

**THE EFFECTS OF HIGH TEMPERATURE ON THE
QUANTITY AND QUALITY OF MITOCHONDRIAL AND
NUCLEAR DNA IN NON-HUMAN SKELETAL REMAINS**

by

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Abstract

A good understanding of DNA preservation is critical for authenticating ancient DNA. However, such knowledge is difficult to obtain from empirical data as every site represents a unique burial environment, often resulting in unpredictable DNA recovery rates.

This study established an artificial DNA degradation model to examine patterns of mitochondrial and nuclear DNA degradation in non-human bone, using time and temperature as controllable degradation factors. The results indicate that DNA degradation increases significantly when temperature is raised from 50°C to 70°C, with a rapid initial reduction in DNA copy number followed by a more gradual period of degradation. It appears that mitochondrial and nuclear DNA undergo similar degradation rates. It is expected that future research will provide more detailed information on DNA degradation patterns, which will be extremely useful for assessing the quality and quantity of retrievable DNA from different recovery contexts, both archaeological and forensic in nature.

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Glossary

Abasic Sites	A lesion in a DNA strand due to the loss of a nitrogenous base (A, T, C, or G). Such sites can lead to strand breakage and incorrect nucleotide incorporation during PCR amplification.
aDNA	Ancient DNA. Generally refers to DNA from archaeological remains; herein it is also used to refer to DNA from degraded remains in any context (i.e. archaeological or forensic).
Amplicon	DNA produced through PCR amplification, targeted by sequence-specific primers.
Hydrolysis (or TaqMan [®]) Probes	Sequence-specific fluorescent probes used in real-time PCR that contain a 5'-end reporter dye and a 3'-end quencher dye. The quencher suppresses the fluorescence of the reporter until the probe anneals to a DNA template and is then excised from the template through the activity of the DNA polymerase, enabling the reporter to fluoresce. The amount of fluorescence can then be compared to known concentration standards for quantification.
Gel Electrophoresis	A method of separating molecules, such as DNA, based on size as they pass through a gel medium with the use of electricity. As the DNA travels through the gel, shorter fragments travel faster and therefore further down the gel.
mtDNA	Mitochondrial DNA. Circular DNA housed within organelles known as mitochondria in eukaryotic cells. There are multiple copies of mtDNA within each cell. It is inherited only from the maternal line.
nDNA	Nuclear DNA. Linear DNA contained within the cell nucleus in the form of chromosomes. Two copies of nuclear DNA are contained within eukaryotic cells, with one copy inherited from each parent.

PCR	Polymerase chain reaction. Method of amplifying specific fragments of DNA through the use of temperature cycles to denature, anneal and extend templates. All PCR reactions must contain the DNA to be replicated, primers for the desired fragment, a DNA polymerase which enables the extension of the DNA template, and deoxynucleotide triphosphates (dNTPs), which are free bases that are used to build the replicated fragment.
Primers	Short fragment of DNA used during PCR amplification to specify the beginning and end of a desired sequence.
Real-time PCR	Works on the same basic concepts of PCR, however analysis of the reaction occurs during the exponential phase of amplification as opposed to end-point analysis, allowing for accurate quantification with the use of different types of fluorescent dyes.

Chapter 1: Introduction

The incorporation of ancient DNA (aDNA) analysis into many archaeological and forensic questions has greatly enhanced research (Alaeddini et al. 2010; Hofreiter et al. 2001; Kaestle & Horsburgh 2002; O'Rourke et al. 2000; Pääbo et al. 2004; Willerslev & Cooper 2005). However, knowledge of the mechanisms of DNA preservation and degradation, in both archaeological and forensic contexts, is limited. The lack of understanding of the processes of DNA degradation is a common thread in articles that include even a limited discussion of aDNA. A greater understanding of how DNA degrades in certain remains and preserves in others would lead to improvements in the application of aDNA, as a good understanding of DNA damage is critical for authenticating degraded and ancient DNA data.

Knowledge regarding when and where DNA is most likely to be preserved will reduce investments of time and money in cases where successful extraction would be unlikely. More importantly, due to the destructive nature of aDNA analysis, this understanding may reduce the loss of irreplaceable archaeological and forensic material that may be inappropriate for DNA analysis. However, such knowledge is difficult to obtain from empirical data due to each burial environment representing a unique combination of physical, chemical and biological conditions (Geigl 2002; Gilbert et al. 2003; Hagelberg & Clegg 1991; Hansen et al. 2006; O'Donoghue et al. 1996; Pruvost et al. 2007; Tuross 1994), often resulting in unpredictable DNA recovery rates.

Most research in the field of aDNA has focused on improving contamination controls (for example, Bouwman et al. 2006; Pruvost et al. 2005; Yang & Watt 2005) and methods of DNA extraction and amplification (for example, Brotherton et al. 2007; Giles & Brown 2008; Pruvost & Geigl 2004; Rohland & Hofreiter 2007b; Tuross 1994). Recent work exploring issues of degradation and preservation has largely been based on empirical data collected during specific excavations (such as, Bollongino & Vigne 2008; Pruvost et al. 2007). Such observational, site-specific data make it difficult to draw consistent conclusions, as burial conditions and taphonomic processes vary from site to site. Unfortunately, little controlled experimentation testing postmortem DNA degradation has been done.

To begin to address this knowledge gap, this research aimed to examine the influence of time and temperature on the rate of DNA degradation in modern sheep skeletal remains, through controlled experimentation. Bones and teeth are often the only elements that persist in the burial environment over extended periods of time and are therefore commonly used in archaeological and forensic investigations. As such, this research focused on DNA degradation patterns in bone. Furthermore, this research focused specifically on temperature because it is often regarded as the key factor influencing DNA degradation (Bollongino & Vigne 2008; Hoss et al. 1996; Poinar & Stankiewicz 1999; Reed et al. 2003; Zhang & Wu 2005). Temperature appears to be the one environmental variable that can be consistently associated with differential aDNA amplification success rates across geographic locations (Bollongino & Vigne 2008; Karanth & Yoder 2010; Reed et al. 2003). DNA extraction has been highly successful from ancient remains found in cold environments (Karanth & Yoder 2010; Mitchell et al.

2005; Schwarz et al. 2009; Smith et al. 2001; Shapiro & Cooper 2003), however there has been little to no success with remains located in tropical climates (Bollongino & Vigne 2008; Karanth & Yoder 2010; Kumar et al. 2000; Reed et al. 2003). Temperature also happens to be one of the most easily manipulated and controllable variables in a lab setting.

Research Objectives

Through a series of experiments, this research was expected to achieve the following three objectives:

- 1) To determine the pattern of degradation in the quantity and quality of DNA in modern skeletal elements exposed to different temperature regimes for various periods of time.
- 2) To assess any differences in the pattern of degradation between mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) within and between heat treatment samples.
- 3) To establish an artificial DNA degradation model examining DNA degradation patterns in bone samples, with the potential to add different factors into the model in future research.

For the first objective, it was expected that as temperature increased, the rate of DNA degradation would increase, with shorter exposures to higher temperatures having a relatively more detrimental effect than longer exposures to lower temperatures. For the second objective, differences in the pattern of degradation between mtDNA and nDNA were anticipated, with nDNA having an increased degradation rate. This expectation is due in part to the notion that the circular structure of mtDNA may confer protection on

the molecule, enabling it to resist degradation (Foran 2006). It is also due to the presence of multiple copy numbers of mtDNA (hundreds of copies per cell as opposed to two copies of nDNA) providing a greater opportunity for retaining the correct, undamaged sequence (Foran 2006; Hofreiter et al. 2001; Hunter 2006; O'Rourke et al. 2000; Pääbo et al. 2004). Analysis of the quality of DNA in heat treated samples was performed through the amplification of DNA fragments of varying lengths, while quantification was accomplished through the use of real-time PCR. The final objective was to validate whether or not the experimentally degraded bone could serve as a functional degradation model for future studies. Although only temperature and exposure period were tested in this study, the observed degradation pattern is expected to lay the foundation for future research involving different materials, such as teeth, soft tissues and plant remains, along with different degradation factors, such as moisture, pH and soil composition.

Deoxyribonucleic Acid (DNA)

DNA is a macromolecule containing the genetic information of an organism. It is composed of two strands of connected nucleotides made up of a deoxyribose sugar molecule, a nitrogenous base, and a phosphate group (Hummel 2003). The two strands carry the same genetic information, but run in opposite directions in a double helix formation (Hummel 2003; Nicklas & Buel 2003). The backbone structure of DNA is comprised of alternating sugar and phosphate molecules held together by phosphodiester bonds, with one of four nitrogenous bases attached to the sugar (Hummel 2003). There are two types of nitrogenous bases – purines, which include adenine (A) and guanine (G); and pyrimidines, which include cytosine (C) and thymine (T). The two anti-parallel

strands are held together by hydrogen bonds between complementary bases – adenine always bonds to thymine with two hydrogen bonds, while guanine bonds to cytosine with three hydrogen bonds (Hummel 2003; Nicklas & Buel 2003). It is the sequence of these complementary bases, known as base pairs (bp), which dictates the genetic information contained within DNA.

Within most eukaryotic cells, there are two types of DNA – nuclear DNA located inside the cell nucleus; and mitochondrial DNA found inside organelles within the cell known as mitochondria. Plant cells also contain chloroplast DNA, located in chloroplast organelles in the cell cytoplasm. Nuclear DNA (nDNA) is linear in structure and organized into chromosomes, with two copies per cell. Mitochondrial DNA (mtDNA) has a circular structure and is generally much shorter in length compared to the nuclear genome, but with multiple copies per cell. While nDNA is inherited from both parents, mtDNA is only passed on matrilineally.

DNA Degradation

DNA in living cells is continuously being degraded through various processes; however living cells are equipped with an arsenal of DNA repair mechanisms that ward off such damage, enabling organisms to maintain their genetic integrity (Handt et al. 1994). Once an organism dies, these repair mechanisms that ensure DNA sequences are preserved cease to function and the DNA quickly degrades unchecked (Lindahl 1993; Shapiro & Cooper 2003). Damage to DNA makes analysis difficult because it creates small fragments due to strand breakage and abasic sites, resulting in fewer amplifiable templates. This damage can lead to amplification inhibition and the incorporation of

incorrect bases into sequences during PCR amplification (Brotherton et al. 2007; Gilbert et al. 2003; Hofreiter et al. 2001; O'Rourke et al. 2000; Pääbo et al. 2004; Willerslev & Cooper 2005). Strand breaks and abasic sites can also cause what is referred to as 'jumping PCR', leading to chimeric sequences in which multiple templates are incorporated into a single fragment due to incomplete extension of the primers (Alaeddini et al. 2010; Brotherton et al. 2007; Handt et al. 1994; Hebsgaard et al. 2005; Pääbo et al. 1990; Willerslev & Cooper 2005). Such amplification artefacts can lead to misidentifications if appropriate sequence authentication measures are not pursued.

Most DNA damage results from hydrolytic and oxidative processes, the activities of microorganisms, and nucleases within dying cells (Alaeddini et al. 2010; Brotherton et al. 2007; Hebsgaard et al. 2005; Hofreiter et al. 2001; Karanth & Yoder 2010; O'Rourke et al. 2000; Pääbo et al. 2004; Willerslev & Cooper 2005; Zhang & Wu 2005). In the burial environment, the extended chemical degradation of DNA is believed to be heavily influenced by hydrolysis and oxidation. DNA hydrolysis results in the breakdown of the N-glycosol sugar-base bonds of DNA in the presence of water (Alaeddini et al. 2010; Burger et al. 1999; Hoss et al. 1996; Lindahl 1993; O'Rourke et al. 2002; Pääbo et al. 2004; Poinar 2003). The sugar-base bond cleavage often results in depurination (the loss of purine bases, A & G), followed by the breakage of the DNA backbone through β -elimination at the abasic sugar-phosphate bond. A similar process can also impact pyrimidines (C & T), however this occurs at a lower rate due to increased stability (Alaeddini et al. 2010). Hydrolysis can also cause base alterations through deamination, which converts adenine, cytosine, 5-methylcytosine (a cytosine residue), and guanine into hypoxanthine, uracil, thymine, and xanthine (respectively) through cleavage of the amino

groups (Alaeddini et al. 2010). These processes result in destabilization of the DNA sugar-phosphate backbone, causing strand breakage and crosslinks, along with incorrect base pair insertion during amplification (Alaeddini et al. 2010; Gilbert et al. 2003; Hofreiter et al. 2001; Hoss et al. 1996; Lindahl 1993; Poinar 2003). Oxidation results in the modification of bases or the structural distortion of DNA through the interaction of reactive oxygen species, such as hydroxyl or superoxide radicals (Alaeddini et al. 2010; Burger et al. 1999; Hoss et al. 1996; O'Rourke et al. 2000; Lindahl 1993; Pääbo et al. 2004). As with hydrolysis, oxidation causes base loss and base pair alterations (with particular influence over pyrimidines), DNA strand breaks and crosslinks, and the creation of PCR inhibitors known as hydantoins through the chemical alteration of thymine and cytosine (Alaeddini et al. 2010; Burger et al. 1999; Hofreiter et al. 2001; Hoss et al. 1996; Lindahl 1993; Poinar et al. 1998). Table 1 provides a brief summary of DNA damage commonly associated with skeletal remains.

Although damage induced by hydrolytic and oxidative reactions are often cited as being most commonly observed, there seems to be no general agreement on exactly which form of such damage is most detrimental to the analysis of degraded DNA. Different authors cite distinct types of DNA degradation as playing the most influential role – from depurination, to deamination, to strand crosslinks, and microbial attack. A key reason for this disagreement is the general lack of knowledge regarding DNA degradation in postmortem specimens, as most research on DNA degradation is based on damage in living tissues, or has been performed *in vitro* on samples in aqueous solution (Bada et al. 1999; Geigl 2002; Götherström et al. 2002; Haynes et al. 2002; Poinar & Stankiewicz 1999). Such studies assume that the chemical processes affecting the

degradation of DNA in living biological systems are the same processes affecting post-mortem remains (Geigl 2002; Götherström et al. 2002; Haynes et al. 2002). However, it is not known whether this is actually the case, or if aqueous studies of DNA can be reliably applied to DNA studies involving postmortem remains.

Table 1 Common degradation pathways in ancient and forensic remains.

Damage Mechanism	Cause	Damage Induced	Resultant Issues for Analysis	Timing
Enzymatic damage	<ul style="list-style-type: none"> •microbial activity •endogenous & exogenous nucleases 	<ul style="list-style-type: none"> •fragmentation •crosslinks 	<ul style="list-style-type: none"> •PCR inhibition 	<ul style="list-style-type: none"> •occurs primarily shortly after death
Hydrolytic damage	<ul style="list-style-type: none"> •interaction of DNA with water molecules 	<ul style="list-style-type: none"> •depurination •strand breaks •deamination 	<ul style="list-style-type: none"> •PCR inhibition •miscoding lesions 	<ul style="list-style-type: none"> •continuous process
Oxidative damage	<ul style="list-style-type: none"> •interaction of DNA with reactive oxygen species 	<ul style="list-style-type: none"> •modification of sugar molecules •modification of bases and the production of hydantoins •abasic sites •crosslinks 	<ul style="list-style-type: none"> •PCR inhibition •miscoding lesions 	<ul style="list-style-type: none"> •continuous process

Compiled from: Alaeddini et al. 2010; Burger et al. 1999; Gilbert et al. 2003; Hebsgaard et al. 2005; Hofreiter et al. 2001; Hoss et al. 1996; Karanth & Yoder 2010; Lindahl 1993; O'Rourke et al. 2000, 2002 ; Pääbo et al. 2004; Poinar 2003; Willerslev & Cooper 2005; Zhang & Wu 2005.

DNA degradation and preservation are believed to be largely dependent on the surrounding environment, with all environmental factors playing a role. The specific taphonomic conditions that each set of remains goes through before and after burial are important in assessing the level of DNA degradation (Geigl 2002; Gilbert et al. 2003; O'Donoghue et al. 1996). Whatever factor particular researchers advocate as the most detrimental and common types of damage in aDNA, most agree that cold temperatures, low oxygen levels, low humidity, and low microbial load allow for the greatest success in DNA extraction and amplification from degraded samples.

Estimating Levels of DNA Degradation

Various methods have been suggested for estimating DNA degradation within skeletal remains. One of the most commonly used techniques is amino acid racemization. This particular method is based on the assumption that depurination is the main form of damage in aDNA, and that the racemization of the amino acid asparagine (the conformational change from the L-enantiomer to the D-enantiomer) occurs at a similar rate and under similar conditions as DNA depurination (Bada et al. 1999; Collins et al. 1999; Elbaum et al. 2006; Götherström et al. 2002; Haynes et al. 2002; Hofreiter et al. 2001; Pääbo et al. 2004; Rollo et al. 2002). Some success has been found in associating the ratio of D:L-enantiomers, however other studies have indicated that this may not be an appropriate proxy for DNA preservation based on the aforementioned assumptions leading to incorrect notions about the availability of DNA in a given sample (Collins et al. 1999; Götherström et al. 2002; Haynes et al. 2002).

Correlations between morphological preservation, both macro- and microscopic, have been used to determine which skeletal remains would be more likely to contain preserved aDNA (Götherström et al. 2002; Haynes et al. 2002; Misner et al. 2009; O'Rourke et al. 2000); however these are not always reliable. Even researchers who find correlations between morphology and DNA preservation often caution that if morphological preservation and environmental conditions appear to be appropriate, it does not guarantee successful DNA amplification (for example, Poinar & Stankiewicz 1999).

It is not only the burial environment that is of concern for the preservation of DNA in ancient remains, as post-excavation storage conditions also play a role. Recently excavated remains have been shown to have increased rates of successful aDNA extractions compared to remains that have been previously excavated and kept in storage for many years prior to analysis (Pruvost et al. 2007). It has been suggested that aDNA preserved in skeletal remains rapidly degrades shortly after removal from the burial environment if precautions are not taken, such as cool storage and not washing the samples (Bollongino & Vigne 2008; Pruvost et al. 2007). Standard cleaning treatments and storage procedures further degrade the aDNA, with cool storage temperatures likely contributing to optimal preservation (Bollongino & Vigne 2008; Burger et al. 1999; Pruvost et al. 2007).

Ancient DNA

The first successful utilization of aDNA was reported in 1984, with mtDNA extracted from museum specimens of the extinct quagga (Higuchi et al. 1984). Shortly after, numerous publications appeared affirming successful amplification of aDNA from other sources, however the results of many of these studies could not be replicated, and it was later concluded that a large portion of the results were likely due to contamination with exogenous DNA (Hagelberg & Clegg 1991; Hebsgaard et al. 2005; Hoss et al. 1996; O'Rourke et al. 2000; Pääbo et al. 2004). Since these early discoveries, a substantial amount of work has been performed with the aims of improving the extraction and amplification procedures employed in aDNA analysis (Giles & Brown 2008; Rohland & Hofreiter 2007b; Tuross 1994), and in reducing contamination issues through the

development of authentication criteria (Hebsgaard et al. 2005; Hofreiter et al. 2001; Kaestle & Horsburgh 2002; Malmstrom et al. 2007; O'Rourke et al. 2000; Pääbo et al. 2004; Poinar 2003; Willerslev & Cooper 2005). Moreover, various types of preserved ancient materials have been examined to determine their potential for further aDNA analysis – from skeletal remains (Götherström et al. 2002; MacHugh et al. 2000; Morin et al. 2007; Pääbo et al. 2004; Rohland & Hofreiter 2007a), coprolites and sediments (Deagle et al. 2006; Haile et al. 2007; Hansen et al. 2006; Hofreiter et al. 2001; Hofreiter et al. 2000; Iniguez et al. 2003; Poinar et al. 2003; Poinar et al. 1998; Rollo et al. 2002), and specimens contained in chemical preservatives (Shedlock et al. 1997; Stuart et al. 2006; Wandeler et al. 2007), to plant remains (Elbaum et al. 2006; Gugerli et al. 2005; O'Donoghue et al. 1996; Parducci et al. 2005; Schlumbaum et al. 2008; Threadgold & Brown 2003), food residues (Hansson & Foley 2008; O'Donoghue et al. 1996) and even parchment and papyri (Marota et al. 2002; Poulakakis et al. 2007).

Due the highly fragmented nature of ancient DNA, short fragments of no more than 200 or 300bp are generally targeted for amplification. Mitochondrial DNA sequences are more commonly the focus of ancient DNA studies rather than nuclear sequences, due to the fact that within a single cell there are only two copies of nDNA compared to hundreds or thousands of copies of mtDNA (Foran 2006; Kaestle & Horsburgh 2002; O'Rourke et al. 2000; Pääbo et al. 2004; Poinar 2003; Willerslev & Cooper 2005). This means that the likelihood of successfully extracting intact mtDNA from ancient and deteriorated remains is greater than extracting intact nDNA. However, with improvements in the field, more and more studies are focusing on extracting the information held within nuclear DNA (Binladen et al. 2006; Hunter 2006; Miloš et al.

2007; Poinar et al. 2003; Stuart et al. 2006). The higher mutation rate of mtDNA also makes it particularly amenable to studies requiring species and genus level identifications (Kaestle & Horsburgh 2002; O'Rourke et al. 2000; Pääbo et al. 2004; Poinar 2003; Willerslev & Cooper 2005).

Although modern DNA was used in this study, ancient DNA extraction and amplification techniques were employed, due to the degraded nature of the samples following exposure to heat. The use of ancient DNA techniques has proven more effective in extracting and amplifying DNA from degraded remains, which is typically fragmented and in low quantity and poor quality due to prolonged exposure to various elements in the burial environment – such as pH, humidity, and temperature (Bada et al. 1999; Burger et al. 1999; Capelli et al. 2003; Deagle et al. 2006; Hofreiter et al. 2001; Kaestle & Horsburgh 2002; O'Rourke et al. 2000; Pääbo et al. 2004; Poinar 2003; Pruvost et al. 2007; Smith et al. 2003). Interestingly, studies have indicated that to a certain degree, the level of DNA degradation is less dependent on the chronological antiquity of the remains than it is on the burial environment, with temperature often identified as the key variable in DNA degradation, as discussed in detail below (Burger et al. 1999; Collins et al. 1999; Karanth & Yoder 2010; Kumar et al. 2000; O'Rourke et al. 2000; Smith et al. 2003; Poinar 2003; Poinar & Stankiewicz 1999; Pääbo et al. 2004; Reed et al. 2003).

Previous Studies Involving Temperature Effects on DNA Degradation

Most experimentation involving ancient DNA preservation and degradation has dealt with what most researchers believe to be the key factor – temperature. A fair

amount of work has been done looking at how DNA is affected by temperature variation, and what temperatures are most conducive to its preservation, however a large portion of this work is based on observational, site specific data. Large syntheses have been performed incorporating all available data on DNA retrieval success and failure rates and the relationship with geographic location (for example see Bollongino & Vigne 2008; Karanth & Yoder 2010; Kumar et al. 2000; Reed et al. 2003). The most prominent factor that stands out with regard to consistent failure of DNA extraction is the temperature of the site at which the remains were found, with little to no success from sites within tropical zones, specifically within 23° north and south of the equator (Bollongino & Vigne 2008; Karanth & Yoder 2010; Reed et al. 2003). In the few cases where aDNA was successfully analysed from such environments, the remains were generally found in high altitude cave sites with lower than average temperatures and stable microenvironments (Bollongino & Vigne 2008; Karanth & Yoder 2010; Kumar et al. 2000; Reed et al. 2003).

Recently, the concept of thermal age has been employed to estimate the amount of DNA degradation and the likelihood of DNA retrieval from ancient remains (Collins et al. 2002; Götherström et al. 2002; Hansen et al. 2006; Ovchinnikov et al. 2001; Poinar et al. 2003; Smith et al. 2001; Smith et al. 2003). Thermal ageing relies on the assumption that DNA depurination (removal of purine bases), which is temperature dependent, is the most influential type of damage (Götherström et al. 2002; Hansen et al. 2006; Smith et al. 2001; Smith et al. 2003). This concept asserts that the actual absolute age of a set of remains (in calendar years) is less important in the degradation of DNA than the thermal age (age based on the average environmental temperature, and the fluctuations around

this average). The absolute, chronological age of a fossil is adjusted based on the specific thermal history of the burial site, by comparing the rate of DNA depurination calculated for the thermal history of a particular site, to that of an ideal site held at a constant 10°C (Smith et al. 2003). Smith et al. (2003) give a maximum fossil thermal age of 19_{kyr@10°C} (19000 years when maintained at a constant 10°C) as an appropriate screening tool for successful ancient DNA amplification, with remains thermally older having a greatly decreased chance of success.

Other authors have argued against the applicability of thermal ageing, suggesting that many sites with thermal ages greater than the limit of 19_{kyr@10°C} proposed by Smith et al. (2003) have yielded amplifiable ancient DNA (Götherström et al. 2002; Hansen et al. 2006; Ovchinnikov et al. 2001; Poinar et al. 2003). Some researchers debate the extreme reliance on depurination being the prime factor in DNA degradation, since many other factors are at work in the degradation process (Götherström et al. 2002; Hansen et al. 2006; Ovchinnikov et al. 2001). Ovchinnikov et al. (2001) and Hansen et al. (2006) both argue that focusing on thermal age could mean that certain remains that could in fact yield informative ancient DNA sequences would be passed over.

Degradation processes of both mitochondrial and nuclear DNA have been studied in light of testing forensic identifications (for example, see Alaeddini et al. 2010; Andréasson et al. 2002; Alonso et al. 2004; Cattaneo et al. 1999; Swango et al. 2006; von Wurmb-Schwark et al. 2003; von Wurmb-Schwark et al. 2004). Many of these studies found that DNA was not retrievable from heat-induced artificially aged remains (Cattaneo et al. 1999; von Wurmb-Schwark et al. 2003; von Wurmb-Schwark et al. 2004). However, these studies often involve testing at extremely high temperatures in the

attempt to replicate events such as house fires or cremation (for example, 800+°C (Cattaneo et al. 1999)) in which the DNA is rapidly degraded, or focus on nDNA markers which are less successfully extracted from degraded samples (for example, von Wurmb-Schwark et al. 2003). Such studies often employ forensic DNA analysis techniques and commercial DNA testing kits, which are considered to be less sensitive than aDNA techniques in the extraction and amplification of degraded DNA (Capelli et al. 2003; Swango et al. 2006). Ancient DNA techniques allow for higher resolution in terms of both methodology (i.e. in targeting very small DNA fragments) and laboratory setup (i.e. strict protocols to reduce contamination).

