MAKE NEW METHODS BUT KEEP THE OLD:

Exploring the use of DNA analysis to study stress in past populations

by

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ABSTRACT

The main aim of this project is to develop and adapt a method currently used in clinical research, DNA methylation analysis of a gene target, to assess stress in past human populations using ancient DNA (aDNA). Specifically, this work will focus on three goals: a) extracting and purifying aDNA from an archaeological source, b) developing a method to amplify and sequence the NR3C1 gene (which encodes the glucocorticoid receptor), including the design and testing of primers that successfully target the gene and optimizing a PCR protocol for use with the primers, and c) applying this method to aDNA extracted from archaeological sources. This preliminary work will allow future researchers to assess differences in methylation levels of NR3C1 and to ascertain whether methylation of that locus can be used to gain additional information on stress in past populations (González Ramírez et al., 2020; Liu & Nusslock, 2017; McGowan et al., 2009).

DEDICATION

This thesis is dedicated to:

Philip Carman Albanese (1927 – 2008)

and to

Andrea Veronique Obulata-Ezeleche, née Allaire (1987 – 2018)

I wish you both could have been here.

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CHAPTER 1: INTRODUCTION

1.1 Purpose

This research occurs at the intersection of anthropology and genetics, in particular bioarchaeology and epigenetics. Bioarchaeology focuses on the study of human remains in the archaeological record and aims to interpret human remains to better understand the lives of those in the past, while epigenetics strives to understand how changes to the DNA structure that do not involve modification of the sequence are created and how they affect gene function (Mazzio & Soliman, 2012; Temple & Goodman, 2014). These two fields are combined in this project because of the insights that can be gained from doing so, namely using established epigenetic relationships between lived experiences and genomic (genetic) information to better understand the past. Understanding the lived experience is a main focus in bioarchaeology research, but existing avenues to explore it are limited. Epigenetics may offer a new avenue of exploration through which bioarchaeologists can learn more about the lived experience through the epigenetic imprints that stressful events can leave on our DNA (Gokhman et al., 2016; Zhenilo et al., 2016).

Stress research can reveal information about patterns of poor health and allow bioarchaeologists to better understand life histories, migration patterns, resource scarcity, and the impact of natural disasters such as famine and disease (Klaus, 2014; Kyle et al., 2020). However, the ability of researchers, whether bioarchaeologists or epigeneticists, to assess the experience of early-life stress in past populations is severely limited compared to modern populations. Stress research is an intrinsic part of health research and can help

researchers understand various internal and external factors affecting health, the ability of people in the past to contend with these factors, and particularly the effect of early-life stress on later life morbidity and mortality (Edinborough & Rando, 2020; Foley, 2020). In short, understanding stress is fundamental to understanding the lives of people in the past, just as it is fundamental to understanding peoples' lives now. Stress experienced early in life can be especially informative about later life health challenges. However, where modern stress research can ask individuals about their experiences and easily collect DNA samples, the study of stress in the past requires more innovative approaches (Higgins et al., 2015). Bioarchaeologists are further limited since bone is not immediately responsive to stress events. Because it takes time for stress to manifest in the skeleton as observable lesions or growth disruptions (Ortner, 2003), it will more quickly affect the DNA structure of certain genes (Watkeys et al., 2018). Recently, bioarchaeologists have developed molecular approaches to answer questions about stress in past populations focusing on ancient proteins (see Scott et al., 2016, 2020; Webb et al., 2010; Webb et al., 2015); however, integrating ancient DNA (aDNA) from human remains has also begun to gain traction in the study of past peoples (see Downes, 2019; Juras et al., 2017; Pääbo, 1985; Slatkin, 2016; Slatkin & Racimo, 2016; Speller, 2018; Weyrich et al., 2017).

1.2 Significance

This research will aim to investigate stress in an innovative way, using a new molecular method to examine signatures of stress in DNA. Specifically, using archaeological tooth samples, aDNA will be extracted, the promoter region of the glucocorticoid receptor gene (NR3C1) will be amplified, and sequenced to examine its methylation levels (glucocorticoids are hormones heavily involved in the stress

response). The NR3C1 locus has been established as an important region for epigenetic regulation and the functioning of the stress response, and this project will allow for the investigation of whether methylation levels of the promoter region can be observed and assessed. Given the strong connection in the clinical literature between methylation of NR3C1 and early-life stress (see Liu & Nusslock, 2017; McGowan et al., 2009; Palma-Gudiel et al., 2015; Turecki & Meaney, 2016; Van Der Knaap et al., 2014; Watkeys et al., 2018), this region is a promising candidate for a new way to assess stress in the past, particularly in individuals who may not show macroscopic signs of stress in their skeletal remains. This study marks the first time an attempt has been made to answer questions about stress in the past using aDNA, as well as the first specific study of the NR3C1 gene in aDNA. Modifying a clinical approach to be used in aDNA research requires an understanding of the unique problems of aDNA; therefore, this study contributes to the literature describing methods for troubleshooting this type of aDNA research and how to gauge the success of various approaches. The application of genetic and biochemical methods to bioarchaeology is fast growing, and this study represents a unique contribution to this interdisciplinary work.

1.3 Objective and specific aims

Modern-day techniques to study early-life or childhood stress have the potential to be adapted for use with past populations, which this research will explore. Researchers looking at the relationships between stress and signatures left on DNA have found that the glucocorticoid receptor gene, in particular, seems to be strongly impacted at the epigenetic levels by early life stressful events (ranging from natural disasters to childhood abuse and trauma) (González Ramírez et al., 2020; Liu & Nusslock, 2017; McGowan et

al., 2009; Palma-Gudiel et al., 2015). The epigenetic changes to the DNA in the glucocorticoid receptor gene may be an accurate proxy for stress experienced in childhood and would represent a novel way to explore periods of duress if identifiable in aDNA samples. Therefore, this project will investigate the following questions:

1. Can aDNA be extracted from 18th century archaeological samples from Atlantic Canada?
2. Can a protocol be developed to amplify the NR3C1 gene in modern DNA?
3. Can this method successfully amplify the NR3C1 gene using aDNA extracted from the archaeological samples?
4. Can bisulfite sequencing performed on aDNA samples produce usable data on the methylation patterns (i.e., stress events) of NR3C1?

1.4 Thesis outline

This thesis contains six chapters. In Chapter One, the purpose and significance of this project has been presented, along with the specific aims and the overall organization of the thesis. Chapter Two is the literature review and will explore the relationship between stress and the stress response, specifically focusing on bioarchaeological approaches. This chapter will also examine the connection between genes and stress, summarize recent literature on the glucocorticoid receptor gene NR3C1, and explore the specific challenges of working with aDNA. Chapter Three outlines the specific protocols used in DNA extraction, primer testing and PCR, and sequencing. The application of these methods to aDNA is then discussed, involving techniques such as agarose gel electrophoresis and Sanger sequencing for DNA analysis and bisulfite sequencing for studying methylation patterns. Chapter Four reviews the results of this project. This

chapter highlights the challenge of low aDNA concentrations in aDNA samples and summarizes the results of the preliminary development and testing of these methods in modern DNA. Chapter Five discusses the results presented in Chapter Four. First, the chapter explores potential causes of the low aDNA concentrations from the six tooth samples, including the possible influence of the burial environment. Next, the chapter discusses the method development and testing using modern DNA, and also addresses the failure of the method to amplify NR3C1 in the aDNA samples. Chapter Six will conclude the thesis by summarizing the content of the previous chapter, and will review the project aims outlined in Chapter One. Finally, the last chapter will also provide some potential directions for future research in this area.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

This literature review will provide the necessary background information on topics relevant to the thesis project, such as stress, genes, and ancient DNA. First, existing bioarchaeological methods of analysing stress will be briefly summarized, with a focus on molecular approaches. Then, the concept of stress and the stress response will be thoroughly explained, including the function of the stress response, stressors, physiological processes, and the roles of the hormone cortisol and the glucocorticoid receptor. Following this will be a section introducing key genetic terminology needed to discuss the gene under study, NR3C1, and exploring the connection between NR3C1 and the experience of stress during the life course. A section on ancient DNA will explain particular issues pertaining to the study of DNA from an archaeological source, including the study of methylation. Finally, this section will conclude with the geographical and historical context of this study's sample population.

2.2 Stress and the stress response

While the term 'stress' is often used non-specifically, clinical stress can be defined as a disruption of the body's normal functioning (or homeostasis), which may be caused by various factors (stressors) that can have numerous impacts on the body (Chrousos, 2009). In bioarchaeology, stress has been defined as a "physiological change caused by strain on an organism from environmental, nutritional, and other pressures" (Reitsema & McIlvaine, 2014, p. 181). This section will briefly summarize bioarchaeological approaches to stress; describe stressors, or the factors which cause

stress; explain the function of the stress response; detail the process of the stress response; and include the significant role of the hormone cortisol.

2.2.1 Bioarchaeological approaches to stress

Archaeologists who are interested in how past populations experienced stress primarily focus on macroscopic osteological observation. They look for characteristic lesions on the bones that result from the activation of the body's stress response system (e.g., Armelagos et al., 2009; Gamble et al., 2017; Kyle et al., 2020; Smith et al., 2018; Sołtysiak, 2015), such as cribra orbitalia and porotic hyperostosis (porosity of the cortical tissue in the eye orbit and back of the skull, resulting from nonspecific stressors), dental enamel hypoplasias (growth arrest lines in the enamel resulting from periods of illness or malnutrition), and periosteal new bone growth (reactive bone formation resulting from inflammation or trauma to the membrane surrounding bone) (Ortner, 2003). However, bone is not immediately sensitive to the activation of the stress response, and not all individuals who experience chronic or acute stress will have macroscopic skeletal evidence of the physiological stress response (Berger et al., 2019). To address this issue, bioarchaeologists have recently attempted to measure stress by assessing molecules produced as part of the stress response, such as the hormone cortisol and the protein osteocalcin (see Collins et al., 2005; Scott et al., 2016, 2020; Webb et al., 2010; Webb et al., 2015). These products, which have been measured in teeth, bone, and hair, are not macroscopically observable and must be measured through biochemical methods. By examining effects of the stress response that occur more quickly than lesions on the bone and teeth, the experience of stress can be more sensitively assessed. According to this

rationale, DNA, as a stable molecule that does undergo changes related to stress, offers a promising target for research.

2.2.2 Purpose of the stress response

The stress response is an adaptive mechanism that enables the body to appropriately respond to environmental threats (Chrousos, 2009; Edinborough & Rando, 2020; Palma-Gudiel et al., 2015). When the stress response system is activated, it puts certain maintenance activities such as digestion or growth on hold, to better distribute resources and energy to vital systems associated with the 'fight or flight' response (i.e., respiration, muscle tension). When more energy is available to fuel these vital processes, the individual is better able to respond to the immediate threat and ensure safety. While these reactions to threats are beneficial in the moment, over the long term the constant activation of the stress response system can result in a chronic disruption to biological homeostasis, which is connected to several health concerns including: decreased bone mineral density, periodontal disease, decreased cognitive function, autoimmune disorders, depression, anxiety, and polycystic ovarian syndrome (PCOS) (Furlan et al., 2005; Gunepin et al., 2018; Marin et al., 2011; Stefanaki et al., 2018).

The factors which cause stress are called 'stressors' and can impact the body's physiological state even if there is only a perceived threat to homeostasis (Chrousos, 2009). Since the threat does not need to be tangible to be harmful, threats can be physiological or psychosocial. Physiological stressors directly threaten the body, such as starvation, injury, or disease. Psychosocial stressors pose threats to social and psychological needs (Kogler et al., 2015), and stressors in this category include social

exclusion, low socioeconomic status, etc. Some stressors are both physiological and psychosocial, such as pregnancy (Kogler et al., 2015; Sapolsky, 2004; Webb et al., 2010).

2.2.3 The physiological stress response

The physiological stress response is a homeostatic process (i.e., aiming to restore homeostasis), and consists of both neural and endocrine adaptations (Chrousos, 2009). It begins within the hypothalamus, which releases a signal to the anterior pituitary, which then signals the adrenal cortex. The adrenal cortex reacts, beginning with an immediate release of the hormone adrenaline, a near-immediate release of glucocorticoid hormones which trigger a cascade of other effects, and ending with a negative feedback loop from the adrenal cortex which stops the signals from the hypothalamus and pituitary (Chrousos, 2009; Oakley & Cidlowski, 2013; Sapolsky, 2004). This collective web of interactions is called the hypothalamic-pituitary-adrenal (HPA) axis, or colloquially as the 'fight or flight' response. According to Sapolsky et al. (2004), most of the compounds released during the activation of the stress response system are glucocorticoids, including cortisol and hydrocortisone. The hormone cortisol plays a key role in the stress response (Adam et al., 2017; Chrousos, 2009; Schür et al., 2018; Zhang & Tian, 2017), influencing the immune system, circadian rhythms, and contributing to physical and mental illnesses, such as cardiovascular disease, depression, fatigue, and general mortality (Adam et al., 2017). Although both hormones play a role in the stress response, only cortisol has been studied to date in an archaeological context, so the next section will focus on cortisol alone.

2.2.4 Role of cortisol in the stress response

The role of cortisol in the stress response is to stimulate the breakdown of molecules like glycogen, which frees up stored energy in response to the immediate stressor (Meaney et al., 1996). However, when levels of cortisol remain too high for too long, these 'breaking down' activities begin to harm the body, specifically the skeleton. Clinical studies have shown that cortisol produced from the body's own stress response is responsible for decreases in bone density, leading to bone diseases such as osteoporosis (Belaya et al., 2018; Furlan et al., 2005; Kim et al., 2018). Cortisol can also have a long-term effect on bone formation and remodelling through its effects on osteoblast cells (Furlan et al., 2005; Hughes, 2020; Webb et al., 2010). Cortisol, and other corticosteroids, decrease the formation of new bone and break down existing bone by both inhibiting the activity of osteoblasts (bone-forming cells) and increasing the activity of osteoclasts (bone-degrading cells), both of which have glucocorticoid receptors (Belaya et al., 2018; Hughes, 2020; Webb et al., 2015). This also has a direct effect on levels of the non-collagenous bone protein osteocalcin, which is produced by the osteoblast cells during regular bone formation (Belaya et al., 2018; Hughes, 2020). Therefore, not only are these compounds proxies for the stress response but they are also precursors to macroscopic bone changes, making them useful and informative to bioarchaeologists interpreting patterns of stress captured in the human skeleton.

2.3 Genes and stress

All the cellular components (signals, receptors, and molecules) involved in the stress response are related to specific genes, which can both influence the stress response and are influenced by it.

2.3.1 Key genetic terminology

Deoxyribonucleic acid (DNA) is the molecule of heredity – it contains information passed from parent to offspring, including information on how to make all the molecules necessary for bodily function (Matisoo-Smith & Horsburgh, 2012). DNA contains units called genes, which contain instructional information for specific products, or proteins. The basic structure of DNA is the sequence of bases - the molecules guanine (G), cytosine (C), thymine (T), and adenosine (A) - which are attached to a 'backbone' composed of a sugar molecule (usually deoxyribose) and phosphate (Alberts et al., 2002). These bases (G, C, T, and A) from two complementary strands attract each other and form a double helix, the secondary structure of DNA (see Fig. 2.1). The length of genes varies greatly and is often measured in bases or base-pairs. For example, a gene that is 25 base-pairs (bp) long could be written as a 25-character sequence of the letters G, C, T, and A. In reality, genes are typically a few thousand to a few hundred thousand base pairs long (often measured in kilobases (kb) – one kb is equal to a thousand base pairs) and contain different subsections (Alberts et al., 2002). Most, but not all genes encode proteins.

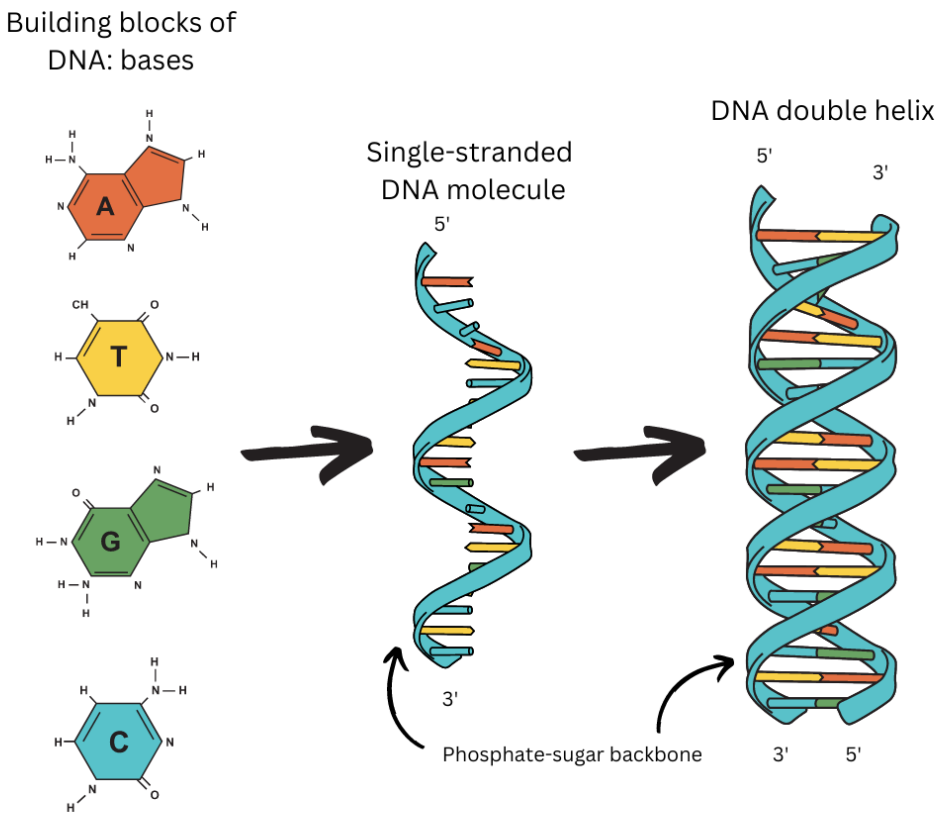

Figure 2.1. Primary and secondary structure of the DNA molecule. Note that the two ends of each strand are not the same and are given the notations 5’ and 3’ (Alberts et al., 2002).

