# Pulling Your Hair Out:

# Exploring the Relationship between Bioarchaeological Cortisol and Carboxylated Osteocalcin to Study Stress in Past Populations.

By

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## ABSTRACT

The objective of this thesis is to investigate and validate the usage of biomolecular methods to study health and physiological stress in past populations through the analysis of osteocalcin and cortisol in human hair and bone. Both hormones play fundamental roles in the acute stress response (ASR) and how the body adapts to metabolic needs when responding to external stimuli (Fukuda and Morimoto, 2001; Moser and van der Eerden, 2019). Archaeological bone and hair samples were collected from 12 individuals from the 18$^{th}$-century Fortress of Louisbourg skeletal collection and quantified using an enzyme-linked immunosorbent assay (ELISA). The results of this study found a meaningful relationship between these two hormones archaeologically. This project significantly aids in expanding the biomolecular study of stress in bioarchaeological contexts through a more targeted and precise measurement of physiological stress than current macroscopic methods of stress analysis (Scott et al., 2016; Webb et al., 2010).

DEDICATION

To My Mom,
I used my best brain

ACKNOWLEDGEMENTS

**The Fortress of Louisbourg lies on the land of U'namaki, the unsurrendered and unceded territory of the Mi'kmaq nation.**

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ברוך אתה יהוה אלוהינו מלך העולם שהחיינו וקימנו והיגינו לאזמן הזה

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# Chapter 1: Introduction

## 1.1 Purpose

Bioarchaeology, the study of human skeletal remains from past populations (Nagaoka, 2023), combined with molecular biology is a novel approach to the study of archaeological stress by investigating the biomolecules involved in the stress response (Cappellini et al., 2018; Hodgson, 2012). Integrating these methods is a powerful tool that can shed light on how stress can be explored through biochemical means. While studying stress in past populations has been a focus of bioarchaeological researchers for decades, current methods leave significant gaps in what questions researchers can ask and the interpretations that can be made (Brien, 2023; Scott et al., 2016). By addressing these limitations, this research advances our knowledge of human health and the lived experiences of those in the past and paves the way for more comprehensive and insightful studies of biomolecular bioarchaeology in the future.

Stress is a universal human experience; as such, bioarchaeologists can use it to explore physiological reactions to impactful events throughout an individual's life (e.g., disease, violence, famine, poor living conditions, etc.) (DeWitte, 2018; Kellner et al., 2022; Klaus, 2014; Roberts and Manchester, 2007; Schaefer, 2017; Scott et al., 2016; Webb et al., 2010). However, because bone is one of the last tissues to respond to external stimuli, the time between a stress event and its manifestation in the skeleton limits research possibilities (Edinborough and Rando, 2020; Reitsema and McIlvaine, 2014; Webb et al., 2010). These limitations have prompted bioarchaeologists to start studying physiological stress on the molecular level to analyze the biochemical signals

that precede macroscopic changes to the skeleton (Brien, 2023; Buikstra et al., 2022; Hughes, 2020; Quade et al., 2021; Scott et al., 2020; Webb et al., 2010).

## 1.2 Significance of Research

This thesis aims to further the subdiscipline of biomolecular bioarchaeology by validating novel biomolecules associated with stress and investigating alternative methods for extracting stress hormones. Specifically, this study assesses the relationship between osteocalcin (a hormone tied to bone turnover and metabolic demands) and cortisol (a hormone that plays an active role in the physiological stress response) to validate osteocalcin as a viable biomarker for assessing physiological stress (Moser and van der Eerden, 2019; Sadoul and Geffroy, 2019). Unlike previous studies that have predominantly relied on cortisol extractions from hair samples, which rarely preserve archaeologically, this research additionally explores cortisol embedded in human cortical bone (Charapata et al., 2021; Quade et al., 2023). This study is the first to integrate osteocalcin with other biomolecules, furthering this new approach in bioarchaeological research. Additionally, this is the first study of its kind to study cortical bone as a cortisol reservoir and the first to study archaeological cortisol, from either hair or hard tissue, in a Canadian archaeological population. The adoption of biomolecular analyses in bioarchaeology is becoming increasingly popular among researchers, and this thesis significantly contributes to expanding the applicability of these innovative methods.

## 1.3 Objectives

Osteocalcin and cortisol have been successfully studied in past archaeological research with promising results; however, several limitations and knowledge gaps need to

be addressed before these biomarkers can be confidently adopted within the discipline (Charapata et al., 2018; Hughes, 2020; Quade et al., 2023; Scott et al., 2020). To address these gaps, this study will use an 18th-century skeletal sample from the Fortress of Louisbourg National Historic Site in Cape Breton, Nova Scotia. The Fortress of Louisbourg was a significant French fortress and town that played a crucial role in the struggle between the British and French for North America (Johnston, 2001, 1984). The site provides a unique opportunity to study the physiological stress of individuals living in a highly stressful environment. This thesis can, therefore, be broken down into four primary research questions:

1. Can osteocalcin be extracted from 18th-century human cortical bone samples from the Fortress of Louisbourg?
2. Can cortisol be extracted from 18th-century hair samples from the Fortress of Louisbourg?
3. Can cortisol be extracted from 18th-century human cortical bone samples from the Fortress of Louisbourg?
4. Is there a measurable relationship between bone osteocalcin and cortisol (extracted from hair and bone)?

From these research questions, it can be hypothesized that 1) based on the success of previous bioarchaeological studies, osteocalcin from bone and cortisol in hair should be able to be successfully extracted; 2) the chemical similarity of cortisol across mammal species should mean that procedures successful in extracting cortisol from animal bone should be successful when attempted on human bone and will likely vary between skeletal elements due to internal factors; and 3) there is likely a measurable connection

between carboxylated (bone) osteocalcin and cortisol, meaning that bone osteocalcin can be confidently used as a biomarker for bioarchaeological stress.

### 1.4 Outline of this thesis

This thesis is divided into six chapters. Chapter One reviews the purpose of this research, its significance, and the key objectives. The second chapter is a literature review that explores biological stress, the hormones involved with stress (i.e., cortisol and osteocalcin), and how bioarchaeologists have previously studied stress. This chapter will also address the applications and limitations of the methods used to study archaeological stress and the molecular analyses applied to address those limitations. A brief history of the Fortress of Louisbourg will also be provided, including some common stressors associated with this site and time period. Chapter Three outlines the materials and methods of this study. Specifically, it contextualizes the skeletal sample, the bone and hair sampling process, the procedure of extracting and quantifying osteocalcin and cortisol, and the measurement of skeletal preservation. Chapter Four presents the collected results, including osteocalcin concentrations, hair and bone cortisol concentrations, and preservation measurements. Chapter Five interprets these results, highlighting possible intrinsic, contextual, and methodological factors that may have influenced the collected osteocalcin and cortisol concentrations. Further, the relationship between cortisol concentrations from the two tissue types (i.e., bone and hair) and the relationship between cortisol and osteocalcin are discussed. Finally, Chapter Six concludes this thesis by revisiting the research questions and hypotheses, summarizing their significance, and providing suggestions for future research.

# Chapter 2: Literature Review

## 2.1 Introduction

This chapter will review the relevant background information of this thesis in three major sections. The first section focuses on stress from a biological perspective and how the body responds biochemically, specifically through the production of hormones (i.e., cortisol and osteocalcin) and how those hormones impact tissues such as hair and bone. The second section focuses on how bioarchaeologists have previously studied stress macroscopically and molecularly. The third section provides a brief history of the Fortress of Louisbourg, the living conditions at the Fortress, and how those living conditions make the Fortress an appropriate site for the bioarchaeological study of stress.

## 2.2 Biological Stress

### 2.2.1 The Acute Stress Response

Despite its ubiquity in our species, stress lacks a universally accepted definition, partially because stress has biological, psychosocial, and environmental components (Henley et al., 2013). Given this dynamic, for this thesis, stress will be approached from a biological perspective and defined as a compensatory response to negative intrinsic or extrinsic stimuli (Yaribeygi et al., 2017). Evolutionarily, stress exists as an adaptation to aid in evading harm and maintaining biological homeostasis (Xin et al., 2020). The cascade of biological changes in response to a stressor is known scientifically as the acute stress response (ASR) (Bloomfield et al., 2019). When we encounter a real or perceived threat to our safety, the ASR initiates biological processes that prioritize survival (e.g., increased heart rate, muscle reaction) and deprioritizes biological functions that do not

serve the immediate goal of threat aversion (e.g., digestion, reproduction) (see Fig. 2.1) (Bloomfield et al., 2019; Trumbull, 2020).

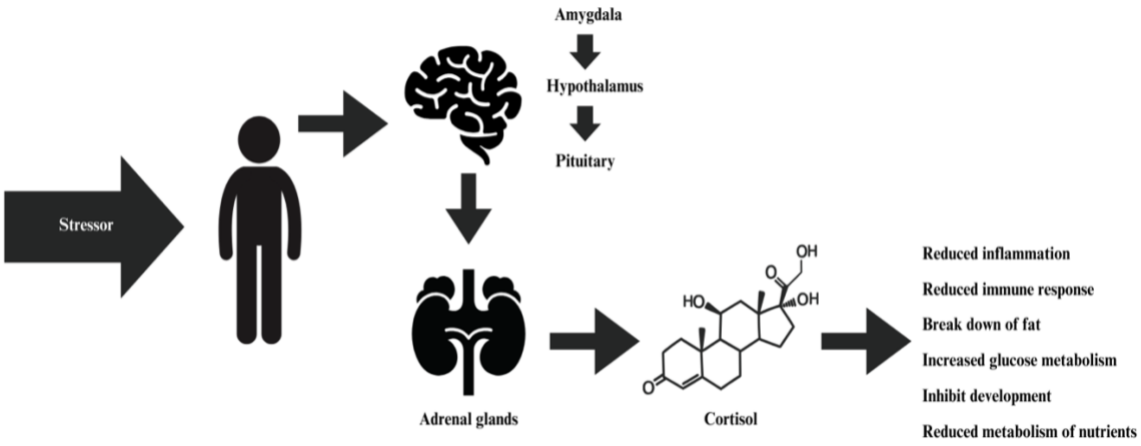


Figure 2.1: A graphic depiction of the series of events of the acute stress response and the activation of the hypothalamus-pituitary-adrenal axis (Modified from East, 2021)

At its foundation, the ASR is a hormonal response controlled by the brain and central nervous system (Almadi et al., 2013; Yang et al., 2013), and its activation is dictated by the hypothalamic-pituitary-adrenal (HPA) axis (Webb et al., 2010). Once the amygdala and hypothalamus perceive a threat, the hypothalamus communicates with the pituitary gland to signal the adrenal glands to begin secreting several hormones, primarily adrenaline, epinephrine, and cortisol that promote essential functions (see Fig. 2.1) (Azmi et al., 2021; Bergendahl et al., 1996; East, 2021; Floriou-Servou et al., 2021; Passeron et al., 2021; Sadoul and Geffroy, 2019). For example, during the ASR, the arteries narrow while the heart rate increases, forcing blood to be pumped harder and faster throughout the body. This is also assisted by inhibiting the inflammatory response, contributing to increased blood flow and, consequently, more oxygen and sugar reaching the muscles (Mariotti, 2015; Yang et al., 2011). Further, converting glycogen into glucose is

promoted, and insulin production is suppressed, which creates ample amounts of chemical fuel to feed the body's cells (Yang et al., 2011).