Until recently, DNA preservation in plant materials has been given less attention than that of other archaeological remains; however it has important implications for questions involving past plant domestication and cultivation practices, and paleoenvironmental reconstructions (Elbaum et al. 2006; O'Donoghue et al. 1996; O'Rourke et al. 2000; Pääbo et al. 2004; Schlumbaum et al. 2008). Controlled experiments have been performed regarding the influence of heat on DNA in plant remains (for example, see Banerjee & Brown 2004; O'Donoghue et al. 1996; Threadgold & Brown 2003), as residues of past cooking practices are commonly found in archaeological contexts, such as desiccated, waterlogged, charred or mineralized seeds (O'Donoghue et al. 1996). However, the relationships between the degradation of plant DNA and faunal DNA are unclear, due to the differences in the respective DNA molecules and cellular structures. Therefore, results from these types of experiments cannot be accurately correlated with faunal remains.

Various experiments have been performed studying the degradation of DNA in aqueous solution after it has been extracted from its originating tissues (Banerjee & Brown 2004; Threadgold & Brown 2003). Although these studies do provide crucial information regarding *in vitro* DNA damage in general, any protection that other cellular structures may bestow upon the DNA is lost due to the degradation experiments being performed after the DNA has been extracted from its source tissue. For example, studies have suggested that the crystalline structure of hydroxyapatite in bones confers protection on the DNA present in the cells (Geigl 2002; Götherström et al. 2002; Salamon et al. 2005). Therefore, the relationship between the rate of degradation determined from DNA in aqueous solution cannot be reliably correlated with the degradation of DNA that remains within the supporting and possibly protective cellular tissues.

Temperature was selected as the degradation factor in this study for various reasons. First, it is often cited as the most detrimental of all environmental influences on the preservation of DNA. Second, it has often been employed as a means of artificially ageing materials (Collins et al. 1999; Dobberstein et al. 2008), which makes data from this study more comparable to earlier results. Third, it is one of the easiest factors to manipulate and control over long periods of time.

DNA Quantification

With the development of the polymerase chain reaction (PCR) in the 1980's (Saiki et al. 1988), it became possible to exponentially amplify minute amounts of DNA, theoretically from as little as a single template. This has been an especially important development for aDNA analysis, as often only small numbers of suitable templates

remain within archaeological samples (Brotherton et al. 2007; Hagelberg & Clegg 1991; Pääbo et al. 2004; Pruvost & Geigl 2004; Willerslev & Cooper 2005). PCR works by running through multiple cycles of temperature fluctuations which enable the initial denaturation of the DNA template, followed by annealing of the primers, and then extension of the template from the attachment site of the primers through the use of DNA polymerase.

Quantification using standard PCR can only be accomplished through endpoint analysis, making the determination of initial template numbers problematic as different primer sets and PCR cycling conditions have differential amplification efficiencies for each reaction (Nicklas & Buel 2003; Shipley 2006). This endpoint analysis generally involves visualization of the amplified fragments on an agarose gel, through the combination of gel electrophoresis and a DNA staining dye (Nicklas & Buel 2003; Shipley 2006). The fluorescent intensity of a particular sample band in the gel can then be compared to that of a known-concentration DNA mass ladder, as the amount of fluorescence should be directly proportional to the amount of amplicon present.

With the advent of real-time PCR, quantification during the exponential phase of the reaction (when amplification efficiency is at its highest) became possible, reducing the reliance on endpoint analysis and post-PCR manipulation of samples. Real-time PCR is based on the same thermal cycling principles as standard PCR, however, it involves the addition of a fluorescent dye to every sample prior to PCR amplification, usually either in the form of a non-sequence-specific intercalating dye (Heid et al. 1996; Pruvost & Geigl 2004; Shipley 2006) that binds to double-stranded DNA (such as SYBR[®] Green) or as a sequence-specific fluorescent probe (such as TaqMan[®] probes) (Adams 2006;

Andréasson et al. 2002; Heid et al. 1996; Orlando et al. 1998; Pruvost & Geigl 2004; Shipley 2006). With every amplification cycle, the amount of fluorescence increases as a product of the increase in the template number. During the first few cycles, known as the baseline phase, amplification is occurring, however, the fluorescence is below the level that is detectable by the real-time software (Shipley 2006). The point at which the level of fluorescence exceeds that of background fluorescence is called the threshold, and is set in the exponential amplification phase (Heid et al. 1996; Pruvost & Geigl 2004; Shipley 2006). The cycle threshold (C_T) for a particular sample is determined when the fluorescence level exceeds that of the background fluorescence and crosses the predetermined threshold (see Figure 1A) (Pruvost & Geigl 2004; Shipley 2006). The level of fluorescence of the reporter dye is measured after each cycle and normalized for any variation between wells using a passive reference dye (Adams 2006; Orlando et al. 1998; Shipley 2006), such as ROX. Comparison of the unknown samples with a set of known-concentration standards allows for the calculation of initial template numbers and the production of a standard curve (see Figure 1B).

The efficiency of a particular real-time PCR assay is determined by the slope and y-intercept of the standard curve and the coefficient of determination (R^2) (Adams 2006). The efficiency, expressed as a percentage, is a reflection of how well the reaction progressed, while the R^2 indicates how robust the fit of the data is to the theoretical line of best fit for the standard curve (Adams 2006). The value of the y-intercept indicates the expected C_T of a one-template sample, and is a reflection of how accurately the initial template numbers are quantified. Ideally, in a 100% efficient reaction, the slope should equal -3.32, with an R^2 value of 1.00 and a y-intercept falling between cycles 33 and 37

(Adams 2006). Real-time PCR is optimized for the amplification and quantification of small fragment lengths, generally of no more than 250bp, so that sequence-specificity and amplification efficiency are maintained (Andréasson et al. 2002; Shipley 2006; Smith et al. 2002).

Both standard and real-time PCR methods were employed in this study, however initial template quantification was calculated using real-time PCR only. Standard PCR was used to analyse the quality of the DNA available through the amplification of fragments of varying lengths; it was not used to estimate template quantities. Quality was determined based on the level of fragmentation observed in a sample, with the successful amplification of longer fragments indicating less fragmentation and therefore better overall quality. No other “quality” factors, such as base alterations, were considered.

The real-time PCR method employed herein relied on the addition of sequence-specific fluorescent hydrolysis probes, often referred to as TaqMan[®] probes. The probes contain a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end (Andréasson et al. 2002; Heid et al. 1996; Orlando et al. 1998; Shipley 2006; Smith et al. 2002). When the probe is free in solution the close proximity of the quencher dye suppresses the fluorescence of the reporter. As the probe anneals to a DNA template and extension of the template begins, the 5'-exonuclease activity of the DNA polymerase excises the probe from the template, releasing both the reporter and quencher dyes, ending the influence of the quencher and enabling the reporter dye to fluoresce (Andréasson et al. 2002; Heid et al. 1996; Orlando et al. 1998; Shipley 2006; Smith et al. 2002).

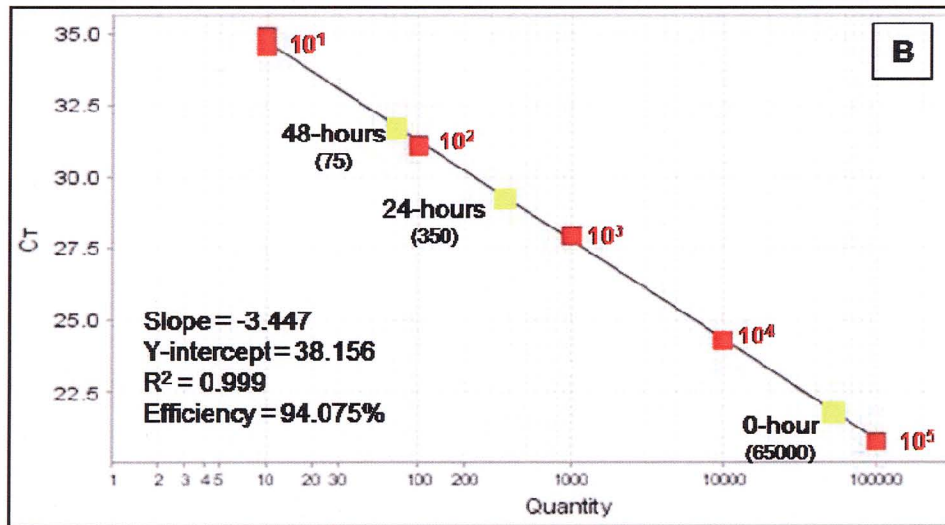
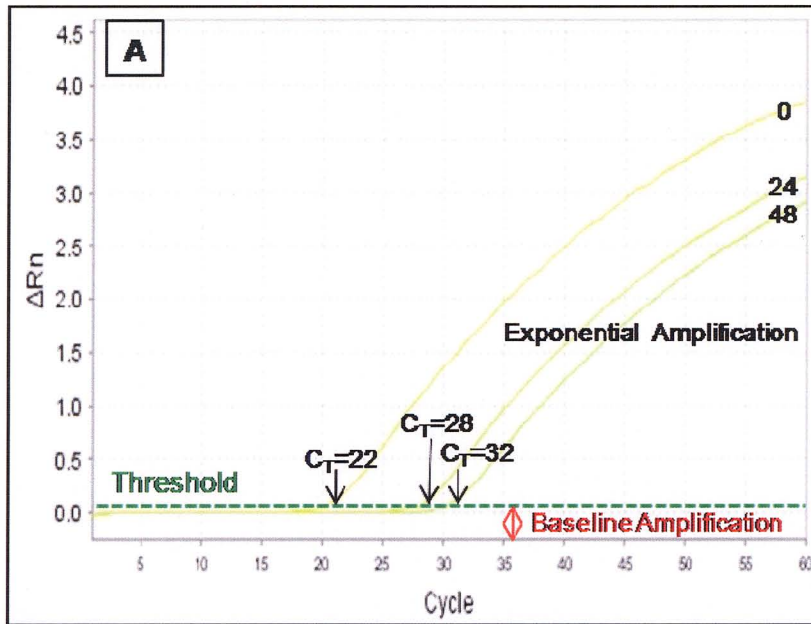


Figure 1 DNA template quantification using real-time PCR. (A) Amplification curve depicting the increase in fluorescence of experimentally degraded samples (labelled 0, 24 and 48 reflecting the number of hours heated) over increasing cycles, using a TaqMan[®] hydrolysis probe. The y-axis indicates the fluorescence of the probe (ΔRn), while the x-axis is the number of PCR cycles. (B) Standard curve quantifying the initial template copy numbers in experimentally degraded samples (shown in yellow), calculated by the real-time PCR software based on the amplification of quantified standards (shown in red). The y-axis indicates the cycle at which the fluorescence of a sample exceeds the background fluorescence or threshold value (C_T), while the x-axis shows the number of DNA templates in a given sample. Both graphs were made using StepOne[™] Software (version 2.0, Applied Biosystems).

Chapter 2: Materials & Methods

Bone Selection and Sample Preparation

Modern sheep (*Ovis aries*) ribs were used for the skeletal elements in this study. Ribs were selected for a variety of reasons, including ease of manipulation for cutting, and consistency of bone texture. Prior to conducting this experiment, the bones were defleshed and boiled for two hours to break down the tissues clinging to the exterior of the bones and the interior marrow, then dried and stored at room temperature for one year. Any desiccated tissue that remained adhering to the bone was scraped off using a scalpel. The ribs were then reduced to fragments (see Figure 2A), using either a hacksaw or pliers. Any visible pieces of dried marrow were removed from the bone, and the proximal and distal ends of each rib were discarded due to high marrow content and adherent cartilage. The resulting rib fragments were then divided between impactor tubes and powdered (see Figure 2B) in a liquid nitrogen grinding mill. The resulting bone powder was mixed together so that inconsistencies in the composition of the bones would not skew the results of any single sample. The bone powder was stored at room temperature (18°C to 20°C) in a sealed tube.

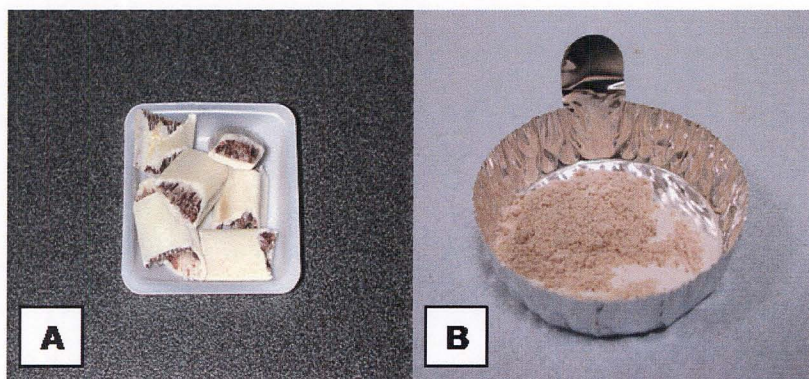


Figure 2 Sheep rib fragments (A) and bone powder (B) used in the experimental degradation. (A) Ribs were first reduced to pieces using either a hacksaw or pliers. The pieces were then broken down further to remove as much of the dried marrow as possible. (B) The rib fragments were then powdered in a liquid nitrogen mill.

Heating

Two different heating methods were used in this study. For the initial trial run, a heat block was employed to heat the bone powder. Later heating was performed using a standard laboratory oven/incubator (Barstead/Thermolyne, Type 19200, Thermo Scientific, Dubuque, Iowa, USA). An initial heating temperature of 70°C was selected. This temperature was chosen as it was low enough to not be completely out of the realm of what may be experienced in certain environments, but high enough so that degradation of the DNA could be tracked in a reasonable amount of time. Once the pattern of degradation was determined for samples heated at 70°C, a shorter run was performed at 85°C to compare the rate of change between the two temperature regimes. This second, higher temperature experiment was performed using the oven/incubator only, as it was determined that the heat block did not provide an accurate measure of the temperature the samples actually reached.

For samples that were placed in the heat block, 1.0g of bone powder was transferred into 2.0mL capped plastic tubes (Sarstedt). One sample was removed from the heat block every 24 hours, for a total of 13 samples (0 to 312 hours of heating). For samples that were heated in the oven/incubator, 1.0g of bone powder was placed in aluminium foil weigh boats (see Figure 2B), which were sealed with aluminium foil. One sample was removed from the oven/incubator every 12 hours, for a total of 26 samples (0 to 300 hours of heating) at 70°C, and 8 samples (0 to 84 hours of heating) at 85°C. All samples were labelled and allowed to slowly cool to room temperature. Once fully cooled, samples were transferred to 2.0mL capped plastic tubes (Sarstedt) and stored at -20°C.

Contamination Control and Detection

Relative to ancient DNA studies, contamination was less of an issue in this study due to the use of modern bones. However, because of the degraded nature of the samples after exposure to the various heat treatments, particularly for those samples that were heated for a significant amount of time at higher temperatures, contamination control was an unavoidable issue. The processing and analysis of these highly degraded bone samples warranted the practice of a series of contamination controls during all phases of the experiment.

Stringent contamination controls were followed in both the Forensic DNA and Post-PCR laboratories. The labs themselves are physically separated, with independent ventilation systems. The Forensic DNA lab is subdivided into three separate labs off a

shared central room, with each room used in a designated step in the extraction process. Dedicated clothing and lab coats are worn inside the lab, and disposable gloves are changed frequently to prevent cross contamination between samples. Equipment and reagents are exposed to UV light whenever possible, to cross-link any contaminating DNA. After every use, the lab workbenches are wiped down with 100% bleach. At the commencement of this study, the Forensic DNA facility had not previously been used for DNA analysis. In the Post-PCR lab, disposable gloves and lab coats are worn over dedicated clothing. Each lab has dedicated equipment, and movement between the labs is only allowed from the Forensic DNA lab to the Post-PCR lab to prevent contaminating the Forensic DNA lab with PCR products.

Extra precautions were taken to prevent contamination from laboratory sources when preparing reagents to be used in the amplification process, such as primers, probes and master mixes. All such products were prepared and aliquoted in the Ancient DNA laboratory at Simon Fraser University to minimize the risk of contaminating the reagents prior to their use in PCR amplification. The decontamination protocols within the Ancient DNA lab are even more stringent than those in place in the Forensic DNA lab, with full Tyvek[™] suits and face masks required to be worn inside the lab. The Ancient lab is also equipped with UV lighting over all workbenches to irradiate any contaminating DNA that might be on the work surfaces. Multiple blank extraction and negative amplification controls were included along with the sheep samples to rule out the possibility of systematic contamination.

DNA Extraction

DNA extraction was performed using a modified silica-spin column technique developed by Yang et al. 1998. Once all samples had been allowed to cool to room temperature, 0.110g of bone powder was transferred to a 15mL tube containing 3mL of lysis buffer (0.5M EDTA, pH 8.0; 0.25% SDS; 1.0mg/mL proteinase K). The samples were then incubated at 50°C overnight in a rotating hybridization oven.

Following incubation, the samples were placed in a centrifuge and spun for approximately 60 minutes, or until a stable interface could be ascertained between the clear lower phase of the supernatant and the foggy upper phase of organic matter. 1.3mL of the clear lower layer of supernatant was transferred to an Amicon Ultra-4 Centrifugal Filter Device (30000 NWML, 4mL; Millipore, USA). The Amicon was then centrifuged for 40 minutes, or until the supernatant was reduced to approximately 100µL. Approximately 500µL of PB buffer (QIAGEN, Hilden, Germany) were added to each Amicon and mixed with the concentrated supernatant. The mixed solution was transferred to a QIAquick Spin Column (QIAGEN, Hilden, Germany) for purification following a modified silica-spin column method (Yang et al. 1998). The spin columns were centrifuged for 1 minute to bind the DNA to the silica membrane, and then placed in a new 2mL collection tube. Each column was washed with 500µL of PE buffer (QIAGEN), followed by an additional 300µL of PE buffer. The columns were centrifuged for 1 minute and placed in new 2mL collection tubes after each wash. The DNA was eluted with the addition of 100µL of EB (QIAGEN) to each column. The samples were incubated in a heat block at 67°C for approximately 7 minutes, or until the membrane began to drip, and centrifuged for 1 minute. A second elution was performed

following the same steps. Both the first and second elutions were then stored at -20°C for further use. Two blank controls were included in every extraction to ensure the results were not due to systematic contamination, either from reagents used or from other sources introduced during the extraction process.

Species and Sex Identification

Prior to designing and employing species-specific PCR primers and probes, the presumed sheep species designation of the bones used in the study needed to be confirmed. This was accomplished through the amplification of a sequence from the D-loop region of mtDNA, using previously designed sheep- and goat-specific primers (see Table 2). The primer set involved the use of a single forward primer which binds to both the sheep and goat fragments, with separate species-specific reverse primers, distinguishing sheep and goat based on the amplicon length, with sheep having a shorter amplicon (125bp) compared to the goat amplicon (148bp).

Table 2 Primers used in species identification of the ribs used in this study.

Primer	Sequence (5'-3')	Species	Amplicon Length
F90	CAC AGA CTT CCC ACT CCA CAA	Sheep	125bp
OA-R214	ACT CGT TTG CAT GTT TAA GAC AG		
F90	CAC AGA CTT CCC ACT CCA CAA	Goat	148bp
R238	GTG TAG GCG AGC GGT GTA AT		

The sex of the remains was identified using primers previously published by Weikard et al. (2006) for the nDNA amelogenin gene (see Table 3). The amelogenin gene is located on both the X and Y chromosomes, however there is a deletion on the Y chromosome which results in a shorter amplicon. For females, only one band of approximately 262bp will be produced, while for males, two bands are produced, one of 262bp from the X chromosome, and a shorter 220bp fragment from the Y chromosome. The sex of the remains was of interest for the later development of a sequence specific amelogenin probe for use in real-time PCR, so that nDNA and mtDNA quantification could be compared.

Primer/Probe and Artificial DNA Design

Primer and Probe Design

Primers specific to the D-loop region of sheep mtDNA were designed for standard PCR so that the quality of DNA could be determined using various amplicon lengths (see Table 3). A set of primers specific to the amelogenin gene of nDNA in sheep was also used (see Table 3), both for sex determination of the remains being used in this study, and also as a comparison for amplification rates between nDNA and mtDNA fragments of similar length.

Table 3 Primers used for PCR amplification.

Primer	Sequence (5' to 3')	Region	Amplicon Length
F90	CAC AGA CTT CCC ACT CCA CAA	D-loop	125bp
OA-R214	ACT CGT TTG CAT GTT TAA GAC AG	D-loop	
OA-rt-F582*	CCA TTC TAG TCA ACA TGC GTA TCC	D-loop	200bp
OA-rt-R781*	GGG AAA GAG TGG GCG ATT TT	D-loop	
OA-F624	CAC GAG CTT GTT CAC CAT GC	D-loop	315, 460 or 556bp
OA-R938	CAG CTA CAA TTC ATG CTC CG	D-loop	315bp (with F624)
OA-R1084	GCG TGT TAA AAA TGG TGA TAA ATA T	D-loop	460bp (with F624)
OA-R1180	TAT GCG TTA TGT ATG TGA CCC AG	D-loop	556bp (with F624)
Amel-F-Bovi*	CAG CCA AAC CTC CCT CTG C	nDNA	262bp (female)
Amel-R-Bovi*	CCG CTT GGC TTG TCT GTT GC	nDNA	220&262bp (male)

NOTE: Primers with an asterisk (*) beside them were used for both standard and real-time PCR.

A large number of other primers were initially designed to be used with the forward primer F90, so that larger fragments from within the same region could be amplified using different reverse primers. However, when PCR amplification was attempted using these primers, a large number of stutter bands of various lengths appeared to have amplified as well (see Figure 3). After numerous attempts to rectify the problem through adjusting PCR conditions, it was discovered that the region amplified by the reverse primers contained multiple sets of highly repetitive sequences. This meant that the reverse primers were annealing to multiple locations instead of just one, causing fragments of varying lengths to be amplified simultaneously. Subsequently, a less repetitive region of the D-loop was located and new primers (those listed in Table 3) were designed so that only the desired fragments would be amplified.

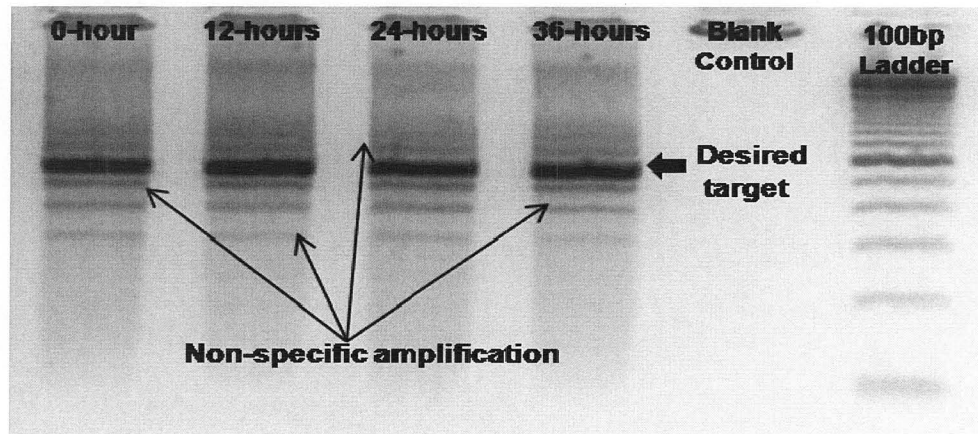


Figure 3 Non-specific amplification of multiple mtDNA fragments using a single primer set. Well labels indicate the amount of time experimentally degraded samples were heated. The primer set used (F90/R589) targeted a 500bp fragment in the D-loop. The reverse primer contained a highly repetitive sequence with multiple annealing locations, as indicated by the numerous bands of non-specific amplification of fragments of various lengths.

A set of primers and a fluorescent probe (see Table 4) specific to a 200bp fragment of the sheep D-loop region were designed specifically for use in the real-time PCR system. This primer set was also used in standard PCR (see Table 3). Similarly, a fluorescent probe (see Table 4) for use in real-time PCR was designed to be used with the amelogenin specific primers used in standard PCR. Both the primers and probe for the D-loop, and the probe for the amelogenin gene were designed using Primer Express 3.0 (Applied Biosystems). The amelogenin gene primers were taken from Weikard et al. (2006). All other primers were designed manually using sheep mtDNA reference sequences from GenBank (accession numbers: AY829418, DQ903281, DQ903286, DQ903292, DQ903301, DQ903302, DQ903303, EF490493). All primers and probes were manufactured by Integrated DNA Technologies[®] (Coralville, Iowa, USA).

Table 4 Fluorescent hydrolysis probes used in real-time PCR amplification.

Probe	Sequence (5'-3')	Region	5' Label	Primer Set
P-OA-638	CCG CGT GAA ACC AAC AAC	D-loop (mtDNA)	56-FAM	OA-rt-F582 and OA-rt-R781
P-Amel	CCA GCA GCC CTT CCA GCC CCA	amelogenin gene (nDNA)	56-FAM	Amel-F-Bovi and Amel-R-Bovi

NOTE: Label refers to the type of fluorescent molecule attached to the 5' end of the probe. Both probes also contained Iowa Black® fluorescence quencher molecules at the 3' end.

Artificial DNA Design for Quantitative Real-Time PCR

A fragment of artificial sheep DNA, 200bp in length, was designed as an exact copy of the D-loop region that was amplified by the real-time PCR mtDNA primers and probe (see Figure 4). The artificial DNA was manufactured by Integrated DNA Technologies® (Coralville, Iowa, USA). The initial concentration of the artificial DNA was measured using spectrophotometry, and converted into the number of molecules present. The concentration of the initial stock of artificial DNA was 14.7ng/μL, or 11.44nM. The desired concentration for simplicity of calculations during dilution was 10nM, so 12.59μL of ultrapure H₂O were added to 87.42μL of the artificial DNA to achieve this concentration. 10nM was converted to 0.01pmol/μL and the number of molecules per μL was calculated to be 6.022x10⁹, using Avogadro's number (equal to 6.022x10¹¹ molecules). A 10X dilution series was prepared by combining 10μL of the artificial DNA with 90μL of ultrapure H₂O. Subsequent dilutions were made by combining 10μL from the previously produced dilution with 90μL of ultrapure H₂O, until reaching 1 molecule per μL.

