-TC5P 07F-TC5Q</td>
</tr>
<tr>
<td><strong>Low pH (A)</strong></td>
<td>08I-A1</td>
<td>08I-A1P 08I-A1Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10I-A3</td>
<td>10I-A3P 10I-A3Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12I-A5</td>
<td></td>
<td>12I-A5P 12I-A5Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14F-A</td>
<td>14F-A1P 14F-A1Q</td>
<td>14F-A3P 14F-A3Q</td>
<td>14F-A5P 14F-A5Q</td>
</tr>
<tr>
<td><strong>Freeze/Thaw (F/T)</strong></td>
<td>16I-FT1</td>
<td>16I-FT1P 16I-FT1Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18I-FT3</td>
<td>18I-FT3P 18I-FT3Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20I-FT5</td>
<td></td>
<td>20I-FT5P 20I-FT5Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22F-FT</td>
<td>22F-FT1P 22F-FT1Q</td>
<td>22F-FT3P 22F-FT3Q</td>
<td>22F-FT5P 22F-FT5Q</td>
</tr>
<tr>
<td><strong>Wet/dry (WD)</strong></td>
<td>24I-WD1</td>
<td>24I-WD1P 24I-WD1Q</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>26I-WD3</td>
<td></td>
<td>26I-WD3P 26I-WD3Q</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28I-WD5</td>
<td></td>
<td></td>
<td>28I-WD5P 28I-WD5Q</td>
</tr>
<tr>
<td></td>
<td>30F-WD</td>
<td>30F-WD1P 30F-WD1Q</td>
<td>30F-WD3P 30F-WD3Q</td>
<td>30F-WD5P 30I-WD5Q</td>
</tr>
</tbody>
</table>

Key: I=intact bones, F=fragmented, TC= test control, FC= fresh control, A= acid, FT= freeze/thaw cycle, WD= wet/dry cycle, 1,3 or 5 = month of extraction, P= phenol extraction, Q= Qiagen extraction.
4.3.1.2 Bones subjected to cycles of wet and dry

To complete a one-month cycle the bones were submerged in one litre of tap water for two weeks, removed and then allowed to dry naturally under a fume hood (20-24°C) for a further two weeks.

4.3.1.3 Exposure to low pH

In order to test the effect of deliberate immersion in a low pH environment, the bones in this test group were submerged in 800ml of sulphuric acid (pH 1). Unlike the other experimental groups, these bones were not cycled through period of submersion and drying but remained submerged within 800ml of sulphuric acid for the allotted time, so that the effect of prolonged exposure could be evaluated.

4.3.1.4 Control groups

Two control groups were used. The first (test control) were dissected at the same time as the subject bones, but not subjected to the conditions outlined above. These bones were housed within a fume hood at 20-24°C for a period of one, three or five months. The purpose of this control group is to allow comparison between bones that were decomposing and degrading naturally and not under the influence of any external taphonomic influences.

The second control group were the fresh bones sourced from the local butcher at the time of each extraction that were not subjected to any taphonomic alterations, nor were they subjected to a period of natural decomposition as for the first control group (see above).

4.3.2 Sampling

Sampling occurred a total of three times across a period of five months. These time points are as follows: after one cycle (one month); three cycles (three months); and five cycles (five months). Molecular extraction across a five-month period allowed both acute and prolonged effects of taphonomic influences on DNA yield to be investigated simultaneously.
Intact bones from each group were sampled and then discarded in an appropriate biohazard bin, the contents of which are incinerated following collection. The fragmented bones in each group were however sampled repeatedly at each of the sampling time points; small sections were cut off the exposed surface of the shaft during each consecutive sampling point. Fragmented bones could be used repeatedly, as further damage did not compromise their integrity due to their already deliberately damaged condition. The time points that each bone was sampled throughout the duration of this study are shown in Table 4.1 (see above).

Each bone is sequentially numbered and labelled with a suffix I or F to indicate intact or fragmented bone, this is then coupled to a code for the treatment group, extraction time and the extraction method. This allocates each sample a unique identifier; for example 01F-FC1, 01 = bone number 1; F= fragmentation and FC1= fresh control femur for extraction at 1 month. It is important to note that segmented bone specimens (simulating fragmentation) were used in more than one treatment group.

The amount of material taken during each sampling time point varied according to the extraction technique. For organic extraction 1g was removed and suspended in 1ml PBS, while 10μg was removed from each bone for the Qiagen extraction method, as it did not required re-suspension prior to extraction.

4.4 DNA extraction and amplification

This section first describes the laboratory equipment and reagents required. This is followed by an explanation of the post-mortem simulations and sampling techniques. The extraction procedures are thereafter described, along with the quantitative techniques, including nanodrop-spectrophotometry and gel electrophoresis. Finally PCR protocol and sequencing of MtDNA and PGH are also accordingly described.
4.4.1 Laboratory equipment

All experimental work was performed in the CFS laboratories at UWA; all equipment was calibrated as required. Table 4.2 below lists the equipment used in this study.

Table 4.2 Inventory of all laboratory equipment used during the study.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanodrop ND-1000 spectrophotometer</td>
<td>Qualify and quantify each sample of DNA following initial extraction</td>
</tr>
<tr>
<td>Siltex (Australian) pty ltd-autoclave</td>
<td>Sterilise all equipment and reagents prior to use</td>
</tr>
<tr>
<td>Memmert- water bath</td>
<td>Incubate samples with proteinase K to ensure cells are lysed and DNA is released</td>
</tr>
<tr>
<td>VOR-MIX- S.E.M- Vortex</td>
<td>Thoroughly mix all samples</td>
</tr>
<tr>
<td>Eppendorf- Centrifuge 5415</td>
<td>Spin and separate samples based on particle density and weight</td>
</tr>
<tr>
<td>1.5ml Eppendorf tubes</td>
<td>Vessel for each reaction</td>
</tr>
<tr>
<td>Socorex- 1000 μl, 200μl 20μl, 10μl – pipettes</td>
<td>Pipettes were used throughout extraction, nanodrop, PCR and gel electrophoresis to move and mix samples where needed</td>
</tr>
<tr>
<td>Bio-Rad- icycler- PCR machine</td>
<td>Run all PCR reactions; programed with specific PCR parameters which are detailed in 4.15</td>
</tr>
<tr>
<td>PCR cabinet</td>
<td>Contamination control during PCR sample preparation and mixing</td>
</tr>
<tr>
<td>QIAGEN® DNeasy® Blood &amp; Tissue Kit (250)</td>
<td>Commercial DNA extract kit which contains all materials required for an extraction, including spin columns, Proteinase K, buffers, and 2 ml collection tubes</td>
</tr>
<tr>
<td>Microwave</td>
<td>Heating agarose gel powder and TBE to ensure mixing and to allow for electrophoresis gel to be poured.</td>
</tr>
</tbody>
</table>

4.4.2 Reagents
Both of the extraction methods used in this study, and the gel electrophoresis, require specific reagents; these are listed and described below.

i. **QIAGEN blood and tissue extraction kit**
All reagents and buffers used during commercial kit extraction were provided within the package. QIAGEN® Buffers AW1 and AW2 necessitated reconstitution with 96% Ethanol as per manufacturer’s instructions.

ii. **Phenol/chloroform extraction**

   *Proteinase K:*
   - 1mg of powdered Proteinase K
   - 1ml of PBS

   *Phosphate buffered saline (PBS pH 8.0)*
   1x TE buffer:
   - 10mM Tris-HCl
   - 1mM EDTA

   *Extraction buffer pH 8.0:*
   - 10mM Tris-Cl (1.211 g)
   - 10 mM EDTA (2.9224g)
   - 100mM NaCl (5.844g)
   - 2% Sodium Dodecyl Sulfate (SDS)
   - 1M hydrochloric acid for correct pH
   - Deionised distilled water to make up to a volume of 1L

iii. **Gel electrophoresis**

   *10x TBE (Tris/borate/EDTA) pH8.0:*
   - 324g Trizma Base powder
   - 165g Boric acid
   - 120ml EDTA
   - 1M hydrochloric acid for correct pH
   - Deionised distilled water to make up to a volume of 1L

4.4.3 DNA extraction protocols
Two DNA extraction methods were selected to address the third aim of this study; a comparison of two established DNA extraction techniques. During each extraction period reagent blanks/ negative controls were used to assess contamination. Each method is described and illustrated below;

4.4.3.1 Organic extraction using phenol/chloroform

Organic extraction using phenol chloroform involved multiple steps to extract sample DNA. Phenol extraction is a standard organic extraction method whereby separation occurred between substances of varied solubility, such as nuclear acids, protein and lipids. Despite the process being cumbersome and involving the use of carcinogenic substances, a phenol extraction produces high purity DNA. The step-by-step process of phenol extraction employed is detailed below, and summarised in Figure 4.2.

Initially, 1 g of sample material (bone marrow) was collected from the medullary cavity and resuspended in 1ml of PBS. Thorough mixing was achieved via vortexing and pipetting the sample. Next, 200μl of the sample was removed and placed within a 1.5ml microcentrifuge tube. 180μl of extraction buffer was then added and the sample was thoroughly mixed via vortexing. 20μl of 20mg/ml proteinase K was added to the mixture and incubated on a water bath at 56°C for 2hrs, to ensure denaturation of proteins within the sample. Every 30 mins the samples were vortexed to ensure the mixture became clear and homogenous.

Following this 800μl of phenol/chloroform/isoamyl alcohol was added to the mixture and vortexed to ensure thorough mixing. The sample was then spun in a centrifuge for 1 minute at 11,000rpm at room temperature. Three phases appeared in the solution: the upper clear aqueous phase containing nuclear material; the white interphase of proteins; and the lower red phenol/chloroform phase. The upper phase was transferred into a clean 1.5ml microcentrifuge tube; 1 volume (equal to the extracted upper phase) of ice-cold isopropanol was then added to the tube and vortexed to ensure mixing (isopropanol precipitates nuclear acids). The sample was then centrifuged for 20 minutes at 13,000 rpm at room temperature.
Following centrifuging the supernatant was discarded and 500μl of 70% ethanol was added and vortexed. Ethanol wash precipitates the nuclear acids and removes contaminants.

The sample was then spun using the centrifuge for an additional 20 minutes at room temperature on 13,000rpm. The supernatant was aspirated to remove as much liquid as possible leaving only a pellet behind in the base of the tube. The pellet was air dried under the fume hood for 30 minutes; this step is essential as it removed excess ethanol from the mixture, which has the potential to inhibit PCR. The final step was to add 200μl of TE buffer to resuspend the pellet. Vortexing occurred to ensure thorough mixing. Following these steps the sample was ready for quantitative assessment using the nanodrop.