It is because of these various responses that the ASR results in feeling more alert and reactive, with many testimonials reporting that individuals are capable of physical feats well exceeding their typical ability when sufficiently stressed (see Bloomfield et al., 2019; Lopes Dos Santos et al., 2020; Passeron et al., 2021; Yang et al., 2013). When activated, the purpose of the ASR is to create the necessary chemical energy for immediate threat survival; however, the body also utilizes chemical energy for several other functions which do not serve the goal of direct threat aversion (e.g., skeletal remodelling, digestion, wound healing, etc.), and thus, these other functions are halted or slowed by the ASR (Antoun et al., 2017; Djurhuus et al., 2004; Fernald and Grantham-McGregor, 2002, 1998; Giustina and Wehrenberg, 1992; Kraemer et al., 2020; Sartin et al., 1994).

The ASR is intended to be a short-term adaption to a temporary threat, and in that acute phase, the ASR fulfills its role incredibly well. However, the impact of the ASR is not sustainable and, when activated for a prolonged period of time, can have adverse effects on the overall health of an individual (Antoun et al., 2017; Benight and Harper, 2002; Jamieson et al., 2013; Yang et al., 2011). For example, sustained hyperglycemia caused by the activation of the ASR can lead to weight gain, anxiety, insomnia, loss of pancreatic function, intense fatigue, and insulin resistance (Janssen, 2021; Kolb et al., 2020; Morales and Schneider, 2014; Sharifi et al., 2022). Additionally, hypercortisolism and chronic stress have been attributed to cardiovascular disease, poor mental development, loss of muscle mass, and several cancers (see Janssen, 2021; Mariotti,

2015; Sharifi et al., 2022). Many studies have also discussed how chronic stress (i.e., persistently elevated cortisol concentrations) during development results in children experiencing lower IQ, stunted growth, higher prevalence of autoimmune diseases in adulthood, and poor fertility (Fernald and Grantham-McGregor, 2002, 1998). To better understand the cascade of biological changes associated with the activation of the ASR, it is important to understand the hormones that trigger this process.

### 2.2.2 Cortisol and Glucocorticoids

Hormones are proteins or lipids that act as chemical messengers in the body, and an essential sub-group of hormones is steroids (Bereshchenko et al., 2018; Zubeldia-Brenner et al., 2016). Steroids are lipid hormones with a cholesterol (fat) group affixed to them, which allows for their message to be taken up quickly by the body's cells (see Fig. 2.2) (Angelousi et al., 2000; Zubeldia-Brenner et al., 2016).

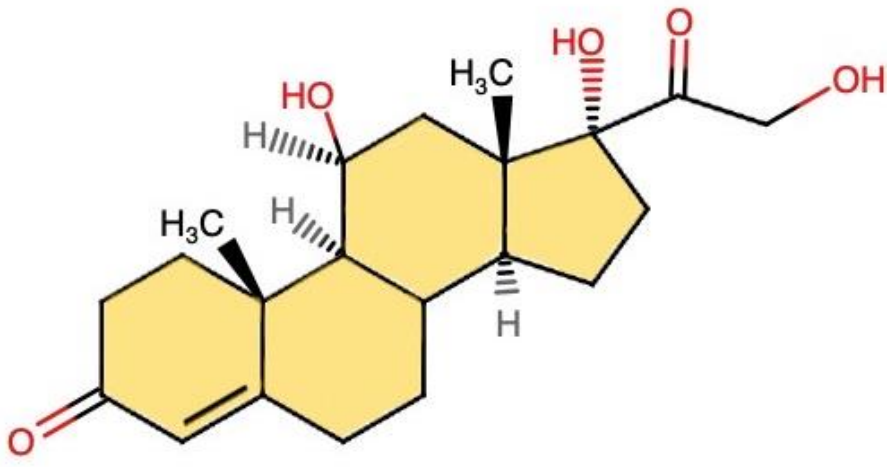


Figure 2.2: A graphic depiction of a human cortisol molecule with the cholesterol (fat) group highlighted in yellow

Specifically, glucocorticoids (i.e., glucose + cortex + steroid) are a pervasive class of steroid hormones whose primary function in the body revolves around metabolism, development, and inflammation (Angelousi et al., 2000; Bereshchenko et al., 2018; Taves et al., 2011). In every living vertebrate, glucocorticoids have pertinent cardiovascular, metabolic, immunologic, and homeostatic functions (Bereshchenko et al., 2018; Shao et al., 2023). Cortisol is the most concentrated glucocorticoid and plays a pivotal role in the body's metabolism of glucose, fats, and proteins to promote energy availability while also suppressing the release of insulin during the activation of the ASR (Adam et al., 2010; Kraemer et al., 2020; Ramamoorthy and Cidlowski, 2016; Rizza et al., 1982; Sadoul and Geffroy, 2019; Shao et al., 2023; Vogelzangs and Penninx, 2007). Additionally, cortisol inhibits other bodily functions such as the immune and inflammatory response, digestive system, and cell turnover in tissues like bone, skin, and stomach lining (Meyer and Novak, 2012; Rizza et al., 1982; Thau et al., 2021; Yamanashi et al., 2016). Outside of its role in the ASR, cortisol is necessary for daily functioning. Cortisol constantly circulates in the bloodstream, mirroring the natural circadian rhythm, spiking when we wake up and reaching its lowest concentrations while we sleep (Mohd Azmi et al., 2021; Smith et al., 1980; Verbeeten and Ahmet, 2018). This natural pattern facilitates the regulation of several bodily functions, such as blood pressure and heart rate, which need to fluctuate throughout the day (Mohd Azmi et al., 2021). This ebb and flow of cortisol in the bloodstream is essential to maintaining homeostasis to the point where it can cause significant health complications (e.g.,

Addison's disease and Cushing's disease) if there is under- or overproduction (Burton et al., 2015; Raff et al., 2014).

#### 2.2.2.1 Cortisol in Hair

Cortisol can be found in several fluids of the body, including blood, sweat, and sebum (Webb et al., 2010). Tissues that receive a constant supply of blood have been found to incorporate cortisol into their protein structure, such as the keratin in hair (Greff et al., 2019; Meyer and Novak, 2012; Raul et al., 2004; Van Uum et al., 2008). Cortisol was first extracted from hair in living participants in 2004 and has become an essential tool for clinical researchers studying how stress biologically impacts the body (see Abell et al., 2016; Greff et al., 2019; Raul et al., 2004). Scalp hair grows on average about one centimetre a month; subsequently, the cortisol found in each hair segment represents the systemic cortisol concentration in the blood while that segment of hair was forming in the follicle (Meyer et al., 2014; Meyer and Novak, 2012; Webb et al., 2010). Hair encapsulating month-to-month cortisol concentrations has allowed researchers to trace stress events that would otherwise be invisible outside of a highly controlled environment (see Meyer and Novak, 2012; Russell et al., 2012; Thomson et al., 2010). Previously, clinical stress studies would require *in vivo* collections of either salivary, urinary, or blood cortisol concentrations, which restricted the type of studies that could be conducted, but the development of hair cortisol extraction has allowed researchers to conduct long term stress studies without participants frequently reporting to a lab setting (Abell et al., 2016; El-Farhan et al., 2017; Greff et al., 2019; Wood, 2009).

While promising, cortisol in hair does not have known concentrations that can be considered medically diagnostic (Wright et al., 2015). With this in mind, however, in the

last twenty years, there has been extensive clinical research that has studied hair cortisol in conjunction with several biological considerations (e.g., age, biological sex, body mass index, geographic region, etc.) (Chan et al., 2014; Gonzalez et al., 2019; Greff et al., 2019; Henley et al., 2013; Manenschijn et al., 2012; Pereg et al., 2011; Raul et al., 2004; Russell et al., 2012; Sauvé et al., 2007; van den Heuvel et al., 2020; Webb et al., 2010). Notable clinical studies have investigated hair cortisol concentrations focusing on both biological and psychological stressors and found elevated hair cortisol in cases such as starvation, heart disease, invasive surgery, heavily physical professions, those suffering from chronic pain, obesity, post-traumatic stress disorder, clinical depression, school pressure, and those living below the poverty line (see Chan et al., 2014; Herane-Vives et al., 2020; Jackson et al., 2017; Morgan et al., 2019; Olstad et al., 2016; Pereg et al., 2011; Psarraki et al., 2021; Stetler and Guinn, 2020; Van Uum et al., 2008). These clinical studies exemplify the complex nature of studying ASR and its relationship to cortisol fluctuations in the body.

#### 2.2.2.2 Hair Biology and its Impact on Cortisol

As opposed to its other pathways in the body (i.e., via blood, saliva, fat, or urine), the cortisol found in hair is susceptible to extraneous environmental factors that can influence its concentration, such as ultraviolet radiation and washing (Cole, 2017; Meyer and Novak, 2012; Otten et al., 2020). Hair is naturally prone to sun bleaching, which is when the radiation from the sun over time breaks down the protein structures in organic tissues (Dario et al., 2015). In hair, this phenomenon can be observed in the loss of melanin pigmentation in the hair shaft, but more importantly, cortisol concentrations from hair constantly exposed to sunlight were found to be statistically lower than those

who were not when chemically extracted (Dario et al., 2015; Meyer and Novak, 2012). Consequently, hair cortisol concentrations at the distal end of hair fibres are statistically lower than that of the proximal end of the hair (approximately 3cm beyond the scalp), arguably influencing the accuracy of recorded concentrations (see Meyer and Novak, 2012; Otten et al., 2022). Hair is also highly permeable, especially when consistently exposed to water or other solvents that may diminish cortisol concentrations over time (East, 2021; Meyer and Novak, 2012; Otten et al., 2020). Multiple studies have found a strong connection between excessive hair washing and lower cortisol concentrations (see East, 2021; Hoffman et al., 2014; Meyer and Novak, 2012). Despite these challenges, however, hair cortisol and the ability to capture varying concentrations over extended periods of time has become a significant data source in the clinical study of stress (Greff et al., 2019; Slominski et al., 2015).

#### 2.2.2.3 Cortisol in Hard Tissues

As previously discussed, tissues with a constant blood supply have been found to incorporate cortisol into their protein structures due to its circulation within the bloodstream (Greff et al., 2019; Webb et al., 2010). While this phenomenon is well-documented in fluids and tissues such as urine, saliva, hair, and adipose fat (see El-Farhan et al., 2017; Kellar et al., 2015; Meyer and Novak, 2012), the extraction and quantification of cortisol from hard tissues remains a relatively novel line of research. A clinical study in 2016 is the first published study to successfully extract cortisol from tooth dentin in humans (see Nejad et al., 2016). Nejad et al. (2016) argue that cortisol embeds itself in the dentin (inner structure of the tooth) through blood flow into the tooth pulp. More recently, zoological researchers have developed a method to study cortisol in

the cortical bone (dense, outer surface of bone) of walruses (see Charapata et al., 2018). Charapata et al. (2018) argue that because skeletal remodelling creates a layered average of present lipids (i.e., steroid hormones), the cortisol extracted from bone represents a greater time depth of stress (i.e., 10-20 years based on average cortical remodelling rate) than other sources of cortisol such as urine and hair (see Charapata et al., 2018; Thau et al., 2021).