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      10      20      30      40      50      60      70      80      90     100
Sheep Ref. CCATTCTAGT CAACATGCGT ATCCTGTCCA TTAGATCACG AGCTTGTTCA CCATGCCGCG TGAAACCAAC AACCCGCTCA GCAAGGATCC CTCTTCTCGC
Exp. Sheep CCATTCTAGT CAACATGCGT ATCCTGTCCA TTAGATCACG AGCTTGTTCA CCATGCCGCG TGAAACCAAC AACCCGCTCA GCAAGGATCC CTCTTCTCGC
OA-rt-F582 CCATTCTAGT CAACATGCGT ATCC-----
OA-rt-R781 -----
P-OA-638 -----
Art.DNA CCATTCTAGT CAACATGCGT ATCCTGTCCA TTAGATCACG AGCTTGTTCA -----CCGCG TGAAACCAAC AAC-----

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.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|
      110     120     130     140     150     160     170     180     190     200
Sheep Ref. TCCGGGCCCA CTAACTGTGG GGGTAACTAT TTAATGAACT TTAACAGGCA TCTGGTTCTT TCTTCAGGGC CATCTCATCT AAAATCGCCC ACTCTTTCCC
Exp. Sheep TCCGGGCCCA CTAACTGTGG GGGTAACTAT TTAATGAACT TTAACAGGCA TCTGGTTCTT TCTTCAGGGC CATCTCATCT AAAATCGCCC ACTCTTTCCC
OA-rt-F582 -----
OA-rt-R781 -----
P-OA-638 -----
Art.DNA TCCGGGCCCA CTAACTGTGG GGGTAACTAT TTAATGAACT TTAACAGGCA TCTGGTTCTT TCTTCAGGGC CATCTCATCT AAAATCGCCC ACTCTTTCCC

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Figure 4 Sequence alignment indicating location and sequences of primers (OA-rt-F582 and OA-rt-R781), probe (P-OA-638) and artificial sheep mtDNA (Art.DNA) used in real-time PCR and the sheep mtDNA sequences used in the design process. Multiple sheep reference sequences were used in the actual development of the artificial DNA, primers and probe; the reference sequence shown was taken from NCBI GenBank, accession number NC_001941. The sequence labelled 'Exp. Sheep' refers to the sequence of the sheep bones examined in this study.

Two separate sets of the dilutions were initially prepared and amplified together to check for consistency between dilutions. The dilutions were then used as known-concentration standards for quantification purposes during real-time PCR. Although the standards were developed using the mtDNA sequence, they were also used in the quantification of nDNA.

DNA Amplification

Both standard PCR and real-time PCR reactions were utilized in this study. Standard PCR only allows for end-point analysis of amplified DNA, while real-time PCR allows for the collection of data during the exponential phase of amplification, enabling more accurate quantification of the initial amount of DNA in a sample through the use of fluorescent dyes. Standard PCR was used to examine maximum fragment length amplification as a proxy for DNA quality, while real-time PCR was used for DNA template quantification.

The PCR cycling conditions employed were the same for both standard PCR and real-time PCR. For amplification of mtDNA D-loop fragments, PCR conditions began with an initial denaturing period for 10 minutes at 95°C, followed by 60 cycles of a denaturing period of 30 seconds at 95°C, an annealing period of 30 seconds at 55°C, and an extension period of 40 seconds at 72°C. All reactions ended with a single final annealing stage of 10 minutes at 72°C after the last cycle. At the completion of the 60 cycles, the samples were held at 4°C until being stored at -20°C. For amplification of the amelogenin fragment of nDNA, similar PCR conditions were applied, however the

annealing temperature was increased to 60°C. At lower annealing temperatures, multiple bands (other than the desired 262bp band) were amplified when the amelogenin amplicons were visualized, likely due to repetitive regions.

After numerous tests, it was determined that mtDNA was amplified best when sample extracts were diluted 2X with ultrapure H₂O (see Figure 5A), possibly due to the presence of inhibitory substances, therefore all reported PCR analyses of mtDNA were performed using a 2X dilution of each DNA sample. This was not the case for nDNA however, as diluting the samples was found to have the opposite effect, reducing amplification success (see Figure 5B). Therefore the reported nDNA data were performed using undiluted sample extracts. For every PCR set up, both standard and real-time systems, at least one negative amplification control, containing only the reagents used in the reaction and no sample DNA, was included in the reaction. Blank extraction controls were included in the initial few reactions after an extraction, and at least once with every primer set, however they were not included in further analysis once they were determined to be clean. Any results that indicated amplification within the blank or negative controls were disregarded and the reactions were entirely redone.

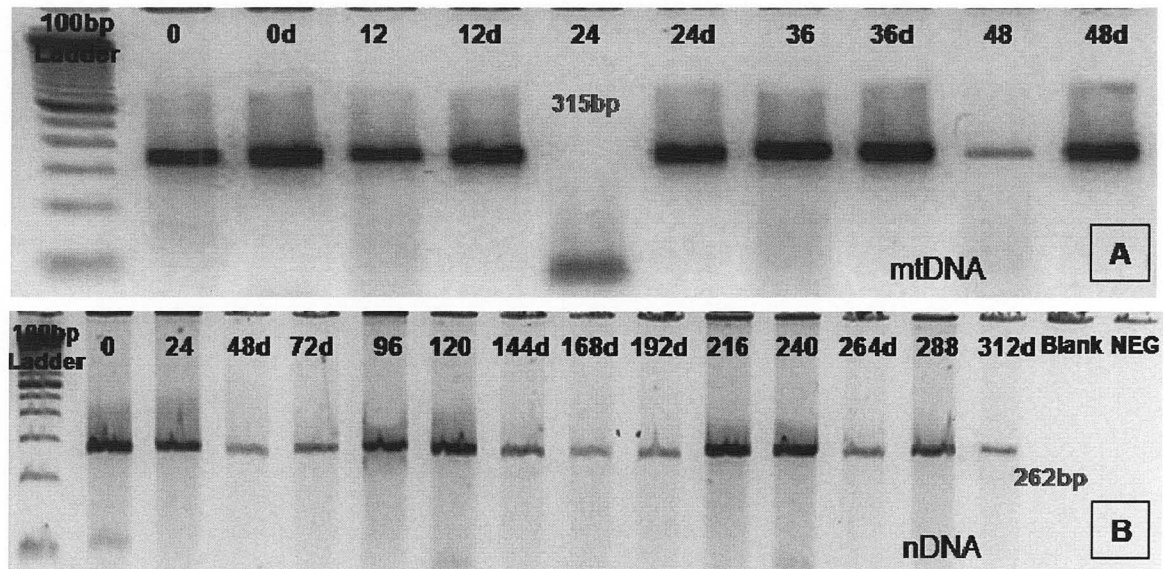


Figure 5 Electrophoresis gels depicting amplification success of diluted (d) versus undiluted samples for both mitochondrial (A) and nuclear (B) DNA. Amplification of mtDNA was improved using a 2X dilution of extracts, however a reduction in amplification success was seen in nDNA amplification of diluted samples. Numbers labelling wells indicate the amount of time experimentally degraded samples were heated. Samples labelled with a “d” were diluted.

Standard PCR

PCR amplification was performed using an Eppendorf Mastercycler Personal (Hamburg, Germany). PCRs were set-up using a previously prepared pre-mix solution containing 1.5X buffer (Applied Biosystems), 1mM MgCl₂, 0.2mM dNTP, 1.0mg/mL Bovine Serum Albumin (BSA). At the time of PCR set-up, 0.3μM of both forward and reverse primers, and 1U of AmpliTaq Gold for every 30μL PCR reaction volume, were added to the pre-mix to create a master mix solution. For every sample, 3μL of DNA were added to the 27μL of master mix.

Amplified samples were then visualized through gel electrophoresis on a 2% agarose gel, by combining the amplified DNA samples with SYBR[®] Green Nucleic Acid Gel Stain (Invitrogen, California, USA), to confirm both successful amplification and the

correct fragment length. Samples were only considered successful if they could be consistently reproduced.

Real-Time PCR

Real-time PCR was performed using a StepOne™ Real-Time PCR System (Applied Biosystems). Real-time PCRs were set up using a 1X concentration of TaqMan® Universal Master Mix (Applied Biosystems). At the time of preparation, 0.3µM of both forward and reverse primers and 0.25µM of probe were added to each 20µL sample reaction volume, along with 2µL of the sample DNA.

Prior to using the 10X dilution series of artificial sheep mtDNA as known-concentration quantification standards, possible differences in amplification efficiencies between the artificial sheep and heat-treated sheep DNA samples were assessed by spiking the artificial samples with either 1µl of ancient goat or whale DNA. The artificial DNA samples were spiked with real DNA so that any other materials that may have been extracted along with the real DNA that could potentially inhibit or decrease the amplification efficiency would be accounted for in the artificial standards. The amplification efficiency was assessed based on whether or not the spiked artificial DNA samples crossed the amplification threshold (C_T) at the same cycles as the non-spiked artificial samples. Once it was determined that the efficiency of the spiked samples did not differ from the un-spiked samples, the artificial DNA dilutions were used as quantification standards.

Quantification standards were included in all reactions in replicates of either two or three. Similarly, all samples were run in duplicate or triplicate so that standard

deviations and average quantities could be calculated, and outliers removed from further analysis. Samples were considered successful if all replicates amplified within the same C_T range and were reproducible. Only real-time PCRs with an R^2 value of at least 0.95 were used in further analyses.

Quantification of the unknown samples was accomplished through comparison of the level of fluorescence of the known-concentration artificial DNA standards and the fluorescence of unknown samples. The fluorescence data were converted into numerical quantities and plotted onto a standard curve using the StepOne™ Software (version 2.0, Applied Biosystems). The sample quantities were then imported into Microsoft Excel 2007 and graphed to visually compare the degradation rate of samples within and between different heat treatments.

DNA Sequencing

Purified PCR products were sent for sequencing at Macrogen (Seoul, Korea). Returned sequences were manually edited in Chromas Lite (version 2.01, Technelysium Pty Ltd., 2005), and a BLAST search was performed through GenBank to determine correct sex and species identification. Returned sequences were aligned using BioEdit Sequence Alignment Editor (version 7.0.9.0, Hall, 2007) and compared to other published sequences on GenBank. The resulting sequences were used for later primer, probe and artificial DNA design.

Determining the Actual Temperature of Samples in the Heat Block

After it was discovered that the level of degradation seen in samples heated in the heat block set at 70°C was not equivalent to the degradation of samples heated in the oven set at 70°C, the actual internal temperature of the samples in the heat block was determined by the method described below. Excess bone powder from each previously heated sample, which was not used up in the extraction process, was stored in the same 2.0mL capped plastic tubes (Sarstedt) in which they had originally been heated. Four of these samples were randomly selected, each containing only slightly less bone powder (less approximately 0.110g as this was the amount used in the extraction process) than when originally heated. The caps of the 2mL tubes were removed and the tubes were then sealed with Parafilm™ so that the stem of the thermometer could be inserted into the bone powder without much manoeuvring. The heat block was set to 70°C, and the four samples were placed sporadically in the wells of the heat block. The internal temperature of the bone powder in each tube was measured using a digital long-stem thermometer inserted into the middle of the bone powder. The temperature was measured for each sample at 24, 48 and 72 hours after being placed in the heat block, and the average temperature over all four samples was calculated. After each temperature measure, the tubes were resealed with Parafilm™.

Comparison of mtDNA and nDNA Degradation Rates

The rate of degradation of both mitochondrial and nuclear DNA within a particular heat treatment was assessed by converting the average template number per sample into percentages, with the 0-hour sample representing 100%. The percentage of

DNA remaining from the initial 100% was determined for every sample, as was the change in DNA amounts between subsequent samples (i.e. from 24 to 36 hours, 36 to 48 hours, and so on). The differences between the change in percentage between mtDNA and nDNA samples were assessed to determine what, if any, differences were observed in the rate of degradation between the two types of DNA. The rates between samples from different heat treatments were also compared to help elucidate the impact of increasing temperature of DNA degradation.

Comparison of experimentally degraded DNA with ancient DNA

A sample of sheep ancient DNA from a previous study was also incorporated into this experiment, to assess the range within which true aDNA might fall in relation to the degradation curves produced. This was done to determine whether it would be possible to actually replicate the damage seen in true aDNA through degrading modern DNA. The ancient sheep sample was only analysed for DNA quantity using real-time PCR, and not DNA quality.

Chapter 3: Results

The results indicate a consistently rapid rate of degradation in both quantity and quality within first few hours/days of heating, followed by a more gradual, plateau-like and inconsistent degradation rate over longer periods of time, particularly for the higher temperature heat treatments. In total, over 170 amplifications were performed, including both standard and real-time PCR. All reported amplification results include only those reactions in which no amplification was observed in all blank and negative controls, unless otherwise stated.

Contamination Detection

Even with the contamination control measures employed, contamination was detected in some samples, although it was sporadic in nature, suggesting isolated events as opposed to systematic contamination. During the preliminary stages of the study, early extractions frequently contained blank controls with positive amplification, indicative of contamination. As modern sheep bones were used, it was not expected that contamination would be such a problem. More stringent decontamination procedures similar to those used in true ancient DNA studies were employed in later extractions, rectifying the problem of positively amplified blank controls and providing a much sounder basis for authentication.

Contamination of negative amplification controls was much less of a problem compared to that of the blank extraction controls, suggested that the contamination occurred during the sample prep or extraction phases. In the rare cases when negative amplification controls were positively amplified, the results were discarded and the PCR was set up and run again, usually with multiple negative controls. Positively amplified negative controls never occurred in consecutive PCR runs, even if the separate PCRs had been set-up together.

As a result, all sample sets in which the blank extraction controls returned positive amplifications were discarded. With the realization that the degraded samples were much more like real ancient DNA than anticipated, the implementation of more rigorous contamination controls was effective in remedying the issue. Similarly, all PCR results with positively amplified negative controls were discarded. The sporadic nature of the false positive negative controls suggests that the contamination was an isolated and not systematic event.

Species and Sex Identification

DNA sequences from the bones used in this study were compared to previously extracted ancient sheep and goat samples (both approximately 200 years old) from an unrelated study, to verify the presumed species identification. Both mtDNA (see Figure 6) and nDNA (see Figure 7) sequences confirmed that that the ribs were indeed from a sheep, species *Ovis aries*. Analysis of the nDNA amelogenin gene also determined that the remains were of a female sheep (see Figure 7).

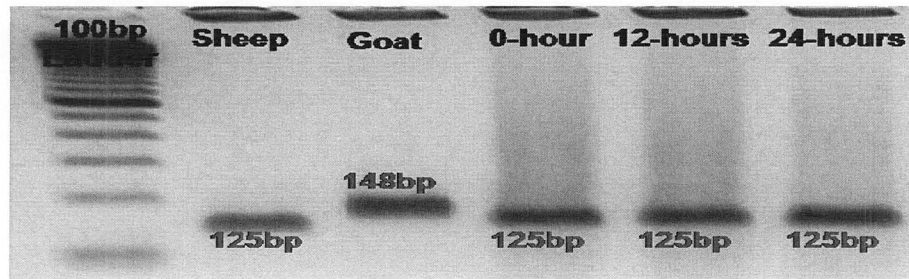


Figure 6 Species determination of ribs used in the experimental degradation. Wells labelled “Sheep” and “Goat” contained previously identified ancient samples, while wells labelled with times contained the experimentally degraded DNA in question.

Variation between Heat Sources

After highly successful amplification of experimentally degraded DNA from the samples that had been heated at 70°C in the heat block for 312 hours, a standard laboratory oven/incubator was used so that a larger number of samples could be simultaneously heated. The exposure period for the first set of samples heated in the oven was increased to 1440 hours in total, well beyond that of the previous 312 hours due to the unexpectedly high success from the heat block samples. After the initial extraction of all the oven-heated samples, 0 to 1440 hours, amplification consistently failed to reach the level of success observed in the previous extractions of heat-block samples.

To test if these samples were inhibited in a way that the previous samples had not been, a selection of the oven-heated samples were spiked with 1 µL of ancient goat DNA (200 years old) from an unrelated study and amplified using the primer sets employed in species determination (see Table 2). As both the goat and sheep specific sequences were successfully amplified (see Figure 8), it was determined that the level of inhibition was not causing the amplification failure. A second extraction was the performed on a limited number of samples to determine if the amplification failure was due to an overall failure

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.....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          10          20          30          40          50          60          70          80          90          100
Sheep X Ref CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC CCCAGTCCAT CCAGCCGCAG CCTCACCAGC CCCTGCAGCC CCTGCAGCCC ATGCAGCCCT
Sheep Y Ref CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCA-- ----- ---GCCACAG CCTCACCAAC CCCTACAGCC CC-----
Amel-F-Bovi CAGCCAAACC TCCCTCTGC- ----- ----- ----- ----- ----- ----- ----- -----
Amel-R-Bovi ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
Exp. Sheep1 ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
Exp. Sheep2 CAGCCAAACC TCCCTCTGCC CGCCCAGCAG CCCTTCCAGC CCCAGTCCAT CCAGCCGCAG CCTCACCAGC CCCTGCAGCC CCTGCAGCCC ATGCAGCCCT

.....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....| .....|.....|
          110         120         130         140         150         160         170         180         190         200
Sheep X Ref TGCAGCCCTT GCAGCCCCTG CAGCCCCAGC CACCCGTGCA CCCCATCCAG CCCTTGCCGC CACAGCCACC TCTGCCTCCG ATATTCCCCA TGCAGCCTCT
Sheep Y Ref ----- ----- -----AGC CACCTGTGCA CCCCATCCAG CGCTTGCCAC CACAGCCACC TCTGCCTTCA ATATTCCCCA TGC AACCGCT
Amel-F-Bovi ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
Amel-R-Bovi ----- ----- ----- ----- ----- ----- ----- ----- ----- -----
Exp. Sheep1 ----- ---GCCCTG CAGCCCCAGC CACCCGTGCA CCCCATCCAG CCCTTGCCGC CACAGCCACC TCTGCCTCCG ATATTCCCCA TGCAGCCTCT
Exp. Sheep2 TGCAGCCCTT GCAGCCCCTG CAGCCCCAGC CACCCGTGCA CCCCATCCAG CCCTTGCCGC GACAGCCACC TCTGCCTCCG ATATTCCCCA TGCAGCCTCT

.....|.....| .....|.....| .....|.....| .....|.....| .....|.....| ..
          210         220         230         240         250         260
Sheep X Ref GCCCCCCATG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA GCAACAGACA AGACCAAGCG GG
Sheep Y Ref GCCCCCTGTG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA GCAACAGACA AGACCAAGCG GG
AMEL-F-Bovi ----- ----- ----- ----- ----- ----- -----
AMEL-R-Bovi ----- ----- ----- ----- ----- GCAACAGACA AG-CCAAGCG GG
Exp. Sheep1 GCCCCCCATG CTTCCTGACC TGCCTCTGGA AGCTTGGCCA GCAACAGACA AG-CCAAGCG GT
Exp. Sheep2 GCCCCCCATG CT-CCTGAC- ----- ----- ----- ----- -----

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Figure 7 Amelogenin sequence of experimentally degraded sheep samples with both sheep X (Sheep X Ref) and Y (Sheep Y Ref) chromosome reference sequences (GenBank accession numbers DQ469591 and DQ469592, respectively). The experimental sheep samples included were sequenced using both forward and reverse primers to check for sequence continuity. Exp. Sheep1 was sequenced with the forward primer (Amel-F-Bovi), and Exp. Sheep2 was sequenced with the reverse primer (Amel-R-Bovi).

of the extraction process, even though the early-hour samples were successful. When the second extraction samples proved to be equally unamplifiable, it was determined that the samples must have been more damaged in the oven/incubator, possibly due to temperature variation between the two heat sources.

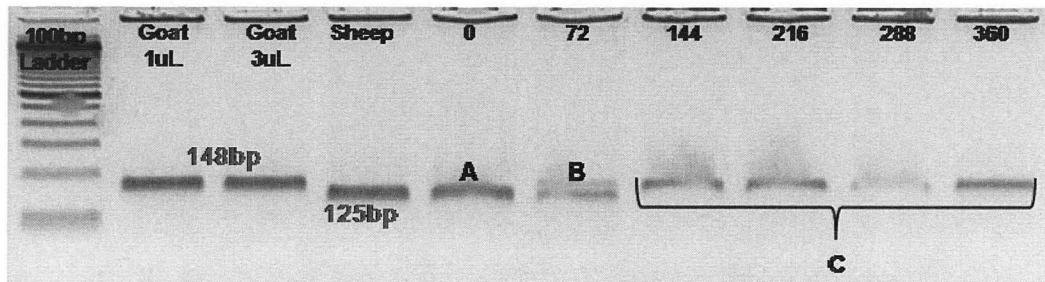


Figure 8 Electrophoresis gel indicating increased DNA degradation was the reason for amplification failure of the initial 70°C oven-heated samples, not inhibition. Experimentally degraded sheep samples (wells labelled with number of hours samples were heated for) were spiked with 1uL of ancient goat DNA to determine if the failure was due to PCR inhibiting substances. Wells labelled with “Goat” and “Sheep” contained positive controls of the specified species. Both band A (amplification of the experimental sheep DNA only) and B (co-amplification of both the experimental sheep and ancient goat DNA) suggest that inhibition was not an issue; while band C (amplification of goat DNA only) indicates significant degradation of the experimental sheep DNA after 144 hours.

The actual internal temperature of the bone powder samples that were heated in the heat block was approximately 50°C (see Table 5); 20°C lower than what the heat block was set to. Only at the very bottom tip of the tube, where the tube was in contact with the surface of the well of the heat block, did the bone powder reach a temperature that even approached the expected 70°C. This explains why such a difference was seen in the level of degradation between samples heated at the same temperature for equal periods of time but in different heat sources. Once it was determined that the samples in the heat block were only reaching an average temperature of 50°C, the samples were

quantified in subsequent analyses along with those heated to 70°C and 85°C in the oven as if they had been intentionally heated in the oven at 50°C.

Table 5 Actual temperature reached by bone powder heated in a 70°C heat block.

Time	Tube 1	Tube 2	Tube 3	Tube 4	Average
24 hours	46°C	48°C	50°C	47°C	48°C
48 hours	48°C	47°C	48°C	49°C	48°C
72 hours	47°C	50°C	49°C	48°C	49°C

NOTE: Temperature readings were taken from the middle of the bone powder in the tube.

Quality Assessment through Standard PCR Amplification

For samples to be considered successfully amplified, the results had to be reproducible in multiple, separate reactions. Samples that amplified inconsistently between reactions were not considered in this or further analysis.

To simplify the comparison of multiple gels, a single composite gel was created from multiple PCR amplifications (see Figure 9, Figure 10 and Figure 12). The gels were produced by incorporating 3µL of PCR product of a single sample from multiple reactions in which the sample was successfully amplified, together into a single tube (i.e. 3µL of each of the 0-hour samples that were successful in separate PCRs for each of the mtDNA fragment lengths – 125, 200, 315, 460 and 556bp – were combined into one 0-hour sample tube). 5µL of the mixed fragment length sample was then combined with SYBR® Green Nucleic Acid Gel Stain (Invitrogen, California, USA), and loaded into a 2% agarose gel. As the different fragment lengths for each heated sample came from separate PCRs, the intensities of the bands (which can also be used for quantification

purposes) should be ignored when looking at gels, and only the presence (indicating successful amplification) or absence (indicating no amplification) of a band should be considered. The variation in intensities is seen because each primer set, although generally used under the same amplification conditions, has rather different amplification efficiencies. It would therefore be misleading to place too much emphasis on the band intensity of different PCR amplifications. All wells are labelled with the number of hours for which a particular sample was heated. For the 70°C mtDNA composite gel, not all 26 samples are shown due to the size limitations of the gel, as the largest gel size contained only 24 wells. The samples shown change from increments of 12 hours after the 84-hour sample, to increments of 24 hours up to the 264-hour sample, followed by a final 36 hours to the final 300-hour sample. Only the composite gels are shown in the main text. For images of the individual amplification gels that refer to the reactions from which the composite gels were assembled, refer to Appendix A.

mtDNA Amplification

Degradation of mtDNA quality was assessed through the amplification of fragments of varying length, from 125bp up to 556bp, within the D-loop region. If samples were successfully amplified consistently, then the level of degradation was considered to be less severe, indicating the target DNA fragment was not completely degraded. The quality of the DNA within a given sample was assessed based on the maximum amplifiable fragment length, with the greater the fragment length the better the quality. The 125bp fragment was amplified using the primer set F90 and OA-R214; the 200bp fragment was amplified using OA-rt-F582 and OA-rt-R781. The 315bp, 460bp

and 556bp fragments were all amplified using the forward primer OA-F624, with separate reverse primers: OA-R938, OA-R1084 and OA-R1180, respectively.

Using the samples from the heat block as if they were intentionally subjected to a 50°C heat treatment, all samples from 0 to 312 hours were successfully amplified by all primer sets up to 460bp in length (see Figure 9). Only in the case of the 556bp fragment was degradation detected, with samples above 72 hours failing to amplify at this length (see Figure 9).

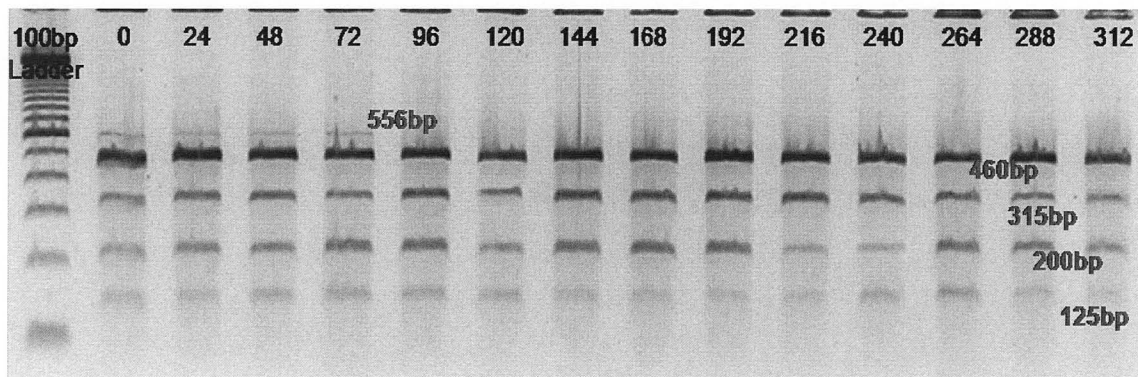


Figure 9 Electrophoresis gel of samples heated at 50°C indicating successful amplification of mtDNA fragments of different lengths for each sample.