![Diagram of phenol extraction steps]

**Figure 4.2** Schematic representing the steps involved in phenol extraction.

### 4.4.3.2 Commercial extraction kit (Qiagen)

The second extraction method used in this study was the commercial QIAGEN blood and tissue kit. This method of extraction employs lysing,
binding and washing to trap nuclear material on a membrane within columns prior to elution. The steps involved for this method of extraction were taken directly from Dneasy blood and tissue handbook (QIAGEN 2006) and are detailed below and illustrated in Figure 4.3.

20μl of proteinase K was added to 10mg of starting material (bone marrow) extracted from the medullary cavity. The volume was adjusted to 220μl with PBS. The sample was then vortexed and incubated for 10 minutes on a heat block at 56°C. 200μl of AL buffer was added and the mixture was vortexed thoroughly and incubated again at 56°C for an additional 10 minutes for until homogenous. Following this, 200μl of 100% ethanol was added and the mixture vortexed. It is essential that the sample material and ethanol is mixed thoroughly to attain a homogenous sample. The entire mixture is then pipetted into a DNeasy mini spin column and centrifuged at 8,000 rpm for 1 minute. The flow through is discarded and the DNeasy column was then placed within a new 2ml collection tube. 500μl of AW1 was then added and centrifuged for 3 minutes on the highest spin cycle and the flow through discarded. The DNeasy column was placed within another 2ml collection tube and 500μl of AW2 was added. The sample was centrifuged for 3 minutes on the highest spin cycle to dry the membrane and the flow through removed; this step is essential as the membrane must be dry to remove residual ethanol that can interfere with subsequent steps.

The DNeasy column was then placed within a new 2ml micro centrifuge tube and 100μl of AE buffer was added directly onto the membrane. This was allowed to incubate for 1 minute at room temperature and spun at 800rpm for 1 minute to elute. The above step was repeated to elute a higher concentration of DNA from the sample, which was then quantified using a nanodrop spectrophotometer.
4.4.4 Quantification Nanodrop spectrophotometer.

Quantification is paramount to assessing the integrity of sample DNA. In order to achieve a reliable and accurate assessment of the quantity of DNA within each sample, two different methods were used. After initial extraction occurred, the samples were immediately measured using a nanodrop spectrophotometer. Spectrophotometers (nanodrop) function to
determine the average concentration of nucleic acids per sample and their purity. The nanodrop measures absorbance of ultraviolet (UV) light at difference wavelengths (Scientific 2008). Nucleic Acids absorb UV light at a wavelength of 260 nanometres. When the beam of UV light is sent through a sample at this wavelength, less light is able to move through and detected by the photo detector at the end of the system; the concentration of the sample can then be determined in ng/ml. Purity is assessed as a ratio of 260-nanometre wavelength against 280-nanometre wavelength. Proteins absorb UV light in the 280-nanometre wavelengths; therefore protein contamination can be determined when the ratio of 260/280 is used. A pure sample of DNA will have a ratio value of 1.8 (Gallagher (2006)).

4.4.5 PCR - DNA amplification

Following DNA extraction and quantification the samples were amplified using PCR. The Australian Genome Research Facility (AGRF) at the Harry Perkins Institute for Medical Research (Perth, - W.A) performed all PCR amplification and subsequent sequencing procedures. Amplification was performed to assess the quantity of extracted DNA from each sample and for visualisation and sequencing purposes. The master mix used for these reactions as well as the protocol used to amplify each of the three desired loci are detailed below.

4.4.5.1 PCR primers

In order to amplify desired regions of DNA, PCR primers were designed to target three specific loci on the genome. Two of these primers were designed to amplify mitochondrial DNA and the third was designed to amplify Pig Growth Hormone (PGH) located on chromosome 12 of nuclear DNA.

Larkin et al. (2010) investigated the utility of DNA degradation as a means of estimating Post Mortem Interval using Pig Growth Hormone as one of the DNA areas of interest. The primer pair is as follows:
PGH-F, 5’ TCA AGC TGA GAC CCT GTG TG 3’

PGH-R, 5’ TTT GGG CCC TTT TTA TAC CC 3’

The final primer pair that was used in the present study targeted porcine mtDNA. MtDNA has been validated in forensic casework and is a recommended means of positive identification where maternal relatives are available for comparison (Budowle 1995). The first primer pair was derived from Yu et al. (2013); this study was designed to investigate the evolution of China’s 88 indigenous pig species. The primer pair is detailed below:

MtDNA-F 5’ AGG AGA CTA ACT CCG CCA T 3’

MtDNA-R 5’ CGC GGA TAC TTG CAT GTG T 3’

The second primer pair was derived from Molnar et al. (2013), for the purpose of analysing relatedness and diversity of Mangalica pig and ancient relatives. The primer pair is defined below:

MtDNA2-F, 5’ CCT ATG TAC GTC GTG CAT TA 3’

MtDNA2-R, 5’ GGA TTG TCG TGC CGG ATC AT 3’

4.4.5.2 Master Mix

In order to facilitate the amplification of selected loci on the genome through PCR, a specific combination of reagents are required to be mixed prior to commencement. This is known as the master mix and contains reagents as well as the DNA template that will be amplified. Promega GoTaq master mix was used in the present study and the specific reagents used are listed below:

- Forward and Reverse primers;
- GoTaq master mix including; Taq polymerase, reaction buffers, dNTP’s (free nucleotides that are incorporated into newly synthesised DNA) and MgCl₂ which acts as a cofactor that increases productivity of Taq polymerase;
• PCR grade water, sterile water used to dilute the mix to its final concentration;
• DNA template (sample).

Each nucleotide is at 200uM, MgCl₂ concentration of 2.5mM and primers at 5uM conc. Samples were normalized to 20ng/μl where possible and 2μls of that was used as a template. Amplification was achieved using Promega GoTAQ master mix (Table 4.3).

Table 4.3 Promega GoTaq master mix standard procedure for a 25μl reaction volume

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GoTaq master mix</td>
<td>12.5</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>2.5</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>2.5</td>
</tr>
<tr>
<td>DNA template</td>
<td>2.0</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>5.5</td>
</tr>
</tbody>
</table>

4.4.5.3 PCR protocol

As stated in Chapter Two, PCR utilises fluctuation in temperature to ensure the denaturation, annealing and amplification of various loci along the genome. Each locus that is amplified requires slightly varied PCR protocols, which are designed according to the primers used. Each of the three primers chosen for this study, and their varied PCR protocols, are detailed in Table 4.4 and Table 4.5 respectively.
Table 4.4 PCR protocol for MtDNA and MtDNA2 primer amplification.

<table>
<thead>
<tr>
<th>PCR cycle</th>
<th>Temp °C</th>
<th>Time (sec)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 PCR protocol for PGH primer amplification

<table>
<thead>
<tr>
<th>PCR cycle</th>
<th>Temp °C</th>
<th>Time (sec)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>56</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>30</td>
<td>35</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>420</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.4.6 Quantification of DNA

Gel electrophoresis was performed to assess both the quantity of DNA obtained following PCR and also to ensure the correct loci were amplified. In order to assess both of these factors a reference ladder was visualised in each gel. This reference ladder was used as a point of comparison whereby band intensity and position of samples could be measured against known band sizes. A 100bp ladder was selected as it can be used to determine the size of each of loci given their expected size. An example of a 100bp reference ladder after it has been run on a 2% agarose gel is shown in Figure 4.4. The ladder ranges from 1500bp in size at the top to 100bp at the base. By running samples adjacent to the ladder, their size
can be determined based on how far they migrate through the gel and along the ladder (Johansson 1972).

![2.0% agarose gel]

**Figure 4.4** A 100bp reference ladder used during gel electrophoresis to determine size of amplified samples.

The expected sizes for each of the target loci are as follows:

- PGH coding region 383-402bp, gene itself 332-500bp;
- MtDNA D-loop region encompassing 1045 base pairs;
- MtDNA2 expected fragment length 714 base pairs.

Electrophoresis was performed using 1% agarose gel, prepared by mixing 100ml of 0.5X TBE buffer with 1mg of UltraPure Agarose powder (Life Technologies, USA). This mixture was then microwaved on high for two minutes, or until all visible crystals had dissolved. After cooling for 15 minutes, 2µl of Ethidium Bromide was added and the mixture was poured into a gel cast and allowed to set. Once the gel had hardened it was placed in the electrophoresis tank along with 250 ml of 0.5x TBE buffer. Next 4µl of sample was mixed with 1µl of 6x Blue/Orange loading dye.
(Promega, USA) on a sheet of Parafilm, which was then loaded into the wells. The system was run for one hour at 100 volts. Following electrophoresis, the gel was placed on a UV transilluminator to visualize the PCR products and photographs were taken using Sony Cybershot HD camera.

4.4.7 Sequencing -mtDNA

The Australian Genome Research Facility (AGRF) performed Sanger sequencing on 54 samples using the three primer pairs described above. Both nuclear and Mitochondrial DNA was therefore sequenced. Sanger sequencing utilised modified dNTP’s that are fluorescently labelled according to the four nucleotide bases (A, T,C,G). Each dye is incorporated into the newly synthesised DNA strand according to their specific base sequence and a pattern of colours is therefore produced which allows for the sequence to be read (Sanger and Coulson 1975).

Following sequencing, DNA quality is derived from three measurements: trace score; QV 20+ value; and continuous read length. Each value is indicative of different measures of DNA quality as detailed below.

i) Trace score (phred)

The first measure of DNA quality is known as a trace (or phred) score. This score is given to each base assignment (or base call) following sequencing and relates to how accurately that particular base was correctly assigned to the sequence. Trace scores of 20 are indicative of a base call with 99% accuracy; scores below 15 generally indicate poor quality DNA, and values between 15 and 20 indicate medium levels of successful amplification (Ewing and Green 1998; Ewing et al. 1998). The overall trace score of a sequence is a mean value of all individual base call accuracies.

ii) QV20+ value
The second measure of DNA quality is closely related to the trace score. QV20+ values indicate the number of bases in a sequence that have achieved a trace score in excess of 20, therefore indicating what portion of the sequence has been read accurately and are thus of a high quality (Technologies 2012)

iii) Continuous read length

The final method of DNA quality measurement is the continuous read length (CRL), which refers to the longest uninterrupted length of DNA that has been read following sequencing. CRL gives an indication of DNA quality and the level of fragmentation. CRL are classified according to three categories: i) short (below 300 base pairs), which is generally indicative of poor quality/highly fragmented DNA; ii) medium, (between 300 and 60 base pairs) read lengths indicate poor quality if the designed length of sequence exceeds 600 base pairs or it can indicate good quality when the desired length of the sequence is within 300 to 600 base pairs; and iii) the final category of CRL is long, (sequences that exceed 600 base pairs) in length that indicate high quality DNA (Technologies 2012).

4.4.8 Statistical analyse

To assess DNA quantity the nanodrop data is compared to four factors; fragmentation of bone, treatment group, time of exposure and extraction method. DNA quality was assessed using trace score, QV20+ values and CRL data against the same four factors outlined above as well as a fifth factor, primer selection.