Cortisol studies on both teeth and cortical bone involve the production of powdered samples and the use of a chemical solvent to extract the cortisol, similar to the extraction of cortisol from hair (Charapata et al., 2018; Nejad et al., 2016; Webb et al., 2015a). Where the two methods differ is that Nejad et al. (2016) quantify their cortisol extractions with an enzyme-linked immunoassay (ELISA), while Charapata et al. (2018) utilize liquid column/ mass spectrometry (LCMS). Studies focusing on hair cortisol have found that while cortisol concentrations quantified with either ELISA or LCMS do differ, they strongly correlate with one another (Russell et al., 2015). Importantly, when compared to other sources of cortisol, such as hair, the extraction of cortisol from teeth and bone requires vastly more substrate to be performed successfully (10 mg of hair compared to 150 mg for teeth and 250 mg of bone) (Charapata et al., 2018; Nejad et al., 2016; Raul et al., 2004). While the study of cortisol in hard tissues remains relatively new, it poses a promising line of research into the biochemical analysis of stress, especially when relating it to other biochemical agents of stress.

### 2.2.3 Osteocalcin

Cortisol is not the sole hormone involved in the ASR, and researchers assessing stress in humans have recently become more interested in osteocalcin, a protein closely

linked to metabolism and skeletal remodelling (Berger et al., 2019; Berger and Karsenty, 2022; Bilotta et al., 2018; Liu et al., 2019; Moser and van der Eerden, 2019). Human bone is a complex web of organic (protein) and inorganic (mineral) components that work in tandem to perform the biological functions of the skeleton (France et al., 2020; Tzaphlidou, 2008). While collagen makes up most of the organic portion of bone, osteocalcin is the largest non-collagen protein found in the skeleton (Moser and van der Eerden, 2019; Tzaphlidou, 2008). Osteocalcin is a peptide (protein) hormone that exists in the body in both active (i.e., decarboxylated osteocalcin) and inactive forms (i.e., carboxylated osteocalcin) based on the absence or presence of attached carboxylic acid to the main osteocalcin structure respectively (see Figure 2.3) (Razny et al., 2017). The former primarily exists in circulating blood serum, and the latter in the skeleton (Berger et al., 2019; Sobel and Berger, 1995). Carboxylic acid has a high affinity to calcium ions, particularly to the inorganic calcium hydroxyapatite found in bone (bioapatite); therefore, when osteocalcin has carboxylic acid attached, it binds to the bioapatite, is rendered inert and stored in the bone matrix (Hauschka et al., 1989; Razny et al., 2017; Wei and Karsenty, 2015; Zoch et al., 2016).

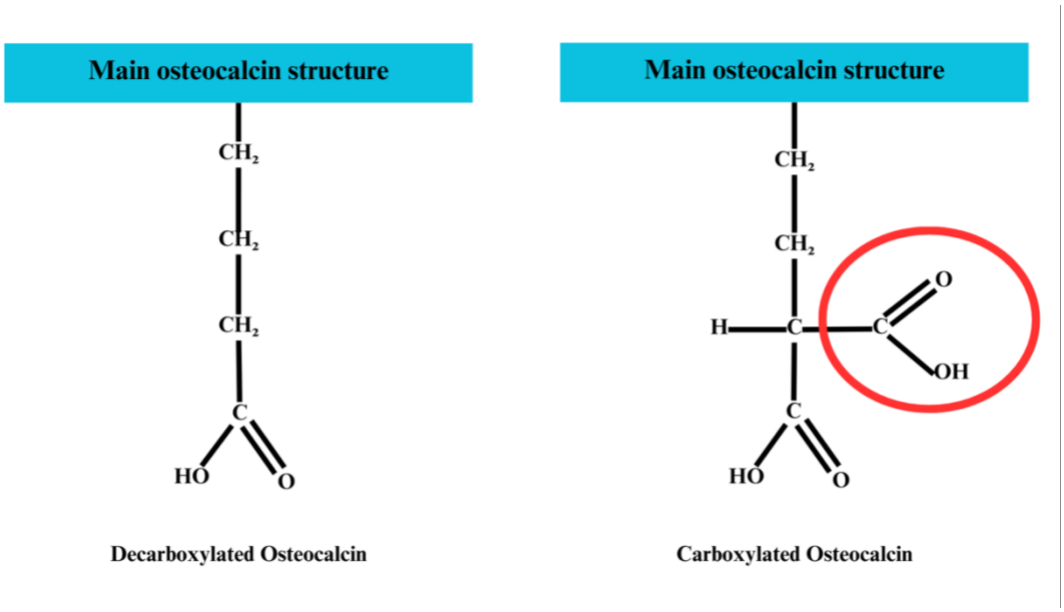


Figure 2.3: A graphic depiction of active-decarboxylated osteocalcin and inactive-carboxylated osteocalcin with the carboxylic acid group setting them apart circled in red

The vital balance between active-serum (decarboxylated) and inactive-bone (carboxylated) osteocalcin is directly controlled by the production of insulin and overall glucose metabolism (Berger et al., 2019; Berger and Karsenty, 2022; Bilotta et al., 2018). In a negative feedback loop with insulin, osteocalcin and bone remodelling are regulated by the metabolic needs of the body at a given time (Berger et al., 2019; Oury et al., 2011). The two primary cells responsible for bone formation (osteoblasts) and bone destruction (osteoclasts) play a role in the regulation of active versus inactive osteocalcin concentrations by either affixing or removing carboxylic acid from the osteocalcin structure (Berger et al., 2019; Ducy, 2011; Razny et al., 2017; Zoch et al., 2016). When insulin is low, and demand for glucose metabolism is high (e.g., chronic stress resulting in lower insulin production and the promotion of fat breaking down into sugar), osteoclasts decarboxylate osteocalcin, allowing it to bind to the insulin-producing pancreatic cells if they are not already blocked by cortisol (Berger et al., 2019;

Kanazawa, 2015; Schernthaner-Reiter et al., 2021). When insulin concentrations are sufficient, that insulin will bind to osteoblasts and encourage the cells to carboxylate osteocalcin and store the protein in the new bone matrix (see Fig. 2.4) (Berger et al., 2019; Hauschka et al., 1989; Lee et al., 2007; Scott et al., 2016; Shu et al., 2016).

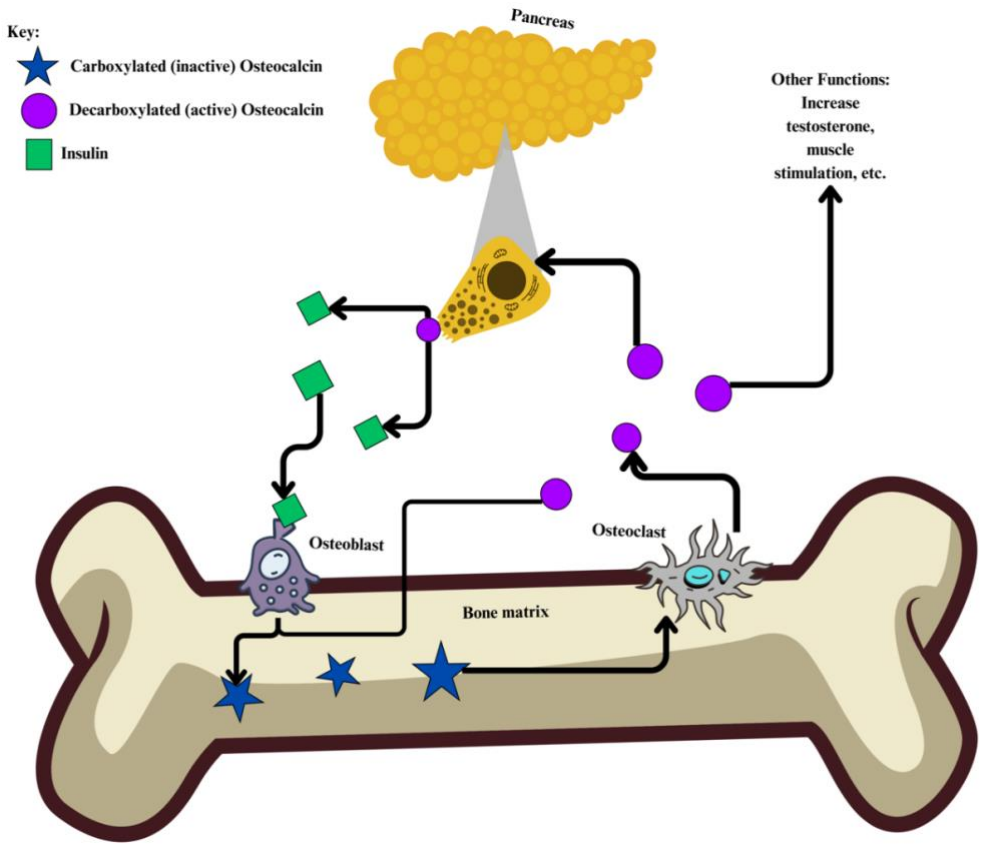


Figure 2.4: A graphic depiction of the negative feedback loop with bone remodelling that regulates the amount of active osteocalcin in circulation

Due to its link with metabolism and bone remodelling, both of which are directly impacted by the ASR, clinical researchers have focused on osteocalcin as a marker for health and biological stress (Berger and Karsenty, 2022; Lee et al., 2000; Nielsen et al., 1991). For example, serum osteocalcin has been found to have a significant relationship with health conditions such as cardiovascular disease, type-2 diabetes, and increased fracture risks (see Liu et al., 2019; Shen et al., 2022; Vergnaud et al., 1997). Additionally,

researchers have seen a significant correlation between insulin resistance (a common symptom of chronic stress), bone remodelling, and elevated osteocalcin in the blood (see Ozkaya et al., 2012; Tonks et al., 2017).

The relationship that osteocalcin has with the ASR has also been extensively researched. For example, higher osteocalcin concentrations in the blood have been strongly associated with reported feelings of depression and stress (see Bartečků et al., 2022; Nguyen et al., 2020). Serum osteocalcin has also been found to significantly correlate with circulating cortisol concentrations in the blood (see Nielsen et al., 1991). When both mice and humans were exposed to stressors and had a measurable stress response, researchers observed a noticeable spike in the circulating concentrations of osteocalcin even in the absence of cortisol (see Berger et al., 2019). Berger and Karsenty (2022) argue that considering the key role that osteocalcin plays in biological functions, suggests that it is an impactful stress hormone. Stress is a highly complex biological process, but understanding its effects on the body and the involved biochemical agents is crucial for bioarchaeologists studying stress in past populations.