For samples exposed to 70°C in the oven, all samples from 0 to 300 hours were successfully amplified for the 125bp fragment (see Figure 10). An earlier heat treatment that included samples heated in the 70°C oven for up to 1440 hours, indicated that this 125bp fragment of mtDNA could still be successfully amplified after 648 hours of heating (see Figure 11). However, the results from this earlier set of extractions may be somewhat suspect as the blank extraction controls were often unexpectedly amplified as well, although that was not the case for the particular PCR in question, as the blanks were both clean as can be seen in Figure 11. The amplification failure of other samples within

this same extraction set also implies that systematic contamination was not an issue. Degradation was observed beginning with the 200bp amplicon, with consistent successful amplification up to 204 hours, followed by sporadic amplification of the remaining samples, with no amplification from 288 to 300 hours. This degradation pattern continues for all larger amplicon fragments, with 300bp being successfully amplified up to 84 hours, 460bp successfully amplified up to 36 hours, and 556bp successfully amplified up to 36 hours, but with the 24 hour sample failing (see Figure 10). The failure of the 24-hour sample at 556bp while the 36-hour sample was successful, suggests that these two samples may have been mistakenly switched by human error at some point during extraction. Similarly, when examining the bands of the 460bp amplicon for both the 24 and 36-hour samples in Figure 10, the 24-hour sample appears to be weaker indicating less DNA. This further suggests that these two samples were reversed. This reversal pattern was also seen when looking at nDNA, discussed later on.

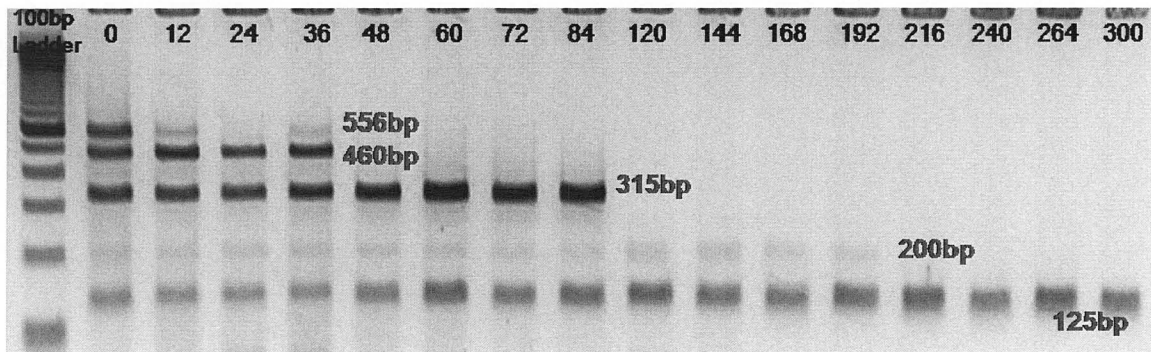


Figure 10 Electrophoresis gel of samples heated at 70°C indicating successful amplification of mtDNA fragments of different lengths for each sample.

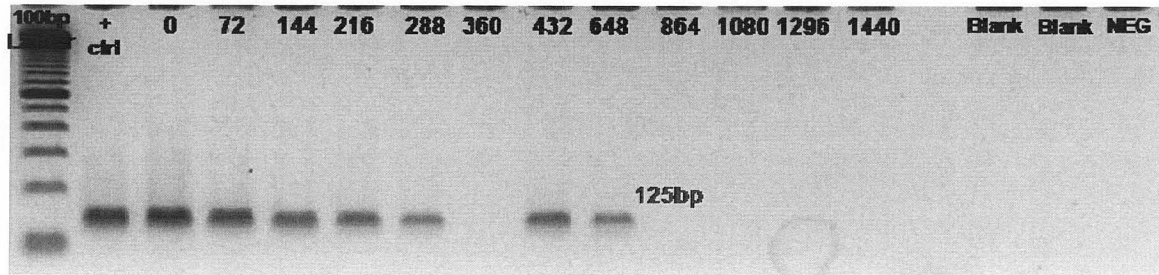


Figure 11 Electrophoresis gel of samples heated at 70°C indicating continued amplification success of the 125bp mtDNA fragment after extensive heating periods. The 360-hour sample, which shows no amplification in this gel, was never successfully amplified in any PCR, indicating extraction failure or possibly sample inhibition.

Samples exposed to the 85°C heat treatment in the oven showed an increased rate of degradation. As with the other heat treatments, the 125bp fragment was successfully amplified from all samples, 0 to 84 hours (see Figure 12). Degradation could be seen beginning with the 200bp fragment, which was successfully amplified up to 36 hours, followed by inconsistent amplification of the 48, 60 and 72 hour samples, and no amplification from the 84 hour sample (see Figure 12). The 300bp fragment could be successfully amplified up to 24 hours, while the longest fragments of 556bp and 460bp were not successfully amplified from any of the heated samples, with the exception of the 0-hour control (see Figure 12).

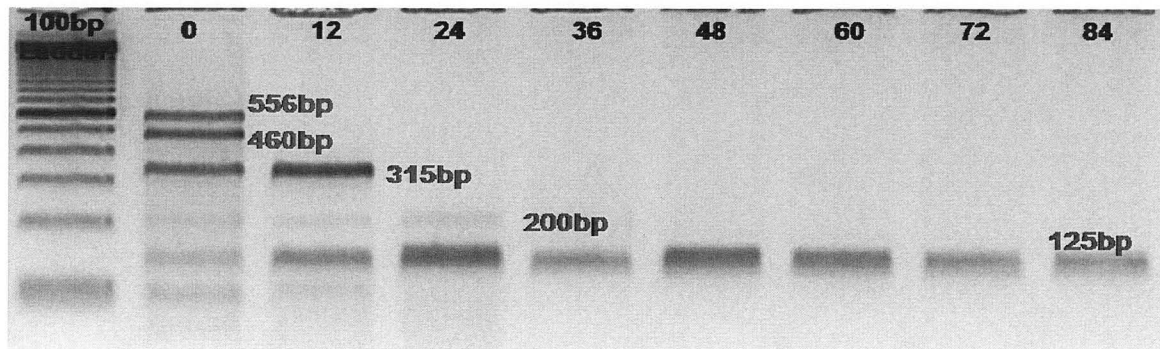


Figure 12 Electrophoresis gel of samples heated at 85°C indicating successful amplification of mtDNA fragments of different lengths for each sample.

nDNA Amplification

Degradation of nDNA was assessed through the amplification of a single 262bp fragment from the amelogenin gene. The quality of the nDNA was based solely on whether or not samples were successfully amplified consistently, with those that did so having a lesser level of degradation. This aspect of the quality assessment differed from that of the mtDNA due to the amplification of only one fragment length.

No appreciable degradation in nDNA was seen in the samples heated to 50°C in the heat-block, with the 262bp fragment successfully amplifying from all samples, 0 to 312 hours (see Figure 13A). Degradation was clearly seen in samples subjected to 70°C in the oven, with the 262bp fragment successfully amplified from 0 to 48 hours, and sporadically at 60 hours, with no successful amplification after this point (see Figure 13B). The rate of nDNA degradation was greatly increased in the samples heated to 85°C, as only the 0-hour control was successfully amplified, with the 12-hour sample only amplifying on one occasion (see Figure 13C).

No appreciable degradation in nDNA was seen in the samples heated to 50°C in the heat-block, with the 262bp fragment successfully amplifying from all samples, 0 to 312 hours (see Figure 13A). Degradation was clearly seen in samples subjected to 70°C in the oven, with the 262bp fragment successfully amplified from 0 to 48 hours, and sporadically at 60 hours, with no successful amplification after this point (see Figure 13B). The rate of nDNA degradation was greatly increased in the samples heated to 85°C, as only the 0-hour control was successfully amplified, with the 12-hour sample only amplifying on one occasion (see Figure 13C).

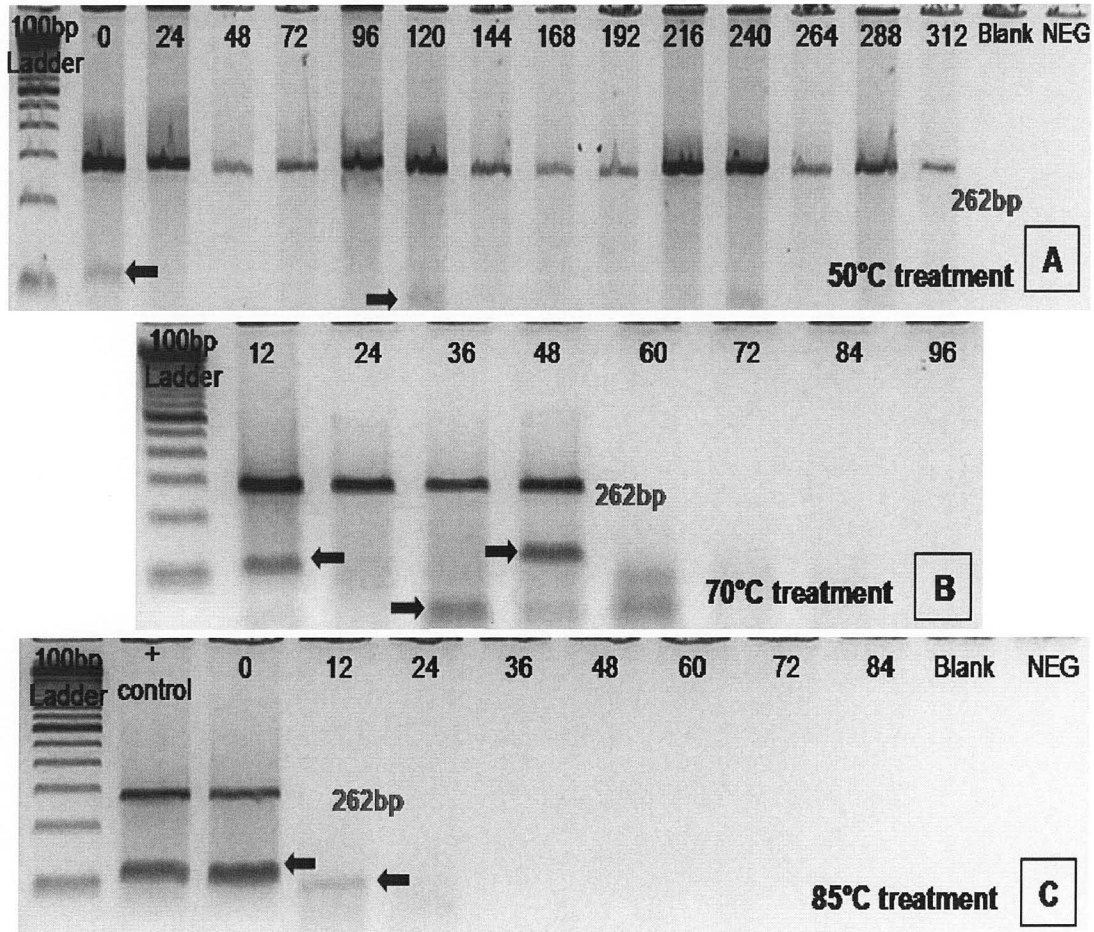


Figure 13 Electrophoresis gel indicating nDNA amplification of samples exposed to 50°C (A), 70°C (B) and 85°C (C). Well labels indicate the number of hours samples were heated. ‘Blank’ wells contained no-template extraction controls, while ‘NEG’ wells contained no-template amplification controls. Lighter bands in A were samples in a 2X dilution with ultrapure H₂O (i.e. 5µL of sample DNA combined with 5µL of ultrapure H₂O). The positive control (+ control) in C was the 70°C 0-hour sample. Arrows indicate non-specific amplification and primer-dimer formation, highlighting the compromised specificity of the primer set.

Calculation of Artificial DNA Standard Concentration

The two separate but equal 10X dilution sets were found to be consistent in their template quantifications. The artificial DNA standards showed no difference in amplification efficiency when compared to those spiked with real ancient goat or whale

DNA, although the standards did exhibit a much cleaner and more ideal amplification curve than true DNA samples (results not shown), suggestive of the presence of some inhibitory substances in biological DNA samples. Therefore, the artificial DNA was deemed sufficient to use as quantification standards for the purpose of creating standard quantification curves during real-time PCR analysis.

Quantitative Real-Time PCR Amplification

To be considered successful, all samples had to successfully amplify in triplicate with approximately the same threshold (C_T) value (within less than one whole cycle difference), and be reproducible. All amplifications that were used in further analysis had an R^2 value of at least 0.95 for the standard curves. Tables of averaged sample initial template amounts, sample cycle threshold values, and standard deviations for both mtDNA and nDNA quantification from multiple real-time PCRs can be found in Appendix B.

mtDNA Amplification

As with standard PCR, samples heated in the heat block were considered to have been heated at 50°C. All 0-hour samples for the different heat treatments had similar initial template concentrations of approximately 65000 copies (see Figure 14). At 50°C all samples, 0 to 312 hours, were successfully amplified, with an average initial template quantity of approximately 11000 for the 312-hour sample (see Figure 14). The pattern of

degradation was quite consistent with the exception of the 192-hour sample which was found to consistently have a much higher than expected template number.

For samples subjected to the 70°C heat treatment, samples from 0 to 84 hours showed a fairly consistent pattern of rapid degradation. This was followed by a period of inconsistent, plateau-like degradation from samples 96 to 276 hours, as indicated by more sporadic amplification success and template numbers hovering between 1 and 2 copies. No amplification was seen in the 288 and 300-hour samples (see Figure 14 and Figure 15). The 24 and 36 hour samples were the only ones within the first 96 hours of heating that showed a reversal in the amount of degradation, with the 36 hour sample having a higher quantity of mtDNA than the 24 hour sample (1353 copies and 570 copies, respectively). As mentioned previously when discussing DNA quality, it appears as though these two samples were mixed up by human error at some point during extraction.

A consistent pattern of rapid degradation was also observed from the 0 to 48 hour samples subjected to the 85°C heat treatment. A more inconsistent pattern was seen with the 60 and 72-hour samples, again as indicated by sporadic amplification success and a copy number of between 1 and 0. The 84-hour sample never successfully amplified, indicating total degradation at the 200bp fragment length (see Figure 14 and Figure 15).

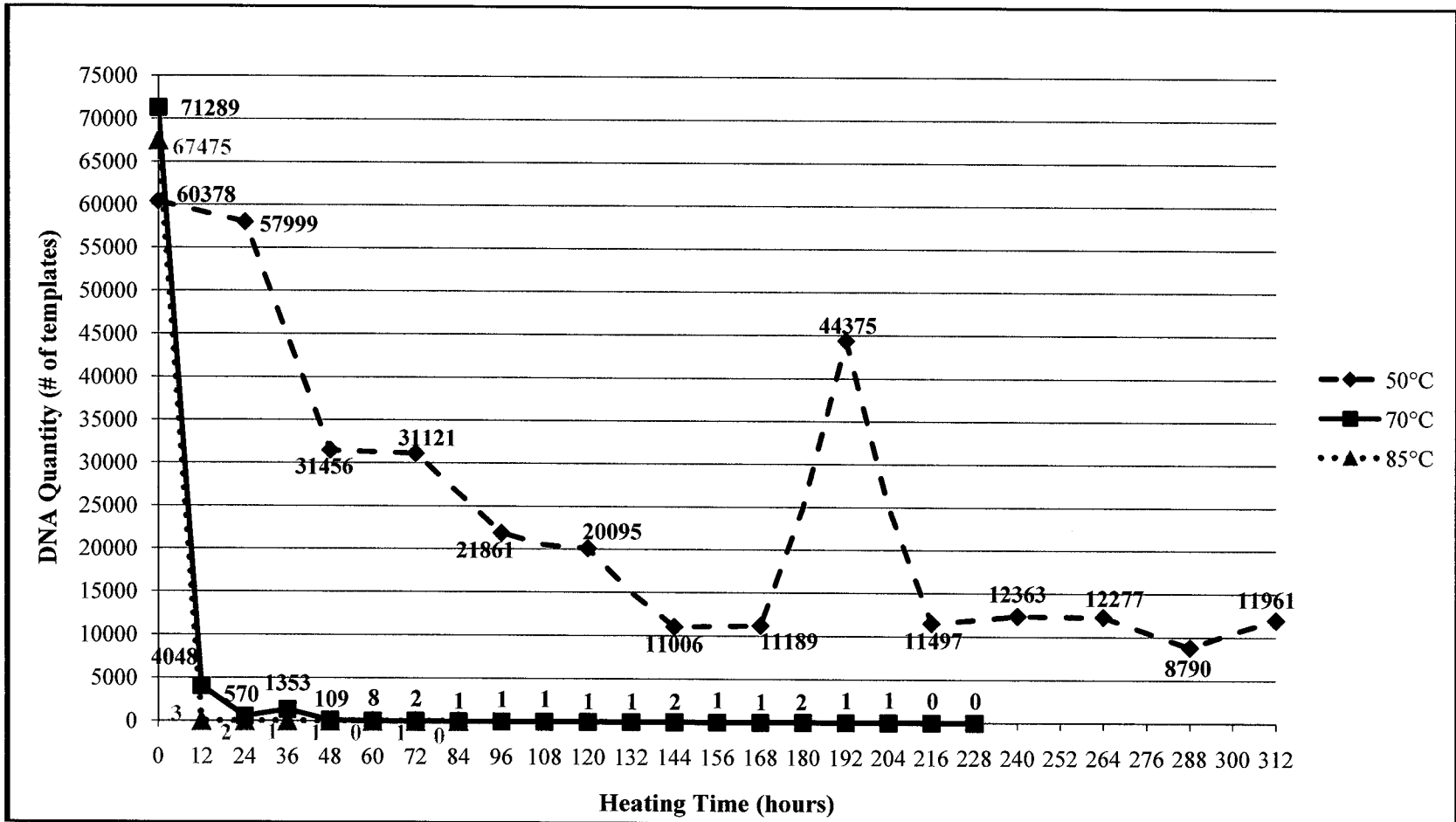


Figure 14 mtDNA degradation of all samples heated at 50°C, 70°C, and 85°C. All template copy numbers were determined by real-time PCR. Once samples for a particular heat treatment consistently reached a copy number of 0, the points were no longer plotted on the graph.

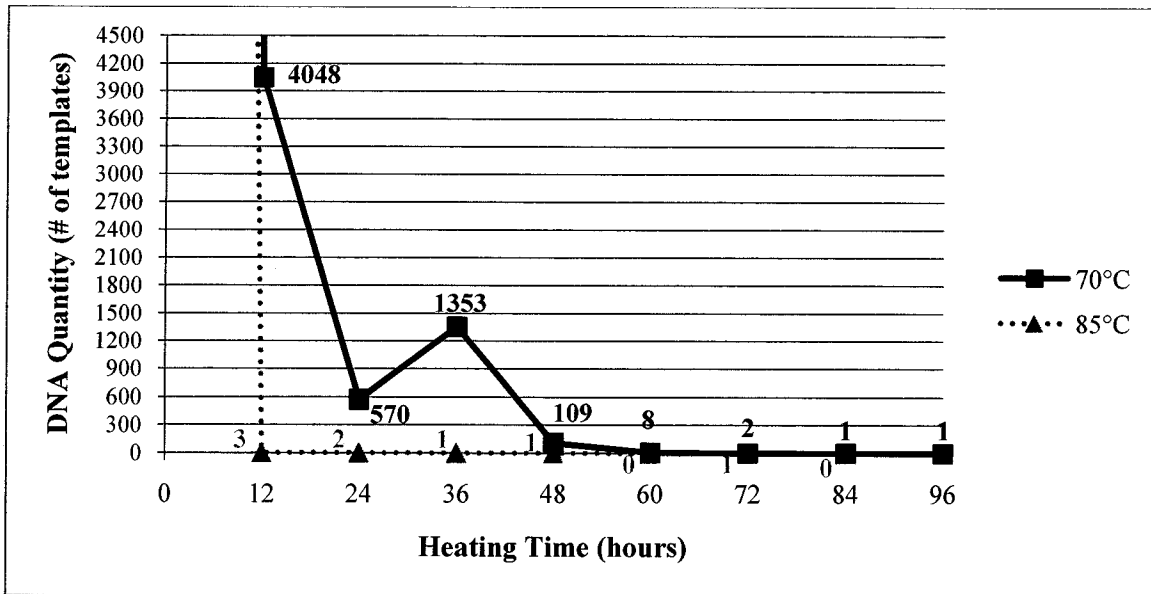


Figure 15 mtDNA degradation of 70°C and 85°C heated samples from 0 to 96 hours of heating. This graph is a zoomed in version of that shown in Figure 14, allowing for a better view of the degradation pattern of the 70°C and 85°C samples.

nDNA Amplification

nDNA was amplified using the same 262bp amelogenin primer set that was employed in standard PCR. For quantification purposes, the previously discussed 200bp artificial sheep mtDNA was used for the known concentration standards. The implications of this will be further discussed in Chapter 4. All 0-hour samples for each heat treatment had a similar initial template concentration of approximately 1300 copies (see Figure 16A). Successful amplification results were the same as discussed for the standard PCR results.

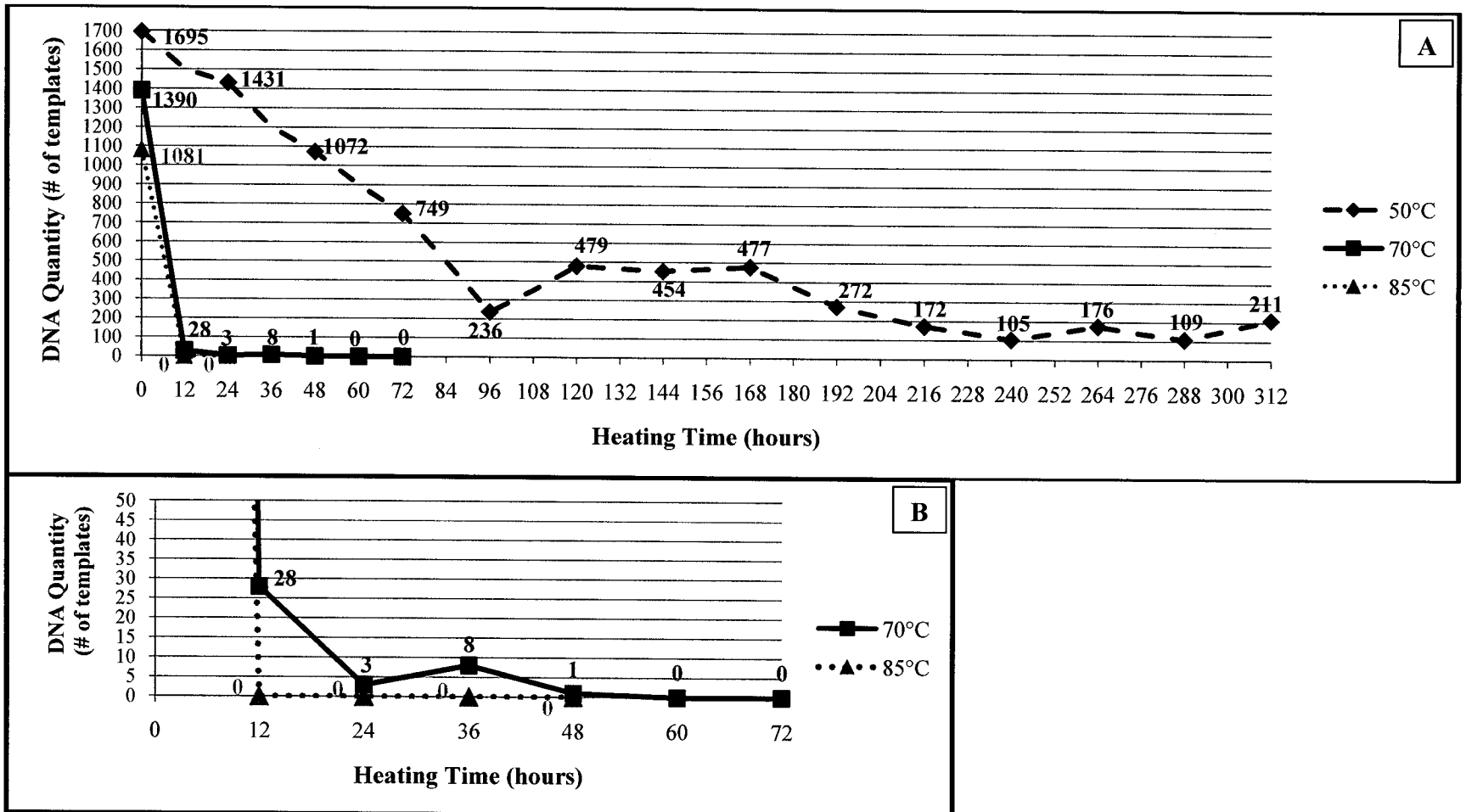


Figure 16 nDNA degradation of samples heated at 50°, 70° and 85°C. All samples for each heat treatment are shown in A. Once samples for a particular heat treatment consistently reached a copy number of 0, the points were no longer plotted on the graph. A close up of the degradation for 0 to 72-hour samples heated at 70°C and 85°C is shown in B.

For the 50°C heat treatment, all samples were successfully amplified, from 0 to 312 hours, with a fairly consistent degradation pattern seen in the reduction of initial template numbers (see Figure 16A and B). Samples 0 to 48 hours were successfully amplified from the 70°C heating regime, with a rapid initial degradation from 0 to 12 hours, followed by a more gradual degradation up to 48 hours, after which no samples successfully amplified, indicating a copy number of 0 at the 262bp length (see Figure 16A and B). As discussed in the results for standard PCR, at 85°C, no samples successfully amplified, with the exception of the 0-hour control, indicating complete degradation of the 262bp fragment length within 12 hours (see Figure 16B).

Comparison of mtDNA and nDNA Degradation

Average initial template copy numbers from multiple real-time PCRs were calculated for all samples and converted into percentages, with the 0-hour samples calculated as 100% for their respective heat treatments. The change in DNA quantity between consecutive samples was also calculated to determine if the rate of degradation changed over time.

50°C Heat Treatment

After 48 hours at 50°C, approximately 50% of the initial mtDNA copy number of 60378 was lost, after which point initial copy number was reduced by approximately 20% every 48 hours, up to 144 hours of heating (see Table 6). All samples from 144 hours and beyond hovered sporadically with only 15% to 20% of the initial mtDNA copy number remaining (see Table 6). The 192-hour sample was the one sample that fell outside of

this degradation pattern consistently quantified with much higher than expected mtDNA template numbers with only a 25% reduction from the 0-hour sample when it was expected, based on similar samples, to have been reduced by approximately 80%.