Within the DNA sequence of a gene, certain subsections perform different functions. The coding sequence contains the information on how to make a specific protein, and the regulatory regions are responsible for adjusting how often the coding sequence is ‘read’ and the corresponding protein made (Alberts et al., 2002). These processes are called transcription (‘reading’ the gene) and translation (making the protein). Promoter sequences of protein-coding genes are one kind of regulatory region,

and their function is to increase the amount of protein being made from a gene. Gene promoters act like dimmer switches for lights – while the dimmer does not turn the light on or off, it controls how much light there is. In the same way, promoters can either increase production of a protein from a gene or decrease production if the promoter is blocked or inhibited in some way. Molecules called transcription factors target the promoter region of a gene to influence how much of the encoded protein is produced (Meaney et al., 1996). When the promoter region changes in shape (structure) and becomes spatially difficult for transcription factors to reach, production of that gene is slowed down or lessened (Mazzio & Soliman, 2012).

The processes of gene regulation, including the action of gene promoters, can be altered by epigenetic changes to DNA (Mazzio & Soliman, 2012). Epigenetic changes are changes that alter the DNA without changing the underlying sequence of bases (Mazzio & Soliman, 2012). For example, methylation is a common type of epigenetic change that can occur in DNA where a small molecular group (a methyl group) is tacked onto one of the four bases. Most often the base affected is cytosine, which changes to 5-methyl-cytosine. Methylation has been found to have effects on gene regulation, or the chance a gene will be transcribed and translated into a protein (Pfeifer & Hahn, 2014). Genes and regulatory elements like promoters can be hypermethylated (more than usual) or hypomethylated (less than usual). Typically, increased methylation causes a more ‘closed’ structure of the gene, making it less accessible to the cell mechanisms (Ladd-Acosta et al., 2014; Paabo et al., 2014); however, it is important to remember that the effects of methylation are location- and gene-specific (Mazzio & Soliman, 2012).

2.3.2 The glucocorticoid receptor gene NR3C1

Since the stress response involves the coordinated action of multiple molecules and receptors acting together, it makes sense that multiple genes are involved in making those molecules. Of these, the most well-studied gene associated with the human stress response is called NR3C1 (Alexander et al., 2018; Cordero et al., 2022; Gardini et al., 2022; Liu et al., 2021; Liu & Nusslock, 2017; McGowan et al., 2009; Meaney et al., 1996; Oakley & Cidlowski, 2013; Palma-Gudiel et al., 2015; Perroud et al., 2011; Schür et al., 2018; Tronche et al., 1999; Turecki & Meaney, 2016; Van Der Knaap et al., 2014; Watkeys et al., 2018; Zhang & Tian, 2017). In humans, this gene is located on chromosome five and is approximately 150 kilobases (kb) long. The NR3C1 sequence encodes the glucocorticoid receptor protein, which has two main forms GR_{α}, which is 777 amino acids long and GR_{β} which is slightly shorter at 742 amino acids and appears to have reduced function (Oakley & Cidlowski, 2013). The NR3C1 gene has multiple exons – which are the portions of the coding sequence that get incorporated into the final protein product – and the difference between the α and β forms of GR comes from the differential exclusion of a portion of exon nine during alternative splicing (Oakley & Cidlowski, 2013). Exon 1 of this gene is a promoter regulatory region and is approximately 475 bp in length. This promoter region of NR3C1 has been the subject of most of the research investigating how regulation of this gene affects the stress response (Alexander et al., 2018; Gardini et al., 2022; Liu et al., 2021; Liu & Nusslock, 2017; McGowan et al., 2009; Schür et al., 2018; Turecki & Meaney, 2016; Van Der Knaap et al., 2014).

The glucocorticoid receptor protein (GR) is a major part of the physiological stress response (Oakley & Cidlowski, 2013). GRs act as signal carriers that initiate and influence the production of hundreds of different proteins based on the signal from outside the cell. The GR protein has sections, or domains, meant to serve different purposes. For example, there is a domain to bind DNA, and another domain to bind incoming glucocorticoids, like cortisol (Escoter-Torres et al., 2019). When cortisol enters the cell, GRs in the cytoplasm bind the cortisol and the complex moves into the nucleus of the cell, to regulate the stress response by binding to gene targets and either stimulating or repressing the expression of multiple genes. GRs do not have a high affinity for cortisol, and only bind cortisol when it is present in high concentrations, as during a physiological stress response when substantial amounts of cortisol are released into the bloodstream (Häusl et al., 2019; Liu and Nusslock, 2017). GRs function as a negative feedback inhibitor of the stress response where once cortisol is bound to the receptor, a signal is sent to stop flooding the system with more cortisol. Accordingly, many studies have found that a lack of GRs is connected to higher circulating cortisol (Chrousos, 2009; González Ramírez et al., 2020; Li et al., 2020; Liu et al., 2021; Tronche et al., 1999; Turecki and Meaney, 2016; Watkeys et al., 2018). The interaction between cortisol and GR can be thought of as a relay race where the cortisol is the torch and the GR the second runner; once the second runner receives the torch the first runner will stop. However, the first runner must pass the torch before they can stop running, so if they cannot find the second runner, they cannot stop. Similarly, when there are much fewer GRs available to bind cortisol, the negative feedback loop is interrupted. Higher circulating cortisol without the negative feedback inhibition provided by the GRs is

associated with PTSD, increased aggression, and chronic stress (González Ramírez et al., 2020; Liu et al., 2021; Liu and Nusslock, 2017; Meaney et al., 1996; Yehuda et al., 2013). This evidence suggests that the gene NR3C1 not only plays an important part in the stress response, but also that regulation of this gene is important in understanding various stress-related illnesses.

2.3.3 Methylation of NR3C1 as a direct result of stress

Stress impacts the body on a molecular level, even down to the DNA structure (Escoter-Torres et al., 2019; Liu & Nusslock, 2017; McGowan et al., 2009; Schür et al., 2018; Van Der Knaap et al., 2014). Key genes involved in the stress response seem to undergo epigenetic changes which alter their function. Of those epigenetic changes, methylation levels in the NR3C1 gene have been well-studied in a modern context. Meaney et al. (1996) in an early study in rats, found that rats who were handled early in their life expressed higher levels of the glucocorticoid receptor (GR) and were better able to respond to stressors in adulthood. Also, among prenatally stressed rats, maternal postnatal care could reverse the effects of a stressful prenatal environment, suggesting that the mechanism for these changes was epigenetic rather than genetic. In other words, the reversal suggested that the mechanism had to do with gene regulation. In the period from 2004 to 2016, 27 studies were published investigating the link between methylation of NR3C1 and stress or the social environment in humans (Turecki & Meaney, 2016). Many studies have found that hypermethylation of NR3C1, particularly in the promoter region, leads to under-expression of the gene. Another study by McGowan et al. (2009) used human brain tissue from deceased individuals to examine tissue specific methylation levels of the NR3C1 gene. They found that among suicide victims, those with a history of

childhood abuse had higher methylation in the promoter region of NR3C1, and lowered gene expression levels. (McGowan et al., 2009). Several other studies have found an association between hypermethylation of the NR3C1 and adverse events (i.e., stressors). Liu and Nusslock (2017) found lower levels of GRs and higher circulating cortisol among study participants who had experienced early life stress, which they hypothesized caused an impaired stress response. Liu et al. (2021) found an association between hypermethylation of NR3C1 and aggression in adult males. This was hypothesized to relate to the 'fight' aspect of the stress response, since aggressive behaviour also activates the HPA axis (Liu et al., 2021). The sensitivity of the NR3C1 promoter region to hypermethylation and under-expression because of stress, especially early-life stress, has become well-established (Agorastos et al., 2019; Palma-Gudiel et al., 2015; Watkeys et al., 2018).

To date, exon 1F of NR3C1 has been studied the most extensively in modern populations and has the strongest association with childhood stress events (Palma-Gudiel et al., 2015; Turecki & Meaney, 2016; Van Der Knaap et al., 2014). Van der Knaap et al. (2014), found that hypermethylation of NR3C1 exon 1F was associated with childhood stress experiences and with traumatic experiences in youth. They note that while prior research indicated that trauma was associated with hypermethylation of this gene, their study indicated that stressful life events that did not meet the criteria of trauma also had this association (Van Der Knaap et al., 2014). In another review of NR3C1 methylation research, 20 out of the 23 papers selected included the exon 1F region in their study, which includes the promoter binding region (Palma-Gudiel et al., 2015). A later review covered 55 studies, where almost half (n=23) investigated links between exon 1F

methylation and childhood trauma (other studies focused on links to other kinds of trauma). All studies examining exon 1F CpG sites 35-47 specifically (n=9) found significant hypermethylation for at least one site in association with childhood trauma. Therefore, the NR3C1 exon 1F region has a significant amount of scholarship indicating the relationship between hypermethylation in this region and stressful life experiences in childhood, making it the best proxy area for investigation (Turecki & Meaney, 2016). Overall, adverse life events that cause stress result in hypermethylation of the GR gene promoter, leading to HPA axis dysfunction and impaired regulation of the stress response.

2.3.4 The process of methylation in DNA

Methylation, which sees the addition of methyl groups to DNA, most often happens in CpG contexts, where the cytosine (C) is methylated and occurs in the form of 5-methyl-cytosine (5mC) then is followed by a G. To identify this epigenetic change in the DNA, bisulfite sequencing can be used to analyze methylation and takes advantage of the chemical differences between cytosine and 5-methyl-cytosine (Frommer et al., 1992). Treating DNA with sodium bisulfite causes a change only in unmethylated cytosines in which they lose the amine (NH3) side group and become the deoxynucleotide uracil (Fig. 2.2). Uracil does not typically occur in DNA, and during PCR, uracil will pair with adenine. In the next cycle, the adenine will pair with thymine to turn an original cytosine-guanine pair into a thymine-adenine pair. Methylated cytosines are unaffected by sodium bisulfite; therefore, any GC pairs remaining in the sequenced DNA represent methylation sites in the original sample (Frommer et al., 1992; New England BioLabs, n.d.). The protocols of bisulfite sequencing are simple. Purified DNA samples are treated with

sodium bisulfite in a low pH setting to remove the amine group of unmethylated cytosines. Then, the pH is raised to remove the sulfite from the cytosine and generate uracil. Bisulfite sequencing remains the gold standard for detecting methylation in DNA (Smith et al., 2015).

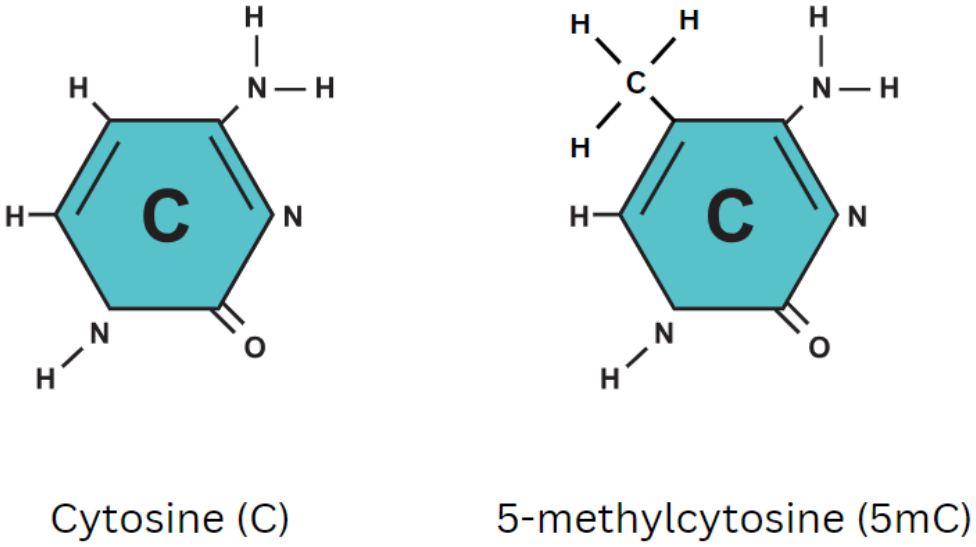

Figure 2.2. Structures of cytosine and 5-methylcytosine (Shock et al., 2011).

2.4 Ancient DNA (aDNA) and methylation analysis in aDNA

DNA recovered from archaeological or paleontological sources is called ancient DNA (aDNA) and undergoes characteristic patterns of damage and denaturing (i.e., unwinding of the DNA double helix). In aDNA, methylation analysis is a new area of research, but with growing interest in the last few years. Gokhman et al. (2014) retrieved global DNA methylation information from Neandertal and Denisovan genomes, the first time that epigenetic markers have been investigated in ancient hominin species. They compared methylation levels to modern human epigenetic data and found that Neandertals had hypermethylated regions in the genes that govern limb size and

structure, which may be one of the factors in morphological limb differences between Neandertals and modern humans (Gokhman et al., 2014). The next year, more researchers attempted to extract methylation information from ancient DNA – one group used methylation-binding-domain enrichment to identify examples of methylation (Seguin-Orlando et al., 2015), while another used bisulfite sequencing (Smith et al., 2015). Smith et al. (2015) were able to recover methylation data from the skeletal remains of 30 Native Americans ranging between 230 to over 4,000 years old. This was an important finding, since previous studies had used indirect methods to estimate methylation, while bisulfite sequencing provides a direct measure. A few programs for aDNA methylation analysis have been created (Hanghøj et al., 2016, 2019), and one study aimed to estimate chronological age from methylation patterns in teeth (Giuliani et al., 2016). More recent studies have also tested the usefulness of Illumina sequencing technologies to detect methylation (Schmidt et al., 2021), explored innovative technologies to account for aDNA-specific issues (Poullet & Orlando, 2020), and analyzed differently methylated regions between ancient and modern humans (Batyrev et al., 2019).

2.4.1 The challenges of aDNA

The study of aDNA presents challenges due to the degraded nature of the DNA. These particular characteristics of aDNA molecules must be considered as most if not all common DNA techniques require modification in order to produce useable results with aDNA. Most of the issues specific to working with aDNA have to do with the age of the DNA itself (Linderholm, 2016; Mitchell et al., 2005; Molak & Ho, 2011; Shapiro & Hofreiter, 2012). First, aDNA is significantly less abundant than modern DNA, regardless of the tissue type. DNA extracted from a modern tooth, for example, will be

present in exponentially higher concentrations than from an archaeological tooth (Fulton & Shapiro, 2012; Shapiro & Hofreiter, 2012). Although some researchers have attempted whole-genome amplification to increase the amount of total DNA available for analysis, results are mixed. For example, Forst & Brown (2016) note that whole-genome amplification did not produce any significant improvement in further analysis, while an earlier study by Suzuki et al. (2010) found the opposite. The amount of aDNA is still the primary limiting factor in research.

Further, aDNA experiences three kinds of characteristic changes that can impair further analysis: 1) fragmentation, 2) increased A or G bases at break points, and 3) CT substitutions at the ends of fragments (Sawyer et al., 2012). Fragmentation of the long strands of DNA into shorter pieces is inevitable, and evidence suggests this process begins shortly after death (Sawyer et al., 2012). To account for this, lab protocols are modified accordingly. For example, several methods for modern DNA analysis perform fragmentation as a preliminary step - this step is skipped when working with aDNA as it is assumed to already be in a fragmented state. When DNA breaks into fragments, both the ‘beginning’ (called the 5’ end) and the ‘end’ (or 3’ end) of resulting fragments are vulnerable to changes (see Fig. 2.1). There are two patterns that are observed in how DNA fragments (i.e., it is not a random process). First is an increased frequency of the bases A or G at the 3’ end of resulting fragments. One proposed mechanism is that the enzymes that break down DNA prefer AT sites, and cut between the A and T bases, resulting in a higher frequency of A bases at the 3’ end of fragments (Sawyer et al., 2012). Another, slower, proposed mechanism targets G bases. The 5’ end is vulnerable to different changes, in particular the change of C bases into T bases. This nucleotide

misincorporation can cause issues with sequencing when a position that originally held a C base is now transcribed as a T base. There are two ways to combat fragmentation-related issues. The first is after sequencing where a certain number of bases (the exact number depends on the sequencing technology used) is cut off from the beginning and end of the 'read' in a process called trimming. The second way is to sequence the same area multiple times to accumulate multiple reads of the same area, which helps determine the true sequence of bases. For example, if in a total of 50 reads, 45 reads list a C in position 15 (and 5 list 'T'), the position is most likely a C. While fragmentation cannot be avoided, some of the effects on sequence accuracy can be mitigated using the above strategies (Shapiro & Hofreiter, 2012).

Polymerase-chain reaction, or PCR, is a common genetics technique used to multiply one particular section of DNA for further analysis (often called amplification) (Shapiro & Hofreiter, 2012). PCR is like a 'gateway' method where almost any analysis that requires discovery of the DNA sequence will use PCR techniques first to ensure there is an abundant supply of the region of interest. Performing PCR with aDNA is difficult because of the much lower extraction yields of aDNA compared to modern samples, often by at least a thousandfold (Fulton & Stiller, 2012). Furthermore, shorter pieces of DNA due to fragmentation also increase the difficulty in amplifying any region of interest. These issues are often mediated by changes to the PCR reaction itself through optimizing primer annealing and extension temperatures, running the reaction for more replication cycles, and designing different short (20 – 25 bp) specific primer sequences to better accommodate the fragmented nature of aDNA (Fulton & Stiller, 2012). Sanger sequencing uses DNA polymerase to extend a primer that matches the target sequence

(similar to PCR), but with some differences. Instead of continually extending the chain until the end of the fragment, Sanger sequencing uses dideoxynucleotides (ddNTPs) to terminate the chain extension process. These ddNTPs lack the 'hook' necessary to latch another nucleotide on to the sequence, and they also possess a visualizable tag which is a different colour for A, C, T, and G, and allows the recording of the sequence (Sanger et al., 1977).

aDNA is also susceptible to contamination from modern DNA, because of its relatively lower abundance (Römpler et al., 2006; Shapiro & Hofreiter, 2012). If aDNA is contaminated with a source of modern DNA, any further analysis is significantly more likely to pick up the abundant modern DNA instead of the limited aDNA. This can result in overestimation and false positives, especially when the contamination is DNA from the same organism. In some instances, studies have reported successful amplification of aDNA, only to later retract results that could not be verified or were determined to be contaminated (Mulligan, 2005). Other factors, such as the presence of endogenous mitochondrial DNA, may also affect contamination estimates (Furtwängler et al., 2018). To combat contamination, clean-lab protocols have been introduced (Fulton & Shapiro, 2012). Experiments are commonly run in triplicate with several negative controls and the elimination of some positive controls to avoid contamination. Since researchers themselves are a potential source of contamination when working with human aDNA, multiple precautions are taken to minimize the risk of contaminating aDNA samples with modern DNA. These precautions include physically separating lab spaces for pre-PCR and post-PCR analysis, appropriate ventilation, disposable full body suits, and sterilizing and decontaminating all materials before and after use (Fulton & Shapiro, 2012).