## 2.3 The Study of Stress in Bioarchaeology

### 2.3.1 Macroscopic Study of Stress in the Skeleton

Historically, macroscopic manifestations of stress in the skeleton have been the primary information source for those studying the ASR in past populations. These indications of stress can be broadly described as the body's adaptive response to outside stressors (e.g., nutritional deficits, disease) that persist in the skeleton (Klaus, 2014; Macintosh et al., 2016; Marciniak et al., 2022; Mays et al., 2017; Roberts and

Manchester, 2007). These lesions are generally caused by either the overproduction or underproduction of bone or tooth tissue in response to these stressors (Klaus, 2014; Temple and Edes, 2022). Enamel hypoplastic lesions, Harris lines, cribra orbitalia, cribra femora, porotic hyperostosis, and periosteal new bone formation are among the most studied and widely researched paleopathological indicators of stress (see Dabbs, 2011; Macintosh et al., 2016; Marciniak et al., 2022). A great deal of research has been done to investigate these skeletal changes, including their causes and the potential information they can provide about the stress process (Biehler-Gomez et al., 2023; Dewitte and Stojanowski, 2015; Mays et al., 2017).

Enamel hypoplastic lesions appear as lines, pits, or grooves on the enamel surface of the teeth, most commonly on the incisors and canines (Brickley et al., 2020b; Goodman et al., 1980; Krishan et al., 2015; Temple, 2018). Caused by the disruption of enamel formation while the permanent dentition develops, enamel hypoplastic lesions have been attributed to childhood stressors such as metabolic deficiency, malnutrition, and disease (see Dabbs, 2011; King et al., 2005; Marciniak et al., 2022; Simalcsik et al., 2014). Enamel hypoplastic lesions develop during the years when the permanent teeth are forming and thus speak to stress events occurring from the ages of birth to approximately ten years of age (Dąbrowski et al., 2021; Goodman et al., 1980; Hillson and Bond, 1997; Krishan et al., 2015). Due to the known rates of permanent teeth calcification (complete at approximately 10 years of age), the size and location of enamel hypoplastic lesions on the teeth can indicate the relative age and duration of the stress event that led to that lesion (Dąbrowski et al., 2021; Edinborough and Rando, 2020; King et al., 2002; Kumari et al., 2022; Simalcsik et al., 2014). Additionally, multiple lesions across the teeth can

potentially represent separate stress events sustained by an individual (King et al., 2005; Simalcsik et al., 2014). Signs of stress found in the dentition have also been studied through microscopic means, utilizing techniques such as electron microscopy, which have allowed researchers to analyze smaller lesions along the dentition—allowing for a finer grain of assessment of these lesions before they develop into larger pronounced marks on the teeth (Hillson, 2014).

Another commonly interpreted sign of lived stress on the skeleton are Harris lines. Appearing on the long bones, particularly on the tibiae and radii, Harris lines are transverse lines of radiodensity that appear lateral to the growth plates as observed on radiographs (Alfonso et al., 2005; Michelman, 2022; Scott and Hoppa, 2015; Temple and Edes, 2022). Harris lines are caused by an arrest of growth and the layer of cartilage along the growth plate mineralizing, creating a thin layer of bone (Alfonso-Durruty, 2011; Mays, 1985; Scott, 2015; Scott and Hoppa, 2015). The formation of Harris lines on the skeleton has been generally attributed to episodes of physiological stress, such as nutritional deficiencies and disease (Alfonso et al., 2005; Beom et al., 2014; Geber, 2014; Michelman, 2022; Scott and Hoppa, 2015). Harris lines form in the years the long bones are growing, approximately from 1-18 years old (Kulus and Dąbrowski, 2019; Scott and Hoppa, 2015). Similar to enamel hypoplastic lesions, because of the known growth rates for each long bone, the relative age of the individual at the time of the stress event can be determined, and multiple Harris lines may represent multiple periods of arrested development (i.e., multiple stress events) (Kulus and Dąbrowski, 2019).

The three major cribriotic lesions: cribra orbitalia, cribra femora and porotic hyperostosis, are among the most studied macroscopic indicators of stress within

bioarchaeology (e.g., Betsinger and DeWitte, 2017; Brickley et al., 2018; Schats, 2021). Cribra orbitalia, cribra femora, and porotic hyperostosis are patches of porosity on the superior surface of the eye orbit, femoral neck, and parietal bone, respectively (Pilloud and Schwitalla, 2020; Rivera and Mirazón Lahr, 2017; Schats, 2021; Stuart-Macadam, 1989). While cribra femora has been far less studied than the other two cribriotic lesions, all three have been associated with many different stressors, with the most common interpretation among researchers being non-specific anemia experienced during childhood (e.g., Gomes et al., 2022; Rivera and Mirazón Lahr, 2017; Schats, 2021; Walker et al., 2009), brought on by a variety of factors such as nutritional deficiency, parasites, or exposure to pathogens (Brickley et al., 2018; Gomes et al., 2022; Gowland and Redfern, 2010). In cases of anemia, those affected often exhibit bone marrow hypertrophy, or an overproduction of blood-producing cells, to compensate for deficient hemoglobin concentrations (Li et al., 2015; Rivera and Mirazón Lahr, 2017). The locations where these porous lesions appear have been tentatively associated with the relative age of onset, where the centres of blood production move from the eye orbit to the cranial vault to the femoral neck. The size of these lesions is presumed to indicate the relative severity of the anemia (see Koontz Scaffidi, 2020; Schats, 2021; Stuart-Macadam, 1989; Wapler et al., 2004).

Affecting the outer layer of cortical bone, periosteal new bone formation is another skeletal change used by bioarchaeologists to study stress in the past (Pilloud and Schwitalla, 2020). Appearing as fine pitting, longitudinal striations, and eventually a plaque of new bone formation on the cortical surface of the bone, an inflammation of the periosteum (i.e., the osteogenic tissue that covers the cortical surface of all skeletal

elements) causes periosteal new bone formation (DeWitte, 2014; Rittemard et al., 2019). This irritation and subsequent inflammation of the periosteum has been associated with several stressors, such as systematic and/or localized infections, hemorrhaging from injury, genetics, nutritional deficiencies, and metabolic conditions (see Chen et al., 2012; DeWitte, 2014; Geber and Murphy, 2012; Paine and Brenton, 2006; Weston, 2008). While having a more extensive age range for onset when compared the other common lesions studied, most periosteal new bone formation lesions have been found to occur before the age of 25 (Rana et al., 2009). Since periosteal new bone formation is generally a reaction to a more acute stressor, researchers have suggested that the degree of remodelling (i.e., healing) could represent those with better health that were able to survive the stress event and produce new bone tissue (see DeWitte, 2014; Mays et al., 2002; Wood et al., 1992). This measurement of survivorship has additionally been extended to other macroscopic lesions, such as enamel hypoplastic lesions, where their presence better represents an individual who was capable of overcoming a stress event (Buikstra et al., 2022; Edinborough and Rando, 2020).

While it is clear that macroscopic manifestations of stress can provide a wealth of information about the lived experience, there are several key and significant limitations to consider when using these indicators to understand stress experiences in the past. First, the skeleton is the last tissue to experience the effects of stress; as a consequence, acute stress events are not captured because stress must be sustained long enough for macroscopic markers to develop (e.g., Goodman and Armelagos, 1989; Scott et al., 2016; Wood et al., 1992). While researchers have tried to account for this through more microscopic analyses of skeletal lesions (e.g., electron microscopy), even those

microscopic changes require considerable amounts of time to develop in regard to the stress event's onset (Elcock et al., 2006; Pilloud and Schwitalla, 2020). Another important consideration is that an individual must survive the stress event for skeletal markers to appear, meaning that stress events that are extreme enough to cause death or took place surrounding the time of death cannot necessarily be seen in the skeleton (Goodman et al., 1988; Klaus, 2014; Webb et al., 2010). These macroscopic indicators are also inconsistent in their longevity and how long they are visible in the skeleton. For example, once the adult teeth are fully calcified, any enamel hypoplastic lesions become permanent. By contrast, Harris lines and periosteal new bone formation will eventually remodel and obliterate with time, effectively deleting those particular stress events from assessment (Alfonso et al., 2005; Edinborough and Rando, 2020; Papageorgopoulou et al., 2011; Wood et al., 1992). The rate at which this remodelling and subsequent obliteration are highly dependent on the rate of mineralization for the impacted element but have been found to be as short as two years after formation (Nowakowski, 2018; Papageorgopoulou et al., 2011). While they do obliterate with remodelling, periosteal new bone formation does leave a long-term appearance of recent healing, which can be used to measure survivorship (e.g., DeWitte, 2014); however, this study is limited by a lack of ability to assign age of the initial condition that led to the original lesion (Pezo-Lanfranco, 2020). Macroscopic indications of stress are also highly vulnerable to inter-observer error both in lesion identification and the scoring of lesion severity due to a level of visual subjectivity when observing these skeletal changes (Assis and Keenleyside, 2019; Biehler-Gomez et al., 2020; Garcin et al., 2010; Rittemard et al., 2019).

The majority of macroscopic indicators of stress have a specific age of onset (i.e., during skeletal development or childhood), meaning that these indications cannot be inherently associated with stress experienced in adulthood (Biehler-Gomez et al., 2023). While studying stress experienced during childhood is an important line of research, it is important to note that using signs of stress that are typically experienced in early development to describe the experiences of adults should be approached with caution. Doing so may lead researchers to draw conclusions that do not accurately reflect the authentic lived experience an individual had as an adult (see East et al., 2022; Wood et al., 1992). Additionally, there have been inconsistencies when attempting to correlate these indicators with one another. For example, Harris lines have been shown repetitively to lack a correlation with other indicators such as enamel hypoplastic lesions (Alfonso-Durruty, 2011; Papageorgopoulou et al., 2011; Pilloud and Schwitalla, 2020). This can be due to a number of factors, such as differing ages of onset and varying aetiologies (Alfonso-Durruty, 2011; Alfonso et al., 2005).

Another major limitation of traditional macroscopic indicators of stress is their non-specificity (Edinborough and Rando, 2020; Reitsema and McIlvaine, 2014; Rothschild et al., 2021). As previously discussed, the aetiologies of these indicators are rather broad and can point to several possible external stimuli, such as disease, trauma, or dietary changes (Brickley et al., 2020a, 2018; Pilloud and Schwitalla, 2020). This presents a challenge to researchers in their ability to accurately predict the factors that contributed to that individual's stress load. A key example of this is periosteal new bone formation, which may be part of a disease process itself or a symptom of another pathology (Assis and Keenleyside, 2019; Lambert, 1993; Rittemard et al., 2019; Weston,

2011, 2008). Moreover, Harris lines are found in seemingly healthy individuals and perhaps represent natural growth patterns better than pathology (Boucherie et al., 2017; Michelman, 2022; Papageorgopoulou et al., 2011). Because the aetiologies of most macroscopic indicators are associated with diet, development, and/or disease, researchers have begun to advocate reconceptualizing these lesions on the skeleton as markers of health and survivorship rather than stress (see Buikstra et al., 2022; Caine et al., 2022; Edinborough and Rando, 2020; Reitsema and McIlvaine, 2014; Temple and Edes, 2022).

In an attempt to address some of these limitations associated with macroscopic stress lesions, researchers have begun to employ molecular and biochemical techniques to better examine stress experienced in the past (e.g., Brien, 2023; Hughes, 2020; Scott, 2015; Scott et al., 2020, 2016; Webb et al., 2015a, 2015b, 2010). All biological responses begin at the molecular level before they lead to macroscopic changes in the skeleton (Grover, 2002; Mariotti, 2015). Therefore, studying stress in bioarchaeological populations using molecular techniques is beneficial as stress can arguably be interpreted more accurately than through macroscopic assessments alone. Essentially, biomolecular methods narrow the gap between when stress triggers the ASR and when visible skeletal changes occur (Scott et al., 2016; Webb et al., 2010).