Table 6 Percent change in mtDNA template copy number of samples heated at 50°C.

Heating Time (hours)	0	24	48	72	96	120	144	168
Template Number	60378	57999	31456	31121	21861	20095	11006	11189
Percent of Initial DNA Remaining (%)	100	96.06	52.1	51.54	36.32	33.28	18.23	18.23
Change From Previous Sample (%)		96.06	54.24	98.94	70.25	91.92	54.77	101.66
Heating Time (hours)	192	216	240	264	288	312		
Template Number	44375	11497	12363	12277	8790	11961		
Percent of Initial DNA Remaining (%)	73.5	19.04	20.38	20.33	14.56	19.81		
Change From Previous Sample (%)	396	25.92	107.53	90.3	71.6	136.08		

For nDNA, an initial increase in the rate of degradation within the first 24 hours of heating at 50°C, followed by a reduced rate of degradation thereafter (see Table 7). The copy number was reduced by approximately 20% every 24 hours within the first 72 hours, after which the rate of degradation slowed, plateauing with approximately 27% of the initial DNA remaining from 120 to 168 hours (see Table 7). After 192 hours and right up to the final 312 hours of heating, a second plateau was observed, with sample copy numbers fluctuating between 6% and 16% remaining of the initial 100% (see Table 7).

The 96-hour sample was lower than expected when compared to the quantities of the previous and subsequent samples, having approximately 14% of the DNA remaining, compared to 27% for the 120, 144 and 168-hour samples (see Table 7). A similar reduction in the quantity of mtDNA in the 96-hour sample was not seen. Although the

192-hour sample was found to have a higher than expected copy number with regards to mtDNA, this was not the case for nDNA, as it fell in line with the overall pattern of nDNA degradation.

Table 7 Percent change in nDNA template copy number of samples heated at 50°C.

Heating Time (hours)	0	24	48	72	96	120	144	168	192
Template Number	1695	1431	1072	749	236	479	454	477	272
Percent of Initial DNA Remaining (%)	100	84.42	63.24	44.19	13.92	28.26	26.78	28.14	16.05
Change From Previous Sample (%)		84.42	74.91	69.87	31.51	203	94.78	106.06	57.02

Heating Time (hours)	216	240	264	288	312
Template Number	172	105	176	109	211
Percent of Initial DNA Remaining (%)	10.15	6.19	10.38	6.43	12.45
Change From Previous Sample (%)	63.24	61.05	167.62	61.93	193.58

70°C Heat Treatment

A rapid initial loss of mtDNA within the first 12 hours of heating was observed in samples heated at 70°C, with only approximately 6% of the initial amount of mtDNA remaining after 12 hours, and only 1% and 2 % after 24 and 36 hours respectively (see Table 8). The reversal in the pattern, with the 36-hour sample retaining a greater percentage of mtDNA compared to the 24-hour sample, again supports the notion that the samples were switched during extraction. When the results for the 24 and 36-hour samples are interchanged, a more consistent degradation pattern is observed (see Table 9). The degradation rate appears to slow after 12 hours of heating, and finally plateaus after 60 hours with samples fluctuating around 0.003% of mtDNA remaining (see Table

8). This plateau continues up to 204 hours of heating, after which point all mtDNA was degraded.

Table 8 Percent change in mtDNA template copy number of samples heated at 70°C.

Heating Time (hours)	0	12	24*	36*	48	60	72	84	96
Template Number	71289	4048	570	1353	109	8	2	1	1
Percent of Initial DNA Remaining (%)	100	5.68	0.80	1.90	0.15	0.011	0.003	0.001	0.001
Change From Previous Sample (%)		5.68	14.08	237	8.06	7.34	25	50	
Heating Time (hours)	108	120	132	144	156 to 204	220 to 300			
Template Number	1	0.5	1	2	0.5 to 2	0			
Percent of Initial DNA Remaining (%)	0.001	0.0007	0.001	0.003	0.003 to 0.0007	0			
Change From Previous Sample (%)									

* denotes samples that were likely mixed up during extraction.

Table 9 Percent change in mtDNA template copy number of samples heated from 0 to 96 hours at 70°C, with the values for the 24 and 36 hour samples switched.

Heating Time (hours)	0	12	24*	36*	48	60	72	84	96
Template Number	71289	4048	1353	570	109	8	2	1	1
Percent of Initial DNA Remaining (%)	100	5.68	1.90	0.80	0.15	0.011	0.003	0.001	0.001
Change From Previous Sample (%)		5.68	33.42	42.13	19.12	7.34	25	50	

* denotes samples that switched, due to a likely mix up during extraction.

A similar pattern was observed with regards to nDNA within samples heated at 70°C, with an initial period of rapid nDNA degradation, with only 2% of the initial amount remaining after 12 hours (see Table 10). The degradation rate again slows after 12 hours, with total nDNA loss after 60 hours of heating. The 24 and 36-hour sample percentages again reflect the sample mix up. If the values for two samples are

interchanged, a pattern emerges after the initial 12 hours of heating in which nDNA is reduced by 30% ever 12 hours up to 36 hours (see Table 11).

Table 10 Percent change in nDNA template copy number of samples heated at 70°C heated samples.

Heating Time (hours)	0	12	24*	36*	48	60 to 300
Template Number	1391	28	3	9	0.5	0
Percent of Initial DNA Remaining (%)	100	2.013	0.216	0.647	0.036	0
Change From Previous Sample (%)		2.013	10.71	300	5.56	

* denotes samples that were likely mixed up during extraction

Table 11 Percent change in nDNA template copy number of samples heated at 70°C, with values for the 24 and 36 hour samples switched.

Heating Time (hours)	0	12	24*	36*	48	60 to 300
Template Number	1391	28	9	3	0.5	0
Percent of Initial DNA Remaining (%)	100	2.013	0.647	0.216	0.036	0
Change From Previous Sample (%)		2.013	32.14	33.33	16.67	

* denotes samples that were switched, due to a likely mix up during extraction.

85°C Heat Treatment

As observed with the 70°C heat treatment, samples heated at 85°C had a rapid loss of mtDNA within the first 12 hours, with less than 0.005% of the initial mtDNA remaining (see Table 12). In other words, 99.99% of the original mtDNA templates were damaged and broken into small fragments of less than 125bp within 12 hours at 85°C. This rapid degradation was followed by a more gradual and fairly consistent loss of mtDNA every 12 hours thereafter, up to 48 hours of heating, with approximately a 50% reduction in mtDNA compared to the previous sample (see Table 12). All mtDNA was lost after 48 hours of heating.

Table 12 Percent change in mtDNA template copy number of samples heated at 85°C.

Heating Time (hours)	0	12	24	36	48	60	72	84
Template Number	67475	3	2	1	0.5	0	0	0
Percent of Initial DNA Remaining (%)	100	0.0045	0.003	0.0015	0.0007	0	0	0
Change From Previous Sample (%)		0.0045	66.67	50	50			

As for nDNA, all DNA was lost within the first 12 hours of heating (see Table 13), indicating a very rapid rate of degradation.

Table 13 Percent change in nDNA template copy number of samples heated at 85°C.

Heating Time (hours)	0	12 to 84
Template Number	1081	0
Percent of Initial DNA Remaining (%)	100	0
Change From Previous Sample (%)		0

The rate of DNA degradation between mitochondrial and nuclear DNA within heat treatments did not differ significantly (see Figure 17) even though amplification failure of nDNA occurred earlier than mtDNA of similar fragment lengths. This earlier cessation of successful amplification can therefore be attributed to the lower copy number of nDNA as opposed to an increased rate of nDNA degradation.

Degradation of both mtDNA and nDNA greatly increased as temperature increased, with a very rapid loss of all DNA in samples exposed to 85°C, as opposed to the much more gradual loss in samples heated at 50°C (see Figure 14, Figure 16 and Figure 17).

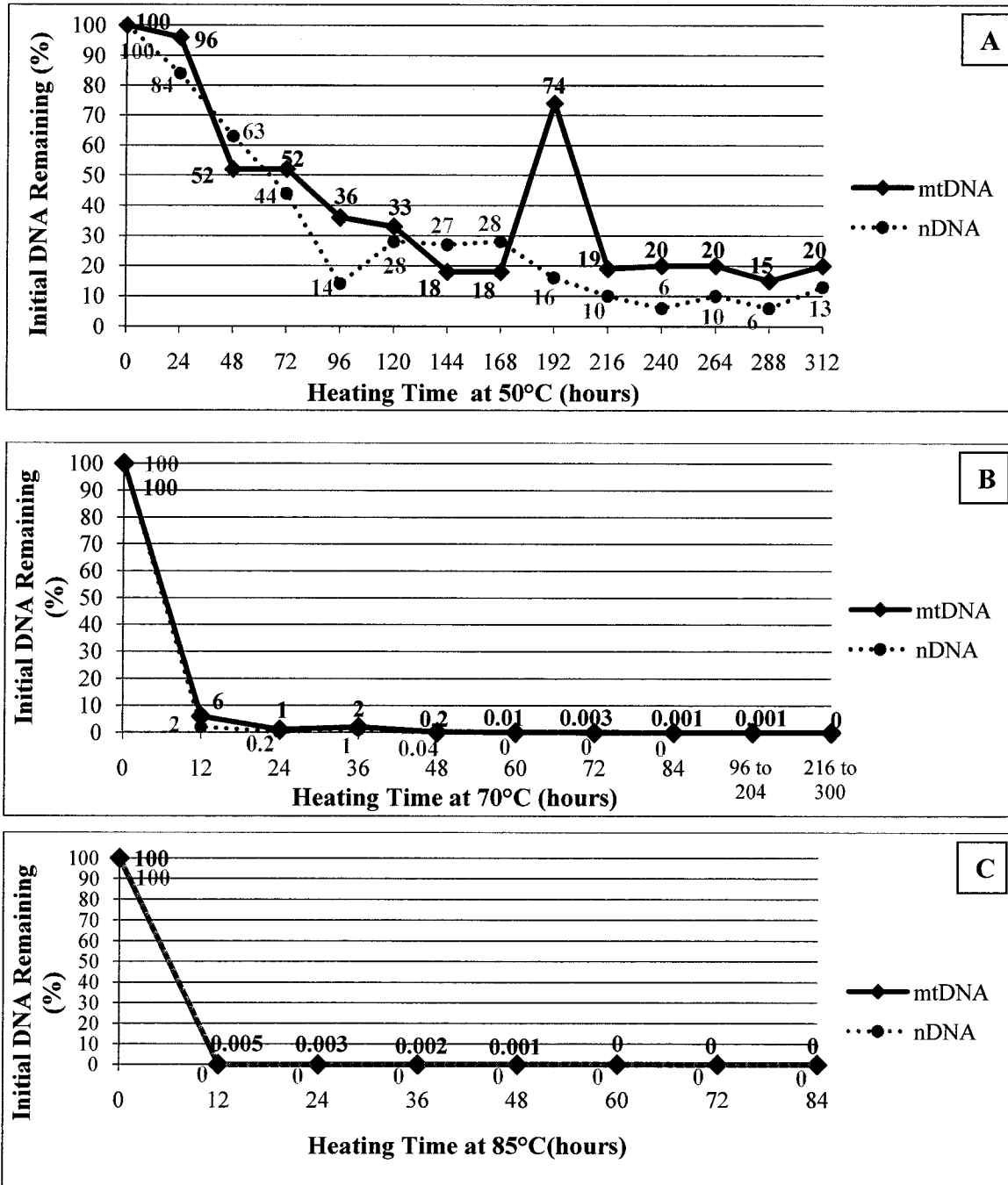


Figure 17 Comparison of the degradation of both mitochondrial and nuclear DNA in samples heated at 50°C (A), 70°C (B) and 85°C (C). All values are expressed as a percentage of the initial, 0-hour template number for each treatment.

Comparison of experimentally degraded DNA and ancient DNA

A previously extracted sample of ancient sheep DNA (approximately 200 years old) from an unrelated study was also examined in order to determine where ancient DNA would fit into the observed experimental degradation pattern, and if aDNA-like samples could be created from modern DNA. With an average mtDNA copy number of 120 templates and a nDNA copy number of approximately 1 template (see Table 14), the ancient DNA sample compared most closely with the 48-hour sample from the 70°C heat treatment, which had average mtDNA and nDNA copy numbers of 109 and 1 templates, respectively. Ideally, more ancient samples from different environmental surroundings should be compared against this artificial degradation pattern to determine a more appropriate comparative picture of how *true* ancient and degraded DNA fit into this pattern.

Table 14 Average initial template numbers and cycle thresholds of mitochondrial and nuclear DNA from a sample of ancient sheep DNA.

Ancient Sheep DNA	mtDNA	nDNA
Template Number	120	0.32
Standard Deviation (+/-)	14.03	0.06
C_T	30	38
Standard Deviation (+/-)	0.18	0.15

Chapter 4: Discussion

The results of this study demonstrate that a simple heat-treatment can be an effective means to degrade DNA in bone to obtain artificially degraded bone samples that are equivalent in template numbers to true ancient skeletal remains. The success of the degradation model in this study should largely be attributed to the use of a dedicated DNA laboratory and the application of vigorous contamination controls. Without such dedicated facilities, it is likely that the highly degraded samples would still have been found to contain significant amounts of DNA because of contamination, proving to be problematic for creating an accurate degradation pattern. The process of clarifying an accurate pattern of degradation proved to be more difficult than anticipated, especially with regard to the higher temperature treatments. With the very rapid degradation process at these temperatures, the high quality of the modern DNA of the 0-hour samples was rapidly transformed into damaged DNA that behaved far more like ancient DNA than modern DNA. For further explanation, see the section on ‘Contamination Controls and Authentication’ below.

As expected, the rate of degradation for both mitochondrial and nuclear DNA increased with exposure to higher temperatures. Samples exposed to higher temperatures for shorter periods of time, showed increased levels of degradation, with a reduction in both the quantity and quality of available DNA templates compared to samples heated for a longer period of time at a lower temperature.

Establishment of the Artificial Degradation Model

The data demonstrate that the degradation patterns can be closely associated with the time and the temperature of the heat treatments. The patterns for all heat treatments were distinct and stable, particularly when the few erroneous samples were removed from analysis in the degradation charts (see Figure 18).

1. At 50°C, bone DNA shows a rather persistent resistance to heat-induced degradation, with approximately 10,000 copies of mtDNA and over 100 copies of nDNA continuing to be recovered even after 300 hours of heating.
2. At 70°C, the DNA in bone shows rapid degradation, a sharp contrast to the 50°C heat treatment even though there is only a 20°C temperature difference. This indicates that there is perhaps some type of DNA preservation threshold that is being broken between 50°C and 70°C.
3. At 85°C, the pattern of rapid DNA degradation was similar to that observed in the 70°C heat treatment. As expected with the higher temperature, the level of degradation was much more severe over a shorter period of time, with almost all mtDNA and nDNA in the bone being destroyed within 48 hours.

Although only three temperature regimes were applied in this study, a clear correlation between temperature and degradation level was observed. This artificial degradation model is functional based on the ability to combine the results into a virtual model in which a change in time or temperature can be used to roughly predict the resulting DNA degradation. The incorporation of other degradation factors in the future, replicating more realistic burial conditions, would greatly enhance this predictive ability.

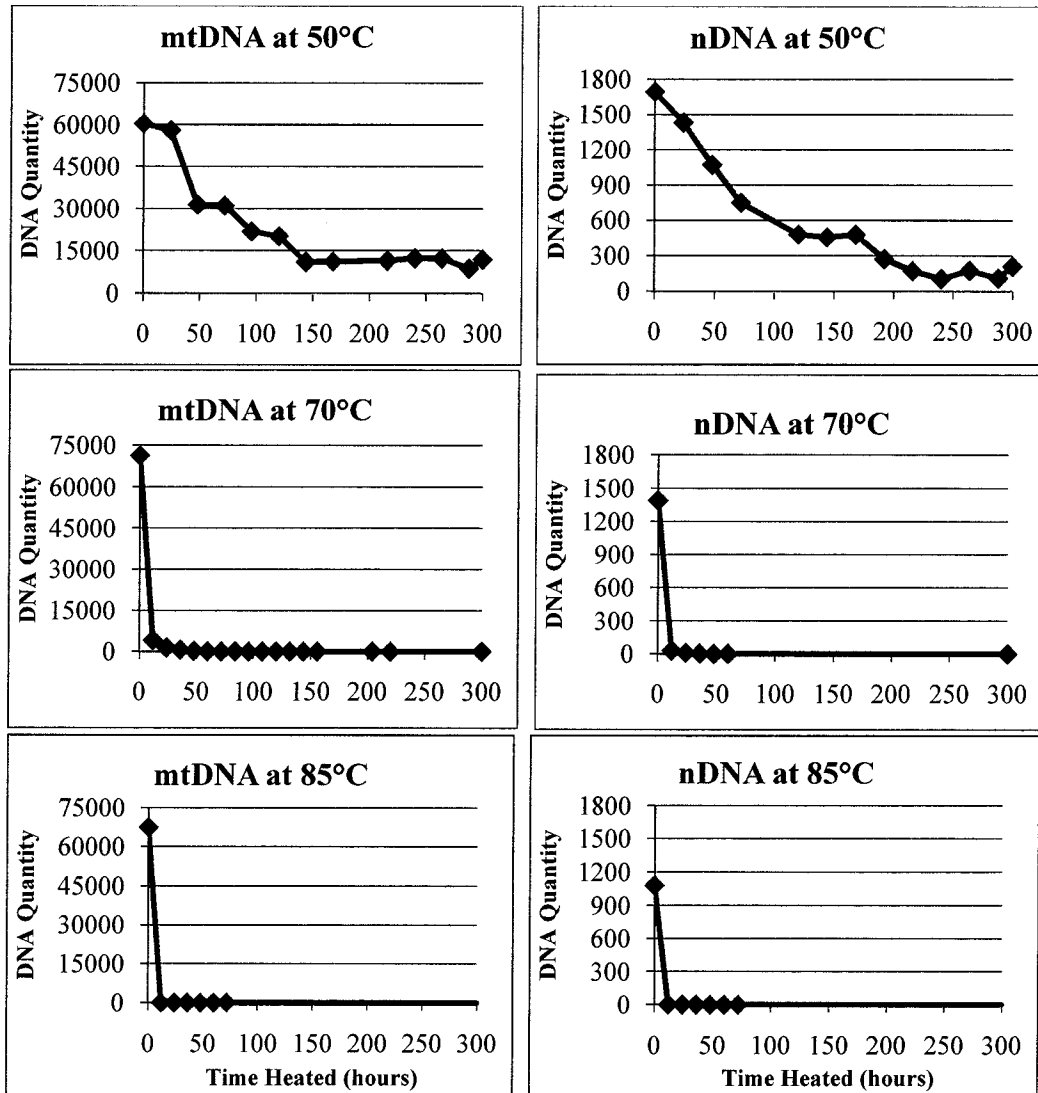


Figure 18 mtDNA and nDNA degradation for all heat treatments. On each chart, the vertical axis indicates the number of mtDNA or nDNA templates preserved after bone samples were exposed to their respective heat treatments, while the horizontal axis reflects the number of hours samples were heated. Some obvious anomalous data were removed or adjusted for the analysis: 24- and 36-hour samples were switched for the 70°C mtDNA and nDNA analysis; 192-hour sample removed from the 50°C mtDNA analysis; 96-hour sample removed from the 50°C nDNA analysis.

New Insights on DNA Degradation

This preliminary study has already revealed many important insights about DNA degradation in bone samples. It was initially expected that nuclear DNA would be found

to degrade at a higher rate than mitochondrial DNA; however this was not the case. Rates of degradation between mitochondrial and nuclear DNA were in fact quite similar, with very little difference found between them within each heat treatment. This similarity suggests that it is largely the increased copy number of mtDNA that makes it more amenable to studies involving degraded samples compared to nDNA, rather than factors of differential preservation. Similar results were reported by Foran (2006), in which little difference was found in degradation rates of mtDNA and nDNA. In this study, the greatest variation in degradation resistance was found between the DNA in different tissue types (muscle, liver and brain tissues) and processing techniques (whole versus homogenized tissue samples). Foran (2006) comes to a similar conclusion that copy number plays a key role in obtaining DNA from degraded remains, with other aspects, such as cellular location and function, as factors further influencing degradation.

The artificial degradation patterns developed here generally showed quite consistent degradation within the first hours/days of heating, followed by one or two periods of more sporadic decay, indicating that degradation had levelled off. This rapid initial DNA loss and slower subsequent degradation is consistent with results from similar studies (i.e. Dobberstein *et al.* 2008; Threadgold & Brown 2003; von Wurmb-Schwark *et al.* 2003; Zimmermann *et al.* 2008). The greatly increased rate of degradation in samples heated at 70°C compared to those heated at 50°C is consistent with results from Zhang & Wu (2005), in which alga cells were heated for 90 minutes at various temperatures. Likewise, the complete degradation of DNA in samples heated at 85°C within approximately 12 hours of heating is consistent with results found in a study performed by von Wurmb-Schwark *et al.* (2003). Von Wurmb-Schwark *et al.* (2003)

artificially degraded femoral bone samples by heating them at 90°C in water for various periods of time. DNA was degraded to very small fragments within 12 hours, with results failing to be reproducible after 36 hours.

Only in one case was the pattern of degradation reversed between consecutive samples in both nDNA and mtDNA within the first few hours of heating – the 24 and 36-hour samples in the 70°C heat treatment. Other reversals were observed in the order of consecutive samples during later phases of heating, generally occurring once DNA quantities had dropped to approximately 1 template with the quantities fluctuating around very low amounts. The consistency in the pattern of reducing quantities in consecutive samples, both within and between heat treatments, along with the fact that the 24 and 36-hour pattern reversal of the 70°C samples was seen in both the mtDNA and nDNA analysis, strongly suggests that this reflects a mix up of the samples due to human error at some point during the extraction process and not the actual degradation pattern.

Regarding the generation of artificially aged skeletal remains, it can be estimated that heat treatments of bone samples (that have not been pulverized prior to exposure) in an oven at 70°C for a period of 12 to 24 hours should be adequate to degrade the DNA down to approximately 1000 copies. Results from this study may overestimate the degradation of DNA in whole bone pieces as the samples used were pulverized prior to heating. Further research is needed, testing other combinations of temperature and/or time, to clarify the pattern. For example, only a few hours of heating at 85°C may be needed to reach the same level of degradation seen after 24 hours at 70°C, which could provide a more manageable time-scale for research purposes.

Heat Source Variation

The extreme temperature difference seen between samples heated at 70°C in the heat block versus those heated in the oven/incubator was not anticipated. Although it was not surprising that the bone powder contained in the tubes in the heat would not necessarily reach exactly 70°C, the 20°C difference between the heat block setting and the actual temperature of the bone powder was somewhat shocking. In a laboratory setting, heat blocks are likely more often used for liquid phase substances through which the heat can more readily dissipate, creating a more constant and accurate temperature. The fact that the samples used in this study were heated as dry bone powder clearly had a detrimental effect on obtaining the expected temperature inside the samples.

A positive outcome of this heat source variation was the ability to use the heat block 70°C samples as if they had been heated in the oven at 50°C. This allowed for an extra heat treatment to be included in the degradation model, and provided insight into the reduced rate of degradation at lower temperatures.

Artificial DNA for Real-Time PCR Quantification

At the beginning of this study, one of the main issues that arose with employing real-time PCR for quantification purposes was what to use as a quantification standard. Standards are required to be as similar as possible, ideally identical, to the sequence of the target of interest, so that amplification efficiencies between the standards and the unknown samples are equal, allowing for accurate quantification (Orlando et al. 1998;

Shiple 2006). The development of such standards is quite simple for modern genetic analyses, as numerous quantification standards have been manufactured for most commercial biochemical assays. However, when working with ancient DNA, this unfortunately is not the case.

Various options were explored as possible sources of quantification, such as extracting DNA from other modern sheep sources (such as blood or non-skeletal tissue samples), and further quantifying the extractions using other methods. However, the intrinsic quantification problem remained unresolved – the total amount of sheep genomic DNA in blood or other tissues can quantify the number of nDNA templates for a given sample, however, it cannot quantify the copy number of mtDNA templates. The ratio of nuclear to mitochondrial DNA can vary significantly among different tissues as the quantity of mtDNA is dependent on cell type (Alaeddini et al. 2010; Durham & Chinnery 2006; Morin et al. 2007), leaving a large amount of guess work involved in calculating mtDNA quantities. Such guess work may not be acceptable when preparing DNA quantification standards with the hopes of accurately quantifying unknown samples.

The simplest solution seemed to be the development of an artificial DNA fragment that was an exact replicate of the sheep mtDNA sequence of interest. Similar techniques have been used in other aDNA studies (for example, see Ottoni et al. 2009), however questions remained regarding the accuracy of quantification based on the artificial DNA, due to the lack of other biological or chemical compounds that may be co-extracted along with true DNA. After designing and quantifying the artificial DNA, changes in the amplification efficiency between samples of *clean* and *dirty* artificial DNA

were assessed by spiking the artificial DNA with real ancient DNA from different species (goat and whale) that had been previously extracted in unrelated studies. No significant difference was observed between the amplification efficiencies of the *clean* and *dirty* artificial DNA samples, even though the amplification curve produced by the artificial DNA (even when spiked) is more ideal in that it reaches higher overall fluorescence levels (results not shown), indicating that some inhibitory factors may be present in the true, unknown samples. However, the lack of impact spiking had on the artificial DNA suggests that whatever inhibitors exist, they were not in great enough a quantity to be significantly detrimental to the amplification of the standards. The standard that was spiked was the 10^4 sample in the dilution series, calculated as having 10000 molecules/ μL . It is possible that standards of lower magnitudes in the dilution series, such as the 10^2 or 10^1 samples, may show changes in the efficiency when spiked due to the reduced template number. Studies have indicated that as initial template number is reduced, the accuracy of real-time PCR quantification also reduces (Ellison et al. 2006), which is something that needs to be kept in mind when dealing with ancient and degraded samples. This reduced accuracy was observed in this experiment, with the amplification of the 10^0 standard (1 molecule/ μL), which was consistently flagged by the real-time software as having high standard deviations in both C_T and copy numbers.