4.4.8.1 One-way ANOVA

A one-way ANOVA is a Univariate analysis tool that investigates mean differences within a data group (dependant variable) and two or more independent variables (Girden 1992). In the present study one-way ANOVA was used to assess if there was statistically significant differences between DNA quantity and quality (dependant variables) and the five independent variables i) a cross a five month period; ii) within each
treatment groups; iii) following each extraction method; and iv) when bone had been fragmented; and v) when different primer sets are used for amplification. The results of one-way ANOVA are expressed as F and P values. P-value indicate statistically significant data i.e. where mean variation between groups has occurred as a result of the influence of the independent variable, p values closer to 0 indicate statistically significant data. An F value is a ratio of the variation between a sample over the variation within the sample, high F-values indicate that variation is greater between samples than within then thus making the data statistically significant (Madrigal 2012).

4.4.8.2 Tukey’s honest significant difference (HSD)

Following the application of one-way ANOVA a post Hoc test, Tukey’s honest significant difference, was applied to determine where within each factor statistically significant differences had arisen. Tukey’s HSD test is a pairwise analysis that investigates the differences between mean values within a given factor to identify where statistically significant data occurred, therefore only factors that have three or more variables can be investigated. In the present study, length of exposure, treatment group and primer were all investigated. Statistically significant data is reported as a p-value, as stated above, p-values approaching zero indicates higher significance.
CHAPTER FIVE

Results

5.1 Introduction

This chapter details the results of the various analyses performed in relation to the aims of this study: to what extent does burial environment negatively affect skeletal DNA quality and quantity? Are these effects more pronounced in fragmented versus intact remains? Finally, which DNA extraction method is preferable for severely weathered skeletal remains? The chapter first presents a visual assessment of the effect each weathering process had on bone morphology. In order to assess DNA quantity, nanodrop spectrophotometry was performed; gel electrophoresis is then used for further quantification. DNA quality sequencing was performed using chromatograms, trace scores, QV 20+ values and continuous read length (CRL) values.

5.2 Morphological changes

As skeletal DNA was the primary focus of the present study, only a basic evaluation of morphological changes and degradation is presented. This was deemed necessary because altered skeletal morphology potentially suggests compromised and/or degraded DNA, and is thus an important indicator when deciding whether to attempt molecular analyses. The fresh bones were originally a vivid white colour, with (after maceration) minimal flesh adhered at the distal and proximal epiphyses (Figure 5.1). The bone had a ‘moist’ quality and also exhibited minimal flexibility. All fresh control samples through the duration of the investigation mimicked this appearance, with no signs of degradation or diagenesis present (see below).
5.2.1 Morphological assessment: one month

At one month there were obvious morphological differences in the test bones, especially those subjected to wet/dry cycling and the sulphuric acid treatment.

5.2.1.1 Test control
At one month there was minimal morphological change relative to the initial fresh appearance. Bones maintained a moist quality and had begun to acquire a yellowish tint (with minimal dark staining from degraded muscle tissue). Bone marrow of the intact remains was still pink, while the fragmented remains exhibited darker marrow at the marrow-atmosphere interface (Figure 5.2). The only indication of degradation was a strong odour apparent when considering both the intact and fragmented remains.
5.2.1.2 Freeze/thaw cycle

At one month the freeze/thaw bones resembled those of the test control group; the bone surface was slimy and slippery, indicating a high moisture content. The soft tissue that remained at the proximal and distal epiphyses had not visibly changed. The bone had a pinkish tint, possibly due to the presence of adhered soft tissue (Figure 5.3). No loss of mineralisation was apparent. Marrow taken from the intact remains resembled that of the fresh control, while the marrow of fragmented remains was a much darker reddish brown colour. The bones were odoriferous, and obviously in an active state of decay.
5.2.1.3  Wet/dry cycle

For bones in the wet/dry environment there was considerable morphological change. The initial moist quality exhibited by the fresh remains (and those of the previous two groups) was not apparent; the bones had a dried out texture, which resulted in flaking across the surface of the bone. Dark red-brown stains were also apparent at the proximal and distal ends where the soft tissue was previously adhered (Figure 5.4). The marrow from the intact remains still resembled that of the fresh control, however the marrow from the fragmented remains was a much lighter pink/white colour, with apparent moisture loss. The fragmented remains presented more flaking than their intact counterparts, with the medullary cavity appearing to be drier relative to any other part of the bone (Figure 5.4).
5.2.1.4 Acidic environment

Bones subjected to the sulphuric acid treatment presented obvious morphological changes; they no longer appeared fresh, but had a pale white colour, with the associated soft tissue having a white/clear colouration (Figure 5.5). There was also evidence of the soft tissue being removed at the point of contact with the sulphuric acid. The bones had a slimy appearance and feel upon removal from the acid medium. The marrow, however, still resembled that of the fresh controls, both in colour and consistency. The fragmented remains presented degradation at their interface with the acid and were a lighter colour compared to the intact remains.

Figure 5.4 Wet/dry cycled bone at one month; bone has been fragmented using a band saw in preparation for DNA extraction.
5.2.2 Morphological assessment: three months

5.2.2.1 Test control
At three months the bones still had a ‘moist’ quality; reddish-black staining was also apparent where soft tissue was previously adhered at the epiphyses, (Figure 5.6). Marrow within the intact remains was light in colour (white/pink) compared to at one month, but it had begun to dry out, as evidenced by a ‘stringy’ consistency. The fragmented remains followed a similar morphosis, but appeared drier in comparison. The marrow within the fragmented remains was also a darker brown and much drier in comparison. These bones were also highly odiferous, indicative of a high level of decomposition.
5.2.2.2 Freeze/thaw cycle

Following three months of freeze thaw cycling the bones maintained a very moist quality and dark red-black staining was apparent across the entire bone, albeit concentrated to regions of adherent soft tissue (Figure 5.7). The marrow within the intact remains was still pink in colour, but it had begun to show signs of moisture loss. The fragmented bones also maintained a glossy wet quality, with dark red-black staining apparent. The marrow within the fragmented remains is darker in colour and had dried out considerably relative to the intact remains.

5.2.2.3 Wet/dry cycle

At three months the bones in the wet/dry environment had dried significantly, with flaking apparent across the entire bone. The epiphyses
were darker compared to the lighter diaphysis (Figure 5.8). The fragmented remains are likewise dried, with heavy flaking evident across the entire bone. The marrow within the fragmented bones was much darker in colour and had a crumbly solid appearance (potentially due to moisture loss), whereas the marrow taken from the intact remains was a white colour.

![Figure 5.8 Freeze/thaw cycled bone at three months.](image)

### 5.2.2.4 Acidic environment

Bones in the acidic environment had substantial mould growth across the surface, causing the remains to take on additional weight and volume. The mould was both white and black in colour. The flesh that remained was gelatinous and translucent in appearance (Figure 5.9). The marrow in the intact bones was white and indistinguishable from bone in transverse section. The fragmented bones followed a similar trend in relation to their general appearance, albeit with darker staining in the medullary cavity (and across the marrow) from visible fungal growth.
5.2.3  Morphological assessment: five months

5.2.3.1  Test control
At five months the intact bones within the test control group had begun to dry out, particularly along the diaphysis, as moisture was concentrated at the epiphyses. Bones were no longer a white-yellow colour and they had taken a dark brown-red stain, possibly from decomposed soft tissue (Figure 5.10). The fragmented remains were drier in comparison (particularly at the interface of the medullary cavity) and flaking was visible across the diaphysis. The marrow was very dark and dry relative to earlier time points.

Figure 5.10 Test control bone at five months prior to DNA analysis.
5.2.3.2 Freeze/Thaw

Following five months of freeze/thaw cycling, the bones had begun to dry out, with flaking apparent across the diaphysis. A dark colour was still evident across the surface of the bone (Figure 5.11). Marrow extracted from the intact remains was stringy and desiccated (difficult to obtain a small sample). Fragmented remains are much drier in comparison and the flaking was more extreme. Dark staining was still apparent, however, less intense. Marrow taken from the fragmented remains was dried, stringy, and darker in colour. Transverse sections of the remains reveal that the bone had begun to take on a crystal-like appearance.

![Figure 5.11 Freeze/thaw bone at five months.](image)

5.2.3.3 Wet/dry cycle

At five months the bones are the driest of any time point. The periosteal surface had heavy flaking, which was lighter in colour than the underlying (beige) bone. The intact remains had cracked at the epiphysis (Figure 5.12). The fragmented remains had likewise cracked, however, it was more severe, with the epiphyseal plate completely detaching from the diaphysis. The appearance of the marrow was no different to that of previous time points.
5.2.3.4 Acidic environment

Following five months in an acidic environment, the mould growth had increased to extend and create a layer across the surface of the acid. Adherent soft tissue had become completely gelatinous and translucent. Fragmentation/cracking was apparent at the epiphyses (Figure 5.13). The marrow taken from within the intact bones had lost all pink colouration and instead appeared grey and stringy. The fragmented bones showed signs of flaking along the diaphysis, in addition to mould growth, which extended into the medullary cavity and infiltrated the bone marrow. The latter caused the marrow to take on a spotted appearance. The marrow was also crystallised, particularly at the bone-acid interface (Figure 5.13).
5.3 Quantification of extracted DNA

DNA was quantified using two methods; nanodrop spectrophotometry and gel electrophoresis. The nanodrop was used initially to provide an indication of DNA quantity prior to sequencing, whereas gel electrophoresis was used to ensure that PCR amplification was achieved.

5.3.1 Nanodrop spectrophotometer

Nanodrop spectrophotometry was performed immediately after each extraction time point of one, three and five months; these intervals were designed to facilitate a measure of both quantity and quality over time. Extraction was performed at each interval using two extraction protocols (see Chapter Four).

Nanodrop spectrophotometer results are shown in Figure 5.14; the graph represents values of DNA quantity measured in ng/μl (Y-axis) for both the phenol chloroform (red column) and Qiagen extraction (blue column) methods. On the X-axis, each column represents a measure of extracted DNA quantity from the intact and fragmented bone samples. The latter is then further categorized to represent the time of extraction (1, 3 or 5 months) and the treatment group. For example, the first red column represents DNA extracted from an intact bone in the fresh control group, using a phenol chloroform extraction method at one month.

Phenol chloroform extraction produced consistently higher DNA quantities compared to the Qiagen extraction method, e.g. the mean value of DNA quantity within the control groups was 80.13ng/μl following phenol extraction and only 12.55ng/μl following QIAGEN extraction. The only instances in which this was not observed occurs for the following: intact freeze/thaw bone at one month, fragmented test control remains at five months, and at three and five months for intact acid bones within an to acidic environment (Figure 5.14).
DNA quantity measured using nanodrop spectrophotometry was lowest (for both extraction methods) in bones subjected to wet/dry cycling (average 30.26ng/μl) and the acidic environment (average 23.46ng/μl). It is also interesting to note that the fresh control bones yielded lower quantities of DNA than both the test control and freeze thaw groups for both extraction methods across the entire five-month period.