### 2.3.2 Cortisol Research in Bioarchaeology

As the primary hormone of the stress response, cortisol is the logical first choice when studying stress biochemically. Considering that cortisol reservoirs such as blood and urine are not available to bioarchaeologists, this means that until recent years, cortisol research in archaeological settings has been restricted to hair analysis. However, hair preservation is unfortunately very rare in bioarchaeological contexts (see Wilson et

al., 2007, 2001). As a result, bioarchaeological research investigating cortisol concentrations in hair has mainly been in regions where individuals are likely to mummify, particularly in Egypt and Peru (see Kellner et al., 2022; López-Barrales et al., 2015; Schaefer, 2017; Tisdale et al., 2019; Webb et al., 2010, 2015b). A common feature among these studies is that with excellent hair preservation, cortisol can be segmented to assess multiple months of stress, expanding the interpretive window of when stress can be evaluated (Schaefer, 2017; Webb et al., 2015a, 2015b, 2010).

Among many of these bioarchaeological studies, there is a recurring theme of integrating hair cortisol with other molecular analyses, such as stable isotopes and other extracted hormones (e.g., Kellner et al., 2022; Schaefer, 2017; Tisdale et al., 2019; Webb et al., 2015a, 2015b). For example, in a number of these studies, hair cortisol was found to be elevated in circumstances of increased mobility, dietary constraints, socio-political upheaval, reduced signs of chemical fertility, and prolonged dying processes (see East, 2021; Kellner et al., 2022; Schaefer, 2017; Tisdale et al., 2019; Webb et al., 2015a, 2015b). This pattern of elevated cortisol in these specific scenarios also aligns closely with clinical examples (Almadi et al., 2013; Bergendahl et al., 1996; Edwards and Mills, 2008; Santa-Cruz et al., 2020; Van Uum et al., 2008; Vogelzangs and Penninx, 2007; Yamanashi et al., 2016), demonstrating that cortisol extracted from hair is an effective and accurate biomarker to study stress bioarchaeologically.

The major benefit of assessing cortisol in hair is the short period of time between the recognition of stress by the HPA, the activation of the ASR, and when the stress event can be observed in the hair (Kapoor et al., 2018; Webb et al., 2010). Additionally, the ability to accurately study the chronological progression of stress through cortisol

analysis is significant, as macroscopic methods alone do not currently allow for this type of nuanced approach (see Schaefer, 2017; Webb et al., 2010).

Despite these benefits, the analysis of cortisol in hair has its limitations. While hair is preserved archaeologically more often than other soft tissues, it is still a scarce data source. This is due to several extrinsic factors that contribute to the long-term degradation of hair samples, including acidity, high water drainage, and soil composed heavily of phosphates and lead (Kootker et al., 2020; Petraru et al., 2020; Tridico et al., 2014; Wilson et al., 2001). Intrinsically, factors such as age, hair thickness, stage of hair growth, and pigmentation have also been found to significantly influence hair preservation (Cole, 2017). Specific preservative agents can also contribute to the preservation of hair archaeologically. For example, the acidic soils at the Fortress of Louisbourg impeded long-term hair preservation; however, burial practices, specifically the use of burial shrouds, have positively impacted preservation. Common in Western Europe until the 18$^{th}$-century, especially in France (Geake, 2023; Gilchrist and Sloane, 2005; Hunter, 2023), burial shrouds were secured using copper or copper alloy pins (Cessford et al., 2022; Janaway, 1998; Mytum, 2014; Scott et al., 2019; Zahedieh, 2013). As copper oxidizes, cupric ions released into the environment inhibit bacterial growth that would otherwise significantly contribute to the decomposition of surrounding biological tissues (Lebow et al., 2020; Michels et al., 2005). As a result, when these pins are recovered near regions of the body where hair is expected (i.e., the cranium), it is usually well preserved (Khademibami and Bobadilha, 2022; Michels et al., 2005; Scott, 2021; Scott et al., 2019).

The various factors impacting hair preservation are likely why the adoption of hair cortisol methods is not as widespread despite the benefits. However, researchers have begun to study cortisol in tissues that are more readily available bioarchaeologically (see Charapata et al. 2018). The pilot study conducted by Charapata et al. (2018) successfully tested a method for extracting cortisol from modern (2014-2016), historical (200-20 BP), and archaeological (>200 BP) walrus bone. This study successfully extracted cortisol from bone as old as 3,500 years old and demonstrated the same range of cortisol availability between modern, historical, and archaeological samples (Charapata et al., 2018). It was proposed that cortisol extracted from bone represents a deeper time depth of lived stress when compared to other cortisol reservoirs such as hair, blood, or fat (Charapata et al., 2018).

While cortisol has not yet been extracted from human bone, Quade et al. (2021) were the first to test whether cortisol could be extracted from other archaeological human tissue besides hair. Following earlier clinical studies (Nejad et al. 2016), Quade et al. (2021) extracted and quantified cortisol found in archaeological tooth dentin and enamel. Unlike Charapata et al. (2021, 2018), Quade et al. (2021) used a commercially available ELISA kit as opposed to liquid-column/mass-spectroscopy (LCMS), a cost-effective quantification method that more closely aligned with previous archaeological cortisol research (see Kellner et al., 2022; López-Barrales et al., 2015; Schaefer, 2017; Webb et al., 2010). Recently, Quade et al. (2023) conducted a similar study focusing on archaeological deciduous teeth and compared that to the deciduous teeth of modern participants. It was found that the amount of substrate (i.e., dentin or enamel) necessary for a detectable cortisol reading was significantly higher than that needed in clinical

samples or their own modern samples (Quade et al. 2023, 2021; Nejad et al., 2016). Not only was the necessary substrate higher, but the readings of cortisol collected from archaeological samples were also significantly lower (see Quade et al., 2023, 2021). Many hypotheses were proposed to explain this phenomenon, ranging from the variability of laboratory practices to the degradation of archaeological remains, which were not directly tested in these studies (Quade et al., 2023, 2021).

While these bone-specific studies show promising and exciting results in the expansion of cortisol research in bioarchaeology, notable concerns and knowledge gaps still limit the applicability of studying cortisol in hard tissues. The primary limitation is the amount of substrate required to quantify cortisol (Quade et al., 2023, 2021). Compared to the analysis of cortisol in hair, teeth and bone require 15 to 20 times more substrate to perform the analysis (Charapata et al., 2018; Quade et al., 2021; Webb et al., 2010). This requirement severely limits the replicability of these types of studies and exemplifies the ethical dilemma between the value of academic inquiry and the destructive nature of molecular bioarchaeological research (see Quade et al., 2021; Smith et al., 2023).

The study of cortisol in hair has coalesced around reporting the data as nanograms of cortisol for every gram of hair (ng/g), while the study of cortisol in hard tissues has not yet achieved this degree of uniformity Chan et al., 2014; Kellner et al., 2022; López-Barrales et al., 2015; Schaefer, 2017; Van Uum et al., 2008; Webb et al., 2010). For example, Charapata et al. (2021, 2018) report their findings as nanograms of cortisol for every gram of lipid extracted via a separate Soxhlet procedure (ng/g $_{lipid}$) (see Schlechtriem et al., 2003). In contrast, Nejad and colleagues (2016) reported their

findings as nanograms of cortisol for every milligram of dentin sampled (ng/mg). In their two papers on teeth cortisol, Quade et al. (2023, 2021) presented their findings in two different units of measurement, the first being in nanograms of cortisol per millilitre (ng/ml) (the original ELISA reading), and the second paper reporting in nanograms of cortisol per millilitre per gram of substrate ([ng/ml]/g). The issue with these inconsistencies is that it is relatively impossible to compare readings of hard tissue cortisol among studies without a universal unit of measurement.

Charapata et al. (2021, 2018) and Quade et al. (2023, 2021) discuss the preservation of osteological material and its potential to impact cortisol readings; however, neither specifically investigates this, and both suggest future studies on the topic. Charapata et al. (2021, 2018) also do not comment on how various skeletal elements or the mechanism of cortisol incorporation in the bone impacts extracted concentrations. This is important to note as these confounding factors: 1) hinder a wide-scale adoption of cortisol assessment in hard tissues, 2) require further analysis of cortisol's relationship to hard tissue preservation, 3) create a need to compare bone cortisol to cortisol from either other substrates such as hair to ensure a co-directionality in evaluated cortisol concentrations, and 4) warrant the comparison of bone cortisol to other biomarkers of stress such as osteocalcin. Ultimately, the limitations faced by bioarchaeological researchers when attempting to study cortisol, such as preservation issues or the large amount of sample required, have prompted the exploration into other stress-related proteins, such as osteocalcin.

### 2.3.3 Osteocalcin Research in Bioarchaeology

Outside of solely studying cortisol, osteocalcin has also been proposed as a biomarker for the assessment of stress (Hughes, 2020; Rich et al., 2022; Scott, 2015; Scott et al., 2020, 2016). As a protein that readily embeds itself in the skeleton with an intimate tie to the body's metabolism and bone turnover, in addition to promising clinical research, osteocalcin can likely be a proxy biomarker of bioarchaeological stress (Scott et al., 2016). Bone osteocalcin was originally tested as a stress biomarker in a study comparing its concentration with macroscopic stress indicators and other observable pathological conditions across different time periods within a single archaeological population (Scott, 2015; Scott et al., 2016). As discussed, macroscopic stress indicators result from either the under-or-overproduction of bone depending on the specific lesion (Klaus, 2014). Since bone osteocalcin is tied to osteoblast activity, extracted osteocalcin was found to be statistically lower when there were signs of degenerative skeletal lesions due to osteoblast inactivity (Scott, 2015; Scott et al., 2016). While not statistically significant, the research conducted by Scott et al. (2016) found that osteocalcin concentrations generally followed trends that align with other factors that result in lower bone mass. These factors are primarily biological sex and age, where osteocalcin concentrations are generally lower in females than males and in older individuals over younger ones (Scott et al. 2016). Further, the Scott (2015) and Scott et al. (2016) studies focused on two different time periods within the same cemetery in Denmark, in which one time period was more socio-economically and environmentally unstable (see Scott, 2015; Scott et al. 2016). Osteocalcin concentrations during the more turbulent period

were found to be statistically lower, suggesting periods of osteoblast inactivity from stress (Scott, 2015; Scott et al., 2016).