The artificial standards also proved to be a useful tool for purposes other than quantification. While running the analysis of spiked standards to examine the possibility of changes in efficiency, whale aDNA samples from an unrelated study that were believed to be inhibited were also mixed with the standards to determine if they were truly inhibited, or if there was another reason for their consistent amplification failure,

such as unsuccessful extraction. The whale samples exhibited a dark brown discolouration after extraction, which is often used as an indicator for the presence of inhibitory substances. As the purportedly inhibited samples also prevented the artificial DNA from amplifying even after diluting the whale samples substantially (data not shown), it was determined that they were in fact inhibited. A second spiking experiment was performed using samples of the degraded sheep that had failed all previous attempts at amplification, to determine whether this failure was due to inhibition or, as hoped, the accumulation of damage. Inhibition was ruled out as the cause for this consistent failure when the spiked samples of quantified artificial DNA successfully amplified, indicating that the DNA had indeed been totally degraded (data not shown).

The use of real-time PCR and the quantified standards also acted as a test of the efficiency of the primers used by comparing the initial number of templates in unknown samples and the frequency of successful amplification. The fact that numerous unknown samples were successfully amplified and quantified as having only one initial template, attests to the high efficiency of the particular assay used. Similarly, the standard containing only 1 molecule/ μL amplified in every PCR it was included in, although with high standard deviations.

Although it is recommended that the known-concentration standards used for quantification are as similar to the desired template as possible (Shiple 2006), the artificial sheep mtDNA standards were also used for the quantification of nDNA in this study. A separate artificial DNA fragment was not designed to replicate the 262bp sequence of the amelogenin gene that was targeted simply because it was not initially known how reliably or accurately the artificial DNA would function as a quantifier. As

such, the actual number of templates may be imprecise, as the efficiencies of the two different primer and probe assays employed (one targeting the nDNA amelogenin sequence for the unknown samples, and the other targeting the mtDNA D-loop sequence of the artificial DNA standards) likely differs. The nuclear primer set indicated a lower efficiency when used in standard PCR compared to that of the mitochondrial primer set, with the amplification of non-specific templates in some nDNA PCRs (see Figure 13), particularly at lower annealing temperatures. The efficiency was increased however at the 60°C annealing temperature that was most often employed, while the efficiency of the mtDNA primers was not reduced when run at the 60°C annealing temperature instead of the optimal 55°C. Although the different efficiencies between the two separate primer sets imply that the specific quantification numbers may be inaccurate, the rate of degradation inferred between samples should be the same, as it was the percent change from the initial 0-hour copy number that was examined and not the absolute amounts.

Sample Decontamination

In aDNA analysis, decontamination of the specimens in question is of utmost concern, as many archaeological and forensic samples will have undoubtedly come in contact with multiple sources of contamination, whether during the excavation and collection of samples or from post-excavation handling (Kaestle & Horsburgh 2002; O'Rourke et al. 2000; Pääbo et al. 2004; Poinar 2003; Willerslev & Cooper 2005; Yang & Watt 2005). Even minimal amounts of exogenous modern DNA can easily overwhelm the damaged endogenous DNA signal. Both chemical decontamination, such as submersion in HCl, and physical decontamination, such as scrubbing the bone surface

with bleach-wetted sandpaper, are often employed when working with ancient samples, however these are generally not employed when performing modern DNA analysis. Due to the modern nature of the sheep bones that were used in this study, specimen decontamination techniques were not employed. Any contaminant DNA that may have been on the bones, would have been outnumbered by the endogenous sheep DNA. As the samples were being intentionally degraded by heating, it is reasonable to assume that any contaminant DNA would also have been degraded and overwhelmed by the sheep DNA during amplification.

Contamination Control and Authentication

Authentication of aDNA results is a major component of all aDNA studies, as contamination is always a possibility, no matter how stringently laboratory protocols are followed (Hofreiter et al. 2001; Kaestle & Horsburgh 2002; O'Rourke et al. 2000; Pääbo et al. 2004; Poinar 2003; Willerslev & Cooper 2005; Yang & Watt 2005). A number of articles have been published which lay out recommended criteria for authenticating results (see Hofreiter et al. 2001; Kaestle & Horsburgh 2002; Malmstrom et al. 2007; O'Rourke et al. 2000; Pääbo et al. 2004; Poinar 2003; Willerslev & Cooper 2005).

With regards to this study, stringent contamination controls were followed in both during all phases of the analysis. The laboratory decontamination techniques discussed in Chapter 2 appear to have successfully limited the amount of systematic contamination from outside sources, as DNA was not amplified in the no-template negative PCR controls. There were issues with successful amplification of DNA in certain no-template

blank controls that were set up during the extraction process. This is reportedly a common issue when working with modern DNA as was used in this study, however the degraded nature of the experimental samples necessitated that the blank controls be clean to authenticate the results. As such, all reported results are only from extractions in which the blank extraction controls were clean and did not amplify (with the exception of those discussed in the Chapter 3 in which the 125bp amplicon was observed in samples up to 648 hours of heating at 70°C).

One of the criteria that has been suggested for determining if aDNA results are indeed authentic or due to contamination is to observe the quality of the DNA amplified. If the data is in fact authentic, then the proportion of small DNA fragments should be much greater than that of large fragments (Alonso et al. 2004; Malmstrom et al. 2007). The results presented here for the decreasing quality of mtDNA over longer periods of heat exposure, as indicated by the longest fragments only amplifying in the earliest samples (as shown in Figures 9, 10 and 12), is indicative that the results are indeed from the bone powder samples in question and not from extraneous sources.

Replication of both amplification and extraction results are also used as criteria for authentication of aDNA data. In order for samples to be considered as successfully amplifying at a specific fragment length, the results had to be reproducible in subsequent PCRs so that anomalous amplification of a particular sample could be ruled out of further analyses. Multiple extractions of the same samples were not directly performed. However, due to multiple heat treatment sessions at the same temperature (two sample sets at 50°C (70°C heat block), and two sample sets at 70°C in the oven, with three extractions for this treatment in total) being performed for various reasons, these separate

extractions for equally degraded samples were taken as extraction reproductions. As the amplification results between equally degraded but separately extracted samples were consistent, this can be taken as evidence of authenticity. The only samples that did not have a second extraction of any form, were those exposed to the 85°C heat treatment. However, as the mtDNA quantification of the 0-hour control for this sample set was found to fall within the range of the other extraction 0-hour controls, the efficiencies of the separate extractions were considered comparable. Moreover, as the 85°C heat treatment samples followed a degradation pattern similar to that observed for the other heat treatments, the results could be considered authentic.

Stable Fragment Length

The 125bp fragment of mtDNA was successfully amplified from all the maximum hour samples for each heat treatment, and even in samples heated in the oven at 70°C for 648 hours, far longer than that any other fragment length, and more than twice as long as the next shortest fragment of 200bp. It is possible that fragments of this length, and perhaps slightly longer, are somehow more stable and therefore more resistant to degradation. Hummel (2003) suggests that the double helical conformation itself may confer protection, with approximately 190 base pairs making up a single loop of the DNA helix. Specifically with regards to nDNA, it has been suggested that the 146bp section that wraps around histones forming the nucleosome core of nDNA, may retain a greater degree of preservation due to this structural conformation (Binladen et al. 2006). Although the 125bp amplicon was only studied in mtDNA which does not contain

histones, a similar type of preservation unit may be responsible for why the fragment could still be amplified over such an extended period of time.

Another possibility for this phenomenon could be the non-random build up of damage at previously deteriorated sites. Perhaps once a specific location on a DNA strand is damaged it becomes more susceptible to further degradation, with damage events occurring preferentially in these locations, and therefore indirectly conferring protection on undamaged sites. This is somewhat similar to the concept of DNA damage “hotspots” which have been found to accrue large amounts of damage at a higher rate than other locations (Banerjee & Brown 2004; Gilbert et al. 2003; Gilbert et al. 2005; Willerslev & Cooper 2005). Clearly, more investigation into the possibility of a specific DNA fragment length with improved stability and damage protection is needed to clarify this relationship.

Although aDNA studies generally rely on amplifying short fragments of approximately 200 to 300bp, it is possible that such fragments are still too long for highly degraded samples. It is quite possible that samples which failed to amplify at a fragment length of 200bp could still be highly successful with fragments of 125 to 150bp.

Applications

Extraction and Inhibition Testing

The modified silica-spin column method of DNA extraction (Yang et al. 1998) was employed in this study, however different extraction procedures are often followed in other laboratories and depending on the types of tissue in question. There is a general

consensus among aDNA researchers that extraction procedures need to be improved, particularly with regard to extraction efficiencies. Studies have been published examining differential success rates between various commonly used techniques (see Bouwman & Brown 2002; Giles & Brown 2008; Rohland & Hofreiter 2007b) with different labs often asserting the effectiveness of certain techniques over others. The artificial degradation of bone samples could be used to generate ideal artificial “ancient remains” to test the differences in efficiency between various extraction techniques, without having to use irreplaceable *true* ancient materials. Changes in the initial template numbers, as determined by real-time PCR, would reflect either improved efficiency with increased template quantities, or lower efficiency with reduced quantities. Moreover, improvements on amplification efficiency could also be achieved through examining the effectiveness of the addition of reagents that are known to reduce the influence of PCR inhibitors, such as bovine serum albumin (BSA) (Andréasson et al. 2002) and N-phenacylthiazolium bromide (PTB) (Poinar et al. 1998).

The methods employed in DNA extraction procedures often require samples to be held at high temperatures (similar to the lower temperature regimes used herein) for various periods of time, so that the necessary chemical reactions can occur (such as during the incubation phase – see Chapter 2: Materials & Methods). These procedures can be a cause of concern when working with degraded remains, as high temperature is known to exacerbate DNA decay. The relatively low levels of degradation in both quantity and quality of DNA observed in the 50°C heat treated samples suggest that the temperatures required during such phases of the DNA extraction process likely do not significantly impact the level of DNA degradation within the samples.

A similar approach to that discussed previously with regards to the use of quantified artificial DNA standards to test for inhibition, could be used to examine variations in the amount of inhibition between samples as well. Analyzing changes in the calculated template numbers between *clean* quantified samples and *dirty* quantified samples spiked with inhibited DNA, would allow for comparison of the level of inhibition, and perhaps provide insight into how to rectify the issue – whether through simple dilution of the samples or through additional purification procedures.

Sample Selection

Sample selection is an important part of any study involving ancient or degraded DNA. This degradation model could be used to assess variation in rates of degradation of different types of bones, enabling more appropriate and educated sample selection. Samples from cortical bone are generally preferentially selected as the increased density is thought to act as protection from both contamination sources and DNA damage (Kaestle & Horsburgh 2002; Misner et al. 2009), although others argue that more DNA can be extracted from cancellous bone (O'Rourke et al. 2000). Direct comparison of both template quantities and degradation patterns between cortical bone and cancellous bone specimens could be accomplished through the development of a model similar to the one used in this study. Likewise, comparison of degradation patterns between specimens of different taxonomic classes would be beneficial, as it is possible that degradation influences the remains of diverse classes in different ways (Haynes et al. 2002).

Sheep rib bones were selected for this study for their ease of manipulation and because ribs have been shown to have a relatively high aDNA extraction success rate (Foran 2006; Misner et al. 2009; Pruvost et al. 2008; Rennick et al. 2005). Due to the

modern nature of the ribs used, the bones were boiled and dried to remove any external soft tissue and to breakdown the internal marrow prior to beginning the artificial degradation experiments. While this means that the DNA within the bones may have already been partially degraded prior to beginning this study, it was a necessary step to ensure that the majority of DNA sampled was in fact from the skeletal material and not from soft tissues. This initial, unmeasured period of degradation should not affect the degradation patterns observed in this study however, as all samples were compared against 0-hour samples which were not exposed to the experimental degradation process. Therefore, the initial level of DNA preservation (after the boiling and drying but prior to the experimental heating) within the samples was equal. The level of degradation was then measured as the change from this initial DNA copy number, and not from an idealized or expected cellular copy number. Although the pre-experimental degradation that the ribs may have undergone during the boiling and drying was less than ideal, it was the only available way at the time to reduce the influence of the interfering DNA in the soft tissues (both on the internal and external surfaces of the bones). As it was the DNA within skeletal remains that was in question in this study, it was a necessary step prior to beginning the experimental degradation regimes.

The bone samples used in this study were milled prior to undergoing the artificial degradation treatments. It is quite possible that this pre-experimental process reduced the potential of protective forces that skeletal tissues may bestow upon the DNA held within their cells, thereby increasing the rate of degradation. However, the choice to pulverize the ribs prior to heat exposure was made with the goal of reducing the potential of rib section sampling bias. The bone powder from all rib segments was combined, and

samples were then separated out into foil weigh boats for heating. In this case, the risk of sample section bias was believed to outweigh the possible reduction in DNA damage resistance.

Maintenance of DNA Samples Pre-Extraction

Recent research has shown the rapid degradation of DNA in fossils after excavation when the appropriate preservation and storage conditions are not met (Bollongino et al. 2008; Bollongino & Vigne 2008; Pruvost et al. 2007; Pruvost et al. 2008; Wandeler et al. 2003). Temperature once again appears to play a key role in this rapid post-excavation decay of DNA, along with cleaning techniques and maintenance of the conditions within the specific burial microenvironment (Bollongino et al. 2008; Bollongino & Vigne 2008; Pruvost et al. 2007; Pruvost et al. 2008). It is recommended that samples which may be destined for ancient DNA analysis not be cleaned, and instead maintained within a portion of their surrounding *in situ* substrate so as to disturb the microenvironment as little as possible, followed by cold storage when possible (Bollongino et al. 2008; Pruvost et al. 2008).

Museum specimens are frequently employed in aDNA analyses concerning phylogenetic relationships, population genetics and conservation biology (Binladen et al. 2006; Morin et al. 2007; Wandeler et al. 2003; Wandeler et al. 2007). It has been well documented that many of the substances used in soft tissue preservation of museum specimens, such as formaldehyde and ethanol, have a negative impact on DNA extraction from such samples (Wandeler et al. 2007; Zimmermann et al. 2008). However, the impact of other dry storage conditions, such as temperature and humidity levels, has been given less attention. The previously mentioned research regarding archaeological

specimen storage has also been recommended for museum curation (Binladen et al. 2006; Pruvost et al. 2008). Likewise, similar precautions should be considered when dealing with the collection and preparation of forensic samples, as many of the chemical and physical maceration techniques employed in removing soft tissues from skeletal elements are detrimental to the preservation of DNA within the bones (Rennick et al. 2005; Smith & Morin 2005; Steadman et al. 2006; Wandeler et al. 2003).

With regard to this current study, the level of degradation observed even at moderately low temperatures (such as 50°C) indicates that greater caution should be taken with regard to sample storage, as DNA decay continues in ancient and degraded samples even at room temperature (Bollongino & Vigne 2007; Pruvost et al. 2008). It is likely that many storage facilities are not equipped with efficient internal environmental control systems, so that fluctuations in storage room conditions likely assist in further DNA degradation (Binladen et al. 2006; Bollongino & Vigne 2007). Moreover, samples that are kept on display in museums are often exhibited in enclosed cases with internal lighting. The temperatures that may be reached within such enclosed and well-lit display cases could easily elicit levels of DNA degradation similar to those observed in the samples subjected to the 50°C heat treatment in this study. Prolonged exposure to such conditions would clearly reduce the successful analysis of DNA from displayed specimens.

This continued, low-temperature degradation should also be of concern to forensic analysts. Material related to forensic cold cases can sit untouched on storage shelves for years before more evidence comes to light. Appropriate conditions should be maintained with regard to the storage of materials which may eventually need to be

reanalysed for trace DNA, so that the integrity of the already damaged DNA is not further compromised.

Maintenance of DNA Samples Post-Extraction

Not only is sample handling and storage prior to DNA extraction important, but storage of samples post-extraction should also be considered. Standard procedures generally involve DNA being stored in solution (usually in either ultrapure H₂O, EDTA or elution buffer) at -20°C. However, studies have indicated that such precautions may not be enough to maintain the integrity of the DNA over extended periods of time (Anchordoquy & Molina 2007; Ellison et al. 2006; Smith & Morin 2005). Storage at even lower temperatures, such as -80°C, has been suggested as more appropriate for long term maintenance, as has the dehydration of the samples followed either by freezing or room temperature storage (Anchordoquy & Molina 2007; Smith & Morin 2005). This has important implications particularly for forensic contexts, as reanalysis of previously extracted DNA samples may be necessary after long periods of time have elapsed since extraction.

It is likely that further storage-induced degradation was observed in samples used in this study, specifically with regard to the initial set of extractions performed on samples heated in the heat block at the beginning of the experiment. Initial amplification was performed using either undiluted or 10X diluted extracts from the heat block samples. When it was determined that the heat block samples could be used as a 50°C heat treatment, reproduction of the previous amplifications were performed using a 2X dilution of the sample extracts for consistency purposes, as this had since been determined to be the optimal dilution factor for successful amplification and was used for

samples heated in the oven. For most fragment lengths, reproduction of previous results was not an issue, however, when amplification of the 460bp and particularly the 556bp mtDNA fragments were attempted, amplification success was much more sporadic than it had previously been.

Approximately twelve months had elapsed since the initial time of extraction of the samples and the subsequent attempts at re-amplification. During this time period, the samples were stored at -20°C in elution buffer (for undiluted samples) or a combination of elution buffer and ddH₂O (for diluted samples). The samples would have undergone a number of freeze-thaw intervals after their initial storage, as multiple PCRs were performed. It is probable that the freeze-thaw cycles, particularly for the 10X dilution set as they had been used more frequently, had a detrimental effect on the subsequent preservation of the extracts. However, as the undiluted extracts used to make the desired 2X dilution would have undergone fewer freeze-thaw cycles, the level of freeze-thaw degradation should have been minimal, however further degradation was clearly observed. This suggests that degradation of DNA quality in the samples continued, particularly at longer fragment lengths, even though they were maintained consistently at -20°C for approximately twelve months.

The model developed in this study could easily be directly manipulated to monitor the continued degradation of previously extracted and stored samples months or even years in the future. Significant reduction in the number of initial templates in a given sample would indicate a high level of subsequent damage. Samples in this study were stored at -20°C either in elution buffer or in a combination of elution buffer and ultrapure H₂O, as in the case of diluted samples. Re-extraction of bone powder left over from the

same samples could be performed and the eluted DNA subsequently stored in different substrates or under different conditions, providing more insight into the continuation of DNA damage post-extraction.

DNA Repair Mechanisms

Another area of interest within the fields of ancient and forensic DNA, is that of postmortem DNA damage repair. In living organisms, damage to DNA is fixed by various repair mechanisms that prevent the damage from continuing and getting out of control. After death, these repair mechanisms obviously no longer function. Research has been done looking into the possibilities of halting and reversing the effects of different kinds of damage seen in ancient DNA, with the hope of increasing both the quality and quantity of successfully extracted and amplified ancient DNA, and decreasing the chances of misidentifications due to postmortem degradation (Alaeddini et al. 2010; Gilbert et al. 2003; Mitchell et al. 2005; Willerslev & Cooper 2005). Various repair mechanisms have been suggested as different types of both samples and damage respond in diverse ways to each technique.

Golenberg et al. (1996) tested the ability to reconstruct damaged and fragmented DNA templates, through experimentally degraded calf thymus DNA. To rebuild damaged DNA templates, reconstructive polymerization was performed through a Taq polymerase extension pre-treatment of the degraded DNA with free nucleotides (dNTPs), MgCl₂ and a reaction buffer, and put through 20 cycles of PCR. The PCR products from the reconstructive polymerization were then used as templates for regular PCR using specific primers. Using a similar reconstructive polymerization technique, Iniguez et al. (2003) were able to piece together and rebuild highly fragmented DNA sequences from

ancient human coprolites. Reconstructive polymerization successfully increased the quality of the DNA that was extracted and amplified from the coprolites, in which unreconstructed fragments showed no successful amplification.

Escherichia coli DNA polymerases have also been used in a similar fashion to repair and reconstruct degraded DNA templates. In this case, the initial polymerization is followed by a ligation step. Ancient DNA was recovered from human burials by Pusch et al. (1998), using *E. coli* DNA polymerase I (Pol I) and T4 DNA ligase. The Pol I is used to first fill in holes in the DNA template by incorporating the correct nucleotides, after which T4 DNA ligase is used to close any gaps in and between fragments. Using radioactively labelled dNTPs, they found that the Pol I and T4 DNA ligase effectively repaired nicks and breaks within degraded DNA, enabling the amplification of higher quality and more informative DNA. Di Bernardo et al. (2002) followed the same procedures, using Pol I polymerase followed by T4 ligase treatment, on horse remains from sites in Pompeii and Herculaneum. The use of both enzymes, or only one or the other, was tested in this experiment, to determine if different forms of DNA damage reacted to different repair techniques. Only one sample was able to be amplified using only one of the enzymes, while all other samples required the use of both Pol I and T4 ligase to be successfully amplified.

A more thorough understanding of DNA degradation and preservation would also allow for more DNA repair methods to be developed and tested (Alaeddini et al. 2010; Mitchell et al. 2005; Poinar et al. 1998). Repair techniques do exist for certain types of damage, however they are not all equally effective, nor do they all work for the same kind of damage in different DNA sources – for example, PTB has been shown to be

effective in breaking DNA cross-links in coprolites and sediments, however it does not appear to be effective when used on bone (O'Rourke et al. 2000). The development of effective, damage-specific repair techniques would also be helpful in reducing the extra opportunities for contamination that are seen with current, multi-step repair techniques (Hansen et al. 2006; Mitchell et al. 2005). The less manipulations a sample has to go through, the fewer the opportunities for contamination.

The artificial DNA degradation model developed in this study could be employed to test the effectiveness of different post-mortem DNA repair techniques on bone. Previously quantified samples could be treated with various repair mechanisms and amplified using real-time PCR to quantify the available templates. Any change in copy number between the treated and untreated samples could then be analyzed to determine whether a particular treatment improved amplification (indicated by increased copy numbers). Different techniques could also be compared to determine if a particular repair mechanism was best suited for repairing DNA in specific materials.

Limitations and Future Studies

There are obviously many obstacles when it comes to experimentation and understanding ancient DNA degradation and preservation. The environments in which remains are found play an infinite number of roles in both the degradation and preservation of DNA, whether in skeletal remains, plant tissues, coprolites, or other materials of interest. The complexity of the interactions between the different elements in the burial environment makes understanding one element difficult unless all other

interrelated aspects are also considered. The most obvious limitation of this study as a whole stems from the fact that only a single degradation factor, temperature, was considered in what is clearly a multivariate process. However, the key objective of this study was to begin to elucidate the full DNA degradation process in skeletal remains, with multiple factors gradually added to the mix once individual factors have been understood in their own right. This objective has therefore, been accomplished, and further understanding can be built upon these results.

One of the limitations of using real-time PCR in conjunction with degraded, low copy number samples such as those examined herein, is the reduction in quantification accuracy. Studies have found that as sample copy number decreases, accuracy of copy numbers calculated by real-time PCR software also decreases (Ellison et al. 2006). With regards to this experiment, reduced accuracy was observed not only in the quantification of artificially degraded samples with low template numbers, but also in the quantification of the 10^0 quantification standard. This standard, which was diluted to a concentration of 1 molecule/ μL , was consistently found to have high standard deviations between samples, both in C_T and copy numbers. This was not a problem with the 10^1 (or 10 molecules/ μL) quantification standard, therefore it seems that the accuracy is reduced when sample template numbers are reduced to less than 10. This inaccuracy could have played a role in the somewhat sporadic nature of the degradation pattern that was observed in samples heated over longer periods of time, particularly with regards to the 70°C heat treatment.

Another key limitation in this study involves the analysis of both quantity and quality of nuclear DNA. Use of a number of primer sets targeting fragments of different lengths, as was performed with mitochondrial DNA, would have greatly improved the

quality assessment of nDNA in this study. Similarly, with regards to quantification, a more appropriate nDNA quantification standard would ideally have been employed for increased precision and accuracy. However, it was initially unclear how well the artificial DNA would function as a standard for either mtDNA or nDNA, as such only one standard was developed and employed. In light of time constraints, another artificial fragment replicating the nDNA amplicon was not developed. However, as previously discussed, although the exact template numbers may not be entirely accurate, the rate of degradation based on the percent change between samples should still be correct.

The quality assessment overall, for both mtDNA and nDNA, could be improved upon through the analysis of different qualitative aspects, such as base alterations, as quality in this study was only assessed based on the level of fragmentation. Cloning and further DNA sequence analysis could be performed in the future to assist in determining the influence of such factors, providing a more complete picture of the degradation in DNA quality.

While the model appears to be functional at this point, there are numerous avenues to be pursued for future research. This study was only the starting point for the development of a larger DNA degradation model. In the future, the current model will be expanded to incorporate multiple degradation factors, such as pH, humidity and soil type, to improve our understanding of the overall degradation process. Moreover, DNA degradation in different tissue types and sample materials, such as teeth and mummified soft tissues, could be integrated into the model and compared. For example, a similar degradation model for plant DNA will be developed based on the current findings of this

model, as there is an equal lack of understanding regarding the DNA degradation process in plants.

Prior to adding more degradation factors into the model, a higher resolution picture of the degradation pattern, particularly for nuclear DNA, should be developed. Even though a rapid rate of degradation was expected for nDNA, the large drop within as little as twelve hours, as seen with heating at 70°C and 85°C, was quite dramatic. A clearer picture of the rate of degradation could be developed through the use of shorter intervals, with samples removed every hour over a period of twelve hours. Similarly, through using multiple primer sets targeting nDNA amplicons of varying lengths, the quality of the nDNA in degraded samples can be better understood through developing a similar pattern using standard PCR as to that indicated in this study for mtDNA (such as those seen in Figures 9, 10 and 12). Quantification of the initial template number could also be more accurately calculated through the design and implementation of known-concentration standards of artificial DNA replicating the desired nDNA target. Not only would this provide a higher resolution picture of degradation, it would also assist in clarifying the correlation between the amount of mtDNA and nDNA within a given sample, so as to better assess the likelihood of successful nDNA amplification of a specific fragment length based on the mtDNA results. Moreover, the incorporation of more ancient samples from various environmental contexts would assist in elucidating where true ancient DNA fits into artificial pattern developed herein.