5.3.2 Statistical significance of Nanodrop results

ANOVA was used to investigate if nanodrop data (quantity) was influenced by the four factors of the study; treatment group, length of exposure (time), fragmentation and extraction method. It was evident that three factors (treatment, time and extraction) all significantly affected nanodrop values and thus DNA quantity (Table 5.1). Further analysis was performed using Tukey's honest significant difference test (HSD) the comparison of DNA quantities within treatment groups. Extraction following treatment within the acidic environments and the wet/dry cycles were lower than the values of the control groups and freeze/thaw group, these differences were shown to be significant (Table 5.2).

Furthermore, the quantity of DNA extracted after one month was shown to be significantly higher than the amount of DNA extracted after five months (Table 5.3). The most obvious difference was between results obtained following phenol extraction compared to QIAGEN extraction, with QIAGEN extraction yielding lower DNA quantity values that were shown to be highly statistically significant (Table 5.1).
Figure 5.14 Nanodrop Spectrophotometry graph; mean values for quantification of DNA post extraction for both Phenol chloroform (red) and Qiagen (blue) extraction across a five-month period (1, 3, 5 months) within each of the test groups.
Table 5.1 Univariate analysis with Nanodrop as the dependant variable assessed against fragmentation, treatment, time and extraction factors.

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Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001

Table 5.2 Tukeys HSD analysis within Treatment groups.

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<td>** NS NS</td>
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Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001
Table 5.3 Turkeys HSD analysis for time.

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<td>3 months</td>
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</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001

5.4 Gel electrophoresis

Further DNA quantification was undertaken using gel electrophoresis. Polymerase chain reaction (PCR) was first performed on 54 samples using three primers (primer design and cycling conditions are detailed in Chapter Four). Two mitochondrial DNA primers were used; amplification of mtDNA (compared to STR DNA) has been shown (in some cases) to be more successful in the context of disaster victim identification (Goodwin et al. 1999). Following PCR, gel electrophoresis was performed; the presence or absence of amplified DNA sections is reported as bright florescent bands in the gel. The location and size of each band is unique to each of the three primers and is indicative of successful DNA amplification.

Three individual gels were run for each of the primers; MtDNA, MtDNA2 and PGH (Figures 5.15-5.17). To facilitate comparison, all of the gels were loaded in the same order as shown in Table 5.4. Appendices Four indicates the successful amplification of each sample based on the primer used, an “x” indicates that amplification was successful for that sample using the specified primer. The results of each gel electrophoresis show that DNA could be amplified to a sufficient level for visualisation. A successfully amplification is shown by a bright/florescent band visible toward the base of the well.
Table 5.4 Electrophoresis gel loading order.

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<th>3</th>
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<td>02F-FC3P</td>
<td>03F-FC5P</td>
<td>04I-TC1P</td>
<td>05I-TC3P</td>
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<td>Ref</td>
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</table>

**Key:** I= intact bones, F= fragmented bones, FC= fresh control, TC= test control, A= acid, FT- freeze/thaw cycle and WD= wet/dry cycle.
In considering the MtDNA results, successful amplification is visible as bright bands for only 9% of the samples (5 of 54); The fresh control bones at one and three months for both extraction types, and the intact test control bone at one month using the phenol chloroform extraction. All other amplifications failed to provide a sequence of sufficient strength to allow them to be visualised as bands on the gel (Figure 5.15).

**Figure 5.15** DNA gel electrophoresis of 54 bone samples amplified using MtDNA primer. Included in the image is a size ladder within the first column of each row.

**Figure 5.16** DNA gel electrophoresis of 54 samples amplified using MtDNA2 primer. Included are ladders within column one of each row used to measure size of amplified DNA.
Interestingly the results following amplification using the MtDN2 primer varied considerably from that of MtDNA primer; amplification was achieved in 79% of the samples (44 of 54). It is also interesting to note that the samples where amplification failed were almost exclusively in the bones subjected to the acidic environment (Figure 5.16).

For the PGH primer, only relatively low levels of amplification were achieved, with just 10% of samples showing strong visible bands (Figure 5.17). The only samples successfully amplified were the fresh control bones from all time points using both extraction methods (Figure 5.17). None of the test bones were successfully visualised, this is interesting because it implies that no PCR product was produced, and thus all subsequent sequencing would also be unlikely to succeed.

5.5 Qualification of extracted DNA

To assess DNA quality the amplified samples were visualised using Sanger sequencing techniques to achieve high throughput results. For each sample a chromatogram was then created, which provided a visual representation of each DNA sequence. Following this, a trace score (phred number) was assigned to each sequence in addition to QV20+.
values and a continuous read length (CRL) number. All three factors are indicative of DNA quality and collectively give an appropriate qualification and understanding of DNA degradation for each sample.

5.5.1 Chromatograms

Chromatograms provide a visual representation of each sequenced sample; they consist of a series of coloured peaks, each representing one of the four-nucleotide bases that constitute DNA. The combination of colours, and therefore bases, allow the sequence to be determined. The accuracy with which each base is assigned is represented by a trace score (this is further explained in section 5.4.2.) that is represented on the chromatogram as a coloured band above each assigned base; blue bands indicate 99% accurate base assignment.

Representative examples of three chromatograms per primer were selected to demonstrate amplification and sequencing success (Appendix 2). To facilitate comparison between primers, the same samples are shown (intact acid treated bones, using phenol chloroform extraction at one three and five months). It is important to note that in some instances sequences ran longer than a single page, however, the success or failure of amplification can be ascertained immediately when considering the first page. Furthermore, since all of the samples were sequenced in dual directions, two chromatograms were produced per sample, however only the forward primer chromatograms are shown.

5.5.1.1 MtDNA chromatograms

Inconsistent results are apparent when considering all of the samples using the MtDNA primer. For samples extracted using phenol chloroform, five samples produced long (above 600 base pairs) sequences, five were medium in length (between 300 and 600 base pairs), and the remaining 45 were short in length (below 300 base pairs). The chromatograms produced were thus of an inconsistent appearance. All of the long (and some of the longer-medium length) sequences produced clean peaks, while the short sequences produced unclear peaks, including dye blobs,
that occur when excess dye that was unable to attach to the appropriate base accumulates. In considering the Qiagen extraction, similar trends emerged, with four samples producing long clear sequences, while the remaining 51 samples were of varied medium lengths that produced inconsistent chromatograms. Furthermore, seven samples were unable to be sequenced using the MtDNA primer irrespective of extraction procedure: These were 10FI_A3; 12FI_A5; 14FF_A1; 18FI_FT3; 07FF_Tc5; 26FI_WD3 and 30FF_WD3.

5.5.1.2 MtDNA2 chromatograms
Chromatograms produced using the mtDNA2 primers were clean with easily discernable peaks that were uniform and consistent in size. Dye blobs were not apparent in any samples. The MtDNA2 primer allowed successful sequencing of the seven samples that could not be sequenced using, the MtDNA primers (Appendix 2). In considering the chromatograms produced using the MtDNA2 primer, certain trends are evident. For the samples extracted using phenol chloroform, 50 produced medium length sequences corresponding to clean chromatograms. Four of the samples produced short sequences that correspond to unclear peaks. For the Qiagen extraction, the results were similar, with only two samples producing short sequences that had unclear peaks. The remaining 52 samples produced medium length clear chromatograms. Overall the MtDNA2 primer produced the most consistent sequences for all of the 54 samples tested.

5.5.1.3 PGH chromatograms
The PGH primer performed the poorest of the primers evaluated. A total of 11 samples failed to be sequenced using both extraction protocols. These were restricted to samples from within the test groups (all of the control samples sequenced). Furthermore, for the readable samples, only short sequences were produced for the remaining samples. The chromatograms of these sequences were not clean and contained numerous dye blobs (see Appendix 2).
5.5.2 Trace score

Associated trace scores according to primer used are shown in Figures 5.18-5.20. Each figure has the same scale to facilitate direct comparison and the samples are subdivided according to test group (see above).

Trace score results across the three primers demonstrated considerable variability. The highest quality DNA sequences was for the MtDNA2 primer, with only 6/54 samples not producing high trace scores (above 20); the low quality scores were largely from the bones subjected to the acidic environment. The latter trend occurred consistently for all three of the primer pairs. Amplification using MtDNA2 also produced consistent results when comparing extraction methods, with both resulting in high quality sequences. Furthermore, there was no significant difference between the results obtained from intact compared to fragmented bone (Figure 5.18).

The raw data for the MtDNA primers was less consistent. Approximately half of the samples (24/54) produced high trace scores, 10/54 produced medium trace scores and the remaining 10 samples had low scores. Furthermore, using MtDNA primers, two samples failed to produce sequences (Figure 5.19). Trace score results for the PGH primer followed a similar trend, with only 20 samples having high trace scores, seven with medium results, and 23 had low scores. Four samples failed to be sequenced (Figure 5.20).
Figure 5.18 Graphic representation of Trace scores for samples amplified using MtDNA primer pair. Data has been first categorised according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
Figure 5.19 Graphic representation of Trace scores for samples amplified using MtDNA2 primer pair. Data has been first categories according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
Figure 5.20 Graphic representation of Trace scores for samples amplified using PGH primer pair. Data has been first categorised according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
5.5.3 Statistical analysis of trace scores

ANOVA was used to investigate if trace scores were influenced by the five factors of the study; fragmentation, treatment, time, extraction and primer. DNA extracted from bones subjected to the acidic environment elicited the lowest trace scores and this difference was found to be statistically significant, furthermore extractions occurring at five months elicited the lowest trace scores compared to the other two time points. The final significant factor was primer, which greatly influenced the quality of DNA sequenced; both MtDNA primers produced higher trace scores compared to PGH (Table 5.5).

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Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001

Tukey’s HSD was performed on all factors deemed statistically significant following ANOVA analyses (Tables 5.6-5.8). It was determined that bones within the acidic environment elicited significantly lower DNA sequence quality yields compared to any of the other treatment groups (Table 5.6). No further significance was found between treatment groups. Time was also shown to have affected DNA sequence quality with extractions occurring at five months resulting in significantly lower trace scores compared to extractions at both one and three months (Table 5.7). Lastly
the quality sequences amplified using each of the three primer pairs was shown to be vastly different in comparison. MtDNA produced the highest quality sequences, followed by MtDNA and finally PGH, which produced the poorest quality sequences (Table 5.8).

Table 5.6 Tukey’s HSD analysis showing significance within treatment groups.

<table>
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<th>Treatment (I)</th>
<th>Treatment (J)</th>
<th>Significance</th>
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<td></td>
<td>Test control</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>**</td>
</tr>
<tr>
<td>Freeze/thaw</td>
<td>Fresh control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>NS</td>
</tr>
<tr>
<td>Wet/dry</td>
<td>Fresh control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>NS</td>
</tr>
</tbody>
</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001
### Table 5.7 Tukey’s HSD analysis showing significance within Time.