2.4.2 The challenges of methylation analysis in aDNA

If all the above issues can be addressed satisfactorily, methylation analysis in aDNA presents another set of challenges. Although bisulfite sequencing remains the gold standard in methylation analysis as a direct measure, there is an issue concerning the use of bisulfite sequencing in aDNA (Shapiro & Hofreiter, 2012). Bisulfite sequencing takes advantage of the chemical differences between cytosine and 5-methylcytosine (Frommer et al., 1992). Treating DNA with sodium bisulfite causes a change only in unmethylated cytosines where they lose the amine (NH_3) side group and become the deoxynucleotide uracil (Fig. 2.3).

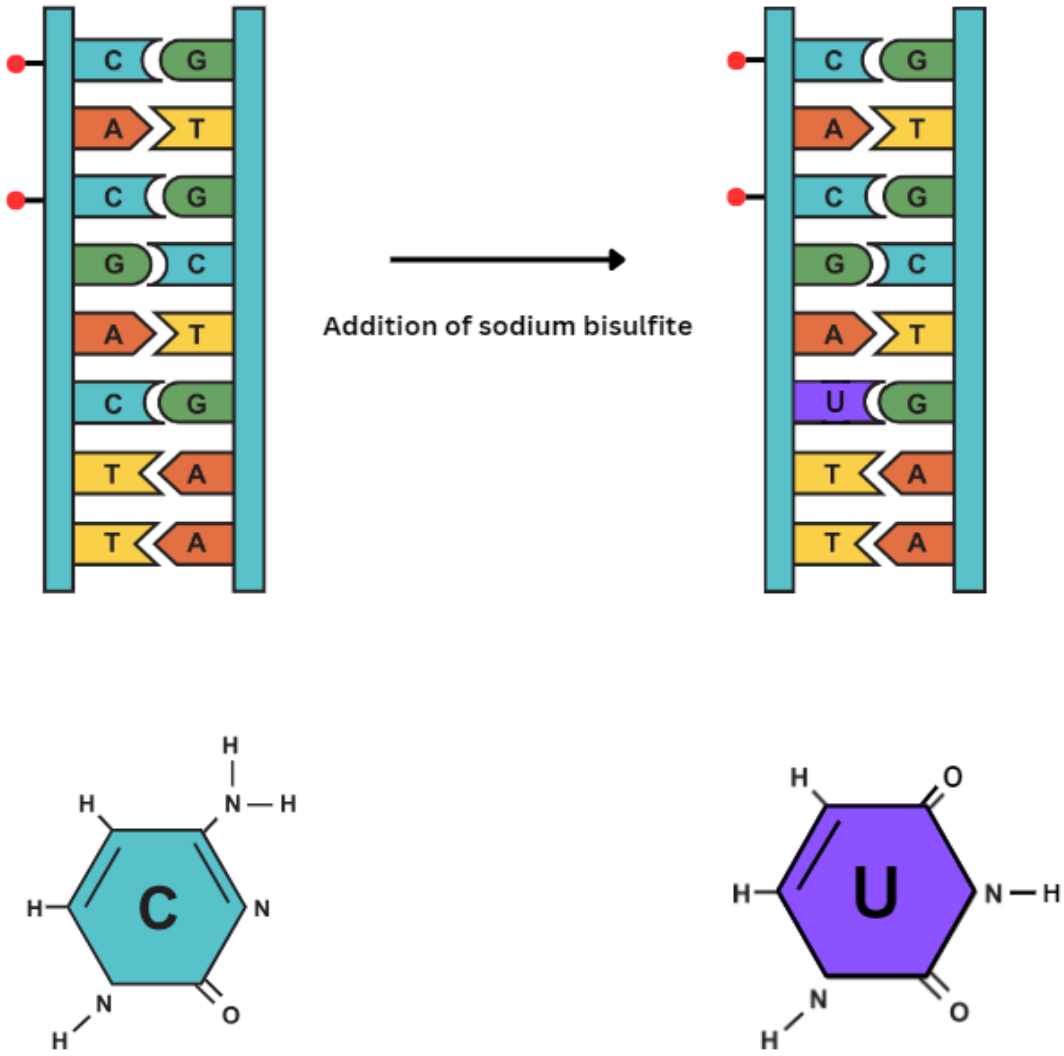

Figure 2.3. Chemical changes to unmethylated cytosines during bisulfite conversion. Red dots indicate methylated cytosines (Frommer et al., 1992).

Uracil does not typically occur in DNA, and during PCR uracil will pair with adenine (A), then thymine (T) to turn an original guanine-cytosine (GC) pair into an adenine-thymine (AT) pair. Methylated cytosines are unaffected by sodium bisulfite, and so any GC pairs remaining in the sequenced DNA represent methylation sites in the

original sample (Frommer et al., 1992; New England BioLabs, n.d.). In aDNA, there is a possibility that the natural degradation process of DNA, including C/T substitutions mentioned earlier, will result in the underestimation of methylation. While sodium bisulfite selectively deaminates unmethylated cytosines only, natural deamination may take place in both methylated and unmethylated cytosines. Methylated cytosines, once deaminated, become thymines and would be indistinguishable from cytosines converted to AT pairs through bisulfite conversion. There is evidence that deamination occurs primarily at the ends of fragments, which could be remedied by trimming the ends of each sequence (Sawyer et al., 2012). Another way to combat this issue, after DNA has been enriched for the target gene, is to apply the following two methods to each sample: 1) bisulfite conversion, followed by sequencing, and 2) only sequencing. This allows a comparison between bisulfite treated and untreated samples, where discrepancies between the two would represent the unmethylated cytosines that were converted. Additionally, having the untreated sequence data for each individual would allow for another approach in the case that damage in the form of age-related deamination is extensive. The approach used by Gokhman et al. (2015) in their study of ancient Neandertal and Denisovan methylation patterns was to calculate the rate of substitution from C to T and use that as a proxy for methylation. Although there are several challenges with aDNA analysis and specifically with bisulfite sequencing, these methods have great potential in bioarchaeology to provide information that is simply not available from other methods.

2.5 Geographic and historical context

Colonial Atlantic North America in the 18th century was a complex patchwork of primarily French and English settlements. In present day Canada, the Treaty of Utrecht, signed in 1713 forced the French from their colonies in Newfoundland and saw their relocation to Cape Breton Island where a new settlement was begun at Louisbourg (McLennan, 1957). From 1713 to 1745, Louisbourg grew into a major trade centre for the French, largely buoyed by its central role in the cod-fishing industry (Johnston, 2004; McLennan, 1957). In 1745, Louisbourg was besieged by a New Englander force in the summer months and was occupied until the return of the fortress to the French by the Treaty of Aix-la-Chapelle in October 1748. Another ten years of French control followed, and in 1758, the English successfully laid siege again and once the French capitulated, the Fortress of Louisbourg was systematically destroyed and eventually abandoned (Johnston, 2001, 2004; McLennan, 1957). A 1960s reconstruction effort revitalized interest in the site (Parks Canada, 2011), and several excavations of the fortress were completed during this period and continue up to the present day (Dunham, 2017).

Throughout Louisbourg's history four main burial sites were used. The earliest cemetery was located in Block 3 (1713 – 1723) and was the original interment site for the first inhabitants and was later moved to Block 34 (1722 – 1726) (Johnston 2004). There was also a hospital burial ground in Block 40 (1723 – 1744), and eventually the main cemetery was moved outside the fortification walls to Rochefort Point (1738 – 1758+) (Johnston, 2004; Scott et al., 2019). Although these were the main burial grounds at Louisbourg, there were also several 'unofficial' burial grounds scattered throughout the settlement (Moore 1974). For example, up to 15 people are reported to be buried on one

individual's property, and at least one individual is buried on Battery Island, in the harbour (Moore 1974). Although the Rochefort Point cemetery likely houses the majority of burials at Louisbourg, it is by no means the only place where human remains are found.

Louisbourg's history has been filled with difficult and challenging circumstances. For example, the two sieges in 1745 and 1758 were highly stressful events (Hobfoll et al., 1991) for both the English and French soldiers as well as the other inhabitants of the fortress. There is also historical evidence for stress related to disease. Treponemal disease (commonly in the form of venereal syphilis) was present at Louisbourg (Thomson, 1996), there were multiple reports of 'flux' (dysentery) in the historical records (de Forest, 1932), not to mention the common incidence of wound infection and sepsis (de Forest, 1932). The above are the immediate conditions of late 1745 but there was also a smallpox outbreak in 1732-33 and again in 1755 (McLennan, 1957). Although living conditions were generally better in the Louisbourg colony compared to urban centres in England and France, there were still periods of food scarcity for the less wealthy residents (Donovan, 1985). Therefore, there is ample evidence that the people buried in the cemeteries at Louisbourg would have been exposed to multiple stressors throughout the life course.

2.6 Conclusion

This literature review has covered bioarchaeological approaches to analysing stress, what 'stress' means, and what physiological processes are associated with stress. This chapter has also highlighted the role of and relationship between genes and stress, as well as the unique circumstances of working with ancient DNA. Finally, this chapter provided a brief overview of the specific historic and geographic context of the

population under study. Research on the genetic aspect of stress has shown that the gene NR3C1 is affected by the experience of stress through the life course in measurable ways. In keeping with the general trend towards the development of biochemical methods in bioarchaeology, NR3C1 could represent a more sensitive and nuanced method to measure stress in past populations. Although working with aDNA is difficult because of natural degradation processes associated with age, with careful consideration of these specific issues and appropriate modification of techniques to address these issues, researchers can improve outcomes for aDNA research.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

This chapter will review the materials and methods used in this research, beginning with permissions and access. Although limited, the available information on the provenience of the samples will be provided, and a rationale for the chosen gene target. Methods will be described in detail beginning with the extraction and purification of ancient DNA (aDNA). Then, the process of method development for targeted amplification of NR3C1 will be described, including primer design, polymerase chain reaction (PCR) testing, sequencing, and the subsequent analysis of sequence data. Finally, the application of the developed method to aDNA will be explained, including primers, PCR, and sequencing.

3.2 Permissions and Access

Six teeth from the Fortress of Louisbourg skeletal collection were selected for aDNA analysis of NR3C1. The collection is currently housed at the University of New Brunswick's Bioarchaeology Research and Teaching Lab (BART). Permission to sample these individuals has been granted by Dr. Amy Scott, Parks Canada, the Diocese of Nova Scotia and Prince Edward Island, and the Catholic Diocese of Antigonish as mandated by Parks Canada Management Directive for Human Remains, Cemeteries, and Burial Grounds. All six samples were sent to the Lakehead University Paleo-DNA Lab for DNA extraction, purification, and sequencing. Modern DNA was used to design and test all methods at UNB (REB 2021-006, Appendix A).

3.3 Tooth sample contextual information

The six teeth used for this project (see Table 3.1) were selected from the Larocque subcollection and most were excavated circa 2000. Both molars and premolars were sampled, which are commonly used in aDNA analysis due to their size and stability (Higgins et al., 2015; Rohland & Hofreiter, 2007). Information for the individuals in this subcollection is limited, which makes an interpretive approach based on context difficult to nearly impossible. Therefore, this small sample, lacking contextual information is ideal for this project as any new method requires testing, DNA extraction is destructive, and these teeth have the potential to help with testing a new method even though they have little potential to inform any other analyses.

Table 3.1. Tooth sample provenience number and tooth type from Fortress of Louisbourg.

PROVENIENCE	Tooth Type
55L1A3.1	Right maxillary Molar 3
55L23H11.1	Right mandibular Molar 3
55L1A1-1	Right maxillary Molar 1
55L24F12.1	Left maxillary Molar 2
55L24A7.2	Right mandibular Molar 3
55L24C24.1	Left mandibular Molar 3

The first two individuals with the provenience numbers 55L1A3.1 and 55L1A1-1 have uncertain origin. For 55L1A3.1, the record associated with the suboperation 55L1A3 only mentions a general location – the “kiln”, which was later identified as not a kiln at all but the house of the Ste. Marie family, on Rochefort Point (M. Moran, pers. comm., April 5, 2023). Excavation of this individual took place in 1980 by Andree Crepeau, the archaeologist at Louisbourg at the time. For 55L1A1-1, the record is also unclear – there is no definite excavation date, but it may have been excavated as early as

1979 or as late as 2000 (M. Moran, pers. comm., April 5, 2023). No information is recorded on the location, arrangement, soil composition, etc. of the burials. Ultimately, interpretation of these remains in context is impossible, as so little is known.

For individuals 55L23H11.1 and 55L24F12.1, slightly more information is available. Both burials are noted in an excavation summary in their respective units (55L23H and 55L24F) on Rochefort Point. The summary from the 2000 excavation states, "55L23H lay approximately one metre north of the trenches. Here, an east-west oriented burial was found adjacent to an east-west oriented, dry-laid, fieldstone wall feature. The burial was covered with a gravelly soil layer." (Dunham 2000, p. 10). No further information is provided about this burial. Individual 55L24F12.1 was similarly described: "another test unit 55L24F exposed a north-south running wall…an east-west oriented burial was found resting directly over the partially dismantled wall-like feature" (Dunham 2000, p. 10). Beyond the soil description and the orientation of the burial, no further information is provided.

For individuals 55L24A7.2 and 55L24C24.1, no information on either the burials or the units they were found in is provided in the summary. A note reads that "Burial features located within the trenches were given unique provenience designations (55L24 or 55L26)" (Dunham 2000, p. 5). Since these excavations were taking place on Rochefort Point, the burials must have been on the Point, however, neither unit 55L24A nor 55L24C appear again in the summary. In sum, the samples used in this study were excavated around 20 years prior (or possibly more) and have limited to no context. Although this severely limits most bioarchaeological analyses of these samples, it also

makes them a better candidate for aDNA sampling as little information can be gained macroscopically.

3.4 Extraction and purification of ancient DNA

DNA extraction and purification took place at the Paleo-DNA Lab at Lakehead University and was carried out by Stephen Fratpietro. Whole genomic DNA (gDNA) was extracted from each tooth sample. Before extraction began, surfaces were cleaned with 3-10% bleach, followed by 70% ethanol, and all equipment was decontaminated before use in accordance with aDNA protocols (Fulton, 2012). Total demineralization protocols were used to obtain aDNA from all six teeth (Loreille et al., 2007), and followed by a silica bead-based purification (Boom et al., 1990).

3.4.1 Pre-treatment of bone samples to remove exogenous DNA

To remove exogenous DNA from the six tooth samples, first the entire surface of the tooth was sanded using an aluminum oxide sanding stone attached to a Dremel tool (Loreille et al., 2007). Next, the samples were sonicated in a 20% bleach solution for five minutes, after which they were rinsed in UV-irradiated water. Then, the samples were sonicated in UV-irradiated water for another five minutes, then sonicated in 100% ethanol. They were then left to air-dry overnight under a fume hood (Loreille et al., 2007).

3.4.2 Extraction of DNA in buffer solution and purification

Each tooth was first powdered, then 1-2g of the tooth powder was incubated overnight in 3mL of extraction buffer (10mM TRIS, pH 8.0; 100mM NaCl; 50mM EDTA, pH 8.0; 0.5% SDS) and 100uL of 20mg/mL Proteinase K. Incubation took place at 56˚C with gentle shaking (Loreille et al., 2007). Purification of DNA followed a

modified silica-based method based on Boom et al. (1990), in accordance with standard practice at the Lakehead University Paleo-DNA lab.

3.5 Method development and testing with modern DNA

The second step of this project was to use a modern DNA source to develop and test a method to be used on the extracted and purified aDNA. Adapting a method used in clinical research with modern DNA (often extracted from blood or saliva), to be used in bioarchaeological research with ancient DNA (extracted through bone or teeth), changes must be made in order to ensure success. These changes can include but are not limited to: primer lengths and sequence composition, PCR conditions including temperatures (denaturation, annealing and extension), times, and number of amplification cycles, types of DNA polymerase used, etc. For this research, this process required multiple experiments to try out different options and approaches, which meant an abundant source of DNA was necessary. Therefore, using modern DNA ensured there was a sufficient amount of DNA available to avoid the unnecessary waste of aDNA from the six tooth samples. The steps in this process were as follows: first, modern DNA was extracted from the source material, NR3C1-specific primers were designed to target the region of interest. Then, the primers were tested on the modern DNA to identify the correct PCR parameters (i.e., optimal number of cycles, time for each PCR step, temperatures, etc.). Once PCR results were successful (determined by gel electrophoresis and UV-visualization of results), the samples were sent to Génome Québec for sequencing to confirm the designed primers were in fact amplifying on-target (i.e., the NR3C1 promoter).

3.5.1 DNA extraction

Modern DNA was extracted from saliva using a Qiagen QiAMP DNA Minikit according to manufacturer instructions with the exception of the following changes. First, the microcentrifuge in the lab space is not able to fit 15ml Falcon tubes – therefore, after adding 4mL PBS, the mixture was separated into four 1.5ml microcentrifuge tubes for centrifugation. After the supernatant was decanted and resuspended in PBS, the volumes were re-combined into one 1.5ml microcentrifuge tube. Second, to improve DNA recovery, the alternate elution protocol was followed where the DNA was eluted with two volumes of 75uL Buffer AE instead of one volume of 150uL.

3.5.2 Primer design

In-vivo DNA replication requires priming sequences (pre-synthetized short DNA fragments) that bind the template molecule (Alberts et al., 2002; Shapiro & Hofreiter, 2012). The PCR approaches take advantage of that natural molecular process allowing us the targeted replication (amplification) of loci of interests (NR3C1 in this case) by designing priming sequences, or simply primers, that specifically match (prime) the target region. Then, primer design is the process of tailoring short sequences of DNA (20 – 30 bp) that match with a target area of interest. To amplify and/or sequence an area of interest in DNA, two primers must be designed that 'bookend' that area: a forward primer and a reverse primer. Together, these forward and reverse primers form a PCR primer pair. Various programs to assist with primer design are available. For example, two options are NCBI Primer-BLAST (Ye et al., 2012) and Primer3 (Untergasser et al., 2012). In this project, both NCBI Primer-BLAST and the software in the genetic analysis program Geneious Prime 2023.0.4 (http://www.geneious.com) were used to design

primer pairs – both are based on Primer3. In total ten primer pairs to amplify regions of the NR3C1 promoter were used in this project, seven were designed and three were taken from literature.

Using the sequence of the human NR3C1 gene (GenBank accession number NM_000176.3) as reference, seven primer pairs designed for this project were GR1, GR2, GR3, GR4, GR5, GR6, and GR7. GR3 and GR4 were designed using NCBI Primer-BLAST and GR4, 5, 6, and 7 were designed in Geneious using Primer3. Once the primer sequences were designed, the primers were ordered from Integrated Data Technologies (IDT). The three primer pairs NR3C1_1, NR3C1_2, and NR3C1_3 were taken from van der Knaap (2014), a study that focused on the connection between methylation of NR3C1 and both childhood and youth stress events. These three primer pairs were also ordered from IDT. Further details on the primer sequences are presented in Table 3.2.