Chapter 5: Conclusions

This study has shown the feasibility and benefits of developing an artificial DNA degradation model to investigate DNA degradation patterns in bone. The data demonstrate that the model is functional, and a relatively stable degradation pattern has been established, particularly with regards to mitochondrial DNA. Although further studies are needed to test other degradation factors, some interesting and potentially very useful insights have already begun to emerge:

1. The analysis and examination of artificially degraded bone samples requires access to dedicated DNA laboratory facilities, along with the implementation of vigorous contamination controls similar to those used in ancient DNA studies. As shown in this study through real-time PCR quantification of DNA templates, DNA recovery and amplification can be accomplished with as few as 1 initial template. The whole process has proven to be very challenging, particularly with the higher temperature regimes, due to the rapid nature of the degradation process, with the 0-hour samples behaving like modern DNA while the 12-hour sample at 85°C behaves just like an ancient DNA sample.
2. The artificial DNA degradation model is functional, although only temperature and time were tested in this study. Obviously many other factors in the burial environment play a role in the preservation of DNA within skeletal elements. Temperature however is often viewed as the most influential on the accumulation

of DNA damage. Future studies will build upon the results of this research through incorporating a greater number of degradation factors into the model.

3. The pattern of DNA degradation is distinct and stable. DNA within bones was shown to consistently resist degradation at 50°C, however it begins to deteriorate dramatically as temperature is increased up to 70°C and 85°C. This drastic increase in degradation suggests that there is some type of DNA preservation threshold that is being broken as the temperature increases above 50°C. As expected, the level of degradation was much more severe over a shorter period of time at higher temperatures, with almost all mtDNA and nDNA in the bone being destroyed within 48 hours at 70°C and 85°C.
4. Both mtDNA and nDNA appear to have undergone similar DNA degradation mechanisms. There is no evidence to support differential mtDNA and nDNA preservation, suggesting that the increased success rate of mtDNA extraction and amplification from degraded skeletal elements is largely due to the greater mtDNA copy number rather than an increased rate of nDNA degradation.
5. This model will provide important information for assessing the quality and quantity of retrievable DNA (both mtDNA and nDNA) in skeletal remains from different recovery contexts, both archaeological and forensic in nature, particularly when artificially degraded DNA samples are extracted along with other naturally degraded bone samples. It also provides critical information regarding optimal sample storage procedures (both pre- and post-extraction) for specimens which may eventually be destined for ancient or forensic DNA analysis.

As with all developing disciplines, research is an important and necessary step in the construction and understanding of methods and concepts. Many aspects regarding DNA degradation and preservation in the environment are still not understood. A full understanding of the processes involved in DNA degradation can only be achieved once all of the interrelated factors are understood in their own right. Further work, including the incorporation of multiple degradation factors into the artificial degradation model and refinement of the nDNA degradation pattern, is needed to in order to elucidate the full picture of post-mortem DNA decay in bones.

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Appendices

Appendix A – Electrophoresis gels for individual PCRs.

The electrophoresis gels of mtDNA amplification included in the results section in Chapter 3 (Figures 9, 10 and 12) for all heat treatments were made by combining 2-3 μ L of various PCR products from multiple PCR reactions into a single tube for each sample. This appendix includes examples of individual gels of some of the samples used in the production of the composite gels. All samples are labelled according to the number of hours they were heated. Blank (no-sample) extraction controls were tested with each primer set but were generally only included in the initial PCR runs of the extraction set they belonged to, while negative (no-sample) amplification controls were included in every PCR. Blank and negative controls are not necessarily shown in all of the following gels as they were sometimes run on separate gels due to spatial constraints (the number of wells available for samples on a single gel) when running multiple gels at a time.

50°C Heat Treatment:

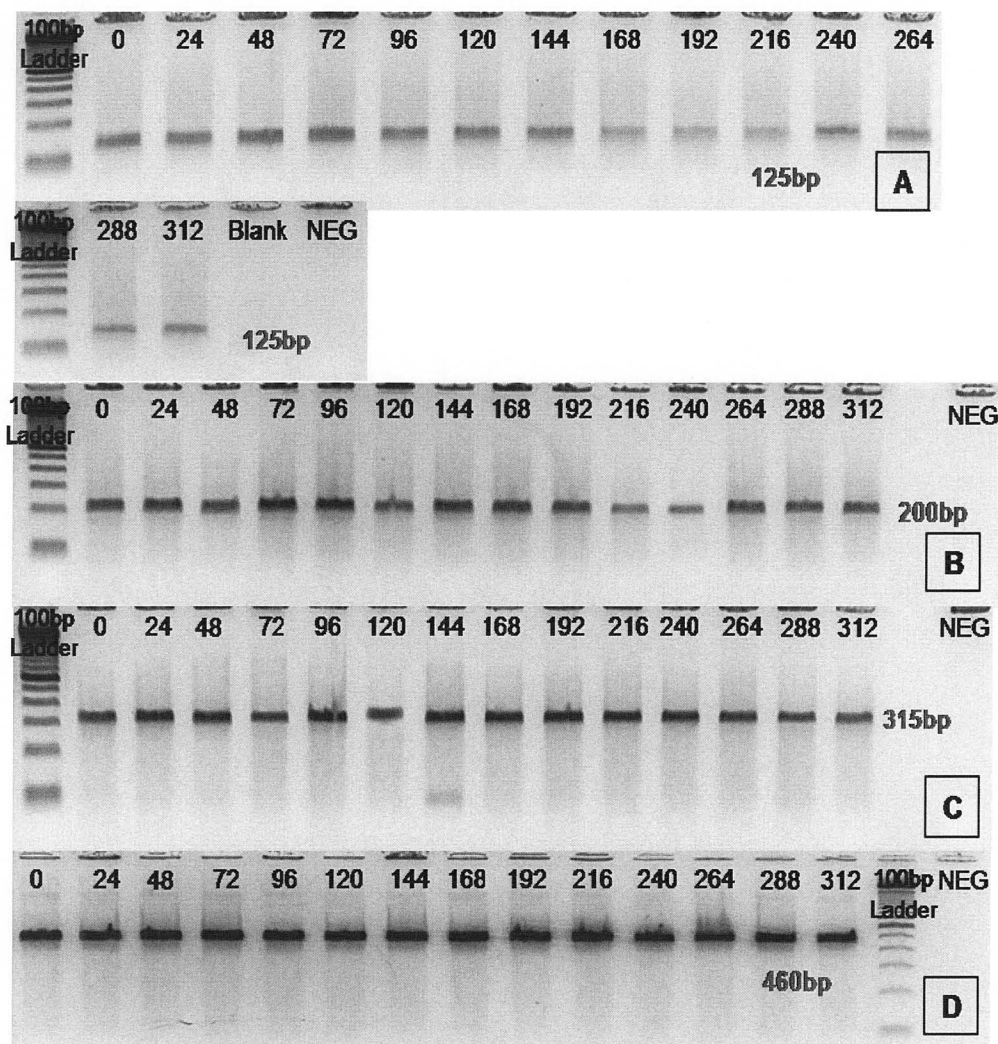


Figure A1 Electrophoresis gels showing samples heated at 50°C that amplified consistently at 125bp (A), 200bp (B), 315bp (C), 460bp (D) and 556bp (E). Wells are labelled with the number of hours heated. Those wells labelled 'Blank' contained no-template extraction controls, while 'NEG' wells contained no-template amplification controls

70°C Heat Treatment:

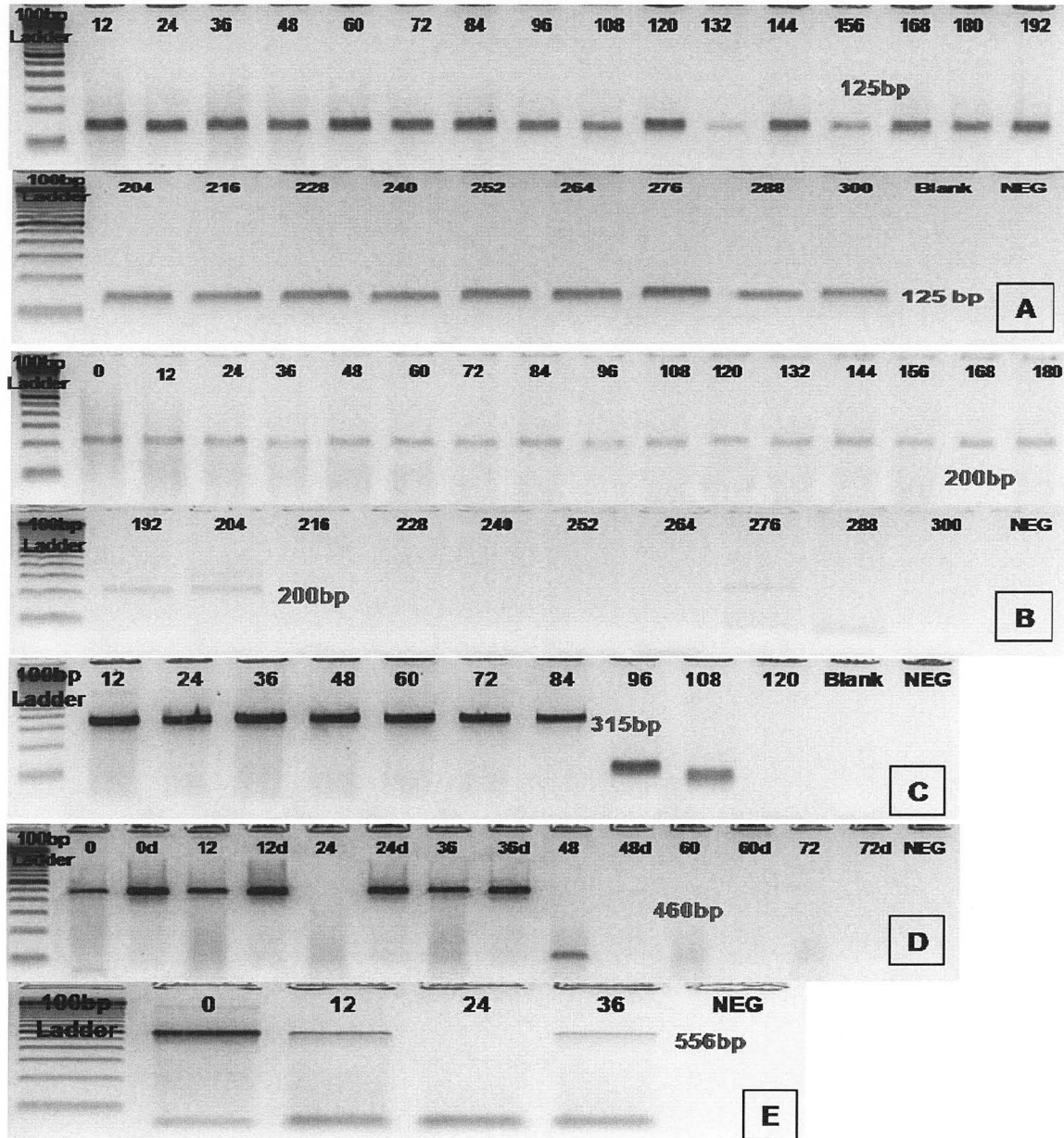


Figure A2 Electrophoresis gels showing samples heated at 70°C that amplified consistently at 125bp (A), 200bp (B), 315bp (C), 460bp (D) and 556bp (E). Wells are labelled with the number of hours heated. Those wells labelled 'Blank' contained no-template extraction controls, while 'NEG' wells contained no-template amplification controls

85°C Heat Treatment:

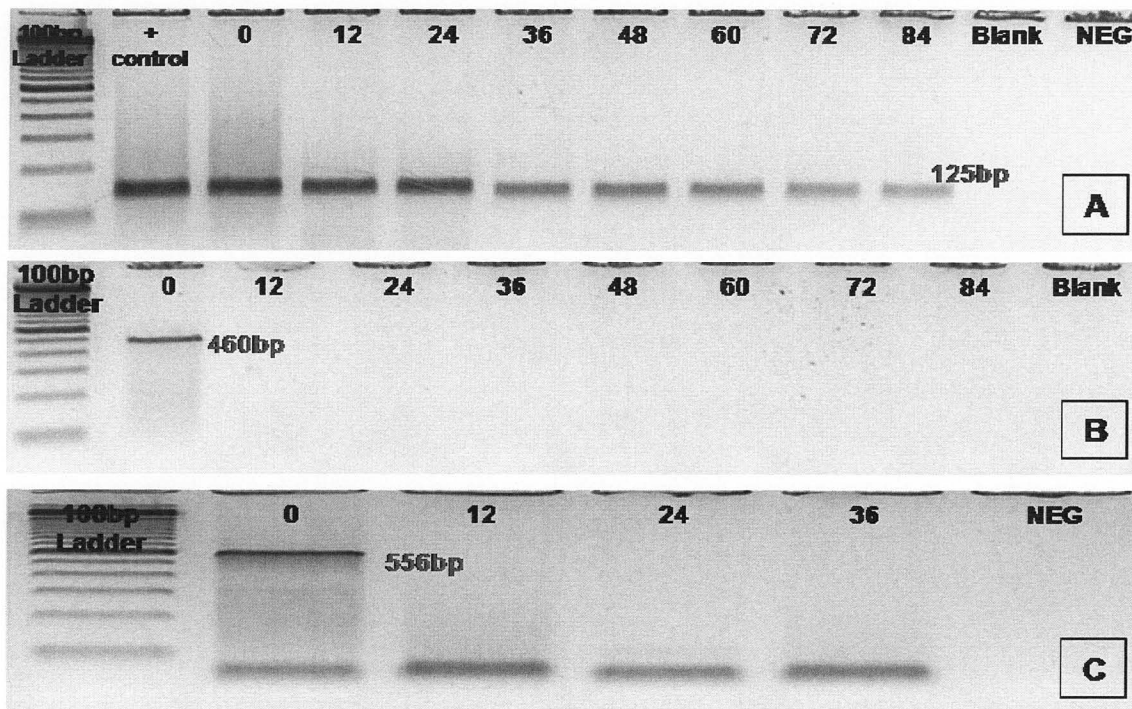


Figure A3 Electrophoresis gels showing 85°C heated samples that amplified consistently at 125bp (A), 460bp (B) and 556bp (C). Wells are labelled with the number of hours heated. Those wells labelled 'Blank' contained no-template extraction controls, while 'NEG' wells contained no-template amplification controls

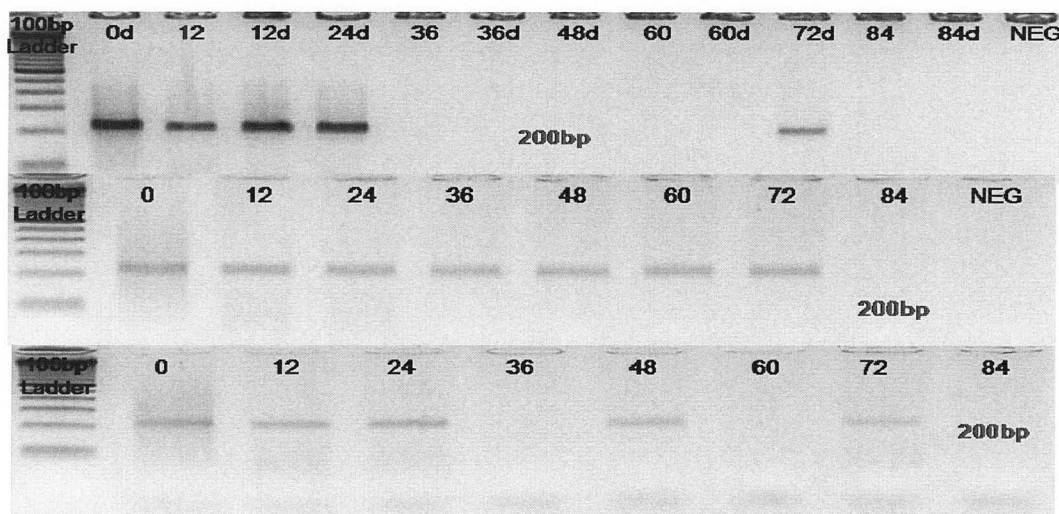


Figure A4 Gel electrophoresis of samples heated at 85°C amplified using OA-rt-F582/OA-rt-R781 primers, showing sporadic amplification of samples after 24 hours. Wells are labelled with the number of hours heated. Those wells labelled 'Blank' contained no-template extraction controls, while 'NEG' wells contained no-template amplification controls

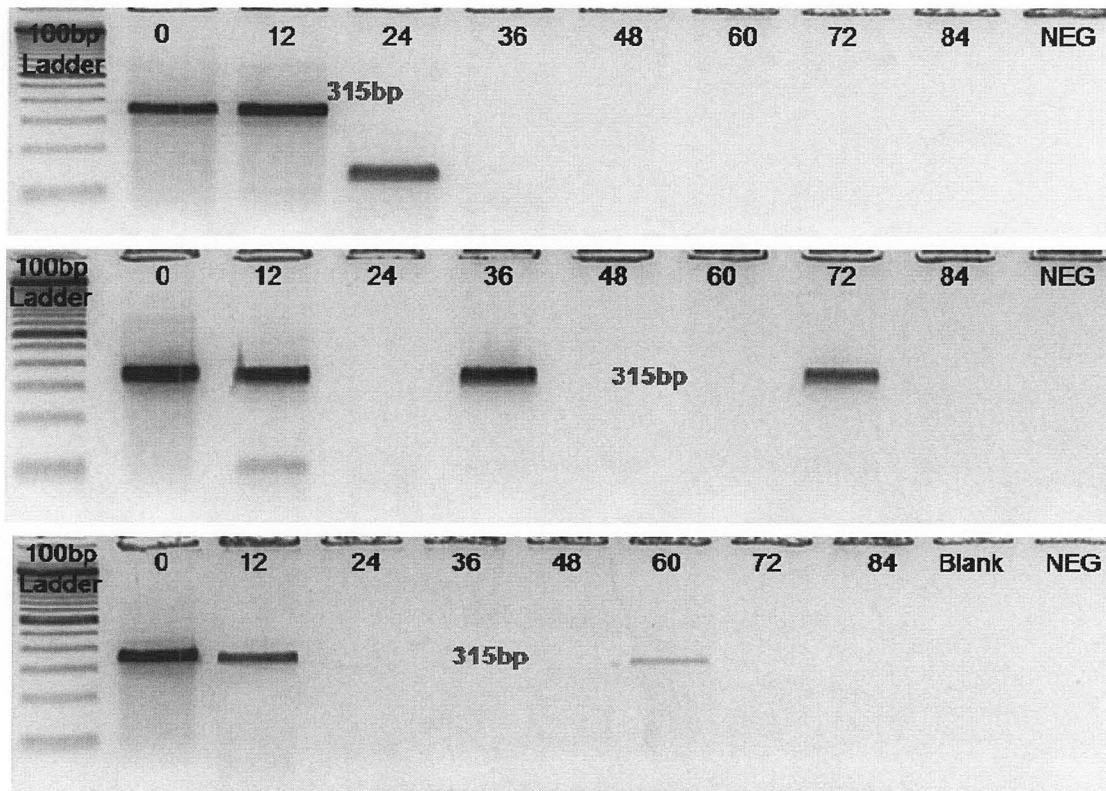


Figure A5 Gel electrophoresis of samples heated at 85°C amplified using F624/R938 primers, showing sporadic amplification of samples after 12 hours of heating. Wells are labelled with the number of hours heated. Those wells labelled 'Blank' contained no-template extraction controls, while 'NEG' wells contained no-template amplification controls

Appendix B – Real-time PCR Data

The following tables contain average template numbers and cycle threshold values for all samples and heat treatments. All reported numbers are averages based on values calculated by the real-time PCR software (StepOne™ Software, version 2.0, Applied Biosystems) from multiple PCR runs. The averages were calculated using both the sample replicates within a single PCR and between duplicate (at least two) PCR runs.

50°C Heat Treatment:

Table B1 Average initial mitochondrial DNA template numbers and cycle thresholds (including value of one standard deviation) for samples heated at 50°C, as determined using real-time PCR.

Heating Time (hours)	0	24	48	72	96	120	144	168	192
Template Number	60378	57999	31456	31121	21861	20095	11006	11189	44375
Standard Deviation (+/-)	8023.29	2981.54	4687.27	5341.84	4919.21	3016.63	1357.88	2142.88	3527.42
C_T	20	20	21	21	22	22	23	23	20
Standard Deviation (+/-)	0.21	0.08	0.22	0.28	0.34	0.24	0.19	0.28	0.13
Heating Time (hours)	216	240	264	288	312				
Template Number	11497	12363	12277	8790	11961				
Standard Deviation (+/-)	2532.58	3291.99	2832.38	1524.06	4505.76				
C_T	23	22	22	23	23				
Standard Deviation (+/-)	0.35	0.47	0.39	0.26	0.7				

Table B2 Average initial nuclear DNA template numbers and cycle thresholds (including value of one standard deviation) for samples heated at 50°C, as determined using real-time PCR.

Heating Time (hours)	0	24	48	72	96	120	144	168	192
Template Number	1695	1431	1072	749	236	479	454	477	272
Standard Deviation (+/-)	454.74	274.92	152.52	120.58	15.83	65.42	77.43	62.89	75.36
C_T	25	25	26	26	28	27	27	27	27
Standard Deviation (+/-)	0.44	0.3	0.22	0.24	0.1	0.2	0.25	0.19	0.44
Heating Time (hours)	216	240	264	288	312				
Template Number	172	105	176	109	211				
Standard Deviation (+/-)	50.28	26.10	52.92	43.13	46.86				
C_T	28	28	28	28	27				
Standard Deviation (+/-)	0.42	0.31	0.45	0.63	0.32				

70°C Heat Treatment:

Table B3 Average initial mitochondrial DNA template numbers and cycle thresholds (including value of one standard deviation) for samples heated at 70°C, as determined using real-time PCR.

Heating Time (hours)	0	12	24	36	48	60	72	84	96	108	120	132	144	156
Template Number	71289	4048	570	1353	109	8	2	1	1	1	0.5	1	2	1
Standard Deviation (+/-)	6288.27	470.47	97.83	162.11	10.88	0.73	0.84	0.19	0.76	0.66	0.27	0.34	0.52	0.76
C_T	20	24	27	26	30	34	36	38	41	40	46	39	36	44
Standard Deviation (+/-)	0.14	0.19	0.27	0.19	0.16	0.15	0.45	0.43	0.71	0.89	0.46	0.21	0.37	0.69
Heating Time (hours)	168	180	192	204	216	228	240	252	264	276	288	300		
Template Number	0.5	2	0.5	0.5	0	0	0	0	0	0	0	0		
Standard Deviation (+/-)	0.44	0.23	0.89	0.67	0	0	0	0	0	0	0	0		
C_T	53	37	47	46	0	0	0	0	0	0	0	0		
Standard Deviation (+/-)	0.63	0.48	0.77	0.83	0	0	0	0	0	0	0	0		

Table B4 Average initial nuclear DNA template numbers and cycle thresholds (including value of one standard deviation) for samples heated at 70°C, as determined using real-time PCR.

Heating Time (hours)	0	12	24	36	48	60	72	84	96	108	120	132 to 300
Template Number	1391	28	3	9	0.5	0	0	0	0	0	0	0
Standard Deviation (+/-)	606.7	6.07	0.84	0.31	0.03	0	0	0	0	0	0	0
C_T	20	24	27	26	30	0	0	0	0	0	0	0
Standard Deviation (+/-)	0.14	0.19	0.27	0.19	0.16	0	0	0	0	0	0	0

85°C Heat Treatment:

Table B5 Average initial mitochondrial DNA template numbers and cycle thresholds (including value of one standard deviation) for samples heated at 85°C, as determined using real-time PCR.

Heating Time (hours)	0	12	24	36	48	60	72	84
Template Number	67475	3	2	1	0.5	0	0.5	0
Standard Deviation (+/-)	7089	0.58	0.38	0.54	0.51	0	0.77	0
C_T	18	34	36	41	43	0	42	0
Standard Deviation (+/-)	0.38	0.38	0.34	0.46	0.39	0	0.69	0

Table B6 Average initial nuclear DNA template numbers and cycle thresholds (including value of one standard deviation) for samples heated at 85°C, as determined using real-time PCR.

Heating Time (hours)	0	12	24	36	48	60	72	84
Template Number	1082	0	0	0	0	0	0	0
Standard Deviation (+/-)	171	0	0	0	0	0	0	0
C_T	28	0	0	0	0	0	0	0
Standard Deviation (+/-)	0.25	0	0	0	0	0	0	0

Appendix C – Sample Information

The following table contains sample reference information for all modern sheep rib samples used in this study.