<table>
<thead>
<tr>
<th>Time (I)</th>
<th>Time (J)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>3 months</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>***</td>
</tr>
<tr>
<td>3 months</td>
<td>1 month</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5 month</td>
<td>**</td>
</tr>
<tr>
<td>5 months</td>
<td>1 month</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td>**</td>
</tr>
</tbody>
</table>

**Key:** NS=not significant, *P<0.05, **P<0.01, ***P<0.001

### Table 5.8 Tukey’s HSD analysis of significance within Primers.

<table>
<thead>
<tr>
<th>Primer (I)</th>
<th>Primer (J)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtDNA</td>
<td>MtDNA2</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>PGH</td>
<td>**</td>
</tr>
<tr>
<td>MtDNA2</td>
<td>MtDNA</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>PGH</td>
<td>***</td>
</tr>
<tr>
<td>PGH</td>
<td>MtDNA</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>MtDNA2</td>
<td>***</td>
</tr>
</tbody>
</table>

**Key:** NS=not significant, *P<0.05, **P<0.01, ***P<0.001

#### 5.5.4 QV20+ values

Another assessment of DNA sequence quality is the QV20+ value, which identifies the quantity of bases within sequence that have a trace value of 20 or more. A large QV20+ value indicates that a high proportion of a sequence was read accurately with correct base assignment (Figures 5.21-5.23). Each figure is representative of the QV20+ values obtained following sequencing from the three primer pairs; a uniform scale has been used across all of the figures to facilitate direct comparison.
When evaluating the QV20+ data across all three-primer groups a number of trends are evident. The highest qualities, and most constant results, occur for samples sequenced using the MtDNA2 primers (Figure 5.22). Only two samples sequenced using the MtDNA2 primer failed to reach a QV20+ value of 400; these were the bones from the acidic environment. High quality sequences were achieved using MtDNA primer (average 578) when considering the control groups, while the test groups had much lower (average 193) QV20+ values (Figure 5.21). PGH produced the lowest QV20+ values for all samples (control groups averaged 116 while test groups averaged only 46) (Figure 5.23). When considering each taphonomic environment, bones subjected to the acidic environment produced the lowest QV20+ values averaging 160, this was followed by the bones subjected to wet/dry cycling averaging 180, while the bones subjected to freeze/thaw conditions produced the highest QV20+ within the test environments with an average of 248. All of these values are however low when compared to both control groups which averaged 424 for the fresh controls and 374 for the test controls.
Figure 5.21 Graphic representation of QV20+ values for samples amplified using MtDNA primer pair. Data has been first categories according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
Figure 5.22 Graphic representation of QV20+ values for samples amplified using MtDNA2 primer pair. Data has been first categories according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
Figure 5.23 Graphic representation of QV20+ values for samples amplified using PGH primer pair. Data has been first categories according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
5.5.5 Statistical significance of QV 20+ values

ANOVA was used to investigate if QV20+ values were influenced by the five factors of the study; fragmentation, treatment, time, extraction and primer. Treatment groups, time of exposure as well as primer selection were all shown to have affected DNA sequence quality (Table 5.9).

Table 5.9 One-way ANOVA using QV20+ value as dependant variable assessed against fragmentation, treatment, time, extraction and primer.

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragmentation</td>
<td>1</td>
<td>0.935</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment</td>
<td>4</td>
<td>9.708</td>
<td>***</td>
</tr>
<tr>
<td>Time</td>
<td>2</td>
<td>11.625</td>
<td>***</td>
</tr>
<tr>
<td>Extraction</td>
<td>1</td>
<td>0.618</td>
<td>NS</td>
</tr>
<tr>
<td>Primer</td>
<td>2</td>
<td>53.196</td>
<td>***</td>
</tr>
</tbody>
</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001

Tukey’s HSD test was performed on the three factors deemed significant by ANOVA analyses. It was determined that the three treatment groups yielded QV20+ values that were significantly lower than the two control groups (Table 5.10). Furthermore, QV20+ values were the lowest when extraction occurred at five months compared to both one and three months (Table 5.11). Finally the three primer pairs were shown to have produced significantly different levels of DNA sequence quality according to QV 20+ data, MtDNA yielded the highest QV20+ values followed by MtDNA and finally PGH which produced the lowest QV20+ values (Table 5.12).
Table 5.10 Tukey's HSD analysis showing significance within Treatment groups.

<table>
<thead>
<tr>
<th>Treatment (I)</th>
<th>Treatment (J)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Control</td>
<td>Test Control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>***</td>
</tr>
<tr>
<td>Test Control</td>
<td>Fresh control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>**</td>
</tr>
<tr>
<td>Acid</td>
<td>Fresh control</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>NS</td>
</tr>
<tr>
<td>Freeze/thaw</td>
<td>Fresh control</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>NS</td>
</tr>
<tr>
<td>Wet/dry</td>
<td>Fresh control</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>NS</td>
</tr>
</tbody>
</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001

Table 5.11 Tukey's HSD analysis showing significance within Time.

<table>
<thead>
<tr>
<th>Time (I)</th>
<th>Time (J)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 month</td>
<td>3 months</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5 months</td>
<td>***</td>
</tr>
<tr>
<td>3 months</td>
<td>1 month</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>5 month</td>
<td>**</td>
</tr>
<tr>
<td>5 months</td>
<td>1 month</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>3 months</td>
<td>**</td>
</tr>
</tbody>
</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001
### Table 5.12 Tukey's HSD analysis of significance within Primers.

<table>
<thead>
<tr>
<th>Primer (I)</th>
<th>Primer (J)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtDNA</td>
<td>MtDNA2</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>PGH</td>
<td>***</td>
</tr>
<tr>
<td>MtDNA2</td>
<td>MtDNA</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>PGH</td>
<td>***</td>
</tr>
<tr>
<td>PGH</td>
<td>MtDNA</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>MtDNA2</td>
<td>***</td>
</tr>
</tbody>
</table>

**Key:** NS=not significant, *P<0.05, **P<0.01, ***P<0.001

#### 5.5.6 Continuous Read Length

The final assessment of DNA sequence quality comes in the form of continuous read lengths (CRL). MtDNA2 primer produced the most consistent read lengths. Only six samples across both extraction methods produced short sequence lengths that did not meet the expected read length of 714 base pairs, these were produced from samples extracted from bones subjected to the acidic environment (Figure 5.24). The remaining samples exceeded length of 714 base pairs. When considering MtDNA primers, inconsistent results were again achieved. Seven samples failed to register a read length and all test bones produced short or medium read lengths that did not meet the required 1045 base pairs. The only long sequences came from the control groups (Figure 5.25). PGH primers resulted in all of the samples producing short read lengths. Since the expected fragment size using this primer pair is 500 base pairs, none of the sample were sequenced to their full potential (Figure 5.26).
Figure 5.24 Graphic representation of CRL for samples amplified using MtDNA primer pair. Data has been first categories according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
Figure 5.25 Graphic representation of CRL for samples amplified using MtDNA2 primer pair. Data has been first categories according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
Figure 5.26 Graphic representation of CRL for samples amplified using PGH primer pair. Data has been first categories according to extraction method, then further subcategorised according to intact and fragmented status, time of extraction and test group.
5.5.7 Statistical significance of CRL data

ANOVA was used to investigate if CRL was influenced by the five factors of the study; fragmentation, treatment, time, extraction and primer. Significant data is noted in Table 5.13 below, time and primer selection were shown to have affected DNA quality significantly, when assessing CRL data.

Table 5.13 One-way ANOVA analysis using CRL values as dependant variables against treatment, time, extraction and primer.

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragmentation</td>
<td></td>
<td>2.143</td>
<td>NS</td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>3.081</td>
<td>**</td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>4.253</td>
<td>**</td>
</tr>
<tr>
<td>Extraction</td>
<td>1</td>
<td>2.739</td>
<td>NS</td>
</tr>
<tr>
<td>Primer</td>
<td>2</td>
<td>125.986</td>
<td>***</td>
</tr>
</tbody>
</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001

Tukey’s HSD test was performed on the time and primer data deemed significant following ANOVA analyses. The results show that read lengths obtained from bones subjected to the acidic environment as well as bones within the freeze/thaw group were lower compared to the other test groups as well as the control groups (Table 5.14). Furthermore MtDNA2 primer was shown to have elicited statistically higher read lengths compared to both MtDNA and PGH primers (Table 5.15).
Table 5.14 Tukey's HSD analysis investigating significance within treatment groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Control</td>
<td>Test Control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>NS</td>
</tr>
<tr>
<td>Test Control</td>
<td>Acid</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Fresh control</td>
<td>NS</td>
</tr>
<tr>
<td>Acid</td>
<td>Freeze/thaw</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Wet/dry</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Fresh control</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>NS</td>
</tr>
<tr>
<td>Freeze/thaw</td>
<td>Wet/dry</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Fresh control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>NS</td>
</tr>
<tr>
<td>Wet/dry</td>
<td>Fresh control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Test control</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Acid</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Freeze/thaw</td>
<td>NS</td>
</tr>
</tbody>
</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.001

Table 5.15 Tukey's HSD analysis investigating significance between Primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtDNA</td>
<td>MtDNA2</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>PGH</td>
<td>NS</td>
</tr>
<tr>
<td>MtDNA2</td>
<td>MtDNA</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>PGH</td>
<td>***</td>
</tr>
<tr>
<td>PGH</td>
<td>MtDNA</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>MtDNA2</td>
<td>***</td>
</tr>
</tbody>
</table>

Key: NS=not significant, *P<0.05, **P<0.01, ***P<0.00
CHAPTER SIX

Discussion and Conclusions

6.1 Introduction

The purpose of this final chapter is to discuss the results of the present study in relation to the three aims. This chapter also considers potential limitations, an assessment of the importance of the study overall, as well as suggestions for future avenues of research in the discipline.

6.2 Gross morphology

Clear morphological alterations were apparent in the bones subjected to the various experimental environmental conditions. The importance of macroscopically quantifying skeletal morphology is that it provides an immediate assessment of remains that facilitates the decision of feasible DNA analysis.

6.2.1 Control environment

The control bones were not subjected to any external diagenetic influences. Morphologically the test control bones experienced the least alteration. Drying commenced only after five months, however flaking was not apparent at any time point investigated. These results indicate that bone diagenesis, and any soft tissue decomposition was slower relative to the bones from the other test environments. Nicholson (1996) investigated the effect that varied environmental conditions had on bone diagenesis over a period of seven years. Remains within neutral soils were found to be the least affected, with no visible signs of altered morphology following soft tissue decomposition. Galloway (1997) investigated 468 deaths within the Arizona-Sonoran Desert and concluded that skeletonisation occurred after a minimum of two months. Furthermore, skeletal decay (bleaching, exfoliation) was evident usually after one year to 18 months following death, but only for those remains found unprotected within the arid conditions.
environment. The results of Galloway's investigation infer that skeletal
diagenesis occurs slowly over a period of years rather than months, and
can be influenced by external factors within the environment.