Further research regarding osteocalcin in archaeological populations has been conducted more recently. For example, Scott et al. (2020) investigated osteocalcin in relation to biological sex, age, and macroscopic signs of stress more extensively with the understanding that remodelling rates of a sampled element significantly impact reported osteocalcin concentrations (Scott, 2015; Scott et al. 2020). This was further explored by Hughes (2020), who found that while there was not a statistical difference in osteocalcin concentrations among skeletal elements, there was a correlation between higher osteocalcin concentrations in areas of the same element that have increased muscle attachment robusticity (i.e., increased localized remodelling). This means osteocalcin concentrations in bone represent an accumulation of the protein over a long period and, therefore, do not reflect serum osteocalcin concentrations at the time of death (Hughes, 2020; Scott et al., 2020; Smith et al., 2005). In 2022, osteocalcin was further studied bioarchaeologically, where statically lower concentrations of osteocalcin were found in both adult and non-adult individuals with signs of destructive or metabolic pathological conditions not previously studied (e.g., rickets) (Rich et al., 2022). This study by Rich et al. (2022) further validated the connection between osteocalcin concentrations and evidence of lesions and diseases associated with metabolic pathology and stress. All these preliminary studies focused on osteocalcin argue for additional molecular validation to truly test its use as a method to assess bioarchaeological stress, such as comparing osteocalcin to cortisol concentrations in both hair and bone (Hughes, 2020; Rich et al., 2022; Scott, 2015; Scott et al., 2020, 2016).

#### 2.3.3.1 Osteocalcin and Diagenesis

A major concern regarding the study of osteocalcin in archaeological bone is its relationship to diagenesis (see Hughes, 2020; Scott et al., 2020; Smith et al., 2005). Diagenesis is the physical and chemical changes occurring to the bone in its burial environment (see Kendall et al., 2018; Nielsen-Marsh et al., 2000; Smith et al., 2023). Bone bioapatite exists in equilibrium with the minerals of the environment, and when the environmental conditions are in flux, the bioapatite crystal chemically exchanges with the environment until the crystal structure of the bone more closely resembles the chemical composition of the surrounding minerals (Nielsen-Marsh and Hedges, 2000; Shemesh, 1990). Due to the tight bond between osteocalcin and the bioapatite crystal, as the bone undergoes diagenesis, osteocalcin concentrations in the bone diminish (Dobberstein et al., 2009; Smith et al., 2005). This indicates that measuring diagenesis is crucial to ensure that comparisons between individuals are authentic and largely unaffected by outside factors (Hughes, 2020; Scott et al., 2020).

A common method in the measurement of diagenesis is through the analytical chemistry instrument Fourier Transform Infrared Spectrometer with attached Attenuated Total Reflection (FTIR-ATR). Subjecting powdered bone samples to a beam of infrared radiation, FTIR-ATR classifies and characterizes heterogeneous materials, such as bone, based on the absorbance of radiation at a range of wavenumbers ($cm^{-1}$), to form a spectrum (France et al., 2020; Smith et al., 2023). Given the known infrared (IR) spectrum of unaltered bone and how bone becomes altered over time, several mathematical measurements have been developed to predict the molecular integrity of a

given sample (see Table 2.1) (Beasley et al., 2014; France et al., 2020; Smith et al., 2023).

Table 2.1: Descriptions of how the Carbonate/Phosphate ratio (C/P), Infrared Splitting Factor (IRSF), and Amide I-Phosphate ratio(AmI/P) measure diagenesis; the mathematical equations using the chemical functional groups and the relative wavenumber peaks for their respective function group

| **Metric** | **How Diagenesis is Measured** | **FTIR Calculation** | **Approximate Wavenumber Calculation** | **Reference** |
|---|---|---|---|---|
| Carbonate-Phosphate ratio (C/P) | Assesses post-mortem alteration to bioapatite via the known ratio between carbonate and phosphate in skeletal material. | (type-B Carbonate) / (Phosphate $v_3$) | $\frac{1415\ cm^{-1}}{1010\ cm^{-1}}$ | (Beasley et al., 2014; Boskey et al., 2016; Grunenwald et al., 2014; Iacumin et al., 2022; Isaksson et al., 2010; Mohd Noor et al., 2020; Smith et al., 2023; Wright and Schwarcz, 1996) |
| Infrared Splitting Factor (IRSF) | The mean crystal length of bioapatite is used as a proxy of bioapatite's order, increased order indicates a higher degree of diagenetic alteration. | (Phosphate $v_4$+Phosphate $v_4$) / (The valley separating them) | $\frac{(605cm^{-1}+565cm^{-1})}{590cm^{-1}}$ | (Beasley et al., 2014; France et al., 2020; Shemesh, 1990; Smith et al., 2023; Tătar et al., 2014; Termine and Posner, 1966; Weiner and Bar-Yosef, 1990) |
| Amide I - Phosphate ratio (AmI/P) | The amide I group of bone collagen is compared to bioapatite's phosphate as a measurement of collagen's integrity. | (Amide I group) / (Phosphate $v_3$) | $\frac{1650cm^{-1}}{1010cm^{-1}}$ | (Isaksson et al., 2010; Scott et al., 2020; Tamara et al., 2022; Trueman et al., 2004) |

The three methods using FTIR-ATR described in Table 2.1 have been used to assess diagenesis in previous archaeological osteocalcin studies (Hughes, 2020; Scott et al., 2020), with results confirming that osteocalcin concentrations generally decrease as the bone becomes increasingly compromised (i.e., undergoes diagenesis). These findings further highlight the relationship between observable osteocalcin concentrations in the bone and preservation, along with the ability to utilize FTIR-ATR as a method to account for diagenesis as a factor when assessing osteocalcin concentrations in archaeological populations.

Stress and health have been topics of interest in bioarchaeology for decades and thus have a vast depth of research. The adoption of biochemical techniques to complement the more traditional macroscopic methods of studying bioarchaeological stress has the potential to immensely broaden the lens through which we can study stress in the past. With that in mind, this thesis aims to further explore stress biochemically by critically evaluating the relationship between cortisol and osteocalcin in a specific 18$^{th}$-century Atlantic Canadian population.

## 2.4 The Fortress of Louisbourg

Located on the northeast shore of Cape Breton Island, Nova Scotia, the Fortress of Louisbourg is a National Historic Site of Canada that has been partially reconstructed on its original archaeological foundations (Johnston, 2001). Situated on unceded Mi'kmaq territory, the Fortress of Louisbourg was established by the French Empire in 1713 once they lost holdings in Newfoundland through the signing of the Treaty of Utrecht (Donovan, 1985; Johnston, 2001; Wall, 1964). Thanks to its strategic location,

Louisbourg became an important military stronghold for the French, along with being a central hub for the cod industry along the Transatlantic trade routes (Welker and Mathwich, 2023; Welker and Quintana Morales, 2022). The wealth and work opportunities from the cod fish industry resulted in Louisbourg becoming a major metropolis within North America, attracting residents from Europe, New France, and New England (Johnston, 1984, 2001).

At its peak, Louisbourg had a population of nearly 7,000 civilians and garrisoned soldiers, along with additional seasonal visitors from the surrounding Mi'kmaq communities, merchants, fishermen, and sailors who came to the Fortress for trade or work (Johnston, 1993, 1984). As a prized possession of the French Empire, the Fortress of Louisbourg was coveted by France's historic rival, Great Britain (Johnston, 1993). The Fortress was besieged twice in its brief history, first by a garrison of New Englanders on behalf of the British Crown in 1745 (accompanied by a four-year occupation), and then again in 1758 by British forces (Johnston, 2008; Wall, 1964). It was after this second siege that Louisbourg was systematically decommissioned and abandoned by the British, ending the French Empire on Cape Breton Island (Johnston, 1984).

The historical and bioarchaeological record of Louisbourg's brief history alludes to a difficult way of life for those who called it home. The Fortress was constructed in a desolate location, notorious for its dampness and harsh Atlantic winds (Donovan, 1982). With little to no insulation in most of the buildings, the residents of Louisbourg were constantly challenged by this cold, damp climate (Donovan, 1982; Krause et al., 1995; Lindsay, 1975). Additionally, the soil at Louisbourg was rocky, sandy, acidic, and low in nutrients, making agriculture laborious and low-yielding (Cann et al., 1963; Donovan,

2006). This meant that the typical diet at Louisbourg lacked nutrient density and consisted mainly of bread, alcohol, salted meat, and cod (Fortin, 2000; Lane-Jonah and Vechambre, 2015; Welker and Mathwich, 2023; Welker and Quintana Morales, 2022). Hygiene at the Fortress was also poor by today's standards; in the 18$^{th}$-century, full-body bathing was deemed socially taboo, with only the occasional washing of the hands and face being seen as appropriate (Donovan, 1985; Tésio, 2008; Tulchinsky and Varavikova, 2014).

These conditions made disease a widespread and constant reality for the residents of Louisbourg. The close quarters, poor diet, and unsanitary conditions were a perfect breeding ground for spreading infectious diseases (Marble, 1997; Scott et al., 2023; Tulchinsky and Varavikova, 2014; Winiwarter et al., 2016). Based on historical documentation and bioarchaeological data, communicable diseases (e.g., typhus, smallpox, malaria, tuberculous, and treponemal disease) along with diseases caused by contaminated food and water (e.g., cholera, dysentery, and parasite infestations) were extremely common (Fonzo, 2019; Forbes, 2021; Marble, 1997; Scott et al., 2023; Tésio, 2008; Tulchinsky and Varavikova, 2014; Winiwarter et al., 2016). With the constant possibility of conflict and military occupation, the harsh and unhygienic living conditions, inadequate resources, and the high prevalence of disease at Louisbourg, it can be safely assumed that the people living there likely experienced a considerable amount of physiological stress, making this population appropriate to study lived stress in the past.

## 2.5 Conclusion

This chapter has focused on stress from a biological perspective. It explored how the body reacts systematically to negative stimuli and two key chemical agents (osteocalcin and cortisol) involved in that response; specifically, the characteristics of these two hormones, their impacts on growth, development, and cell turnover, their relationship to one another, and extraneous factors that control their concentrations. The applications and limitations of previous macroscopic methods for studying stress were also discussed, and how molecular analysis can potentially address these limitations. This chapter then explored current methods, applications, and gaps in bioarchaeological research using molecular techniques. Finally, this chapter looked at the 18$^{th}$-century Fortress of Louisbourg, its history, living conditions, and how it relates to the topic of studying stress bioarchaeologically.

# Chapter 3: Methods and Materials

## 3.1 Introduction

This chapter will begin with the permissions required for this research and a brief contextual history of the archaeological site at the Fortress of Louisbourg that makes up the study sample. This chapter will also review the methods used for the extraction and quantification of osteocalcin (i.e., demineralization, filtering, total protein quantification, ELISA, and FTIR-ATR) along with the methods employed for the extraction and quantification of cortisol in hair and bone (i.e., sampling, processing, chemical aliquoting, and ELISA).

## 3.2 Permissions

Permission to sample individuals from the Fortress of Louisbourg collection has been granted by Dr. Amy Scott, Parks Canada, the Anglican 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 (*Human Remains, Cemeteries and Burial Grounds*, 2000). All sampling and extraction of osteocalcin from bone samples were completed at the University of New Brunswick's (UNB) Bioarchaeology Research and Teaching (BART) laboratory, while the extraction of cortisol from hair and bone were completed in collaboration with the UNB Department of Biology under the guidance of Dr. Dion Durnford.