Heat Treatment	Sample Name	Time Heated (hours)	Bone Powder (g)		Number of Extractions	PCR/rtPCR Repeats	Sequenced
			Heated	Extracted			
50°C* (70°C heat block) 11 samples	T1-0	0	0.0	0.111	2	28/3	D-loop
	T1-6	6	1.0	0.111	1	4	NO
	T1-12	12	1.0	0.111	1	5	NO
	T1-18	18	1.0	0.110	1	4	NO
	T1-24	24	1.0	0.111	2	7	NO
	T1-30	30	1.0	0.111	1	4	NO
	T1-36	36	1.0	0.110	1	5	NO
	T1-42	42	1.0	0.111	1	4	NO
	T1-48	48	1.0	0.111	2	7	NO
	T1-54	54	1.0	0.112	1	4	NO
	T1-60	60	1.0	0.111	1	7	NO
50°C* (70°C heat block) 14 samples	T2-0	0	0.0	0.111	2	6/5	D-loop /AMEL
	T2-24	24	1.0	0.111	2	5/3	NO
	T2-48	48	1.0	0.111	2	18/3	D-loop
	T2-72	72	1.0	0.112	1	30/4	NO
	T2-96	96	1.0	0.111	1	9/3	NO
	T2-120	120	1.0	0.112	1	4/3	NO
	T2-144	144	1.0	0.111	1	30/4	NO
	T2-168	168	1.0	0.112	1	4/2	NO
	T2-192	192	1.0	0.111	1	10/3	D-loop
	T2-216	216	1.0	0.111	1	27/4	NO
	T2-240	240	1.0	0.112	1	6/3	NO
	T2-264	264	1.0	0.111	1	5/3	NO
	T2-288	288	1.0	0.112	1	8/3	NO
T2-312	312	1.0	0.111	1	31/3	AMEL	
70°C ^A (oven) 21 samples	T3-0	0	0.0	0.111	2 ^s	13/2	NO
	T3-72	72	1.0	0.111	2 ^s	8	NO
	T3-144	144	1.0	0.111	2 ^s	9	NO
	T3-216	216	1.0	0.111	2 ^s	8	NO
	T3-288	288	1.0	0.112	1	12	NO
	T3-360	360	1.0	0.111	1	9	NO

Heat Treatment	Sample Name	Time Heated (hours)	Bone Powder (g)		Number of Extractions	PCR/rtPCR Repeats	Sequenced
			Heated	Extracted			
70°C ^Δ (oven – cont'd)	T3-432	432	1.0	0.111	1	8	NO
	T3-504	504	1.0	0.112	1	5	NO
	T3-576	576	1.0	0.113	1	8	NO
	T3-648	648	1.0	0.112	1	7	NO
	T3-720	720	1.0	0.112	1	5	NO
	T3-792	792	1.0	0.111	1	6	NO
	T3-864	864	1.0	0.113	1	9	NO
	T3-936	936	1.0	0.112	1	3	NO
	T3-1008	1008	1.0	0.111	1	2	NO
	T3-1080	1080	1.0	0.113	1	4	NO
	T3-1152	1152	1.0	0.112	1	4	NO
	T3-1224	1224	1.0	0.111	1	2	NO
	T3-1296	1296	1.0	0.111	1	3	NO
	T3-1368	1368	1.0	0.112	1	2	NO
T3-1440	1440	1.0	0.111	1	3	NO	
70°C ^Δ (2 nd extraction of T3 samples)	T4-0	0	0.0	0.111	2 [§]	7/9	NO
	T4-72	72	1.0	0.111	2 [§]	7/8	NO
	T4-144	144	1.0	0.111	2 [§]	7/8	NO
	T4-216	216	1.0	0.112	2 [§]	7/7	NO
70°C ^{ΔΔ} (oven) 26 samples	T5-0	0	0.0	0.111	1	20/6	NO
	T5-12	12	1.0	0.111	1	14/6	NO
	T5-24	24	1.0	0.112	1	25/8	NO
	T5-36	36	1.0	0.112	1	19/7	NO
	T5-48	48	1.0	0.112	1	20/6	NO
	T5-60	60	1.0	0.111	1	14/6	NO
	T5-72	72	1.0	0.111	1	16/9	NO
	T5-84	84	1.0	0.111	1	9/3	NO
	T5-96	96	1.0	0.110	1	19/5	NO
	T5-108	108	1.0	0.110	1	5/3	NO
	T5-120	120	1.0	0.111	1	7/4	NO
	T5-132	132	1.0	0.112	1	4/3	NO
	T5-144	144	1.0	0.111	1	9/7	NO
	T5-156	156	1.0	0.111	1	3/3	NO
	T5-168	168	1.0	0.111	1	9/4	NO
	T5-180	180	1.0	0.110	1	3/3	NO
	T5-192	192	1.0	0.111	1	8/3	NO
	T5-204	204	1.0	0.111	1	4/3	NO
T5-216	216	1.0	0.111	1	12/7	NO	
T5-228	228	1.0	0.111	1	4/3	NO	

Heat Treatment	Sample Name	Time Heated (hours)	Bone Powder (g)		Number of Extractions	PCR/rt-PCR Runs	Sequenced
			Heated	Extracted			
70°C ^{ΔΔ} (oven – cont'd)	T5-240	240	1.0	0.112	1	8/3	NO
	T5-252	252	1.0	0.111	1	10/3	NO
	T5-264	264	1.0	0.111	1	10/3	NO
	T5-276	276	1.0	0.110	1	6/3	NO
	T5-288	288	1.0	0.112	1	13/4	NO
	T5-300	300	1.0	0.111	1	14/3	NO
85°C (oven) 8 samples	T6-0	0	0.0	0.112	1	18/5	NO
	T6-12	12	1.0	0.111	1	15/4	NO
	T6-24	24	1.0	0.112	1	17/5	NO
	T6-36	36	1.0	0.111	1	11/4	NO
	T6-48	48	1.0	0.111	1	12/5	NO
	T6-60	60	1.0	0.112	1	12/4	NO
	T6-72	72	1.0	0.111	1	11/5	NO
	T6-84	84	1.0	0.112	1	10/4	NO

* samples heated in the heat block set at 70°C, but which actually only reached 50°C.

^Δ first set of samples heated in the laboratory oven at 70°C, prior to realization that heat block samples did not actually reach 70°C.

^{ΔΔ} samples heated in the laboratory oven at 70°C after it was discovered the heat block samples did not reach 70°C.

[§] indicates direct re-extraction of previously extracted samples, done to test for extraction failure.

Reference List

- Adams, P. S.
2006 Data analysis and reporting. In *Real-time PCR*, edited by M. T. Dorak, pp. 40-62. Taylor & Francis Group, New York.
- Alaeddini, R., S. J. Walsh and A. Abbas
2010 Forensic implications of genetic analyses from degraded DNA: A review. *Forensic Science International: Genetics* 4(3):148-157.
- Alonso, A., P. Martín, C. Albarrán, P. García, O. García, L. F. de Simón, J. García-Hirschfeld, M. Sancho, C. de la Rúa and J. Fernández-Piqueras
2004 Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Science International* 139(2-3):141-149.
- Anchordoquy, T. J. and M. C. Molina
2007 Preservation of DNA. *Cell Preservation Technology* 5(4):180-188.
- Andréasson, H., U. Gyllensten and M. Allen
2002 Real-Time DNA quantification of nuclear and mitochondrial DNA in forensic analysis *BioTechniques* 33(2):402-411.
- Bada, J. L., X. S. Wang and H. Hamilton
1999 Preservation of key biomolecules in the fossil record: Current knowledge and future challenges. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 354(1379):77-87.
- Banerjee, M. and T. A. Brown
2004 Non-random DNA damage resulting from heat treatment: Implications for sequence analysis of ancient DNA. *Journal of Archaeological Science* 31(1):59-63.
- Binladen, J., C. Wiuf, M. T. P. Gilbert, M. Bunce, R. Barnett, G. Larson, A. D. Greenwood, J. Haile, S. Y. W. Ho, A. J. Hansen and E. Willerslev
2006 Assessing the fidelity of ancient DNA sequences amplified from nuclear genes. *Genetics* 172(2):733-741.
- Bollongino, R., A. Tresset and J.-D. Vigne
2008 Environment and excavation: Pre-lab impacts on ancient DNA analyses. *Comptes Rendus Palevol* 7(2-3):91-98.

- Bollongino, R. and J.-D. Vigne
 2008 Temperature monitoring in archaeological animal bone samples in the Near East arid area, before, during and after excavation. *Journal of Archaeological Science* 35(4):873-881.
- Bouwman, A. S. and T. A. Brown
 2002 Comparison between silica-based methods for the extraction of DNA from human bones from 18th to mid-19th century London. *Ancient Biomolecules* 4(4):173-178.
- Bouwman, A. S., E. R. Chilvers, K. A. Brown and T. A. Brown
 2006 Brief communication: Identification of the authentic ancient DNA sequence in a human bone contaminated with modern DNA. *American Journal of Physical Anthropology* 131(3):428-431.
- Brotherton, P., P. Endicott, J. J. Sanchez, M. Beaumont, R. Barnett, J. Austin and A. Cooper
 2007 Novel high-resolution characterization of ancient DNA reveals C > U-type base modification events as the sole cause of post mortem miscoding lesions. *Nucleic Acids Research* 35(17):5717-5728.
- Burger, J., S. Hummel, B. Hermann and W. Henke
 1999 DNA preservation: A microsatellite-DNA study on ancient skeletal remains. *Electrophoresis* 20(8):1722-1728.
- Capelli, C., F. Tschentscher and V. L. Pascali
 2003 "Ancient" protocols for the crime scene?: Similarities and differences between forensic genetics and ancient DNA analysis. *Forensic Science International* 131(1):59-64.
- Cattaneo, C., S. DiMartino, S. Scali, O. E. Craig, M. Grandi and R. J. Sokol
 1999 Determining the human origin of fragments of burnt bone: A comparative study of histological, immunological and DNA techniques. *Forensic Science International* 102(2):181-191.
- Collins, M. J., A. Angerbjorn and K. Liden
 2002 Bone preservation and DNA amplification. *Archaeometry* 44(3):395-404.
- Collins, M. J., E. R. Waite and A. C. T. van Duin
 1999 Predicting protein decomposition: The case of aspartic acid racemization kinetics. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 354(1379):51-64.
- Deagle, B. E., J. P. Eveson and S. N. Jarman
 2006 Quantification of damage in DNA recovered from highly degraded samples: A case study on DNA in faeces. *Frontiers of Zoology* 3:11-21.

- Di Bernardo, G., S. Del Gaudio, M. Cammarota, U. Galderisi, A. Cascino and M. Cipollaro
 2002 Enzymatic repair of selected cross-linked homoduplex molecules enhances nuclear gene rescue from Pompeii and Herculaneum remains. *Nucleic Acids Research* 30(4):e16.
- Dobberstein, R. C., J. Huppertz, N. von Wurmb-Schwark and S. Ritz-Timme
 2008 Degradation of biomolecules in artificially and naturally aged teeth: Implications for age estimation based on aspartic acid racemization and DNA analysis. *Forensic Science International* 179(2-3):181-191.
- Durham, S. E. and P. F. Chinnery
 2006 Mitochondrial DNA analysis. In *Real-time PCR*, edited by M. T. Dorak, pp. 183-204. Taylor & Francis Group, New York.
- Elbaum, R., C. Melamed-Bessudo, E. Boaretto, E. Galili, S. Lev-Yadun, A. A. Levy and S. Weiner
 2006 Ancient olive DNA in pits: Preservation, amplification and sequence analysis. *Journal of Archaeological Science* 33(1):77-88.
- Ellison, S., C. English, M. Burns and J. Keer
 2006 Routes to improving the reliability of low level DNA analysis using real-time PCR. *BMC Biotechnology* 6(1):33-44.
- Foran, D. R.
 2006 Relative degradation of nuclear and mitochondrial DNA: An experimental approach. *Journal of Forensic Sciences* 51(4):766-770.
- Geigl, E. M.
 2002 On the circumstances surrounding the preservation and analysis of very old DNA. *Archaeometry* 44(3):337-342.
- Gilbert, M. T. P., A. J. Hansen, E. Willerslev, L. Rudbeck, I. Barnes, N. Lynnerup and A. Cooper
 2003 Characterization of genetic miscoding lesions caused by postmortem damage. *The American Journal of Human Genetics* 72(1):48-61.
- Gilbert, M. T. P., B. Shapiro, A. Drummond and A. Cooper
 2005 Post-mortem DNA damage hotspots in Bison (*Bison bison*) provide evidence for both damage and mutational hotspots in human mitochondrial DNA. *Journal of Archaeological Science* 32(7):1053-1060.
- Giles, R. J. and T. A. Brown
 2008 Improved methodology for extraction and amplification of DNA from single grains of charred wheat. *Journal of Archaeological Science* 35(9):2585-2588.

- Golenberg, E. M., A. Bickel and P. Weihs
1996 Effect of highly fragmented DNA on PCR. *Nucleic Acids Research* 24(24):5026-5033.
- Götherström, A., M. J. Collins, A. Angerbjörn and K. Liden
2002 Bone preservation and DNA amplification. *Archaeometry* 44(3):395-404.
- Gugerli, F., L. Parducci and R. J. Petit
2005 Ancient plant DNA: Review and prospects. *New Phytologist* 166(2):409-418.
- Haile, J., R. Holdaway, K. Oliver, M. Bunce, M. T. Gilbert, R. Nielsen, K. Munch, S. Y. Ho, B. Shapiro and E. Willerslev
2007 Ancient DNA chronology within sediment deposits: Are paleobiological reconstructions possible and is DNA leaching a factor? *Molecular Biology and Evolution* 24(4):982-989.
- Hagelberg, E. and J. B. Clegg
1991 Isolation and characterization of DNA from archaeological bone. *Proceedings: Biological Sciences* 244(1309):45-50.
- Handt, O., M. Höss, M. Krings and S. Pääbo
1994 Ancient DNA: Methodological challenges. *Cellular and Molecular Life Sciences* 50(6):524-529.
- Hansen, A. J., D. L. Mitchell, C. Wiuf, L. Paniker, T. B. Brand, J. Binladen, D. A. Gilichinsky, R. Ronn and E. Willerslev
2006 Crosslinks rather than strand breaks determine access to ancient DNA sequences from frozen sediments. *Genetics* 173(2):1175-9.
- Hansson, M. C. and B. P. Foley
2008 Ancient DNA fragments inside Classical Greek amphoras reveal cargo of 2400-year-old shipwreck. *Journal of Archaeological Science* 35(5):1169-1176.
- Haynes, S., J. B. Searle, A. Bretman and K. M. Dobney
2002 Bone preservation and ancient DNA: The application of screening methods for predicting DNA survival. *Journal of Archaeological Science* 29(6):585-592.
- Hebsgaard, M. B., M. J. Phillips and E. Willerslev
2005 Geologically ancient DNA: fact or artefact? *Trends in Microbiology* 13(5):212-220.

- Heid, C. A., J. Stevens, K. J. Livak and P. M. Williams
1996 Real time quantitative PCR. *Genome Research* 6(10):986-994.
- Higuchi, R., B. Bowman, M. Freiberger, O. A. Ryder and A. C. Wilson
1984 DNA sequences from the quagga, an extinct member of the horse family. *Nature* 312(5991):282-284.
- Hofreiter, M., H. N. Poinar, W. G. Spaulding, K. Bauer, P. S. Martin, G. Possnert and S. Pääbo
2000 A molecular analysis of ground sloth diet through the last glaciation. *Molecular Ecology* 9(12):1975-1984.
- Hofreiter, M., D. Serre, H. N. Poinar, M. Kuch and S. Pääbo
2001 Ancient DNA. *Nature Reviews: Genetics* 2(5):353-359.
- Hoss, M., P. Jaruga, T. H. Zastawny, M. Dizdaroglu and S. Pääbo
1996 DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Research* 24(7):1304-1307.
- Hummel, S.
2003 *Ancient DNA Typing: Methods, strategies and applications*. Springer, Germany.
- Hunter, P.
2006 Ancient DNA research goes nuclear. *EMBO Reports* 7(2):136-139.
- Iniguez, A. M., A. Araujo, L. F. Ferreira and A. C. Vicente
2003 Analysis of ancient DNA from coprolites: A perspective with random amplified polymorphic DNA-polymerase chain reaction approach. *Memorias do Instituto Oswaldo Cruz* 98 Supplement 1:63-65.
- Kaestle, F. A. and K. A. Horsburgh
2002 Ancient DNA in anthropology: Methods, applications, and ethics. *American Journal of Physical Anthropology* 119(S35):92-130.
- Karanth, K. P. and A. D. Yoder
2010 Geographic origins of ancient DNA samples and prospects for ancient DNA studies in the tropics. In *Molecular Markers, PCR, Bioinformatics and Ancient DNA: Troubleshooting and Applications.*, edited by G. Dorado. In Press. Science Publishers, New York.
- Kumar, S. S., N. Ivane, S. R. Walimbe and S. Mark
2000 Brief communication: Discouraging prospects for ancient DNA from India. *American Journal of Physical Anthropology* 113(1):129-133.

- Lindahl, T.
1993 Instability and decay of the primary structure of DNA. *Nature* 362:709-715.
- MacHugh, D. E., C. J. Edwards, J. F. Bailey, D. R. Bancroft and D. G. Bradley
2000 The extraction and analysis of ancient DNA from bone and teeth: A survey of current methodologies. *Ancient Biomolecules* 3(2):81-102.
- Malmstrom, H., E. M. Svensson, M. T. Gilbert, E. Willerslev, A. Götherström and G. Holmlund
2007 More on contamination: The use of asymmetric molecular behavior to identify authentic ancient human DNA. *Molecular Biology and Evolution* 24(4):998-1004.
- Marota, I., C. Basile, M. Ubaldi and F. Rollo
2002 DNA decay rate in papyri and human remains from Egyptian archaeological sites. *American Journal of Physical Anthropology* 117(4):310-318.
- Miloš, A., A. Selmanović, L. Smajlović, R. L. M. Huel, C. Katzmarzyk, A. Rizvić and T. J. Parsons
2007 Success rates of nuclear short tandem repeat typing from different skeletal elements. *Croatian Medical Journal* 48(4):486-493.
- Misner, L. M., A. C. Halvorson, J. L. Dreier, D. H. Ubelaker and D. R. Foran
2009 The correlation between skeletal weathering and DNA quality and quantity. *Journal of Forensic Sciences* 54(4):822-828.
- Mitchell, D., E. Willerslev and A. Hansen
2005 Damage and repair of ancient DNA. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 571:265-276.
- Morin, P. A., N. M. Hedrick, K. M. Robertson and C. A. Leduc
2007 Comparative mitochondrial and nuclear quantitative PCR of historical marine mammal tissue, bone, baleen, and tooth samples. *Molecular Ecology Notes* 7:404-411.
- Nicklas, J. and E. Buel
2003 Quantification of DNA in forensic samples. *Analytical and Bioanalytical Chemistry* 376(8):1160-1167.
- O'Donoghue, K., A. Clapham, R. P. Evershed and T. A. Brown
1996 Remarkable preservation of biomolecules in ancient radish seeds. *Proceedings: Biological Sciences* 263(1370):541-547.

- Orlando, C., P. Pinzani and M. Pazzagli
1998 Developments in quantitative PCR. *Clinical Chemistry and Laboratory Medicine* 36(5):255-269.
- O'Rourke, D. H., M. G. Hayes and S. W. Carlyle
2000 Ancient DNA studies in physical anthropology. *Annual Review of Anthropology* 29(1):217-242.
- Ottoni, C., H. Koon, M. Collins, K. Penkman, O. Rickards and O. Craig
2009 Preservation of ancient DNA in thermally damaged archaeological bone. *Naturwissenschaften* 96(2):267-278.
- Ovchinnikov, I. V., A. Götherström, G. P. Romanova, V. M. Kharitonov, K. Liden and W. Goodwin
2001 Reply: Not just old but old and cold? *Nature* 410(6830):772.
- Pääbo, S., D. M. Irwin and A. C. Wilson
1990 DNA damage promotes jumping between templates during enzymatic amplification. *Journal of Biological Chemistry* 265(8):4718-4721.
- Pääbo, S., H. Poinar, D. Serre, V. Jaenicke-Despres, J. Hebler, N. Rohland, M. Kuch, J. Krause, L. Vigilant and M. Hofreiter
2004 Genetic analyses from ancient DNA. *Annual Review of Genetics* 38(1):645-679.
- Parducci, L., Y. Suyama, M. Lascoux and K. D. Bennett
2005 Ancient DNA from pollen: A genetic record of population history in Scots pine. *Molecular Ecology* 14(9):2873-2882.
- Poinar, H., M. Kuch, G. McDonald, P. Martin and S. Pääbo
2003 Nuclear gene sequences from a Late Pleistocene sloth coprolite. *Current Biology* 13(13):1150-1152.
- Poinar, H. N.
2003 The top 10 list: Criteria of authenticity for DNA from ancient and forensic samples. *International Congress Series* 1239:575-579.
- Poinar, H. N., M. Hofreiter, W. G. Spaulding, P. S. Martin, B. A. Stankiewicz, H. Bland, R. P. Evershed, G. Possnert and S. Pääbo
1998 Molecular coproscopy: Dung and diet of the extinct ground sloth, *Nothrotheriops shastensis*. *Science* 281(5375):402-406.
- Poinar, H. N. and B. A. Stankiewicz
1999 Protein preservation and DNA retrieval from ancient tissues. *Proceedings of the National Academy of Science USA* 96(15):8426-8431.

- Poulakakis, N., A. Tselikas, I. Bitsakis, M. Mylonas and P. Lymberakis
2007 Ancient DNA and the genetic signature of ancient Greek manuscripts. *Journal of Archaeological Science* 34(5):675-680.
- Pruvost, M. and E.-M. Geigl
2004 Real-time quantitative PCR to assess the authenticity of ancient DNA amplification. *Journal of Archaeological Science* 31(9):1191-1197.
- Pruvost, M., T. Grange and E. M. Geigl
2005 Minimizing DNA contamination by using UNG-coupled quantitative real-time PCR on degraded DNA samples: Application to ancient DNA studies. *BioTechniques* 38(4):569-575.
- Pruvost, M., R. Schwarz, V. B. Correia, S. Champlot, S. Braguier, N. Morel, Y. Fernandez-Jalvo, T. Grange and E. M. Geigl
2007 Freshly excavated fossil bones are best for amplification of ancient DNA. *Proceedings of the National Academy of Science USA* 104(3):739-744.
- Pruvost, M., R. Schwarz, V. B. Correia, S. Champlot, T. Grange and E.-M. Geigl
2008 DNA diagenesis and palaeogenetic analysis: Critical assessment and methodological progress. *Palaeogeography, Palaeoclimatology, Palaeoecology* 266(3-4):211-219.
- Pusch, C. M., I. Giddings and M. Scholz
1998 Repair of degraded duplex DNA from prehistoric samples using *Escherichia coli* DNA polymerase I and T4 DNA ligase. *Nucleic Acids Research* 26(3):857-859.
- Reed, F. A., E. J. Kontanis, K. A. Kennedy and C. F. Aquadro
2003 Brief communication: Ancient DNA prospects from Sri Lankan highland dry caves support an emerging global pattern. *American Journal of Physical Anthropology* 121(2):112-116.
- Rennick, S. L., T. W. Fenton and D. R. Foran
2005 The effects of skeletal preparation techniques on DNA from human and non-human bone. *Journal of Forensic Sciences* 50(5):1016-1019.
- Rohland, N. and M. Hofreiter
2007a Ancient DNA extraction from bones and teeth. *Nature Protocols* 2(7):1756-1762.
2007b Comparison and optimization of ancient DNA extraction. *BioTechniques* 42:343-352.

- Rollo, F., M. Ubaldi, L. Ermini and I. Marota
2002 Otzi's last meals: DNA analysis of the intestinal content of the Neolithic glacier mummy from the Alps. *Proceedings of the National Academy of Sciences* 99(20):12594-12599.
- Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich
1988 Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239(4839):487-491.
- Salamon, M., N. Tuross, B. Arensburg and S. Weiner
2005 Relatively well preserved DNA is present in the crystal aggregates of fossil bones. *Proceedings of the National Academy of Sciences* 102(39):13783-13788.
- Schlumbaum, A., M. Tensen and V. Jaenicke-Després
2008 Ancient plant DNA in archaeobotany. *Vegetation History and Archaeobotany* 17(2):233-244.
- Schwarz, C., R. Debruyne, M. Kuch, E. McNally, H. Schwarcz, A. D. Aubrey, J. Bada and H. Poinar
2009 New insights from old bones: DNA preservation and degradation in permafrost preserved mammoth remains. *Nucleic Acids Research* 37(10):3215-3229.
- Shapiro, B. and A. Cooper
2003 Beringia as an Ice Age genetic museum. *Quaternary Research* 60(1):94-100.
- Shedlock, A. M., M. G. Haygood, T. W. Pietsch and P. Bentzen
1997 Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin-fixed museum specimens. *BioTechniques* 22(3):394-400.
- Shiple, G. L.
2006 An introduction to real-time PCR. In *Real-time PCR*, edited by M. T. Dorak, pp. 1-31. Taylor & Francis Group, New York.
- Smith, C. I., A. T. Chamberlain, M. S. Riley, A. Cooper, C. B. Stringer and M. J. Collins
2001 Neanderthal DNA: Not just old but old and cold? *Nature* 410(6830):771-772.
- Smith, C. I., A. T. Chamberlain, M. S. Riley, C. Stringer and M. J. Collins
2003 The thermal history of human fossils and the likelihood of successful DNA amplification. *Journal of Human Evolution* 45(3):203-217.

- Smith, S. and P. A. Morin
2005 Optimal storage conditions for highly dilute DNA samples: A role for trehalose as a preserving agent. *Journal of Forensic Sciences* 50(5):1101-1108.
- Smith, S., L. Vigilant and P. A. Morin
2002 The effects of sequence length and oligonucleotide mismatches on 5' exonuclease assay efficiency. *Nucleic Acids Research* 30(20):e111.
- Stuart, B. L., K. A. Dugan, M. W. Allard and M. Kearney
2006 Extraction of nuclear DNA from bone of skeletonized and fluid-preserved museum specimens. *Systematics and Biodiversity* 4(2):133-136.
- Swango, K. L., M. D. Timken, M. D. Chong and M. R. Buoncristiani
2006 A quantitative PCR assay for the assessment of DNA degradation in forensic samples. *Forensic Science International* 158(1):14-26.
- Threadgold, J. and T. A. Brown
2003 Degradation of DNA in artificially charred wheat seeds. *Journal of Archaeological Science* 30(8):1067-1076.
- Tuross, N.
1994 The biochemistry of ancient DNA in bone. *Cellular and Molecular Life Sciences* 50(6):530-535.
- von Wurmb-Schwark, N., M. Harbeck, U. Wiesbrock, I. Schroeder, S. Ritz-Timme and M. Oehmichen
2003 Extraction and amplification of nuclear and mitochondrial DNA from ancient and artificially aged bones. *Legal Medicine* 5 Supplement 1:S169-S172.
- von Wurmb-Schwark, N., T. Schwark, M. Harbeck and M. Oehmichen
2004 A simple Duplex-PCR to evaluate the DNA quality of anthropological and forensic samples prior short tandem repeat typing. *Legal Medicine* 6(2):80-88.
- Wandeler, P., P. E. A. Hoeck and L. F. Keller
2007 Back to the future: Museum specimens in population genetics. *Trends in Ecology & Evolution* 22(12):634-642.
- Wandeler, P., S. Smith, P. A. Morin, R. A. Pettifor and S. M. Funk
2003 Patterns of nuclear DNA degeneration over time: A case study in historic teeth samples. *Molecular Ecology* 12:1087-1093.
- Weikard, R., C. Pitra and C. Kühn
2006 Amelogenin cross-amplification in the family Bovidae and its application for sex determination. *Molecular Reproduction and Development* 73(10):1333-1337.

- Willerslev, E. and A. Cooper
2005 Ancient DNA. *Proceedings: Biological Sciences* 272(1558):3-16.
- Yang, D. Y., B. Eng, J. S. Wayne, J. C. Dудар and S. R. Saunders
1998 Improved DNA extraction from ancient bones using silica-based spin columns. *American Journal of Physical Anthropology* 105(4):539-543.
- Yang, D. Y. and K. Watt
2005 Contamination controls when preparing archaeological remains for ancient DNA analysis. *Journal of Archaeological Science* 32(3):331-336.
- Zhang, L. and Q. Wu
2005 Single gene retrieval from thermally degraded DNA. *Journal of Biosciences* 30(5):599-604.
- Zimmermann, J., M. Hajibabaei, D. Blackburn, J. Hanken, E. Cantin, J. Posfai and T. Evans
2008 DNA damage in preserved specimens and tissue samples: A molecular assessment. *Frontiers in Zoology* 5(1):18.