Surabian (2012) further emphasised the above, proposing that bone
diagenesis was primarily influenced by a number of extrinsic factors, such
as soil pH, temperature and water content; in the absence of the latter,
intrinsic factors (e.g. porosity) have the greatest influence, with bone
mineral density being among the most important. Given the results of the
present study, it is evident that bone diagenesis occurred much slower for
the test control bones in comparison to any of the test environments, with
minimal signs of altered gross morphology.

6.2.2 Freeze/Thaw environment

Bones exposed to the freeze/thaw environment over the course of five
months also exhibited minimal change to their natural morphological
appearance. Periosteal flaking and a dried appearance were evident at
five months for both the fragmented and intact remains. Within the
medullary cavity a crystalline appearance was evident after the bones
were sectioned. The results of the present study would indicate that bone
diagenesis had been slowed compared to the control bones, with only a
minimal affect on skeletal morphology (Figure 5.11). This inference is
supported by Tersigni (2007) who was also able to show that human long
bone fragments subjected to freezing temperatures (0°C) for prolonged
periods (21 days), experienced sign of diagenesis with apparent micro-
cracking. At 0°C micro-cracks formed around the centre of Haversian
systems as well as across the periosteal surface, leading to compromise
structural integrity.

Further support is evident in the works of Karr (2012a); Karr (2012b) who
demonstrated that the freeze/thaw cycle slows bone diagenesis (relative to
control bones) with only micro-fractures and flaking apparent on bones
exposed to -20°C after 20 weeks of exposure. Based on the results of the
present study, and in consideration of previous research, it is evident that
freezing leads to a decrease in moisture content, which in turn affects the collagen content of bone; this then leads to chemical decay and diagenesis (Andrade et al. 2008; Karr 2012a; Karr 2012b).

6.2.3 Wet/dry environment

The bones in the wet/dry environment underwent significant morphological change; the drying out process resulted in heavy flaking across the diaphyseal surface in both the fragmented and intact remains. The most notable changes, however, were in the metaphysis, where cracking was apparent on the intact remains, and complete detachment had occurred in the fragmented bones. Similar results were empirically demonstrated by Behrensmeyer (1978), who identified longitudinal stressors across the diaphyseal surface of mammalian bones that were subjected to natural wetting and drying processes of a lake bed over a period of two years. An environment of fluctuating water levels increases the diagenesis of bone through leaching of its mineral components, most notably nitrogen into the surrounding environment, as equilibrium is never met between environment and bone (Cox 2000; Hedges 2002). This influences the structural integrity of the bone, which in turn increases diagenesis that leads to fractures, periosteal flaking, and increased porosity (Franco Rollo 2002; Nielsen-Marsh 2000; Pike et al. 2001). In the present study, these effects were apparent after three months, with the disarticulation of the metaphysis occurring at five months.

6.2.4 Acidic environment

Unlike the three other test groups, the bones within the sulphuric acid (pH 1) matrix experienced markedly distinct morphological changes. Extensive mould growth occurred across the atmospheric-acid interface, which included bone. This mould was evident after the second extraction period at three months and increased in magnitude until the end of the five-month test period. These results are supported by Marchiafava et al. (1974), who examined the effect of fungal growth on buried fragments of human
vertebra at 20°C. Growth was evident after 45 days, with tunnelling evident from hyphae penetration. The conclusions drawn from that study suggest that fungus has an osteoclastic activity, which allows for hyphae penetration, which in turn leads to erosion and degradation of skeletal remains.

The above findings are further emphasised by Nicholson (1996) who investigated the effect of soil pH across 18 burial locations. Acidic soils accounted for eight of the 18 burial sites, which varied in pH between 3.2-4.5. Following seven years of exposure, extensive fungal mycelium covered the skeletal remains within the low pH environments. Morphologically these bones exhibited surface rippling, scalloping and tunnelling caused by fungal hyphae penetration. That study thus indicates that fungal colonisation aids in the decomposition of bone through fungal hyphae penetration and metabolite secretion. When considering that data in relation to the present study, extensive mould growth considerably altered the physical appearance of bone. Hyphae penetration was not macroscopically apparent, however, there were obvious colour and texture changes.

6.3 Quantification of extracted skeletal DNA

Two measures of quantification were utilised during the present study: i) spectrophotometry to determine the relative quantities of DNA per sample; ii) a relative measure of quantity using gel electrophoresis.

6.3.1 Nanodrop

Nanodrop spectrophotometry was performed to provide a measure of DNA quantity immediately following DNA extraction. Due to the time at which extraction occurred, the nanodrop data was assessed against four factors: time (length of exposure); environment; extraction method; and fragmentation.
6.3.1.1 Time
Length of exposure was shown to decrease DNA quantity, between one and five months. The present utilised a diminished time frame in comparison to other contemporary studies but still paralleled their results. For example, Hochmeister et al. (1991), extracted skeletal DNA from femora of cadavers in advanced stages of decay (between one and six weeks after death), in addition to remains submersed within the Rhine River for 18 months and a mummified leg dating to 1979. The results of this study demonstrated that, despite advanced decay and prolonged environmental exposure, DNA could be successfully obtained from each set of remains. The femora that had entered into advanced decay produced quantities of DNA between 25ng and 3.3μg, the submerged remains produced 50 ng of DNA, while the mummified leg yielded 500 ng of DNA. The latter thus indicates that time was not an important factor for determining DNA quantity of skeletal remains, which contradicts the results in the present study. It should be noted, however, that DNA quality for the remains within this study was severely diminished; this is discussed further below (see section 6.4.2)

6.3.1.2 Environment
Environmental condition was also shown to have a direct, and significant impact on DNA quality. Bone in the acidic environment as well as the wet/dry treatment group varied the most compared to the control groups, with significantly lower DNA quality from three months. Since these groups had experienced the most morphological change the DNA results were somewhat expected and follow those of Zehner (2007), whom in the analysis of skeletal DNA from the SEA Boxing Day Tsunami was unlikely to successfully amplify profiles following prolonged exposure to wet and then dry conditions.

6.3.1.3 Extraction method
A comparison between organic phenol chloroform and the Qiagen DNA extraction kit demonstrated that phenol chloroform consistently extracted higher quantities of DNA at each extraction time point. The latter is
supported by previous research (Ferreira et al. 2013; Iyavoo et al. 2013; Jian Ye 2004; Piglionica et al. 2012) who similarly demonstrated that phenol chloroform extraction consistently yields the highest quantity and quantity of DNA, especially for scenarios in which DNA yield is expected to be low (e.g. harsh environmental conditions, natural disasters, prolonged periods of time). Iyavoo et al. (2013) used a pig bone model to compare extraction efficacy of five DNA extraction techniques. Four of the extraction methods were commercially available (one of which was the Qiagen blood and tissue kit used in the present study) the final extraction method was a traditional phenol chloroform protocol. The results of Iyavoo et al. (2013) mirror that of the present study; phenol chloroform extraction produced the highest yields of quantitative DNA.

6.3.1.4 Fragmentation

Fragmentation was the only factor of the study that did not influence the likelihood of obtaining high quantities of skeletal DNA. This could be due to the short time frame of the study or to the fact that despite fragmentation skeletal material still affords bone a durable barrier between the medullary surface and the environment. The results of the present study support previous work in particularly studies addressing DNA based identification from skeletal remains from mass disasters, associated with high levels of fragmentation. For example, Holland et al. (2003) investigated the development of a high throughput method for skeletal DNA extraction and identification for victims of September 11. This scenario required the extraction of DNA from 13,000 bone fragments towards identifying 2,700 individuals. The results of that investigation demonstrated the successful extraction of DNA in 65.7% of cases. In some instances more than 200 fragments of bone were attributed to the same individual; fragmentation, therefore, did not have a significant role in determining likelihood of obtaining sequenceable DNA, but rather the conditions of the remains (charring, exposure to the environment, and prolonged time spent decomposing) were confounding post-mortem factors.
6.3.2 Electrophoresis

Gel electrophoresis was performed on all samples using each of the three primer pairs and demonstrated amplification with varied success rates according to the primer pair chosen. A total of 79% of samples amplified using the MtDNA 2 primer produced strong gel bands; the MtDNA and PGH primers, however, only resulted in the successfully amplification of 9% of samples. This would indicate that the affinity between sample and primer was poor within the later primer pairs. Interestingly, sequencing was still successful for the samples that did not produce a strong PCR product (strong bands).

Some factors have the potential to influence sample migration and thus affect its visualisation. The two most notable for the present study are dye concentration and gel pore size (Kim and Morris 1994; Voet 1999). Dyes have the potential to influence the rate of migration of samples within a gel; for example, increasing DNA dye ratios slow the migration of samples through the gel, which has the potential to alter the trajectory of the sample (Kim and Morris 1994). Furthermore, fluorescence intensity is also affected depending on the DNA/dye ratio. For example larger samples with smaller ratios are known to produce less intense bands (Kim and Morris 1994).

In further explaining the electrophoresis results of this study, gel pore size is likely an associated factor. Larger pore sizes do not sieve smaller DNA fragments to a degree sufficient enough to facilitate strong separation of the sample, and thus gel resolution is less (Voet 1999). In the present study, nuclear DNA is believed to have a much higher degree of fragmentation than the mitochondrial DNA, as per the results of the continuous read length analysis. For this reason a standard 1% gel may not facilitate pore sizes that are small enough to adequately sieve the sample, and thus are factors in the poor resolution observed (especially for the PGH gel electrophoresis).
6.4 Qualification of extracted skeletal DNA sequences

DNA sequence qualification was achieved following Sanger sequencing and represented using three approaches: i) visually using chromatograms; ii) trace score/ QV20+ values; and iii) continuous read lengths.

6.4.1 Chromatograms

One main trend was obvious after interpreting all of the chromatograms; primer selection clearly had the greatest influence on sequence clarity of all of the study factors quantified. Both mitochondrial DNA primers provided peaks that were clearer and cleaner than the PGH primers. As all samples were derived from the same bone specimen, and extracted under the same conditions at the same time, it is feasible to suggest that primer selection is pertinent to achieving clean peaks and readable/usable sequences. This further suggests that mitochondrial DNA extraction (and thus survival) was achieved over nuclear DNA. Fragmentation, time and extraction method, were shown to be factors that did not influence the visual representation of any group of sequences. Treatment group however, appeared to influence the outcome of the chromatograms, with bones from the acidic environment having the lowest peaks, suggesting that DNA degradation was the highest for samples extracted from the bones subjected to that environment. This trend is evident throughout all of the qualification tests and is discussed further below.

6.4.2 Trace scores and QV20+values

After assessing trace scores and QV20+ values, three factors were found to have significantly affected DNA sequence quality; time (length of exposure); treatment group; and primer selection.