Table 3.2. Primer names, sequences, and expected product lengths.

PAIR NAME	PRIMER NAME	SEQUENCE (5' TO 3')	PRODUCT LENGTH	T_M
GR1	GR1F	GTCGGGGACGGATTCTGTGG	285	60.0
	GR1R	GCCGCAGCCGAGATAAACAAC		59.0
GR2	GR2F	CCATTTTGCAGAGCTCGTGTC	310	56.0
	GR2R	TTGTGAACGCAGAAGGAAGCAG		57.0
GR3	GR3F	CCACTCACGCAGCTCAGC	256	59.0
	GR3R	CTCCCCAGGAAAAAGGGTGG		58.0
GR4	GR4F	CACTCACGCAGCTCAGCC	261	59.0

	GR4R	CCCCAACTCCCCAGGAAAAA		
GR5	GR5F	AAAGTACGTATGCGCCGACC	160	57.7
	GR5R	CCGAGTTGCGTGAAGTGTGT		58.0
GR6	GR6F	ACACTTCACGCAACTCGGC	199	58.5
	GR6R	TTCTCGCTACCTCCTTCCCG		58.6
GR7	GR7F	GGGAAGGAGGTAGCGAGAAAA	120	56.5
	GR4R	CCCCAACTCCCCAGGAAAAA		57.0
NR3C1_1	NR3C1_1F	AAAGGGGTTATTTAGAAATTTTAGG	427	47.0
	NR3C1_1R	AAAATCCTAACCTCTTTTCTCCCC		53.0
NR3C1_2	NR3C1_2F	TTTTTGAAGTTTTTTTAGAGGG	322	45.0
	NR3C1_2R	AATTTCTCCAATTTCTTTTCTC		44.0
NR3C1_3	NR3C1_3F	TTTTTAGTTTAAGGGGAAGGGAAT	392	50.0
	NR3C1_3R	CTTCAAAATATCAAAACAAAAAAAACC		47.0

3.5.3 Polymerase chain reaction (PCR) testing

In any analysis after purified DNA has been obtained, the DNA in the sample will be nonspecific – i.e., it will contain parts of the whole genome. In order to sequence NR3C1, the polymerase chain reaction (PCR) needs to amplify the specific fragments in the sample belonging to NR3C1. PCR consists of three main stages: template denaturing, primer annealing, and replication extending; plus an initial denaturing step and a final extension step. To optimize PCR, adjustments can be made to the DNA polymerase used in this experiment, temperature at any of the steps, number of cycles, and time at each temperature. For this project, four different commercial DNA polymerases were tested: New England BioLabs (NEB) OneTaq, NEB LongAmp, Invitrogen PlatTaq, and Qiagen

HotStarTaq. Each DNA polymerase requires a slightly different PCR 'recipe' (see Appendix B). For the seven designed primers, each primer pair began optimization with a baseline PCR protocol outlined in Appendix B, then adjustments were made in subsequent PCR reactions based on prior results. For the three primers from van der Knaap et al. (2014), PCR protocols for thermocycling were taken directly from the literature. These specific parameters are also summarized in Appendix B. Each primer pair underwent multiple rounds of PCR testing and optimization. All PCR experiments were performed using a BioRad C1000 thermal cycler. Final optimized PCR protocols for each primer pair are presented in Appendix B.

After PCR, all samples were initially visualized by performing agarose gel electrophoresis. All gels were made using a 70mL 1% agarose gel, with 7uL 1X SyBr Safe dye in the gel to assist with visualization. PCR products were mixed with a loading dye (New England BioLabs Gel Loading Dye, Purple, 6X) with a ratio of 3uL PCR product to 1uL dye, and the 4uL mix was pipetted into each well. To assist with size estimation of PCR products, 'ladders' with DNA fragments of known size were added to the gel – the standard referemce used was ThermoFisher's GeneRuler 1kb ladder. Gels were first visually inspected with the lab's UV transilluminator (Clare Chemical Research DarkReader transilluminator). If results were significant, higher resolution images were obtained using the UNB Department of Biology's BioRad XR+ gel documentation system.

3.5.4 Sequencing and analysis of sequence data

Samples with a successful PCR result (expected amplicon size) were sent to Génome Québec for Sanger sequencing. Sanger sequencing was developed in the 1970s

and was the first DNA sequencing method widely used in the original human genome project (Lander et al., 2001; Sanger et al., 1977; Venter et al., 2001). Although shotgun sequencing and various next-generation sequencing methods have been developed, Sanger sequencing retains the advantage of longer read lengths and is still used widely in non-genomic studies.

Upon completion of sequencing, Génome Québec classified each sequence as 'OK' or 'Failed'. This classification is based on the quality score of each base position. By default, Génome Québec 'masks', or hides base positions with a quality score of less than 15 (97% base call accuracy). Once sequences were downloaded, further analysis was performed using the genetic analysis program Geneious Prime (http://www.geneious.com). Typically, DNA sequences are 'trimmed' as a quality control step to remove low-quality sequencing results and primer sequences. For this research, all sequences were trimmed using default program settings with an error probability limit of 5%, and trimmed regions were automatically removed from the sequence. Sequences with a percentage of high-quality bases (%HQ) lower than 50% were excluded from further analysis.

The estimation of consensus sequences from multiple sequencing reads of the same area of the genome is a common method to remove ambiguity from results. For this research consensus sequences were aligned with two different algorithms depending on how many sequence reads covering the area needed to be aligned: if only two, a pairwise alignment algorithm was run, using global alignment with free end gaps and a 70% cost similarity matrix (65% was the default). When there were more than two sequence reads to align, the Geneious multiple alignment algorithm was used with the same parameters.

Since sequence results contained both ‘forward’ and ‘reverse’ directional sequences, all forward sequences were aligned together, and all reverse sequences were aligned together. NCBI’s Basic Local Alignment Search Tool (BLAST), available in Geneious, was run on consensus sequences to ensure they matched recorded sequence data for the NR3C1 gene. Default parameters were used for the BLAST search. Alignment scores were generated from this search, where matches in the sequence increased the score and mismatches or gaps decreased the score. An inverse measure of the score, called the E-value (number of expected hits of similar BLAST score that could be found just by chance), was also provided. The smaller the E-value, the more significant the matching sequences were. Consensus sequences were judged as having successfully amplified NR3C1 if the BLAST search returned greater than 99% identity with NR3C1, and the E-value was less than 1.0e-10.

3.6 Application of method to ancient DNA

Once method optimization was complete using modern DNA, testing with aDNA samples proceeded. Only primers that successfully amplified modern DNA were selected to move forward to testing with aDNA. Primer sequence data and primer-specific protocols were sent to Stephen Fratpietro at Lakehead University so that fresh primers could be ordered. PCR was performed by the Lakehead Paleo-DNA lab according to the protocols that were developed in modern DNA with one change: instead of a volume of 1uL DNA, 3uL DNA was used and the amount of nuclease-free water adjusted accordingly. Once PCR was performed all successful reactions underwent Sanger sequencing, also at the Lakehead Paleo-DNA Lab. The following data was returned from these protocols: labeled images of agarose gel electrophoresis, to ascertain success of

PCR in amplifying sequences of the correct length; and sequencing results, to judge whether high-quality sequences were able to be obtained from PCR results.

3.6.1 Agarose gel electrophoresis and Sanger sequencing

The agarose gel images were visually inspected to check the size of DNA fragments and band brightness. Typically, when performing agarose gel electrophoresis, a 'ladder' with DNA fragments of known size is loaded onto the gel along with the samples, so that size can be estimated for resulting sample bands. Band brightness indicates the amount of DNA present – faint bands can indicate PCR was unsuccessful in generating enough DNA to move forward with sequencing. Agarose gel images were also inspected for the presence of extra bands, which may indicate contamination, non-specific amplification or primer dimer, which results from primers attaching to themselves instead of to the sample DNA.

Analysis of sequence data from Sanger sequencing of the aDNA samples aimed to confirm that the sequences matched the exon 1F region of NR3C1. First, sequences were trimmed according to above parameters (i.e., to remove low-quality bases and primer sequences). If there was enough high-quality sequence to continue, sequences were BLASTed against the NCBI database to investigate identity with NR3C1.

3.6.2 Bisulfite sequencing

Methylation, the addition of methyl groups to DNA, most often happens in a CpG context. That is, the dinucleotide combination of cytosine followed by guanine is most often where methylated DNA is found in the form of 5-methyl-cytosine (5mC). The protocols of bisulfite sequencing are simple: purified DNA samples are treated with sodium bisulfite in a low pH setting to remove the amine group of unmethylated

cytosines. Then, the pH is raised to remove the sulfite from the cytosine and generate uracil. Typically, a first step of fragmentation is performed, but this step can be omitted since aDNA is already in a fragmented state. Bisulfite sequencing remains the gold standard for detecting methylation in DNA (Smith et al., 2015). Bisulfite sequencing was intended to be performed on aDNA samples, but due to the small volumes of aDNA available, this step was not able to be completed.

3.7 Conclusion

This chapter has provided a detailed description of the methods used in this project, including how aDNA was extracted and purified from six archaeological teeth for this project. Then, method development and optimization using modern DNA was explored, including DNA extraction from modern DNA, designing primer pairs, PCR testing, sequencing of PCR products, subsequent analysis of sequence data, and the quality control procedures for these steps. Finally, the optimized method developed using modern DNA was applied to the sample aDNA, and this process was described, including the quality control steps that were used to verify and assess the results.

CHAPTER 4: RESULTS

4.1 Introduction

This chapter will include the results of the methods outlined in Chapter 3, beginning with the results of ancient DNA extraction and purification. Next, the results of method development will be summarized, including primer design, testing, and sequencing of primer products. Finally, the results from application of the method to aDNA will be presented.

4.2 Extraction and purification of ancient DNA

DNA extraction was performed on the six tooth samples from the Fortress of Louisbourg at Lakehead University's PaleoDNA Lab. DNA extraction was successful in five out of the six samples. The concentrations of DNA extracted from the six samples is summarized in Table 4.1 (volume for all samples was 25uL). Individual 55L24A7.2 had the highest DNA concentration after extraction at 564 pg/uL, whereas individual 55L23H11.1 had the lowest DNA concentration after extraction at 0.0987 pg/uL. DNA concentrations are typically measured in nanograms (ng), where 1ng = 1000 pg; therefore, these reported concentrations are considered very low.

Table 4.1. Summary of tooth sample DNA concentrations after extraction.

PROVENIENCE #	**TOOTH**	**DNA CONCENTRATION**
55L1A3.1	Right maxillary third molar	0.223 pg/uL
55L23H11.1	Right mandibular third molar	0.090 pg/uL
55L1A1-1	Right maxillary first molar	0.407 pg/uL
55L24F12.1	Left maxillary second molar	0.164 pg/uL
55L24A7.2	Right mandibular third molar	564 pg/uL
55L24C24.1	Left mandibular third molar	*No detection*

4.3 Method development and testing with modern DNA

4.3.1 Primer design and testing

Four rounds of primer testing were conducted with a total of ten primer pairs tested. In the first round, GR1 and GR2 were tested; in the second round, NR3C1_1, NR3C1_2 and NR3C1_3 from van der Knaap et al. (2014) were tested; in the third round, GR3 and GR4 were tested; and in the fourth round GR5, GR6, and GR7 were tested. All primer sequences are given in Chapter 3, Table 3.2.

GR1 and GR2 were the first two primer pairs designed for this project. These two pairs underwent multiple rounds of PCR testing. PCR with gel electrophoresis either produced gels that had no bands, or gels that had multiple bands (Fig. 4.1). However, one experiment produced promising results for GR2 with no extra bands (Fig. 4.2); therefore, GR2 moved on to the method application step with aDNA.

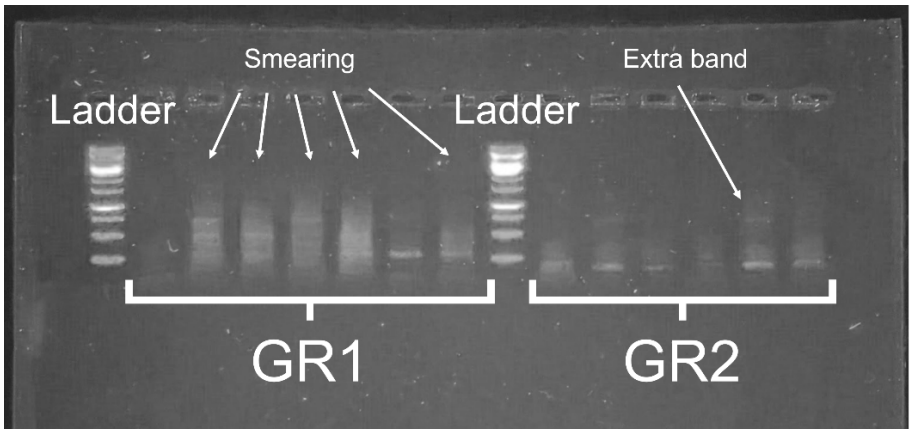

Figure 4.1. Gel electropherogram of PCR amplification with primer pairs GR1 and GR2. The lanes with GR1 show significant smearing, and the lanes with GR2 show extra bands.

Figure 4.2. Gel electropherogram of PCR amplification with primer pairs GR1 and GR2. Note the results for GR1 continue to show smearing but the extra bands for GR2 are no longer present.

The next pairs tested were NR3C1_1, NR3C1_2, and NR3C1_3. These primer pairs were taken from a publication by van der Knaap et al. (2014). These three pairs were tested multiple times, beginning with a PCR protocol taken directly from the publication. There was no amplification for NR3C1_2 and NR3C1_3, and only faint amplification for NR3C1_1. The following was attempted to fix these issues: increasing the extension time during PCR to allow for more amplification; increasing the amount of DNA used in the reaction; adjusting the annealing temperature, including a gradient PCR; trying a different DNA polymerase (with associated temperature changes); contacting the original authors for assistance. None of the attempted fixes were successful, and the

decision was made to design additional primer pairs for testing. A typical gel electropherogram showing no amplification is presented in Figure 4.3. The faint bands seen are the result of primer dimer when primers attach to each other.

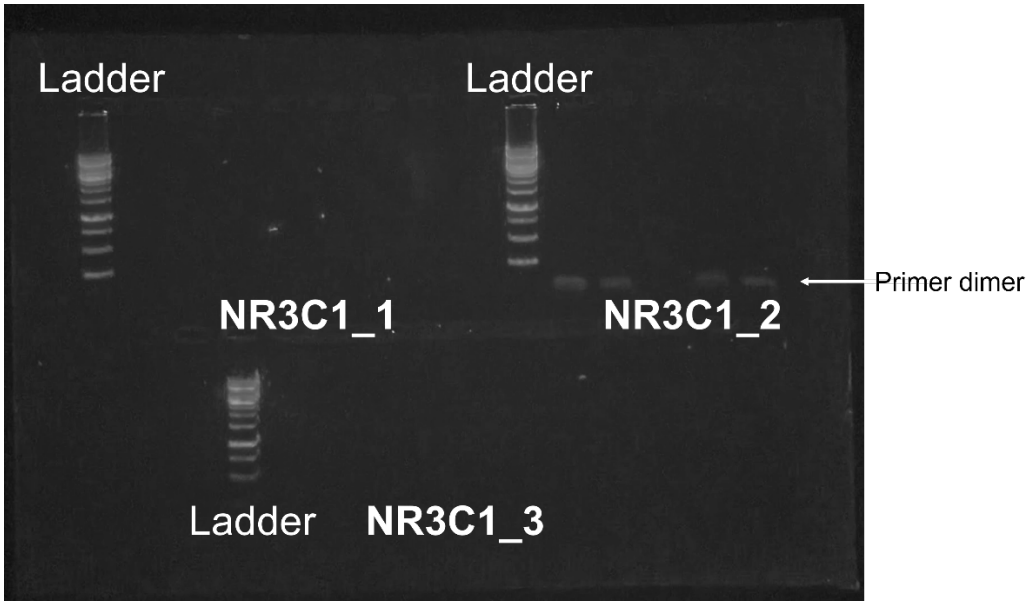

Figure 4.3. Gel electropherogram of PCR amplification with primer pairs NR3C1_1, _2, and _3. Other than the primer dimer seen for NR3C1_2, all the lanes are empty and have no bands.

After the failure of the primers from van der Knaap et al. (2014), two more primer pairs were designed: GR3 and GR4. These primer pairs showed strong amplification of the target DNA fragment without additional troubleshooting (see Fig. 4.4). PCR products from this reaction were sent for sequencing with results from sequencing discussed in the next section.

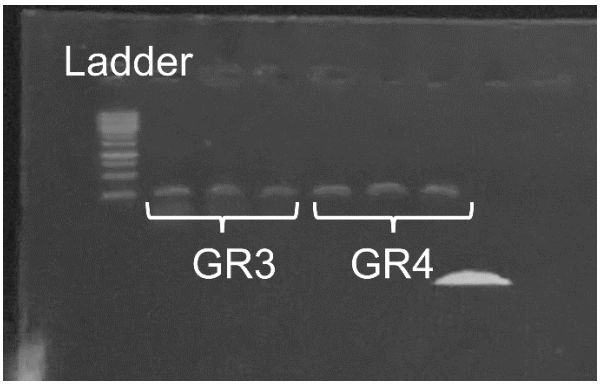

Figure 4.4. Gel electropherogram of PCR amplification with primer pairs GR3 and GR4. Each lane has a strong, single band with no smearing or contamination.

After the success of primers GR3 and GR4, another three primer pairs were designed and tested: GR5, GR6, and GR7. After some troubleshooting, a successful PCR result was obtained for GR5 and GR7. Later, another PCR experiment also produced a successful result for GR6, and all bands in this experiment were the expected size for each primer pair (Fig. 4.5). PCR products from these primers were also sent for sequencing.