## 3.3 The Rochefort Point Cemetery

Located on a peninsula directly east of the Fortress of Louisbourg, the Rochefort Point Cemetery was established as early as 1738 (Johnston, 1984). While in use before

the first siege in 1745, both historical and isotopic data tell us that the cemetery was used extensively by the New Englander forces who occupied Louisbourg between 1745 and 1749 (Johnston, 1984; Scott et al., 2023, 2022). Following the return of Louisbourg to the French Crown, documentation suggests the French continued to use Rochefort Point as a burial ground, particularly for military personnel (Johnston, 1984; Moore, 1974). The cemetery remained in use during the second British occupation of the Fortress until the formal decommissioning of the site in the early 1760s (Johnston, 1984; Scott et al., 2023, 2018). In the short period of its usage, historical records suggest that upwards of 1,100 individuals were buried in the Rochefort Point Cemetery (Moore, 1974; Scott et al., 2022, 2019).

The Fortress of Louisbourg has faced significant coastal erosion in recent years, and with the Rochefort Point Cemetery being situated only four metres above sea level, the safety of these burials has become increasingly dire (CBCL Limited, 2010; Dunham, 2014; Scott et al., 2018; Taylor et al., 2011). This severe coastal erosion has prompted rescue excavation efforts that have been ongoing since 2017. As of 2023, over 200 burials have been excavated with all skeletal material temporarily curated in the UNB BART Lab. While the burials excavated to date could represent any period of cemetery use, the majority were likely interred after 1745 as they were recovered on top of the Carrerot property, which was in use and standing until just before the first siege (Duggan, 2010; Scott et al., 2022).

## 3.4 Sample Overview

Given the topic of this thesis, the primary criteria for selecting individuals to be included in this study were those with both hair and skeletal material present. Additionally, only adult individuals were sampled as non-adult individuals (i.e., those who have not gone through the biological changes associated with puberty) (see Inglis and Halcrow, 2018; Lewis, 2022), had limited skeletal material present and could not be sampled consistently. Adults for this study were considered those who had both passed through puberty and were socialized as adults, which at Louisbourg was approximately 7-8 years of age (Belshaw, 2020; Johnston, 2001, 1984; Lewis, 2022; Rubinger, 1980). With this consideration in mind, no individual younger than age 12 was sampled in this study. Research to this point has found no statistical difference in osteocalcin concentrations from sampled elements (see Hughes, 2020; Scott et al., 2016) and no research regarding cortisol has been conducted up to this point; however, to eliminate as many extraneous contributing factors as possible, sample uniformity was prioritized. For sample uniformity across all individuals, osteocalcin and bone cortisol were taken from the parietal bone of the skull or the femur when the parietal was not preserved. For each individual, biological sex and age were assessed using standard macroscopic methods based on the development of dentition, pelvic shape, and the fusion of growth plates where possible (AlQahtani et al., 2010; Brooks and Suchey, 1990; Buikstra and Ubelaker, 1994; Christensen et al., 2019; Lovejoy et al., 1985; Phenice, 1969; Schaefer et al., 2009; St. Hoyme, 1989; Walker, 2005).

## 3.5 Sampling and Pre-treatment

### 3.5.1 Bone

Previous research shows a direct correlation between osteocalcin concentrations and areas of muscle attachment (see Hughes, 2020); therefore, a location with minimal muscle attachment was chosen on the ectocranial region of the parietal, specifically the posterior-lateral portion. In cases where the parietal could not be sampled or was missing, a femur sample was taken from the superior, posterior diaphysis, lateral to linea aspera. To collect these bone samples, a Dremel (model 300) rotary tool with the rose burr was used to clear away an approximately $3cm^2$ section of cortical bone. The burr of the rotary tool was then cleaned with isopropanol and sanitized with an ethanol flame. Once the cortical outer surface of the bone was cleared away, ten milligrams of inner cortical bone powder was collected and transferred to an Eppendorf Lo-Bind 2 millilitre (ml) tube. Bone samples to test for cortisol were procured using the same sampling method; however, 250-260 mg of bone powder was collected for each individual.

### 3.5.2 Hair

As discussed in Chapter 2, section 2.3.2, hair is a biological tissue not typically preserved during decomposition, especially in cases where the individual was not mummified (Kootker et al., 2020; Wilson et al., 2007). At the Fortress of Louisbourg, the preservation of hair can be almost entirely ascribed to copper shroud pins and the preservative properties found with this type of metal (Hunter, 2023; Lebow et al., 2020; Mytum, 2014; Zahedieh, 2013). Unfortunately, while copper is a preservative agent, it only works to preserve the tissue in close contact with it (see Lebow et al., 2020);

therefore, the retrievable hairs were approximately the length of these shroud pins - about two centimetres on average. Tweezers were used to remove hair from any associated shroud pin(s) to sample this hair for cortisol. Approximately 10mg of hair was collected from each individual, and in cases with longer hair preservation, an additional 10mg of hair was collected for each month of hair growth (approximately 1cm per month) (see Chapter 2, section 2.2.2.1) (Meyer et al., 2014; Meyer and Novak, 2012; Webb et al., 2010).

When capturing month-to-month hair samples, it is important to understand the biology of human hair and how its growth impacts cortisol concentrations. Hair only receives blood flow while it is forming in the scalp, meaning that besides external contamination, cortisol can only embed itself in hair while the strand is forming in the follicle – providing a month-to-month snapshot of stress (via cortisol concentrations) that can be captured within one strand of hair (Kapoor et al., 2018; Meyer and Novak, 2012; Otten et al., 2020; Schaefer, 2017; Webb et al., 2010). Therefore, it is imperative to identify the directionality of hair growth to ensure a sequentially accurate timeline of cortisol variability (Otten et al., 2022). When the hair root is absent, making it difficult to determine the newest growth, the cuticle pattern of the hair structure can be used. Human hair has a characteristic regular-flattened imbricate pattern (Yang et al. 2014), and thus, the directionality (direction of growth) of a hair strand can be determined by assessing a casting of the hair under a compound microscope (see Fig. 3.1) (Koch, 2004).

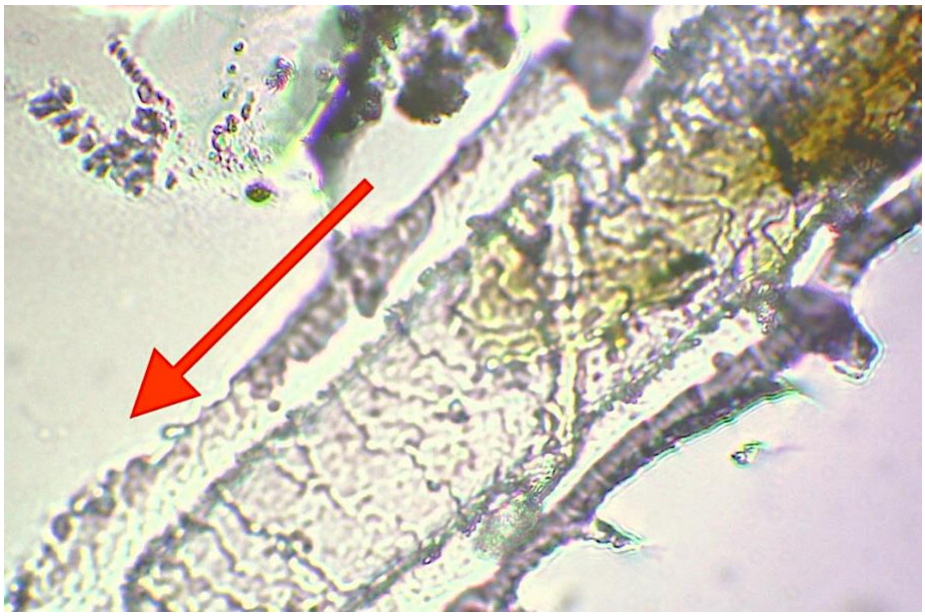

Figure 3.1: A scale cast photo of the direction of hair growth indicated with a red arrow (RP 3/2017)

To assess scale casting for all samples longer than 1 cm, a thin layer of clear-coat nail polish was added to a microscope slide and a cast mould of each hair sample was taken. Each mould was then observed under a compound microscope for the imbricate cuticle pattern to determine the direction of hair growth. However, while scale casting can establish which end of the hair is the newest, it cannot determine how close that segment of hair was to the scalp without a root present. Therefore, while variations in cortisol concentrations can be sequentially assessed, their relationship to the time of death cannot be definitively determined (East et al., 2022; East, 2021; Yang et al., 2014).

To assess cortisol concentrations in each 1cm hair segment, all samples were cut with a scalpel and placed in separate 2ml tubes with a pair of tweezers. Before the chemical extraction, the hairs were gently washed with 500 microlitres (µl) of isopropyl alcohol to remove surface contaminants and possible extraneous cortisol (Greff et al., 2019). When conducting a chemical extraction, maximizing contact between the solvent

(methanol) and the substrate (hair) is tantamount; therefore, to increase the surface area as much as possible, each hair sample was finely minced with a pair of sterilized dissection scissors (Greff et al., 2019).

## 3.6 Extraction and Processing

### 3.6.1 Osteocalcin

As a calcium-binding protein, osteocalcin requires demineralization of the bone material to be extracted and quantified (Hughes, 2020; Scott et al., 2016). To begin the demineralization process, ten percent weight by volume of ethylenediaminetetraacetic acid (EDTA) was added to each sample, proportional to the exact weight of each sample. The samples were then oscillated in a tube rotator in the fridge (5$^{o}$C) for 24 hours. Samples were then centrifuged at 14,000g for 10 minutes, separating the solid pellet from the liquid substrate.

As a corrosive agent, EDTA should not be present above 6.1mM for the quantification method to remain functional. By the end of the demineralization process, however, the concentration of EDTA was at 342.2mM and thus had to be diluted through filtration. Four hundred microliters of the liquid substrate was transferred to an Amicon Ultra–0.5 Centrifugal Filter device inside a filtering tube. All samples were then centrifuged for 10 minutes at 14,000g to reduce the sample volume to 50µl. Following this, three more cycles of dilution and filtration were completed by adding 450µl of a 1X phosphate buffered solution (PBS) to the filter containing the substrate and spinning at 14,000g in the centrifuge for ten minutes (1:10 dilution). In the final filtration, samples were only spun for about two minutes so that the final volume of each sample was

approximately 100µl. The final samples were then collected by centrifuging each filter upside down in a new filter tube at 1,000g for two minutes. The final elution volume for each sample was brought up to 200µl using 1X PBS, making the final concentration two times that of the original substrate.

### 3.6.2 Cortisol

Procedures designed to extract cortisol use organic solvents to penetrate the substrate's structure and dissolve cortisol as a supernatant (Van Uum et al., 2008). Methanol, in particular, has been among the most popular choices for the solvent to extract cortisol due to the high solubility of cortisol and the relative volatility of methanol, allowing for easier drying over other solvents such as water (Russell et al., 2012; Van Uum et al., 2008; Webb et al., 2010). For this study, each bone sample was eluted in 1.46ml of methanol solvent while gently shaken at 200 rotations-per-minute (rpm) for at least 18 hours (Charapata et al., 2018). After the extraction period, all samples were centrifuged for 10 minutes to avoid suspended contaminants and the supernatant methanol was transferred to a new tube to be dried down. Methanol in cortisol extractions is typically dried under a stream of nitrogen to both expedite the drying process and prevent the oxidation of cortisol (see Greff et al., 2019; Sauvé et al., 2007); however, due to instrumentation available in the UNB Department of Biology, a vacufuge was used as a comparable method to dry out and concentrate the cortisol extraction. A vacufuge is a centrifuge that operates under a vacuum which aids in expediting drying and preventing oxidation, addressing the two major concerns of cortisol extraction and has been used in other published experiments involving cortisol

extractions (Greff et al., 2019; Slominski et al., 2015). The cortisol pellet from each extraction was reconstituted in PBS at a pH of 7.6 to stabilize the solution for long-term storage and to ensure the pellet was chemically compatible with the method of quantification used in this study (Schaefer, 2017; Webb et al., 2010, 2015a, 2015b). The extraction of cortisol from each hair followed the same process as bone, with the only variation being that the hair was eluted in one millilitre of methanol as opposed to 1.46ml as per standard protocol (see Charapata et al., 2021, 2018; Slominski et al., 2015; Webb et al., 2010).