6.4.2.1 Time

DNA sequence quality significantly diminished when comparing results obtained from one month to those after five months. Clearly prolonged
exposure to extreme environments increased DNA degradation, which in turn adversely affects the possibility of extracting high quality sequenceable DNA. The latter is particularly evident for the nuclear DNA, as it exits as a one helix cell (Butler 2005). As stated above, Hochmeister et al. (1991) conducted an study in which skeletal DNA was extracted from remains that had experienced varied levels of decay (between one and six weeks), as well as a set of remains that had been submerged within the Rhine for 18 months and mummified lower limbs from 1979. While length of exposure to the environment did not influence the success of extracting high quantities of DNA, sequence quality was (at times), severely affected. Only three of the ten samples taken from the decaying remains produced high-molecular weight (high quality and non fragmented) DNA samples, while neither the submerged or mummified remains produced any high-molecular weight DNA. The results of this investigation show that high quality DNA sequences were achieved from skeletal remains experience decay congruent with both prolonged exposure and environmental stressors.

6.4.2.2 Treatment group (environment)

All test environments were shown to have yielded DNA that was significantly lower in sequence quality compared to both control groups. Bones subjected to the acidic environment represented the most significantly different group with an average QV20+ value of 160 compared to both the fresh control and test control groups with averages of 424 and 374 respectively. The latter is likely attributed to two primary factors; Firstly, the extensive mould growth across the surface of the bone influenced bone diagenesis through hyphae infiltration and thus DNA degradation was adversely effected; this is supported by Nicholson (1996) who showed that fungal growth increased diagenesis of skeletal remains within low pH environments. Secondly, the other potential cause of low trace scores and QV20+ values for bones subjected to the acidic environment are the accumulation of PCR inhibitors that occur within low pH environments. Burger et al. (1999) demonstrated that the dentition
accumulated PCR inhibitors following burial within acidic environments, and thus DNA analysis was unsuccessful for identifying those remains.

Based on their QV20+ values the bones subjected to wet/dry conditions were also shown to have lower levels of DNA sequence quality compared to the control bones with an average QV20+ value of 180. The results of the present study indicate that fluctuating water levels had the effect of lowering sequence quality, especially when comparing both MtDNA and nuclear DNA. Samples amplified using the PGH primer within this group resulted in low quality sequences (average QV20+ value of 36). Edwards et al. (2004) found very similar patterns of DNA quality values; following the attempted sequencing of 14 samples from an ancient river within the Bercy region of France that experienced seasonal waterlogging and drying, readable sequences were only obtained from 23.1% of those samples. PCR inhibition and DNA degradation are attributed to the low success rate of that study and the same relationship is likely to hold true for the present study.

Finally bones subjected to freeze/thaw cycles also yielded lower DNA sequence quality compared to both control groups. Royere et al. (1988) investigated the affect that freeze/thawing had on spermatozoa. Cycles were shown to decrease both surface, as well as native DNA, thus indicating that freezing and thawing lowers the quantity and quality of DNA. Within the present study the lower trace scores and QV 20+ values within this group, relative to the control, demonstrated the same relationship.

6.4.2.3 Extraction method

A comparison between the two extraction methods applied did not result in any significant difference in DNA sequence quality. Therefore, it can be concluded that, in relation to DNA sequence quality, extraction method is not a defining factor affecting successful sequencing. Obviously, the latter only applies to the methods tested within this study and inferences about other protocols would require empirical testing.
6.4.2.4 Fragmentation

Fragmentation was again not shown to have impacted on the likelihood of achieving successful DNA amplification from skeletal material following prolonged exposure to varied environmental stressors. The quality of the DNA sequences extracted from remains that were intact, relative to those that were fragmented, was not significantly different, thus indicating that fragmentation was not a factor influencing DNA sequence quality. These results are supported by the findings of Holland et al. (2003), which were discussed above (see section 6.3.1.).

6.4.2.5 Primer

Primer selection was shown to be the most significant factor determining amplification success. The importance of primer selection is a well-established factor influencing the likelihood of successful amplification (Butler 2005; Gill 2005). In the present study the MtDNA 2 primer produced the most consistent results when considering trace scores and QV20+ values; the MtDNA primer was the next most successful and the PGH primer produced the lowest (and least consistent) DNA yields. Therefore both mitochondrial primers elicited high quality DNA compared to nuclear DNA (PGH primer). There are three proposed reasons for this that are supported by the published literature; i) High copy number of MtDNA compared to nuclear DNA (hundreds of thousands of MtDNA can be held within one cell compared to the single helix of nuclear DNA per cell)(Bender et al. 2000). ii) MtDNA is haploid (half the number of usual chromosomes) therefore sequencing is simpler compared to diploid (paired chromosomes) nuclear DNA by a factor of 2 (Butler and Levin 1998), and iii) MtDNA has been shown to be resistant to extreme environments over long time periods e.g. years (Hagelberg et al. 1991; Shook and Smith 2008)

6.4.3 Continuous Read Length

Continuous read length (CRL) is the final measure of DNA sequence quality measured in the present study. This refers to the longest
uninterrupted section of DNA sequenced. It is therefore, a measure not only of sequence quality, but also potentially of DNA fragmentation. Samples that fail to produce read lengths that match the desired segments (as per the primer used) have been damaged or degraded in some capacity. CRL is considered in the context of each of the experimental parameters below.

6.4.3.1 Time
Interestingly time was not shown to have significantly influenced the quality of DNA sequences extracted from the bones when considering CRL values. This could be attributed to the fact that all samples sequenced using PGH primers failed to reach the minimum read length, thus rendering the factor of time insignificant when assessing it as an overall factor. As stated above, within the present study the length of exposure appears to have influenced the quality of DNA sequences (trace score and QV 20+ data) but not the quantity of DNA extracted from the experimental remains. These findings accord with Hochmeister et al. (1991), who was able to show that high quantities of DNA could be extracted from skeletal remains, that had experienced varied levels of decay over time (between one week and 18 months) but that DNA sequence quality was diminished with prolonged exposure.

6.4.3.2 Treatment group (environment)
Environment had a significant influence in the likelihood of sequencing high quality skeletal DNA. The bones subjected to the acidic and wet/dry environment elicited the shortest read lengths and thus produced the lowest quality DNA sequences compared to the other environmental groups. The DNA extracted from these bones failed to meet the expected primer length. These results indicate that DNA damage and fragmentation likely occurred resulting in shorter read lengths and effecting sequencing.

Krajden et al. (1999) similarly demonstrated that freezing of blastocyst caused cell apoptosis and DNA fragmentation. Furthermore, Burger et al. (1999) demonstrated that acidic environments were detrimental to DNA preservation as humic and fulvic acids were shown to inhibit PCR and
cause oxidation and hydrolysis. This leads to high fragmentation, degradation and a severely impacts the possibility of successfully sequencing samples from acidic environments. The results of the latter research support the findings of the present study, whereby environment was shown to have a significant influence in the likelihood of acquiring high quality intact sequenceable DNA from weathered skeletal remains.

6.4.3.3 Extraction method
There were no statistically significant differences between DNA sequence qualities obtained from phenol chloroform compared to the QIAGEN extraction kit. Therefore, it can be concluded that (in terms of CRL values) either extraction method is effective in producing high quality DNA from weathered skeletal samples. Davoren et al. (2007), supported this finding by showing that DNA extracted from femora using silica based methods (QIAGEN) compared to organic extraction (phenol) both resulted in high quality sequenceable DNA.

6.4.3.4 Fragmentation
Fragmentation was again shown to be a non-significant factor in relation to extracting sequenceable DNA. As already discussed, despite fragmentation the skeletal structure still appears to provide sufficient protection from the external environment for the marrow/DNA. Holland et al. (2003) and Zehner (2007), both were able to obtain sequenceable DNA from highly fragmented remains (World Trade Center) and SEA (Boxing day Tsunami).

6.4.3.5 Primer
Following the trends shown for trace score and QV20+ values, the continuous read length values also indicated that primer selection was the most influential factor determining successful DNA amplification and sequencability. Results obtained from primer specific sequencing showed that only samples sequenced using the MtDNA2 primer achieved read lengths exceeding, or of equal length, to the expected 714 base pairs; seven samples failed to reach the required length, all of which were sampled from bones subjected to the acidic environment. Only the test
control bones (and a single bone within the freeze/thaw treatment group) met the expected 1045 base pair length requirement for the MtDNA primer, while none of the samples reached the 400-500 base pairs required for PGH primer. These results are likely due to a combination of poor primer pair specificity, as well as high fragmentation of DNA following extensive time spent within each treatment group; this is especially true for nuclear DNA amplified using PGH primer. The higher copy number of MtDNA compared to nuclear DNA, in addition to the added protection afforded by the mitochondrion, has resulted in differential degradation between the two nuclear sites, resulting in divergent results between the two MtDNA, and the one nuclear DNA, primers (Foran 2006). The disparate results between the two-mitochondrial primers can be attributed to different primer specificities, which has a significant impact on all subsequent DNA sequencing and analyse (Chauhan 2009). This is shown by the present study where the same samples have produced very different CRL values according to the primer pair chosen MtDNA and MtDNA2.

6.5 Forensic significance of the present research

The outcomes of this study have implications within the field of forensic identification (e.g. victim identification following natural or deliberate mass victim scenarios) and also archaeology. When skeletonised remains are located, an anthropological assessment is first undertaken and a biological profile is established to aid towards establishing identification (Cattaneo and Baccino 2002). During large-scale disaster victim identification (DVI) scenarios, a molecular analysis of skeletal remains is often the subsequent method for positive identification (Lee et al. 2008; Prinz et al. 2007).

Within the United States, a principal set of 13 STR loci are traditionally used for identification (Budowle 1999). The use of nuclear DNA as the sole means of identification is, however, complicated when DNA quality and quantity is compromised through taphonomic process associated with
mass disasters (Budowle et al. 2005). This is clear in the present study, as there is a statistically significant difference between the mean data obtained from sequences derived from nuclear DNA (PGH) compared to mitochondrial DNA (MtDNA and MtDNA2) in relation to QV20+ values and CRL. MtDNA is used in forensic investigations involving remains subjected to prolonged exposure to extreme taphonomic processes (Butler and Levin 1998) or for cases that involve closed groups (e.g. identification of the deceased Romanov family) (Gill et al. 1994). In the present study sequencing using mtDNA primers resulted in higher quality DNA than the PGH primers (nuclear DNA) when considering all of the test groups. The highest quality and most consistent results occurred when comparing trace scores, QV 20+ values and CRL’s from samples amplified using MtDNA2 primers.

This study was, therefore, able to show that MtDNA does not undergo significant degradation following exposure to extreme environments. The preservation of MtDNA is believed to be due to the high copy number of MtDNA within the mitochondrion compared to nuclear DNA in addition to its circular shape, which aids preservation of mtDNA over nuclear DNA (Bogenhagen and Clayton 1974; Budowle et al. 2005). Within a forensic context, however, MtDNA is rarely used as the primary means of identification. In the wake of the World Trade Centre attacks on September 11, positive identification of remains was only obtained through the combined sequencing of nuclear DNA and MtDNA (Biesecker et al. 2005; Budowle et al. 2005). Given the results of this investigation, a combined STR and MtDNA approach would appear to provide the most comprehensive database from which positive identification in a forensic context could be achieved.