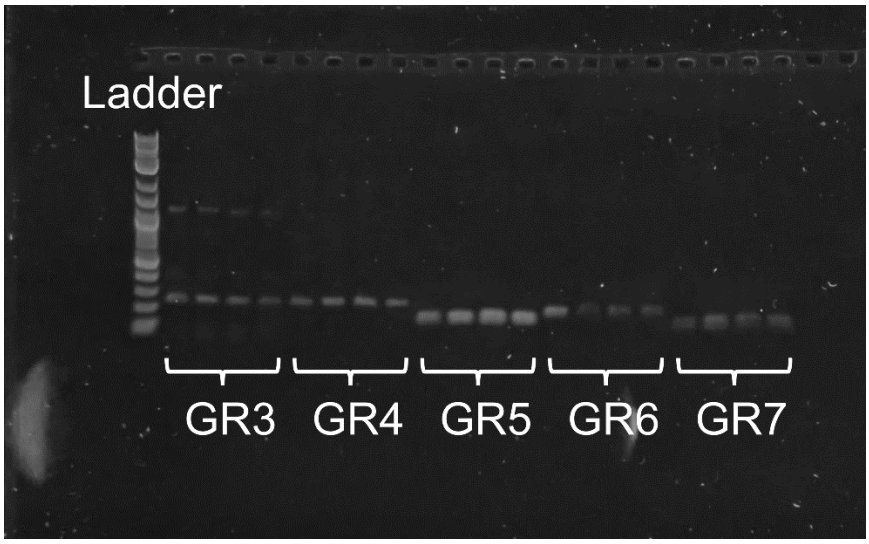

Figure 4.5. Gel electropherogram of PCR amplification with primers GR5, GR6, and GR7**.** Although the GR3 lanes appear to have an additional band, the lanes with GR5, 6, and 7 all have strong single bands.

4.3.2 Sequencing PCR products using modern DNA

PCR products from experiments with modern DNA were sent for sequencing to confirm that the primers were amplifying the correct region of DNA. Of the primer pairs tested in modern DNA, only five pairs produced PCR products that were sent for sequencing. These were the pairs GR3, GR4, GR5, GR6, and GR7. Samples were sent for sequencing three times. The first set of samples (sent December 2022) was lost by Génome Québec, and included samples amplified with GR3 and GR4. The second set of samples (sent January 2023) all had failed sequencing reactions, and included samples amplified with GR3, GR4, GR5, and GR7. The third set of samples, sent February 2023, produced usable results, and included samples amplified with GR3, GR4, GR5, GR6, and GR7.

After analysing the failed sequencing results from January, it was hypothesized that the concentration of the samples might be affecting the success of sequencing. Specifically, Genome Quebec recommends that sample concentrations be between 1 ng/uL and 10 ng/uL. Therefore, after running another PCR experiment with the same primers and protocols, an Invitrogen Qubit 2.0 fluorometer was used to measure the concentration of DNA in all the resulting PCR products. The results of fluorometry are included in Table 4.2. Highlighted samples were higher than the recommended concentration and were therefore diluted by a factor of 1:10 before being sent to Genome Quebec.

Table 4.2. Concentration of DNA in PCR products

SAMPLE	CONCENTRATION (ng/uL)	PRIMER PAIR USED
DNA V.I[1]	21.20	n/a
DNA V.II	16.88	n/a
DNA V.III	10.94	n/a
1A	10.96	GR3
1B	6.52	GR3
1C	7.54	GR3
1D	8.62	GR3
1E	7.26	GR4
1F	7.02	GR4
1G	6.78	GR4
1H	3.32	GR4
2A	12.66	GR5
2B	21.00	GR5
2C	22.8	GR5
2D	17.12	GR5
2E	10.4	GR6
2F	3.80	GR6
2G	4.26	GR6
2H	2.98	GR6
3A	5.90	GR7
3B	7.02	GR7
3C	5.80	GR7
3D	4.36	GR7

[1]The first three lines of this table show the base concentrations of the DNA extractions, to compare to concentrations after PCR.

Furthermore, the PCR product of the primer pair GR6 was sent for sequencing this time based on the successful PCR result (see Fig. 4.4). Upon receiving results from Genome Quebec, twenty-five out of thirty-two samples had been successfully sequenced. The successful sequence results were used to create consensus sequences for each primer and then compared using BLASTn against the NCBI database to determine identity with NR3C1 (see Table 4.3). Although there were not enough usable sequence results to assess the success of the GR7 primer pair, GR3, GR4, GR5, and GR6 were all determined to have amplified the target section of NR3C1 in modern DNA.

Table 4.3. Results of primer consensus sequence BLAST against NCBI database.

Primer	BLAST-search % sequence identity with	E-value	No. sequences used to form consensus
GR3F	99.5	3.95e-91	3
GR3R	99.5	9.46e-93	3
GR4F	99.5	7.86e-94	2
GR4R	99.5	7.86e-94	2
GR5F	100.0	2.34e-38	6
GR5R	100.0	4.03e-42	3
GR6F	99.5	3.68e-64	3
GR6R	99.6	1.82e-61	3
GR7F	N/A	N/A	0
GR7R	N/A	N/A	0

Of the ten primer pairs tested, five pairs were successful at amplifying the promoter region of NR3C1 in modern DNA and moved forward to application in ancient

DNA (GR2, GR3, GR4, GR5, and GR6). The three pairs from the literature (NR3C1_1, NR3C1_2, and NR3C1_3) as well as two of the pairs I designed for this project (GR1 and GR7) did not move forward from method testing to application in aDNA. A full overview of all experiments performed at this stage is provided in Appendix C.

4.4 Application of method to ancient DNA

Following the method development and testing stage, the next step was to attempt to perform the method using archaeological aDNA samples. Three separate PCR experiments were run with all six aDNA samples each time, and sequencing was subsequently performed on promising PCR results. The first experiment was run with the primer pair GR2 in duplicate (i.e., two reactions for each sample). The results from this experiment are shown in Figure 4.6. Note that for simplicity, samples are referred to by a portion of their provenience number (see Table 4.4). Several bands were observed in some lanes (samples 1A3, 24A), while other lanes had unexpected band sizes (1A1, 24C).

Table 4.4. Sample provenience numbers and short names.

SAMPLE PROVENIENCE NO.	SHORT NAME
55L1A3.1	1A3
55L23H11.1	23H
55L1A1-1	1A1
55L24F12.1	24F
55L24A7.2	24A
55L24C24.1	24C

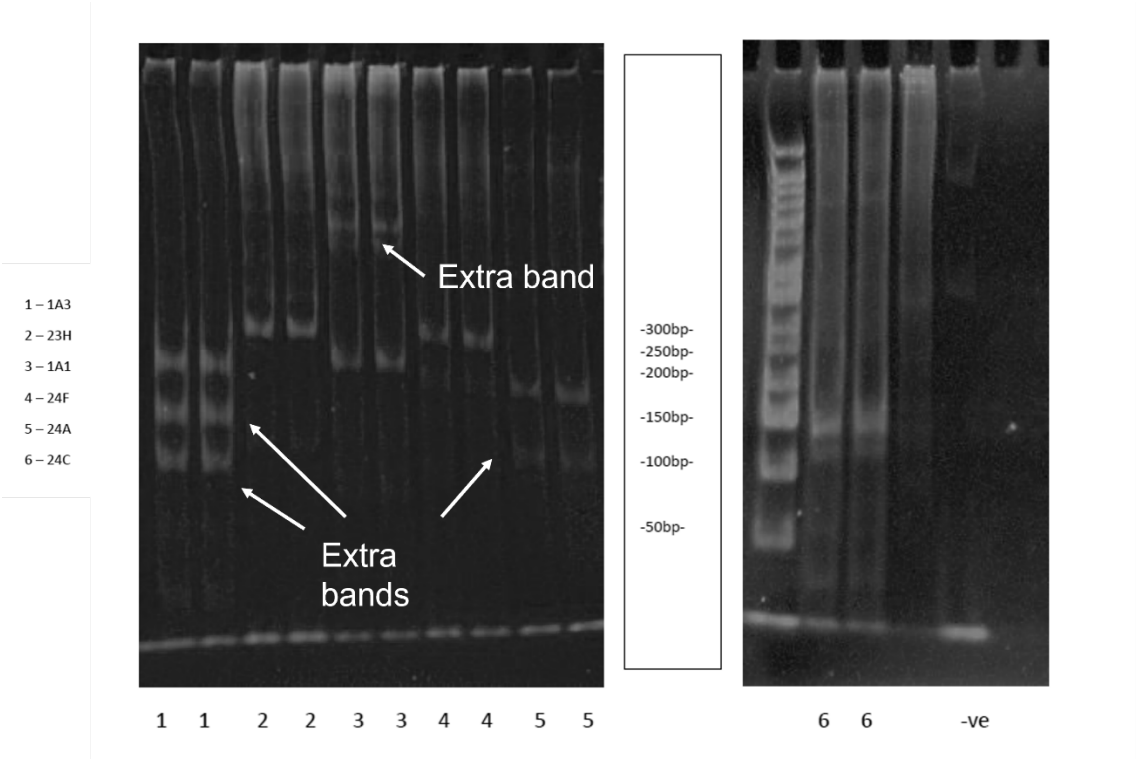

Figure 4.6. Gel electropherogram of PCR results in aDNA with GR2. Note the significant smearing in most lanes and that bands are all different sizes. GR2 was supposed to produce a band around 286 bp long. The absence of positive controls aims to limit contamination.

The PCR products with the most promising results, from samples 23H, 1A1, and 24F, were sequenced for more information. Unfortunately, the sequences were low-quality. In particular, the issue with these sequences was that amplification appears to have been not specific enough – instead of a high concentration of the target fragment, there was more than one fragment, which produced multiple inconsistencies in the sequence. Sequencing technology works by reading a chemical signal unique to each base (A, C, G, and T) – when more than one signal comes through for one position, the result is recorded as 'N',

to indicate that the result is indeterminate. Sequences with too many ‘N’s are unable to be used for any further analysis.

The second PCR experiment was run with the GR4 primer pair, also in duplicate with all six samples. The results of this experiment are presented in Figure 4.7. Samples 1A3, 1A1, 24A, and 24C had no amplification. Samples 23H and 24F had off-target amplification, where a band appears in the wrong position for the primer pair used. Additionally, the positive control failed. Sequencing was not performed for the second experiment due to the poor PCR results.

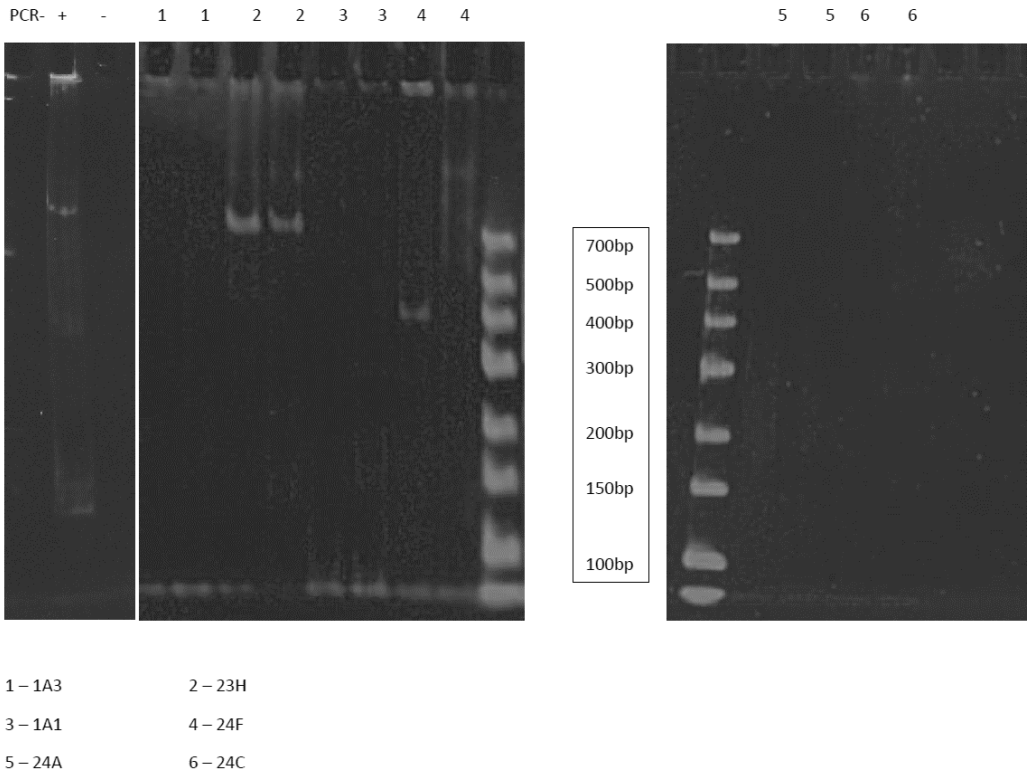

Figure 4.7. Gel electropherogram of PCR results in aDNA with GR4. GR4 should produce a DNA fragment about 260 bp long. Sample 23H has a band above 700 bp, and

sample 23F has a band around 400 bp. None of the lanes produced a band of the correct size.

The last PCR experiment was done with the primer pairs GR3, GR4, GR5, and GR6. This experiment was not performed in duplicate due to the lack of remaining aDNA from the extraction step, meaning only 24 reactions could be performed (not including positive and negative controls). The results of this experiment are presented as gel electropherograms in Figures 4.8a, b, and c. Many lanes had multiple bands (e.g., Lane 10 in Fig. 4.8a), and some lanes had no amplification at all (e.g., Lane 4 in Fig 4.8a). Lane 7 in Figure 4.8a, which used DNA from Individual 55L1A1 with the primer pair GR3, was considered the most promising out of all the reactions. Therefore, we decided to proceed with sequencing for this reaction only. The sequencing results were high-quality enough to proceed with analysis (see Table 4.5 for sequence data). Q20, Q30, and Q40 scores were used to determine quality. Q20 indicates an accuracy of 99% (error rate 1 in 100 bases), Q30 an accuracy of 99.9%, and Q40 an accuracy of 99.99%. However, the sequences did not appear to align to any known region of the genome and did not align with the target section of DNA.

Table 4.5. Sequence data for 55L1A1 amplified with GR3.

SEQUENCE NAME	SEQUENCE	% OF BASES AT LEAST Q20
1A1_GR3_F	GTTGGCCTGGCCGGGTGCNNCGTCTCNATGTGNNTTC NGAAGGNGNNNTGCCATGACTGCGGTCTGGGCTGCT CNNATCNNGNCATCNNCNGGGGTTTTNCNTGGNNNT TACNANNANCCCCCNANNCCGGGCGAANCNCGNGCN GCNAANANTGNNCTCATGCCNNCNANCATGNATC TGTTCTGNCGGGNCNCCCTTTTTNCNGGGGGANG	53.7%
1A1_GR3_R	GANNGANNTTNNCNCCGCGCGCGCTTCNCCCGGTAT CGGGGGATCCTCGTANCTTCCNCGCAAAACCCCNNG NGANNNNNNNATCCNAGCNNCCCNNACCNNANTCN TGGCAATTTCACCTTCCNAATGNNNNTCNANACNTT TNNNNCCNGNNNNGNCANNCTCNCCCTGGGACGTT TGCNANCANGA	45.4%

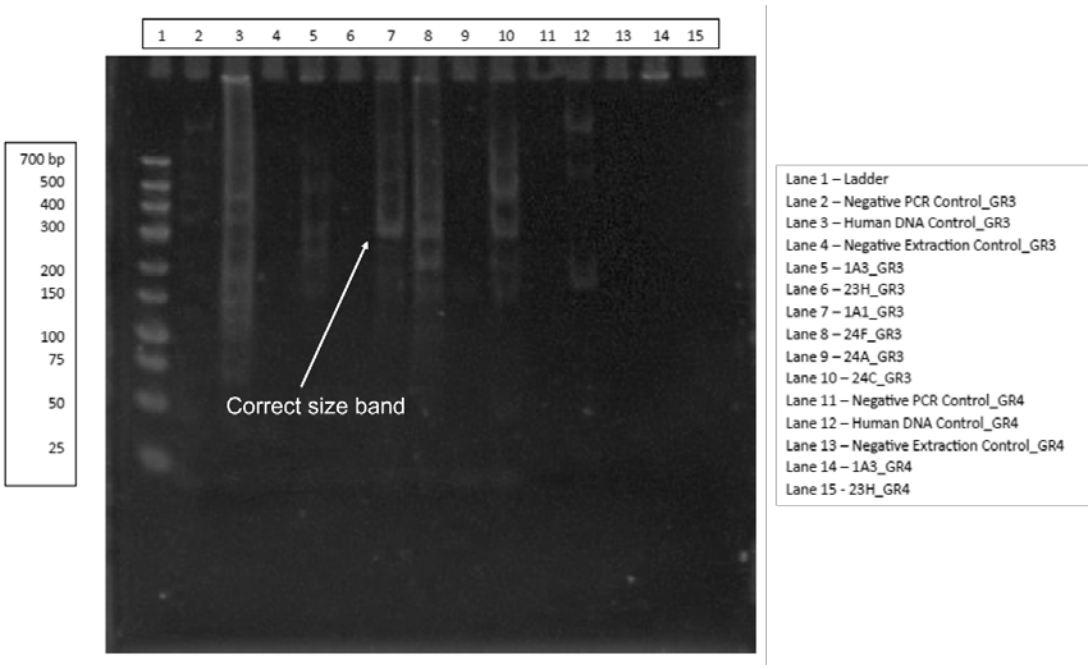

Figure 4.8a. Gel electropherogram of PCR results in aDNA with GR3 and GR4. Note that Lane 7 has one single band of the correct size for GR3 (286 bp). Lanes 8 and 10 also have bands of the correct size but have more than one band overall in the lane. Although Lane 12 is meant to be the positive control, we did not obtain a band of the correct size.

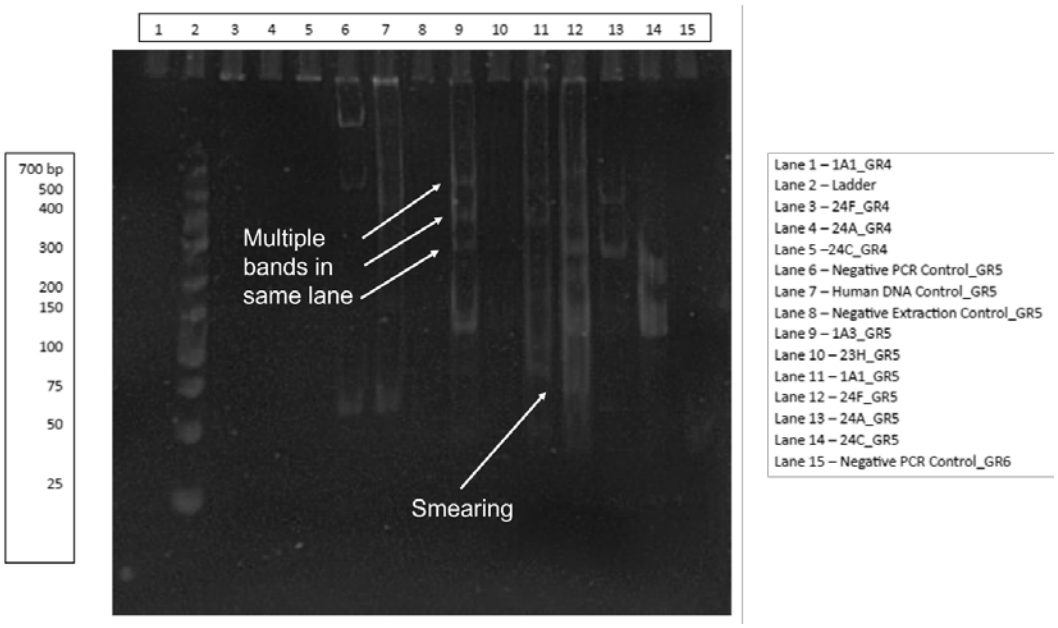

Figure 4.8b. Gel electropherogram of PCR results in aDNA with GR4, GR5, and GR6. Lanes 6, 7, 8, 11, 12, 13, and 14 have multiple bands, and lane 12 has smearing. Other lanes, like 3, 4, 5, and 10, have no amplification at all.