6.6 Potential limitations of the present study

Certain limitations need to be addressed in relation to this study in order for the results to be valid and applicable to forensic casework. First (and foremost) within Australia and New Zealand the use of human tissue for
scientific experimentation is strictly limited to medical research. Furthermore, the use of human tissue for experimentation is performed under strict guidelines relating to personal consent, privacy and confidentiality (Wooldridge 2007). Research involving decomposition is, therefore, performed using human substitutes, such as Sus Scrofa (domestic pig). This project utilised domestic pig hind limbs in place of human long bones, and while domestic pig specimens are used internationally in place of human tissue across a wide variety of disciplines (Early and Goff 1986; Eberhardt and Elliot 2008; Grassberger and Frank 2004; Voss et al. 2009) the divergent species should be noted as a potential limitation of the study.

Structurally human and porcine bone structure is similar and therefore assumptions can be extrapolated from one about the other. Differences between the two arise when considering the age of the specimens used. The bones sampled for this project were taken from Linley Valley (a commercial abattoir) and as such the age of the specimens used ranged from 3 to 5 years. The degree of skeletal development for each pig, therefore, varied slightly, with evidence of recent epiphyseal fusion generally still visible (a faint line) in some bones. While this did not affect the structural integrity of the bones during the early stages of the project, the later stages saw cracking and disarticulation around the epiphysis, which was potentially aided by this weakness. Any inferences made regarding morphology of adult human remains under the same environmental stressor should thus take this into consideration.

Another caveat that should be acknowledged is the time frame of the study and the controlled laboratory setting. A five-month period was essential given the time frame of experimentation afforded by a one-year Master thesis, however a study conducted over a number of years has the potential to result in an increased level of DNA damage given the extended period of exposure. In order to attempt to control this factor, the present research attempted to simulate prolonged exposure by varying the time each set of remains spent within any given condition. While this does
accelerate the level of morphological damage visible in the bone, as well as damage to the genetic material within, a prolonged experiment utilising a longer time period will afford a more comprehensive analysis and understanding of the effect that prolonged exposure to extreme environments has on skeletal material.

Furthermore, a laboratory setting was necessary for this project; this was done to limit any potential miscellaneous environmental stressors that would otherwise be present in an outdoor setting. The benefit of the latter approach is that specific parameters relating to the weathering process can be investigated in isolation (e.g. the wet/dry, freeze/thaw cycles or low pH) and become the sole environmental stressor acting on the remains. This approach does, however, eliminate all naturally occurring environmental stressors such as temperature fluctuations, humidity, predation and rainfall which have the potential to also effect the likelihood of obtaining high quality DNA from skeletal remains this factor should therefore be considered when assessing the results of the present study and its applications on forensic investigative context.

6.7 Recommendations for future research

The present study showed that exposure over time to extreme environments causes skeletal DNA degradation, which in turn affected the extractability of high quality sequences, required to achieve positive identification. As this study was only a preliminary investigation into the effects that taphonomy has on skeletal DNA, a number of avenues could be further explored to build on the knowledge applicable to victim identification.

From the results of this investigation, primer selection, and the apparent preferential degradation of nuclear DNA over mitochondrial DNA, would be the most beneficial avenue of further investigation. Replication using a set of established loci would be important because, as the present study was only able to use a single nuclear primer targeted at PGH. As stated above, within the United States a set of 13 loci are used for positive identification.
A study designed to directly compare the efficacy of a large set of loci against MtDNA sequencing would facilitate a more detailed insight of nuclear compared to mitochondrial DNA degradation over time.

Another avenue of investigation could be the inclusion of taphonomic conditions pertinent to local institutions. In 2010, the Commonwealth Scientific and Industrial Research Organisation (CSIRO) stated that within Australia annually, bushfires affect approximately 50 million hectares of land resulting in one of the largest causes of loss of life (Booth 2009). Noting the morphological changes to bone experienced during fire, and how that affects the extraction of high quantities of quality DNA from skeletal material, is a highly relevant avenue of future investigation.

### 6.8 Conclusions

Burial environment was shown to accelerate diagenesis of bones, particularly those exposed to sulphuric acid and wet/dry environments. Morphologically these bones experienced marked changes across five months, resulting (in certain circumstances) in the fragmentation of remains. The quality and quantity of skeletal DNA extracted from those bones was also affected, with prolonged exposure resulting in both a decrease in DNA quantity and quality compared to the control bones. The difference between DNA quality extracted from the control bones, and the DNA extracted from bones subjected to the experimental environments was also found to be statistically significant. Therefore, burial environment was shown to have a significant influence on the ability to extract high quality sequenceable DNA from skeletal material.

Fragmentation did not affect the extraction of high quantities of quality DNA and was not a significant influence when considering any of the test parameters for both quantity (nanodrop) and quality (Trace score, QV20+ value and CRL). Therefore, it is concluded that DNA extracted from fragmented skeletal remains did not undergo accelerated degradation compared to that extracted from intact remains.
A comparison between the two selected DNA extraction techniques (organic extraction using phenol chloroform and a commercial DNA Qiagen extraction kit), resulted in statistically significant quantities of DNA extracted from each bone. Control groups averaged 80.13 ng/μl for phenol extraction and only 12.55 ng/μl for QIAGEN extraction, similarly when considering the test groups as a collective, following phenol extraction the average amount of DNA extracted was 50.71 ng/μl compared to only 14.82 ng/μl extracted using QIAGEN kit. There was however no significant difference when considering the quality of DNA extracted. Overall, phenol chloroform extraction was shown to elicit higher quantities of DNA compared to Qiagen extraction kit, however both methods of extraction yielded equivalent levels of DNA quality. It is thus concluded that DNA extraction technique, within the context of the present study, did not influence the ability to elicit high quality, sequenceable DNA from skeletal remains.

Overall, the most significant factor influencing the successful amplification of skeletal DNA was primer selection, which was found to be significant for all qualitative results. Primer selection is crucial for eliciting clean readable DNA sequences. For this study the MtDNA primers out performed PGH (nuclear DNA primer), with MtDNA2 eliciting the highest quality and cleanest sequences overall.

The present study concludes that attempts to establish positive identification following prolonged exposure of skeletal remains to extreme taphonomic conditions can result in the successful amplification of skeletal DNA. This will most likely be achieved if the MtDNA is targeted, with primer selection proving to be crucial to sequencing success. The results of the present study hope to influence the field of victim identification, for both natural and deliberate mass death scenarios, when identification via DNA analysis is questioned, thus bridge the gap between forensic anthropology and molecular biology.
REFERENCES


APPENDIX 1- ETHICS APPROVAL

NOTIFICATION OF USE OF ANIMAL TISSUE / CADAVER

The scientific use of animals undertaken at The University of Western Australia by University staff or students must comply with the requirements of the Australian code for the Care and Use of Animals for Scientific purposes 5th Edition 2013 (The Code) and with the Animal Welfare Act WA (2002).

In certain instances where animals are obtained as cadavers or where only animal tissue or samples are obtained from third parties (e.g. samples from abattoir, animal tissue derived from other AEC approved projects) and where no trapping or handling of any live animal has occurred to obtain the tissue samples or cadaver, a full AEC application may not be required.

If the tissue is derived from bio-banked stock collected as part of a past scientific project, which has since closed, or from a non-current commercial activity, exemption from a full AEC application to use the tissue may also be granted.

In all circumstances it is strongly recommended you contact the Animal Ethics Office for determination as to whether you will be able to apply for exemption for work being considered – email: aec@uwa.edu.au

OFFICE USE ONLY (July 2013) 

FILE REF: RA/3/5007

Date: 11/3/14

1. CHIEF INVESTIGATOR DETAILS

Title, last name, first name: Staff / Student number (UWA only)
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2. CO-INVESTIGATOR / STUDENT DETAILS

Title, last name, first name: Staff / Student number (UWA only)

Miss, Swift, Lauren 21276021

Work mailing address / school (include UWA MBDP)
Centre for Forensic Science, M420
Email: 21276021@student.uwa.edu.au Phone Mobile: 0413039727

2. DESCRIPTION OF THE PROPOSED STUDY

Must be written in lay language
This project will assess the effect that season wet/dry and freeze/thaw patterns have on the quality of DNA from pig bone. Extraction will occur after various cycles of freeze/thaw and wet/dry directly from the pig bone involved. The outcomes have direct relevance to forensic practice.

Proposed start date: (dd/mm/yyyy) 17/3/14 Proposed completion date: (dd/mm/yyyy) 17/3/15

3. DETAILS OF WHERE THE STUDY IS TO BE CARRIED OUT (location)
4. ANIMAL SPECIES AND NUMBERS TO BE USED - species name and common name
The animal species required for this project is the pig (Sus scrofa) - a total of approximately 4 pigs will be required. The only elements of the animal required are extremity bones (femur, tibia, humerus). The pigs are sourced from a commercial abattoir and are not sacrificed for the purpose of this study - the material provisioned is a by-product of the human commercial food consumption chain.

5. SUPPLIER OF ANIMAL TISSUE / CADAVER - Please provide details of where tissue will be sourced. Include the name of abattoir, name of farm owner etc.

AEC approved project: Chief Investigator name and AEC approval number RA/3/
Abattoir: Lincoln Valley Abattoir, Wooroloo, Western Australia. x
Tissue biopsies (conservation programs): 
Commercial breeding supplier:
Farm:
Fishers:
Other (please specify):

6. METHOD OF DISPOSAL
Medical waste disposal bin following the appropriate UWA OHS guidelines. After cleaning and stabilization some of the bones will also be retained for teaching purposes.

7. HEALTH AND SAFETY - If this project involves the use of materials that are of concern for the health and safety of other staff/students and/or other animals, ensure that the appropriate approvals have been obtained. For information, refer to the University Safety and Health Website www.safety.uwa.edu.au/

I have read, and will abide by, the Standard Operating Practice for Tissue Use at www.research.uwa.edu.au/staff/biological/tissue-use

Attached are copies of required permits, approvals, and assessments. Yes [ ] No [x ]

8. DECLARATION

Chief Investigator:

PRINT NAME: [redacted] SIGNATURE: [redacted] DATE: 11/03/19

Please forward one (1) signed copy to: Animal Ethics Committee - M499 or aso@uwa.edu.au
APPENDIX 2- CHROMATOGRAMS

MTDNA PRIMER
MTDNA2 PRIMER
# APPENDIX 3- RAW MEAN DATA

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APPENDIX 4

Gel electrophoresis data, each sample has been assessed based on the three primers used. An x indicates that PCR amplification was achieved and visualised with a strong bright band.

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