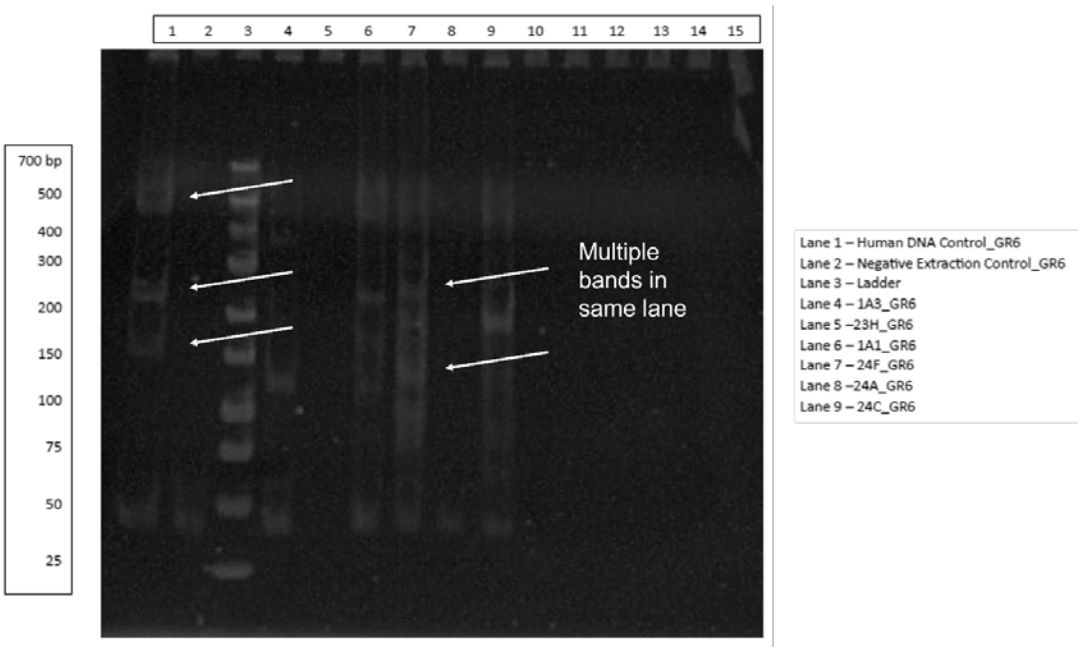

Figure 4.8c. Gel electropherogram of PCR results in aDNA with GR6 and GR7. Note that the positive control (lane 1), which should give one single band at the correct size, also has multiple bands.

The plan was to continue with bisulfite sequencing after performing PCR with the aDNA samples. However, due to both the poor performance of the primers in the aDNA samples and the limited amount of aDNA remaining, this plan was no longer feasible.

4.5 Conclusion

This chapter has reported the outcomes and results of this project. Extraction and purification of ancient DNA from archaeological sources was successful in five out of the six tooth samples, even though concentrations were low. Out of the ten primer pairs tested, six were able to reliably amplify the correct region of DNA using modern sources, and five were confirmed with sequencing. The last stage of this project, which was to

apply the method to aDNA, did not succeed as no experiment was able to amplify the promoter region of NR3C1. Therefore, bisufite sequencing could not be completed.

CHAPTER 5: DISCUSSION

5.1 Introduction

This chapter will discuss the implications of low aDNA concentrations, including typical extraction concentrations and recommended minimum concentrations for downstream analyses. Next, the results of the method testing stage will be explored, including difficulties encountered in troubleshooting primers and the varied results of sequencing. Finally, this chapter will explore the difficulties encountered in applying this method to aDNA and potential explanations for these challenges.

5.2 Extraction and purification of aDNA

The results obtained from the extraction and purification of aDNA at the Lakehead Paleo-DNA Lab reflect a common issue in aDNA work. The concentrations of the six archaeological samples were very low (<1 pg/uL), except for individual 55L24A7.2 (564 pg/uL). For context, commercial DNA extraction kits such as the Qiagen MiniKit report typical extraction concentrations between 5 - 15 µg with modern DNA. A concentration on the minimum end of this range, 5 µg, would still be equivalent to 5,000 ng or 5,000,000 pg. This is higher than the highest concentration obtained from the individual 55L24A7.2 tooth sample (564 pg/uL) by a factor of 10^4. Furthermore, the aDNA concentrations in this project were still low compared to typical aDNA yields. The aDNA extraction protocol used in this project, from Loreille et al. (2007), reports their method yielding aDNA concentrations ranging from 25 pg/uL to 109,980 pg/uL. Only one tooth sample in this project yielded an aDNA concentration within this range. There are many potential implications of these low concentrations of aDNA, which will be explored in the relevant sections of this chapter.

One possible reason for the low concentrations of aDNA in this study is the characteristics of the burial environment. Although some degeneration of DNA is expected due to the age of the samples (Molak & Ho, 2011), there are other factors influencing degeneration in the burial environment, including temperature, pH, and the concentrations of specific chemical compounds. Chemical aDNA damage processes like oxidation and hydrolysis are temperature- and pH-dependent (Gaeta, 2021; O'Rourke et al., 2000). While these reactions also take place within living cells, DNA repair mechanisms continuously address the damage; however, after death these processes remain unchecked. Generally, in archaeological contexts lower temperatures in the burial environment are associated with decreased reaction speed and less damage to DNA, with one study noting that degradation can be up to 25x slower with a decrease of 20˚C (Höss et al., 1996; O'Rourke et al., 2000). Smith et al., (2001) introduced the concept of thermal age, which combines temperature and age in years to determine DNA preservation. Essentially, aDNA is more likely to be recovered from sites that have lower temperatures, even if the sample is chronologically older (Smith et al., 2001). This indicates that temperature is just as important to consider as the age of the sample when analysing aDNA preservation. Additionally, certain soil-derived compounds (e.g., tannins, humic acids, and fulvic acids) can act as PCR inhibitors (O'Rourke et al., 2000). Although these compounds can be removed from the sample through adjustments to the aDNA extraction process, small amounts can remain in the sample and interfere with the PCR process.

Although it is difficult to make definitive statements about the specific burial environment of the tooth samples used in this study, we can examine the temperature, pH, and soil conditions at the Fortress of Louisbourg. The Fortress of Louisbourg is

located on Cape Breton Island in Nova Scotia and faces the North Atlantic Ocean. Between 1981 and 2010, the average yearly maximum temperature at Louisbourg was 9.5°C, and the average minimum was 1.9°C (Environment Canada). Although Louisbourg experiences cold weather, the average temperatures over that 30-year period are not low enough to significantly contribute to aDNA preservation. Soil pH on Rochefort Point is on average acidic at pH 6.3 (Scott & Fonzo, 2019). More alkaline values at the site are associated with better skeletal preservation (Scott & Fonzo, 2019), and it has been established within the literature that better skeletal preservation is further associated with better aDNA preservation (Campos et al., 2012; Lindahl, 1993). Scott and Fonzo (2019) also note that pH is variable throughout the small area of Rochefort Point that was tested, suggesting that soil pH may vary throughout other areas as well. Although not as influential as temperature and pH, salt concentrations are also known to increase the rate of aDNA degradation (Campos et al., 2012; Lindahl, 1993; Smith et al., 2003). Louisbourg in general and Rochefort Point in particular, are in close proximity to the Atlantic Ocean, and during winter storms it is common for seawater to flood the Point, which would increase salt concentration in the soil. Since so little is known about the provenience of the tooth samples in this study, it is not possible to definitively say whether the depositional environment played a role in the preservation level of the aDNA, and consequently the low concentrations. However, considering what is known about the conditions at Louisbourg, it certainly seems possible that the temperature, soil pH, and salt concentrations could have caused the low aDNA concentration in the archaeological tooth samples.

5.3 Method development and testing with modern DNA

5.3.1 Primer design and testing

Method development and testing with modern DNA encountered several roadblocks, especially when optimizing primer protocols to amplify modern DNA. The primer pair GR1 had no success, while GR2 had some success after multiple rounds of troubleshooting. The main issues encountered were non-specific amplification (extra bands), and PCR failure (no bands). Non-specific amplification, or the presence of extra bands, can mean that one or more of the reagents have been contaminated. When this issue was encountered, fresh reagents were made and used in the next experiment, according to which reagent was most likely to have caused the contamination. For example, if only one lane showed contamination, that would likely require fresh primer stock solution. Whereas if all lanes showed contamination, the issue was much more likely to be another reagent used in all lanes, such as nuclease-free water. The issue of PCR failure, when no bands were observed in the lane (this occurred with both GR1 and GR2), could have been the result of several factors, including an incorrect annealing temperature, too few PCR cycles, not enough DNA, or degraded DNA. To check if the annealing temperature was incorrect, a thermal gradient PCR was run where each sample in a row of eight used an incrementally higher annealing temperature than the last. The best-performing sample helped determine the annealing temperature used in future PCR experiments. The number of PCR cycles was increased, from 35 to 40. Finally, to check if the DNA sample was at fault, either more DNA was used in the next reaction (typically increasing volume by 0.5uL), or a new DNA extraction was performed.

The primer pairs NR3C1_1, NR3C1_2, and NR3C1_3 were the only three pairs taken from the literature (Van Der Knaap et al., 2014). Although the protocol provided in the supplementary materials of their article was followed identically, the authors did not include the specific DNA polymerase that was used with their parameters to achieve the outlined results. In the absence of this information, three DNA polymerases were trialled: OneTaq, LongAmp, and PlatTaq, with no success. An attempt was made to reach out to the corresponding author of the study to ask which DNA polymerase had been used, but with no reply. It is possible that these primers may have had more success if the appropriate DNA polymerase had been used, as annealing temperatures are typically calibrated to each specific DNA polymerase.

In the case of primer pairs GR3 and GR4, very little troubleshooting was required – the first PCR experiment ran with these primers produced clear, bright, single bands of the correct size. After the initial success, however, there were a string of several failed experiments. When later experiments encountered issues, they were either a) contamination from old reagents or b) degradation of the DNA extracts. In these cases, either new reagents were made, or a new DNA extraction was performed. These types of issues are common in PCR troubleshooting. Whether individual reagents (including DNA) have expired, been improperly stored, were contaminated by pipetting error, or have simply stopped working for an unknown reason, the first attempted fix is to make new reagents, either from safely stored stock solutions or by doing another extraction (Khrapko, 2006; Schrick & Nitsche, 2016).

Primer pairs GR5, GR6, and GR7 were made to test whether shorter DNA target fragments (<200 bp long) would improve results from application of the method to

aDNA. All the previous primer pairs were designed to produce fragments between 250 bp and 300 bp. These primers also required minimal troubleshooting before a successful PCR result was obtained with modern DNA. Primer testing in modern DNA faced typical troubleshooting issues, mainly with reagents or protocol details rather than inherent limitations of the DNA.

5.3.2 Sequencing PCR products using modern DNA

The three attempts to sequence PCR products from modern DNA can be summed up as follows: one lost, one failed, and one mostly successful. Between the second and third batches sent, additional measures were taken to ensure the success of sequencing, namely decreasing DNA concentrations. The second batch of samples sent for sequencing all failed, meaning that the sequencing process was unable to 'read' the DNA fragment with enough certainty to generate a sequence. This batch of samples had not been quantified before sending them to Genome Quebec, so the DNA concentrations were unknown. After the failure of the second batch, it was hypothesized that the concentration of the samples might be affecting the success of the sequencing reactions. The recommended maximum concentration for Sanger sequencing from unpurified PCR samples was 10 ng/uL, and it was possible the samples were too concentrated. Therefore, before sending the third batch of samples for sequencing, they were quantified to determine the concentration of DNA in the samples after PCR, and samples with concentrations above 10 ng/uL were diluted by a factor of 1:10. The undiluted samples were also sent, to see whether dilution increased sequencing success.

Twenty-five out of thirty-two samples were successful. Analysing the seven failures, two of them used undiluted samples with DNA concentrations of 22.80 ng/uL

and 17.12 ng/uL, amplified with the primer pair GR5. The high DNA concentration was likely at fault for these two failures, since ten other samples using GR5 and a lower DNA concentration succeeded. Four of the failures used undiluted samples with concentrations in the recommended range (5.90 ng/uL, 7.02 ng/uL, 5.80 ng/uL, and 4.36 ng/uL). However, these samples were all amplified with the primer pair GR7, so there may have been a separate issue relating to primers, as none of the samples amplified with GR7 succeeded. The last sequencing failure was a diluted sample amplified with GR6 (original concentration 10.40 ng/uL). This reaction had been done in duplicate (i.e., two reactions with the exact same reagents, including the 1:10 dilution of the original DNA sample), and its duplicate succeeded. Therefore, this last failure may be due to inherent limitations in the sequencing technology, such as old or mishandled reagents, loss of sequencing extension products, or a blocked capillary, and it does not appear to be an inherent fault of either the DNA concentration or the primers.

5.3.3 Future recommendations for method development and testing

Overall, primer testing took up most of the time dedicated to this project. Since the aim was to develop a method for use with aDNA, the limitations of testing with modern DNA should be considered in any related future endeavours. Some potential changes that could be made to a primer testing phase in the future would be a) diluting concentrations of modern DNA to typical aDNA levels, b) testing whether any benchtop processes could replicate the fragmented state of aDNA, c) testing multiple primer pairs at once, and d) limiting troubleshooting time of any specific primer pair.

The first two recommendations have to do with replicating the conditions of working with aDNA in modern DNA. Although this was not considered in this project,

future work could attempt to alter modern DNA to more closely resemble aDNA, which would help with optimizing protocols for their intended purpose (i.e., to amplify aDNA). First, diluting the concentrations of modern DNA to aDNA levels could help identify concentration-related issues and adapt the protocol without doing extensive work with limited aDNA samples. Second, testing should be done to determine whether benchtop processes (such as inversion or vortexing) could produce fragmentation patterns similar to aDNA. Vortexing is already used in certain cases to create smaller fragments of DNA (see Koetsier & Cantor, 2021). If so, this would provide a better proxy DNA source for testing, and potentially avoid some of the issues encountered when protocols were applied to aDNA samples.

The other two suggestions have to do with efficiency in testing, which was a major issue in this project as a great deal of time was spent troubleshooting primer issues using modern DNA. Thirdly, multiple primer pairs should be tested at once. Although testing aDNA samples with only a few primer pairs is preferable due to the limited volume of aDNA, there is no such limitation for working with modern DNA. When troubleshooting the method in modern DNA, multiple primer pairs should be tested at the same time to increase efficiency, and more quickly identify primers that do and do not work. Finally, troubleshooting for any particular primer pair should be limited. Although this issue is partially addressed by the previous recommendation to test multiple (>2) primer pairs at once, it is also important to discard primers that require extensive troubleshooting in order to work.

5.4 Application of method to aDNA

Although the primers were successful in amplifying the correct region of DNA when used with modern DNA, applying the method to aDNA produced significantly different results. Specifically, the results of applying this method to aDNA produced off-target amplification (i.e., bands of incorrect size), variation in band size between samples using the same primers, and multiple bands in the same lane. Furthermore, even if the PCR results appeared successful (i.e., a strong single band of the correct size), sequencing revealed that the amplified DNA fragment did not line up with the target region of the NR3C1 gene. There are several potential explanations for this: low aDNA concentrations, the level of damage in the aDNA samples, and the sequencing technology used.

5.4.1 Low aDNA concentrations

Some of the results seen could be explained by the low aDNA concentrations discussed earlier. Four out of the five samples with detectable aDNA concentrations had less than 1 pg/uL of aDNA. Low DNA concentrations increase the chances of both PCR failure (no bands) and non-specific amplification (extra bands), since the scarcity of template DNA can make amplification of exogenous DNA more likely, especially in aDNA (Lindahl, 1993). This could explain the results where no bands were detected in lanes, i.e., there was simply not enough aDNA in the sample for amplification to work. Total PCR failure occurred in 45.8% of all PCR reactions, over all primer pairs used (see Table 5.1). However, as one study notes, it is not uncommon for aDNA research to have failure rates over 50%, due to low concentrations, contamination, and PCR mishaps (Kaestle & Horsburgh, 2002). Finally, another study notes that aDNA PCR often requires

optimization (i.e., calibration of temperatures, times, and number of cycles) for each DNA extract, even between aliquots of one extract (Fulton & Stiller, 2012). All in all, the results seen, including the failure rates, are not unusual for aDNA research. However, the low aDNA concentrations do not account for all the results. Interestingly, the sample with the highest concentration of aDNA (564 pg/uL) had the most total PCR failures, indicating there are other contributing factors to these negative results.

Table 5.1. Total PCR failures by sample, in order of increasing aDNA concentration.

Sample Name	**aDNA Concentration (pg/uL)**	**No. of total PCR failures (no band in lane)**	**Total PCR failures (all experiments)**
55L24C24.1	*No detection*	4/8	50.0%
55L23H11.1	0.0897	4/8	50.0%
55L24F12.1	0.164	2/8	25.0%
55L1A3.1	0.223	4/8	50.0%
55L1A1-1	0.407	3/8	37.5%
55L24A7.2	564	5/8	62.5%
OVERALL	/	**22/48**	**45.8%**

Also, although the bisulfite sequencing step was not completed due to limited aDNA, one study noted that the bisulfite sequencing results were much more consistent when aDNA concentration reached above 0.015 ng/uL (15 pg/uL) (Smith et al., 2015). It is likely that even if the bisulfite sequencing step had been performed, the results would be inconsistent because of low aDNA concentrations.

5.4.2 Damaged aDNA

Another contributing factor is the quality of the aDNA, or how damaged it was. Although the aDNA samples were not evaluated for damage levels, it is a safe

assumption when working with aDNA that some postmortem damage has occurred (Jónsson et al., 2013; Lindahl, 1993). While many of the issues seen in this project can be attributed to the low concentrations of aDNA, issues with the preservation of the aDNA extracts can still significantly impact PCR success. Even at archaeological sites very conducive to preservation (e.g., permafrost), DNA fragments longer that 300 bp rarely survive (Fulton & Stiller, 2012; Stiller & Fulton, 2012). As some of the primer pairs (i.e., GR2, GR3, and GR4) amplify fragments close to that size (between 250 – 300bp), it is possible that damage to the DNA from the burial environment fragmented it into pieces too small for the primers to target. We attempted to address this issue with primer pairs that targeted smaller DNA fragments, specifically, GR5, GR6, and GR7 (between 100 – 200 bp). However, these primer pairs were also unsuccessful.

Apart from fragment length, other patterns of damage to aDNA can impact PCR success. As mentioned previously, C > T substitutions are prevalent in aDNA, and cause mismatches in the annealing phase of PCR (Brotherton et al., 2007). Mismatches can affect PCR specificity – although primers are designed to amplify one target sequence only (in this case, the promoter region of NR3C1), but enough mismatches could cause the primer to bind a different place in the genome and amplify the wrong target DNA. This effect, in particular, could explain some of the off-target results (i.e., bands of the wrong size). All PCR experiments with aDNA in this project used the high-fidelity DNA polymerase Invitrogen PlatinumTaq, where ‘high-fidelity’ means that mismatches are not tolerated in order to provide better specificity. Although this was intended to ensure that the primers annealed only to the target area of aDNA, is it possible that enough

substitutions in the aDNA sequence could have caused the primers to anneal to an off-target area, and produce the bands seen on the gel electropherograms.

5.4.3 Sanger sequencing versus next-generation sequencing

This project made the choice to use Sanger sequencing instead of next-generation high-throughput sequencing methods for the following reasons. First, Sanger sequencing tends to be more reliable when working with smaller target sequences of DNA, which is the case for this project. Second, Sanger sequencing is more affordable for non-genomic studies where only a small portion of DNA needs to be sequenced. Thirdly, and perhaps most relevant, is the fact that the Lakehead Paleo-DNA laboratory does not have the capability to perform next-generation sequencing (NGS). However, Sanger sequencing also has significant drawbacks: primarily that only one sequence can be generated from each sample, limiting its applicability to genome-wide studies.

While several studies in aDNA still use Sanger sequencing (see Ramirez et al., 2021; Shin et al., 2017; Van Dissel et al., 2019), next-generation sequencing technologies are also widely used (see Kaestle & Horsburgh, 2002; Khairat et al., 2013; Linderholm, 2016; Poullet & Orlando, 2020; Römpler et al., 2006; Weyrich et al., 2017). In fact, NGS is thought to have constituted something of a revolution within aDNA research because the conditions required for NGS, particularly the widely-used Illumina technology (150 – 250 bp reads) correlate well with the common conditions of aDNA. First of all, most NGS sequencing technologies require DNA to be in small fragments, which is the default for aDNA already. Since Illumina technology is optimized for small fragments, the fragmentation of aDNA is less of an issue. Although Sanger sequencing is limited to producing one sequence from one sample, NGS methods can produce multiple short

sequences, or ‘reads’ from one sample using massively parallel sequencing, which makes it an especially good choice for genome studies (Linderholm, 2016). Although the reasons for using Sanger sequencing in this project still apply, the fact remains that a different sequencing method, specifically an NGS method, may have produced better results.

5.5 Conclusion

This chapter has contextualized the results presented in Chapter 4 and has explained some potential causes of these negative results. First, this chapter discussed issues with the extraction step, primarily that the concentrations of aDNA in the samples were very low, which may have been impacted by the burial environment. Next, the challenges of method development were analyzed, including the efficiency of primer testing, DNA concentrations affecting sequencing failures, and the potential for future studies to consider altering modern DNA to behave as a better proxy for aDNA. In the last section, the application stage results were discussed, focusing on potential reasons for the failure of this stage. In particular, the low aDNA concentrations, levels of aDNA damage, and the choice of sequencing method were all explored as potential factors contributing to these results.

CHAPTER 6: CONCLUSION

6.1 Objectives and Aims of the Project

The goal of this project was to explore a novel method for assessing stress in bioarchaeology by adapting a method used in modern clinical research. Specifically, this project explored whether the NR3C1 gene could be isolated in aDNA and then examined for evidence of methylation that may speak directly to periods of early childhood stress. This chapter will begin by revisiting the specific objectives that were outlined in Chapter One, explore potential avenues of future research, and highlight the significance of this research to method development and bioarchaeological interpretations of stress in the past.

#1 Can aDNA be extracted from 18th century archaeological samples from Atlantic Canada?

Yes, aDNA was able to be extracted from five out of six tooth samples. Additionally, although the sixth tooth sample showed no detection when the aDNA extracts were quantified, some of the PCR experiments performed showed amplification with the sample, indicating there was at least some aDNA present. Likely the amount of aDNA was too small for the equipment to concretely measure, but after PCR, there was enough amplified aDNA to show up on a gel electropherogram. However, despite five of the six samples producing appreciable amounts of aDNA, most of the concentrations were low, with the exception of individual 55L24A7.2 which had a concentration within the typical range for the extraction method used.

#2 Can a protocol be developed to amplify the NR3C1 gene in modern DNA?

Yes, the protocol developed in this project was able to successfully amplify the target region of NR3C1 when using modern human DNA. This finding, while not particularly surprising given the previous success of other studies (see González Ramírez et al., 2020; Liu et al., 2021; Van Der Knaap et al., 2014), was nonetheless significant as new primers were used to perform the amplification. Since these primers were designed for this project and not taken from the literature, the confirmation of their success is a notable result.

#3 Can this method successfully amplify the NR3C1 gene using aDNA extracted from the archaeological samples?

This method was not able to successfully amplify the NR3C1 gene in aDNA. Several differences between modern and ancient DNA made this step particularly challenging, including the low concentrations of aDNA in the six tooth samples, the expected fragmentation, and damage patterns of the extracted aDNA, and the need for troubleshooting with an appropriate proxy for aDNA samples. Although this project was not successful in amplifying the NR3C1 gene in aDNA, the endeavour is not necessarily futile. With an established clinical relationship between NR3C1 and stress, and several successful studies in modern DNA, the issue likely lies in the specific characteristics of aDNA (and possibly the specific characteristics of this project's samples) and not with the gene target.

#4 Can bisulfite sequencing performed on aDNA samples produce usable data on the methylation patterns of NR3C1?

Unfortunately, the bisulfite sequencing portion of the project was not completed due to the limited volume of aDNA extracted from the six tooth samples and this question was not able to be answered. It is still an outstanding question whether methylation data of NR3C1 can be obtained from aDNA samples. It has been confirmed by multiple studies (see Van Der Knaap et al., 2014 in particular) that methylation data can be obtained from NR3C1 in modern DNA, so future aDNA studies may consider revisiting this question.

6.2 Future research

This project highlights some of the current issues in aDNA research, and further exploration into the following suggestions could ameliorate some of the identified challenges. Of particular interest is finding an appropriate proxy for aDNA to use in the testing phase, and additionally developing a reliable approach for single-gene targets in aDNA.

6.2.1 Finding a reliable proxy for aDNA

Arguably, one of the most important aspects of future research will be the establishment of an alternative proxy for aDNA during method testing, possibly through the alteration and/or modification of DNA from a modern source. This project has highlighted that modern DNA may not be a reliable proxy for aDNA, unless modified intentionally. As discussed in Chapter 5, it would be useful to have a method that can reproduce the fragmentation and low concentrations of aDNA, using modern DNA. One of the major roadblocks to developing new methods in aDNA is the limited amount of aDNA available for testing and troubleshooting. Since it is unlikely that any new method will work right away, some testing and troubleshooting will need to be done. In this

project specifically, the amount of PCR experiments performed using aDNA could have been reduced if modern DNA could be altered to more closely resemble aDNA, as per the recommendations in Chapter 5. This would have enabled more of the troubleshooting to be performed using modern DNA while still providing useful information about how well the method would work in aDNA.

6.2.2 Single-gene nuclear targets in aDNA research

In addition to developing a reliable aDNA proxy, another valuable question for future research is how to approach single-gene nuclear targets in aDNA, to better gain insights on individual genes and their research significance. Many studies in aDNA have focused on genomic approaches, where the entire genome is sequenced using an NGS method (Hanghøj et al., 2019; Lindqvist & Rajora, 2019; Poullet & Orlando, 2020). Additionally, many studies focus on mitochondrial DNA which is exponentially more abundant, increasing the chances of success (e.g., Juras et al., 2017; Oswald et al., 2019). Very few studies, on the other hand, focus on the analysis of single-gene targets in the nuclear genome, and when they do (e.g., Bolnick et al., 2012), it is often a gene that has more than one copy. Essentially, when nuclear genes are targeted, they are chosen based on likelihood of amplification rather than any particular gene's relevance to the research question. This is indeed the best choice when the goal is to show that amplification can occur, but as the field develops, researchers will need an established approach for investigating a given nuclear gene for its research significance. There are various technologies that may hold the answer to this issue, although the viability of each of these technologies should be investigated. Since the cost-effectiveness of short-read NGS (e.g., Illumina) does not hold when the target is small, Sanger sequencing may be the better

choice for investigating particular single-gene nuclear targets. However, an approach that uses Sanger sequencing will have to address the issues outlined earlier with fragmentation in aDNA. Another possible avenue for investigating single-gene nuclear targets in aDNA is finding a more cost-effective NGS method. For example, NGS can be the more cost-effective method if the sample size was increased, which would require running more reactions, and reduce the cost-per-sample (Arriola et al., 2023; Poethe et al., 2023).

6.3 Research significance

This thesis has contributed to the understanding of the challenges facing researchers hoping to integrate epigenetics and bioarchaeology and has some specific contributions to the field of stress research in bioarchaeology, specifically in the exploration of new methods. This project aimed to integrate modern clinical methods to answer archaeologically relevant research questions. It represented a new entry in the approach of integrating modern clinical research and bioarchaeology, by beginning with a well-established clinical relationship (i.e., methylation of the NR3C1 promoter region and early-life stress) and attempting to develop a method to assess one part of that relationship (methylation) to learn about the other (early-life stress). This approach aimed to close the gap in bioarchaeological stress research between the onset of stress and the current macroscopic methods bioarchaeologists currently use to explore stress. This research represented the first attempt to measure stress in past populations using aDNA and has contributed novel primers to target the NR3C1 gene promoter that can be used and further expanded in future research. Although multiple challenges were faced and not all objectives could be completed, the results of this project can give bioarchaeologists a

better understanding of the challenges faced in aDNA research and how to better integrate modern biomolecular methods into our interpretations of the past.

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APPENDIX A: RESEARCH ETHICS BOARD APPROVAL

From: David John Coleman
To: Natassja Ellen Brien
Cc: Ethics, Department of; Clara Evangeline Simpson; Amy Scott
Subject: REB 2021-006
Date: November 10, 2022 1:26:57 PM

Natassja Brien, Graduate Student
Faculty of Interdisciplinary Studies
University of New Brunswick Fredericton

To Natassja Brien:

I have reviewed your request for a modification of your previously approved application to the UNB Fredericton Research Ethics Board ("Extraction and sequencing of mtDNA from self" -- REB #221-006) for its compliance with Tri-Council Policy (TCP) and with UNB Policy (UPRIH). On the basis of the review, I am pleased to inform you that, in my opinion, your project appears to be still in compliance with TCP and UPRIH. Accordingly, please consider this E-mail to represent notification of the continuation of the previous REB approval of your project.

This approval is based on my understanding that the underlying nature of the research itself has not changed, and that — while your proposed modification still involves targeted amplification and sequencing of a portion of DNA — the target has changed to a gene in the nuclear genome instead of in mitochondrial DNA.

Please note that, in the future, if you find that you must make any changes to your protocol, any such changes must be considered and approved by the REB before they are implemented.

Best wishes for the successful completion of your research project.

— David

David Coleman
Chair • Research Ethics Board
Professor Emeritus • UNB Fredericton
T 506 453-5189 E dcoleman@unb.ca

UNB campuses are open with strict adherence to New Brunswick Public Health requirements. Our priority continues to be the health and safety of our community. The Office of the Vice President (Research) and the Office of Research Services continue to work remotely with minimal staffing in the office. Access to these offices is by appointment ONLY. To contact our staff, visit our website at https://www.unb.ca/research/contact.html. Some delays may occur. We appreciate your patience.

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UNIVERSITY OF NEW BRUNSWICK

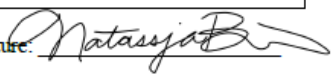
University of New Brunswick – Research Ethics Board

Application for Modification of Research Project Involving Humans

REB File Number: REB #2021-006. Application Date: 11/07/2022 (mm/dd/yyyy)

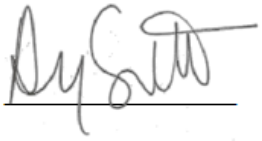
Project Title:

Extraction and sequencing of mtDNA from self

Principal Investigator Name: Natassja Brien Signature: 

Email Address: nbrien@unb.ca

Department/Faculty: Interdisciplinary Studies

Supervisor Name(s): Amy Scott Signature(s): 
(Note: Only when PI is a student)

Summary of Requested Modifications:

Will the risk of harm or deception/partial disclosure change? Yes ☐ No ☑
Will the nature or the objectives of the research change? Yes ☐ No ☑
Will the changes to the research design be: Major ☐ Minor ☑ None ☐
Will the changes to the ethics protocol be: Major ☐ Minor ☐ None ☑
Which sections of the approved REB application will be affected by the modification(s)?
**List all affected Sections (e.g. Recruitment, Consent, Data Security etc.) below.

The objectives of the research are the same - targeted amplification and sequencing of a portion of DNA - but the target has changed to a gene in the nuclear genome instead of in mitochondrial DNA.

Detailed Description of the Requested Modifications:
Outline the planned changes to the research design and/or ethics protocol (status before the changes and after the changes) along with justifications and implications.

The research plan has changed after the original plan became infeasible due to lack of required equipment. The new research plans to target a nuclear gene, NR3C1, instead of the whole mitochondrial genome. Risks for the research remain low to nonexistent. The principal investigator (myself) continues to be the only participant, and I understand the research and consent to my own DNA being used in this manner.

- If more pages are needed, please attach them and check here:
- Please append the approval notice from your department or faculty Ethics Review Committee should one exist.
- Original signatures are required.

From: David Coleman
To: Natassja Ellen Brien
Cc: Ethics, Department of; Elizabeth Harrison; Jason Addison; Amy Scott
Subject: REB 2021-006
Date: January 21, 2021 11:42:58 AM

Natassja Brien, Graduate Student
Faculty of Interdisciplinary Studies
University of New Brunswick Fredericton

To Natassja Brien,

As Chair of the Research Ethics Board (REB), I have reviewed your ethics application for the project entitled "Extraction and sequencing of mtDNA from self" which has been assigned the file number REB #2021-006. On the basis of the review, I consider your project to be eligible for delegated review, since any risk to participants that might exist appears not to exceed the "minimal risk" outlined in the Tri-Council Policy Statement, 2nd edition (TCPS2). I am also pleased to inform you that, in my opinion, your project is in compliance with TCPS2 and the University Policy on Research Involving Humans (UPRIH). Accordingly, please consider this E-mail to represent official notification of REB approval of your project for a period of three years from the date of this E-mail.

Please note that, in the future, if you find that you must make any changes to your protocol, those changes must be considered and approved by the REB before they are implemented. Please submit the REB Case Modification Request form, available online through the Research Ethics page of the Office of the VP (Research).

Annual Reports for this project are due on the 15th of January each year, provided that this date is at least six months after the date of project approval. Final reports are due 90 days after project completion. Form templates for both of these reports can be found on our website at https://www.unb.ca/research/vp/ethics.html.

Best wishes for the successful completion of your research project.

— David

David Coleman
Chair • Research Ethics Board
Professor Emeritus • UNB Fredericton
T 506 453-5189 E dcoleman@unb.ca

UNB campuses are open with limited access as we continue to work toward ensuring the health and safety of our community. More information is available here.

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FOR REB USE ONLY:
File No: _____
Date Complete App'n. Rec'd: _____
Approved/Approved with: _____
Modification/Rejected: _____
Date: _____

University of New Brunswick
Application for Review of Research Involving Humans

Principal Investigator(s): Name(s); Academic Status (Faculty, Undergraduate Student or Graduate Student); Academic Unit, e-mail Address, Office Telephone, Home Telephone:

Natassja Brien, Graduate Student, Interdisciplinary Studies, Natassja.brien@unb.ca, N/A, 204-229-9869

Title of Proposed Research:

Extraction and sequencing of mtDNA from self

Commencement Date: **December 14, 2020** Completion Date: **Jan 13, 2020**

Co-Investigator(s): Academic Unit, e-mail address, Office Telephone

Dr. Jason Addison, Biology, jaddison@unb.ca

Supervisor(s) (if Principal Investigator is a student); Academic Unit, e-mail address, Office Telephone:

Dr. Amy Scott, Anthropology, amy.scott@unb.ca

The undersigned parties certify that they have read, and undertake to comply fully with, the Tri-Council Policy Statement "Ethical Conduct for Research Involving Humans." (*Original signatures required*)

X Natassja Brien
Natassja Brien

Principal Investigator(s): _____
_____

Co-Investigator(s): _____

X _____
Dr. Amy Scott
Thesis Supervisor

Supervisor(s):_____

The undersigned certifies that the proposed research has been reviewed by, and is acceptable *in all respects* to, the academic unit(s) responsible. (*Original signature required*)

Dean/Director/Chair(s) Typed Name Dion Durnford, Chair

Signature _____

Date submitted to the REB:

1. Summary: Provide here, in approximately 300 words, a summary of the proposed research, indicating clearly the role of the research subjects and any procedures to which they will be subjected.

Natassja Brien will extract her own mtDNA from hair, blood, and saliva for purification and sequencing. Dr. Jason Addison will provide instruction and guidance to help Natassja develop the skills necessary to complete this work independently in the future. This will serve as coursework for the ANTH 6003 practicum course, intended as skills-building for the completion of Natassja's thesis project.

2. Risk: In your opinion, does this research pose more than minimal risk (Tri-Council Policy, Chapter 2, Section B) to participating subjects? *Yes* *No X*

If yes, provide here a statement that describes in detail the aspects of the research procedure that pose a risk to subjects, and provide your assessment of the risk of harm (probability and severity). Note that not only physical injury but also anxiety or embarrassments are included in the concept of harm. Describe means adopted to minimize risk, and means (such as provision of counseling) to deal with harms, which subjects may experience. Describe as well the potential benefit, which will result from this research, which justifies the above risk of harm.

3. **Deception:** Does this research involve deception or partial disclosure? *Yes* *No X*

If yes, refer to the Tri-Council Policy, Chapter 3, specifically Article 3.7 and subsequent commentary, and provide here an explanation of how you plan to comply with the requirements of that Section for debriefing. Describe as well the potential benefit, which will result from this research, which justifies waiving the normal requirements for full disclosure.

4. **Funding:** Has funding been received for this research? *Yes* *No X*

If yes, *from* what agency and for what period?

If yes, *from* what agency and for what period?

5. **Research Subjects:**

5.1 Number of Subjects: How many subjects will participate in this research? **1**

5.2 Recruitment: How will they be recruited, and from what population?

The only participant is Natassja Brien, also the applicant.

6. **Informed Consent:**

6.1 Informing Subjects: How will the nature of the research be explained to potential subjects, in compliance with Chapter 3, specifically article 3.2 and the subsequent commentary of the Tri-Council Policy? Attach a copy of any document(s), such as an explanatory letter, to be used for this purpose.

The nature of the research is known to the only participant, since she is conducting the research.

6.2 Consent: If written evidence of informed consent will be obtained, attach a copy of the consent form. (See Requirements for Informed Consent Forms.) **If written evidence of informed consent will not be used, explain here, in detail, how you intend to comply with the requirements of Chapter 3, Article 3.12 and the subsequent commentary of the Tri-Council Policy:**

6.3 Children as Research Subjects: If the proposed research involves children as subjects, provide here a statement indicating how compliance with Chapter 3, Section C, and specifically with Articles 3.9 and 3.10 of the Tri-Council Policy, will be achieved.

6.4 Incompetent Adults as Research Subjects: If the research involves adults of diminished competence as subjects, provide a statement indicating how compliance with Chapter 3, Section C, and specifically with Articles 3.9 and 3.10 of the Tri-Council Policy, will be achieved.

7. Inducements: Will any inducements (money, grade points, etc.) be offered to encourage participation? *Yes* *No X*

If yes, indicate here how compliance with Chapter 3, Article 3.1 and the subsequent commentary of the Tri-Council Policy (concerning voluntariness) will be achieved. If academic rewards are to be used, give details of alternative means of achieving equivalent rewards.

8. Private Information: Does the proposed research involve accessing identifiable personal information about subjects by means of surveys, questionnaires, etc.? *Yes* *No X*

If yes, indicate here, in detail, how you propose to meet the requirements of the Tri-Council Policy, Chapter 5, Section B and C. A copy of any questionnaire, survey document or interview schedule to be used should be attached as well.

9. Feedback: Describe the measures, which you propose for providing feedback to research subjects concerning the outcome of the research.

The outcome of the research will be known to the only participant since she is also the primary researcher.

10. Data Security: Describe the measures, which you propose for ensuring the security of any identifiable personal data, which will be retained after completion of the research.

NA

11. Continuing Review: All research requires brief annual reports and a brief report upon completion of the research. Suitable report forms are included at the end of this file. **Research involving more than minimal risk may require additional measures for continuing review.** If your research involves more than minimal risk, describe here the measures you propose for facilitating continuing review of this research, in compliance with Article 2.8 of the Tri-Council Policy.

12. Additional Information: Please feel free to append any additional information, which you feel may be helpful to the REB in evaluating this application.

The training for this practicum course was originally meant to take place at the aDNA lab at Lakehead University, but due to COVID this is no longer possible at this time, so this is the best alternative. This practicum is a requirement for Natassja's graduate degree.

Checklist for Attachments to Application for Review of Research Involving Humans

For items that are attached, indicate X; for items that are not applicable, indicate N/A.

Provide the following attachments where applicable:

X or N/A	
N/A	**Where the academic unit responsible for the research has a process of formal ethics review,** a copy of the approval notice from that process, together with any substantive comments provided by the reviewers.
N/A	**If external funding has been sought or obtained** for this research, **one copy only** of the complete application form as well as **two copies** of any reviewers' comments which have been received.
N/A	**For student research**, one copy of the full research proposal if one has been submitted to the relevant academic unit.
N/A	**A copy of any proposed information letter and/or informed consent form.** (Do not duplicate if already included in above material.)
N/A	**A copy of any questionnaire(s), survey documents or interview schedules** to be used in the research. (Do not duplicate if already included in above material.)
N/A	**A copy of any debriefing material to be provided to subjects.**
N/A	**For research under the jurisdiction of more than one institution**, an indication of which other REBs will review the research, and the results of such review if available (see Tri-Council Policy, Chapter 8, Section A).
N/A	**For all research (including student research) that exceeds minimal risk, which has not been approved by a sanctioned peer review process**, the applicant must recommend two reviewers competent to undertake a "scholarly review" of the proposed research. "Scholarly review" in this context refers to the process of determining whether the design of the research project is capable of addressing the questions being asked in the research.
N/A	**In all cases,** a full description of the proposed research, if this is not already contained in the material listed above.
X	**In all cases,** an electronic text version of the Summary (Item 1 on the application form), via e-mail to ethics@unb.ca on the UNBF campus or to reb@unb.ca on the UNBSJ campus.

Please append this checklist to the application form

APPENDIX B: PCR SPECIFICATIONS

Table B1. PCR reaction mix recipe by DNA polymerase.

	Total volume of mix	**Ingredient**	**Concentration**	**Amount**
OneTaq	25uL	OneTaq MasterMix	2X	12.5uL
		Forward primer	10uM	0.5uL
		Reverse primer	10uM	0.5uL
		Template DNA	various	1uL
		Nuclease-free water	n/a	10.5uL
LongAmp	25uL	LongAmp buffer	5X	5uL
		dNTPS	10mM	0.75uL
		Forward primer	10uM	1uL
		Reverse primer	10uM	1uL
		LongAmp DNA Pol		1uL
		Template DNA	various	1uL
		Nuclease-free water	n/a	15.25uL
PlatTaq	25uL	PlatTaq Buffer, -Mg	10X	2.5uL
		$MgCl_2$	50mM	0.75uL
		dNTPs	10mM	0.5uL
		Forward primer	10uM	0.5uL
		Reverse primer	10uM	0.5uL
		Template DNA	various	1uL
		PlatTaq DNA Pol		0.1uL
		Nuclease-free water	n/a	19.15uL
Qiagen Multiplex PCR Kit	25uL	Multiplex PCR Master Mix	2X	12.5uL
		Forward primer	10uM	0.5uL
		Reverse primer	10uM	0.5uL
		Q solution	5X	2.5uL
		Template DNA	various	1uL
		RNAse-free water	n/a	8uL

Table B2. PCR protocol details by primer pair.

PCR parameters	1. Initial denaturing	2. Denature	3. Anneal	4. Extend	No. Of cycles (repeat step 2 – 4)	5. Final extension
Base-line	95˚C for 2 min	95˚C for 30 sec	Use labeled annealing temp.	65˚C for 30 sec	30	72˚C for 5 min
			Optimized protocols			
GR1	95˚C for 3 min	95˚C For 30 sec	57˚C for 30 sec	68˚C for 1 min	35	68˚C for 5 min
GR2	95˚C for 3 min	95˚C for 30 sec	55˚C for 30 sec	68˚C For 1 min	35	68˚C For 5 min
GR3	95˚C for 2 min	95˚C For 30 sec	60˚C for 90 sec	72˚C for 30 sec	40	72˚C for 5 min
GR4	95˚C for 2 min	95˚C For 30 sec	60˚C for 90 sec	72˚C for 30 sec	40	72˚C for 5 min
GR5	95˚C for 5 min	95˚C For 30 sec	60˚C for 90 sec	72˚C for 30 sec	35	72˚C for 10 min
GR6	95˚C for 5 min	95˚C For 30 sec	60˚C for 90 sec	72˚C for 30 sec	35	72˚C for 10 min
GR7	95˚C for 5 min	95˚C For 30 sec	60˚C for 90 sec	72˚C for 30 sec	35	72˚C for 10 min
NR3C1_1[1]	94˚C for 15 min	94˚C for 20 sec	˚C for 30 sec	72˚C for 1 min	45	72˚C for 3 min
NR3C1_2[1]	94˚C for 15 min	94˚C for 30 sec	63-54˚C[2] for 30 sec	72˚C for 1 min	10	
	then	94˚C for 30 sec	53˚C for 30 sec	72˚C for 1 min	35	72˚C for 5 min
NR3C1_3[1]	94˚C for 15 min	94˚C for 20 sec	˚C for 30 sec	72˚C for 1 min	45	72˚C for 3 min

[1]All PCR parameters taken from van der Knaap et al., 2014.
[2]The protocol for NR3C1_2 had two sets of temperatures: a touchdown PCR protocol (meaning that the annealing temperature decreased every cycle by one degree Celsius) for the first ten cycles, then steady at 53C for the remaining 35 cycles.

APPENDIX C: PCR EXPERIMENTS

Primer pair	Taq used	Protocol used	Results	Details	Solutions tried	Possible issues or next steps
GR1, GR2	OneTaq	GR1, GR2	None	Gel issue		Need new running buffer
GR1, GR2	OneTaq	GR1, GR2	Poor	Only one lane showed a band	Made new TBE	Visualization problem?
GR1, GR2	OneTaq	GR1, GR2	None	Gel issue	Tested different voltage	Use 1X TBE instead of 5X
GR1, GR2	OneTaq	GR1, GR2	Adequate	Good amplification of GR2, poor amplification of GR1	Used 1X TBE	
GR1, GR2	OneTaq	GR1, GR2	Adequate	Bands too faint		Too few PCR cycles?
GR2	OneTaq	GR2	Adequate	Bands too faint	Increased no. of cycles	Temperature ?
GR2	OneTaq	GR2	Poor	Bands too faint, showing off-target bands		Raise annealing temp?
GR1, GR2	OneTaq	GR12GRAD	Poor	Barely any amplification		Increase DNA?
GR1, GR2	OneTaq	GR1, GR2	Poor	Barely any amplification	Increased amt of DNA	
NR3C1_1, NR3C1_2, NR3C1_3	OneTaq	NR3C1, VDK1	None	No amplification		Voltage too high?
NR3C1_1, NR3C1_2, NR3C1_3	OneTaq	VDK2	Poor	Primer dimer for NR3C1_1, no amplification otherwise	New DNA extraction	Temperature ?

Primer pair	Taq used	Protocol used	Results	Details	Solutions tried	Possible issues or next steps
NR3C1_2	OneTaq	NR3C1_2	None	power outage. PCR null		
NR3C1_1, NR3C1_2, NR3C1_3	OneTaq	NR3C1_1, NR3C1_2	Poor	One band for NR3C1_1, otherwise no amplification	Temp gradient	Touchdown PCR?
NR3C1_2	OneTaq	NR3C1_2	None	No amplification	Touchdown PCR	Increase DNA? Increase primers?
GR1, GR2	OneTaq	GR1, GR2	None	No amplification		DNA compromised? Test with known primers
ND1, CYTB, ATP6, COX1, HVS	OneTaq	MTGRAD	None	No amplification	Tested DNA with known primers	DNA compromised
CYTB, ATP6	OneTaq	CYTB, ATP6	Excellent	Strong single bands in all lanes	New DNA extraction	
GR1, GR2, NR3C1_1, NR3C1_2, NR3C1_3	OneTaq	GR1, GR2, NR3C1_1, NR3C1_2	Poor	No bands for GR2, NR3C1_2, NR3C1_1; faint bands for NR3C1_3, GR1		Enzyme?
GR1, GR2	LongAmp	GR1, GR2	Poor	Smeared bands for GR1, primer dimer for GR2	Different enzyme	Too much DNA? (Smearing)

Primer pair	Taq used	Protocol used	Results	Details	Solutions tried	Possible issues or next steps
GR1, GR2, NR3C1_1, NR3C1_2, NR3C1_3	LongAmp	GR1, GR2, NR3C1_1, NR3C1_2	Adequate	Good amplification of GR1, faint bands for GR2, no amplification for NR3C1_X pairs	thermal gradient; less DNA	*sent to Stephen for testing
GR2	PlatTaq	(Lakehead)	None	No amplification		Design new primers
GR3, GR4	LongAmp	GR34	Excellent	Strong single bands in all lanes		*sent to Stephen for testing
GR3, GR4	PlatTaq	(Lakehead)	None	No amplification, positive control failed		Difference in enzymes? Troubleshoot PlatTaq @UNB
GR3, GR4	PlatTaq	GR34GRAD	None	No amplification	Temp gradient	Possible power outage? Try with LongAmp positive control
GR3, GR4	PlatTaq, LongAmp	GR34	None	No amplification	Positive control with LongAmp	DNA issue?
GR3, GR4	LongAmp	GR34	Poor	Multiple bands, off-target	New DNA extraction	Primer contamination?
GR3, GR4	LongAmp	GR34	Excellent	Strong bands in all lanes	N/A	None
GR3, GR4	PlatTaq	GR34	None	No amplification		Temperature?
GR3, GR4	PlatTaq	GR34 GRAD	None	No amplification	gradient protocol	Unsure. Try positive control with LongAmp

Primer pair	Taq used	Protocol used	Results	Details	Solutions tried	Possible issues or next steps
GR3, GR4	PlatTaq, LongAmp	GR34	None	No amplification, even of LongAmp		Primer contamination?
GR3, GR4	LongAmp	GR34	Poor	Multiple bands, off-target	New DNA extraction	DNA or primer contamination?
GR3, GR4	LongAmp	GR34	Good	Faint bands, but on-target	New primer dilutions	LongAmp? Try OneTaq or order more LongAmp
GR3, GR4	LongAmp/OneTaq	GR34	Poor	Off-target bands; contamination	Compare LongAmp + OneTaq	Contamination: new DNA extraction, New approach: try Q solution
GR3, GR4	N/A	N/A	Good	Did new DNA extraction		
GR3, GR4	HotStarTaq (Q kit)	Q34	Adequate	Bands brighter, one lane has two bands	New enzyme protocol	Try comparison between different enzymes
GR3, GR4	OneTaq, Q solution, PlatTaq	Q34	Adequate	No amplification with PlatTaq, OneTaq best overall results	Comparison between different enzymes	Issue with DNA ladder and gel dye. Possible difficulty visualizing bands due to gel dye.

Primer pair	Taq used	Protocol used	Results	Details	Solutions tried	Possible issues or next steps
GR3, GR4	LongAmp	GR34	Good	Bands faint but on target	Replace gel dye, try LongAmp once more	Try to test again with PlatTaq and Q solution now that results from May have been reproduced; optimize temp for Q solution
GR3, GR4	HotStarTaq (Q kit)	QGRAD	Poor	Contamination	Temperature gradient	New primer stock - are primers contaminated? amount of Q solution to be added
GR3, GR4	Q solution, OneTaq	Q34	N/A	Thermocycler broken	N/A	Redo this PCR
GR3, GR4	HotStarTaq (Q kit)	Q34	Good	Some off-target effects; 50% of results adequate for sequencing	Adjust amount of Q solution added	Redo primer dilutions, adjust pipetting technique
GR3, GR4	HotStarTaq (Q kit)	Q34	Good	50% of results adequate for sequencing	New dilutions, different pipetting technique	Pipetting technique adjustments worked well - continue for next PCR

Primer pair	Taq used	Protocol used	Results	Details	Solutions tried	Possible issues or next steps
GR3, GR4	HotStarTaq (Q kit)	Q34	Good	50% of results adequate for sequencing	New pipetting technique	Still some off-target effects, although much improvement. Make sure to deep-clean workstation in case that is the source of contamination.
GR3, GR4	HotStarTaq (Q kit)	Q34	Excellent	All results adequate for sequencing, clear bands, no off-target effects	Workstation deep-cleaned in addition to new pipetting technique.	Accumulated enough samples to send for sequencing - ship samples to Genome Quebec
N/A	N/A	N/A	N/A	N/A	Sent samples to Genome Quebec for sequencing	Sent samples to Genome Quebec for sequencing
N/A	N/A	N/A	N/A	N/A	Genome Quebec reports samples lost. Redo PCR.	Genome Quebec reports samples lost. Redo PCR.
N/A	N/A	N/A	N/A	N/A	Did new DNA extraction	Did new DNA extraction

Primer pair	Taq used	Protocol used	Results	Details	Solutions tried	Possible issues or next steps
GR3, GR4, GR5, GR6, GR7	HotStarTaq (Q kit)	Q34	Excellent	No amplification for GR6, clear on-target amplification for all other pairs	New primers tried	Redo GR6 on a temp gradient to investigate possible issues with annealing temp; redo GR5 to get enough sample to send to Genome Quebec
GR5, GR6	HotStarTaq (Q kit)	Q1 (gradient)	Good	Amplification for GR5 but not GR6	Temperature gradient	Send to Genome Quebec
GR3, GR4, GR5, GR7	N/A	N/A	Poor	All sequences failed	Sequencing results from Genome Quebec	possibly too much or too little DNA sent - quantify samples using Qubit
GR3, GR4, GR5, GR6, GR7	HotStarTaq (Q kit)	Q34	Excellent	All primers worked, including GR6 which didn't work before		Quantitate samples and dilute if necessary, before sending to Genome Quebec
N/A	N/A	N/A	N/A	Sent samples to Genome Quebec		32 samples sent
GR3, GR4, GR5, GR6, GR7	N/A	N/A	Good	25 out of 32 samples succeeded, GR7 samples all failed		*send to Stephen for testing (not GR7)

CURRICULUM VITAE: NATASSJA BRIEN

Education

2020 – Present	Master of Interdisciplinary Studies in Bioarchaeology and Genetics, University of New Brunswick, Fredericton, New Brunswick
2020	Bachelor of Science (Honours) in Genetics, University of Manitoba, Winnipeg, Manitoba
2017	Bachelor of Arts (Honours) in Anthropology, University of Manitoba, Winnipeg, Manitoba

Presentations and Publications

2019 **Brien, Natassja E**. and the Living Prairie Mitogenomics Consortium. The complete mitochondrial genome of the North American pale summer sedge caddisfly *Limnephilus hyalinus* (Insect: Trichoptera: Limnephilidae). *Mitochondrial DNA Part B 4*(1), 413 – 415.

2017 **Brien, Natassja**. You Are What You Eat: A Biocultural Analysis of Canada's Arctic and the Inuit. Presented at the Manitoba Anthropology Students' Conference, University of Manitoba.

Academic Awards

2023 IGSS Travel Award - $400

2022 Third place, Images of Research Competition - $150

2021 SSHRC Joseph Armand Bombardier Canada Graduate Scholarship - $17,500

2020 Graduate Research Award, Faculty of Interdisciplinary Studies - $5,600

Graduate Research Award, Department of Anthropology - $5,000

Graduate Research Award, Department of Biology - $5,000

2019 Faculty of Agriculture and Plant Sciences Endowment Fund - $4699.30