## 3.7 Quantification

### 3.7.1 Osteocalcin

An enzyme-linked immunosorbent assay (ELISA) is a common method for accurately identifying and quantifying proteins and other key molecules and was used to quantify osteocalcin in all bone samples. By using the natural immune response to a foreign body, ELISAs use an engineered antibody to bind to the desired protein or steroid (Engvall, 1980). By tying this binding to the release of a coloured dye, researchers can quantify the concentration of a sample by comparing the richness of the sample hue to a control ladder (Engvall, 1980; Porstmann and Kiessig, 1992). While other methods exist, an ELISA was the ideal choice for this thesis due to its ability to test multiple samples at once, the accessibility of the technique and required instrumentation, and the cost-effectiveness of prefabricated ELISA kits. These benefits have made the use of ELISAs standard in paleoproteomic research.

When the concentration of a sample lies outside the ELISA control ladder (e.g., 0-69 ng/ml for commercially available osteocalcin kits), the reading of that concentration lacks the accuracy guaranteed by the ELISA manufacturer. Based on previous research into osteocalcin in archaeological bone, it is possible for osteocalcin concentrations to fall outside this accuracy range (see Hughes, 2020); therefore, all collected samples were diluted to ensure accurate results. To do this, a preliminary ELISA was performed with samples being highly concentrated (1:10 dilution) and highly diluted (1:100 dilution) using 1X PBS to determine the dilution that would place the readings near the centre of the ladder where concentrations are the most accurate (Hughes, 2020; Pandey et al., 2019; Thakur et al., 2015; Waritani et al., 2017). Following this preliminary test ELISA, it was determined that a 1:30 dilution fell most consistently near the centre of the ELISA calibration curve. As such, a 1:30 dilution was used for all samples.

Osteocalcin for this project was quantified using a commercially available, prefabricated ELISA kit (ALPCO: 43-OSNHU-E01) with the manufacturer protocol followed. Twenty-five microliters of standards, controls, and samples were pipetted into each microwell, and 200 µl of antibody working solution was then added to each well. Protecting the microplate from exposure to light, the plate was rocked at 350 rotations-per-minute (rpm) for an hour. Any freely floating osteocalcin was bound to the immunoglobulins pre-coated at the bottom of the well plate during this one hour. The wells were then emptied, aspirated, and washed five times with 350µl of wash solution to clear away any contaminants or unbound osteocalcin. Two hundred microlitres of anti-human osteocalcin conjugated horseradish peroxidase (HRP) were added to each well, beginning the colour change reaction while being shielded from light exposure. After 20

minutes, 50µl of tetramethylbenzidine (TMB) substrate was added to each well to stop the colour change reaction, turning the blue-coloured reaction to yellow for quantification. The plate was then read on a Fisher Scientific Multiskan FC Microplate Reader Version 1.01.16 at 450nm. The optical densities from the plate were translated to concentrations using a 4-parameter logarithmic regression. Intra-observer error was accounted for by running all samples in duplicate wells and calculating the coefficient of variance (CV) as is standard within the discipline.

#### 3.7.1.1 Bicinchoninic Acid Assay

When analyzing the relative concentration of osteocalcin in a sample, it is crucial to consider the osteocalcin level in relation to the total protein present in the sample (Hughes, 2020). This is because the quantity of substrate (bone powder) used for extracting osteocalcin is not a reliable indicator of the relative concentration of osteocalcin in the sample (Hughes, 2020; Scott et al., 2020). An additional assay with the extracted samples was necessary to quantify the total protein present. Bicinchoninic acid (BCA) assays work by chemically reacting with the protein in a sample to produce a purple hue where its richness is directly proportional to the total protein in the sample (Otieno et al., 2016). A commercially available (Thermo-Scientific: Pierce BCA Protein Assay) prefabricated BCA kit was used to measure the total protein present in each neat (undiluted) osteocalcin sample. Control samples and 25µl of neat osteocalcin samples were added to the microplate in duplicate. Following this, 200µl of the chemical solution that elicits the BCA's colour-change reaction was added to each well. The plate was then set on a hot plate for thirty minutes at $37^0$C, creating a colour change reaction from green

to purple to reflect the total protein concentration in the sample. The plate was read on a Fisher Scientific Multiskan FC Microplate Reader Version 1.01.16 at 562 nm and translated to concentrations with a 4-parameter logarithmic curve. Intra-observer error was accounted for by running all samples in duplicate wells and calculating the coefficient of variance (CV) as is standard within the discipline.

#### 3.7.1.2 Fourier-Transform Infrared Spectroscopy and the Assessment of Diagenesis.

As previously discussed in Chapter 2, sections 2.3.2 and 2.3.3.1, the analysis of osteocalcin and bone cortisol in archaeological samples requires careful consideration of diagenesis. For this thesis, FTIR-ATR was used to measure diagenesis to ensure appropriate comparisons between osteocalcin and cortisol concentrations and assess the overall molecular preservation of these samples. A Bruker Alpha II FTIR spectrometer with an ATR accessory was used to create IR spectra for each sample in this study. Twenty-four absorbance scans were collected for each sample between 400-4,000 $cm^{-1}$ with a resolution of 8 $cm^{-1}$ to form a composite spectrum of the average absorbances. All collected spectra received an automatic baseline correction of absorbance peak heights in the Opus FTIR software.

The three FTIR-ATR metrics discussed in Table 2.1 in Chapter 2, section 2.3.3.1 (C/P, IRSF, and AmI/P) were used to measure diagenesis based on the collected IR spectra. To make the most authentic assessment of diagenesis for each sample, the absorbance reading at the absolute peak per individual spectrum was used for the mathematical calculations of these FTIR metrics (see Smith et al., 2023). To assess the severity of diagenesis for each sample, the scales for C/P (0.05-0.32) and IRSF (2.5-4.0)

established by (France et al., 2020) were used; for the assessment of AmI/P, the percent collagen was calculated from Trueman et al. (2004)'s regression, and the collagen cut-off value (3% by weight, i.e., AmI/P <0.069) from Chesson et al. (2021) was used.

### 3.7.2 Cortisol

For the quantification of cortisol, a commercially available, prefabricated ELISA kit was also used (ALPCO: 11-CORHU-E01-SLV) following the manufacturer's protocol. Fifty microliters of calibrator, control, and sample were pipetted into each microwell, followed by 100µl of anti-human cortisol conjugated horseradish peroxidase (HRP) conjugate working solution. The plate was then incubated on a shaker for 45 minutes at ambient temperature. The excess liquid was disposed of, and the plate was washed with 300µl of buffer solution three times to remove any unbound cortisol or other contaminants. Following the last wash, 150µl of tetramethylbenzidine (TMB) substrate was added to each well, eliciting the colour change reaction. The colour change reaction was allowed to progress for 15-20 minutes until the blank calibrator became a dark blue colour. At that time, 50µl of stop solution was added to each well, causing a colour change from blue to yellow. The plate was read through a Fisher Scientific Multiskan FC Microplate Reader Version 1.01.16 and read for absorbances at 450nm and 405nm. These optical densities were then translated to concentrations using a 4-parameter logarithmic curve. Intra-observer error was accounted for by running all samples in duplicate wells and calculating the coefficient of variance (CV), which is standard within the discipline.

## 3.8 Analysis

### 3.8.1 Intra-Observer Error

When quantifying proteins using an ELISA, there is natural variation between sample duplicates due to the individual differences in the colour change reaction (Reed et al., 2002; Watson and Musher, 1993). Consequently, metrics have been adopted to measure the amount of variance and acceptable ranges of variance within a sample set. The coefficient of variance (CV) is a percentage of how much the standard deviation makes up the average of a given set of replicates (Reed et al., 2002). The CV has become the discipline standard when using quantitative assays, where researchers and manufacturers generally agree that per sample, the CV should not exceed approximately 20% and the CV grand mean per assay should not exceed 15% (Kinn Rød et al., 2017; Lexmond et al., 2011; Reed et al., 2002; Watson and Musher, 1993). For each tested sample for osteocalcin, cortisol, and total protein content (BCA), the CV was calculated to ensure intra-observer is not a significant contributing factor to collected concentrations using ELISA.

### 3.8.2 Interpretation of Readings

The raw ALPCO ELISA readings for osteocalcin are reported in nanograms of osteocalcin per millilitre (ng/ml), which means that osteocalcin readings need to be mathematically converted to nanograms of osteocalcin per total micrograms of protein in the sample (ng/μg) (Scott et al., 2020). To accomplish this, the following mathematical equation was used (Hughes 2020):

$$\frac{A}{1} \times \frac{B}{C} \times \frac{D}{1} \times \frac{1}{E} = F$$

In this equation, A stands for the original ELISA reading in ng/ml, B represents the dilution factor (which is 30X in this study), C is the filtration ratio (0.5 in this study), D stands for the elution volume (0.2 ml in this study), E is the total protein readings obtained from the BCA assay, and F is the final osteocalcin concentration reported in the appropriate ng/μg unit of measurement (Hughes, 2020).

The ALPCO ELISA quantifies cortisol in nanograms per millilitre (ng/ml); however, the reporting standard for cortisol in hair is nanograms per gram of substrate (ng/g) (Meyer et al., 2014; Sauvé et al., 2007; Thomson et al., 2010; Van Uum et al., 2008; Webb et al., 2010). While there is yet to be a universal unit of reporting cortisol concentrations from bone or other hard tissues, the decision was made to also report these findings in ng/g to allow for proper comparisons between bone *and* hair concentrations of cortisol (Charapata et al., 2021, 2018; Quade et al., 2023, 2021). Therefore, both sample types needed to be converted using the mathematical equation adapted from Meyer et al. (2014):

$$\frac{A}{B} \times \frac{C}{D} \times E \times 1{,}000 = F$$

The letter A is the cortisol concentration determined from the ALPCO ELISA (ng/ml), and B is the weight of hair or bone used in the extraction (mg). C represents the volume of methanol (ml) used to perform the cortisol extraction, and D is the amount of methanol (ml) transferred to a new tube to be dried down. The amount of PBS (ml) used to reconstitute the dried-down methanol is represented by the letter E; the 1,000 value accounts for unit conversion. This produced the final output (F) in the unit picogram per

milligram (pg/mg), which is mathematically interchangeable with ng/g (Meyer et al., 2014).

### 3.8.3 Statistical Analysis

Statistical analyses were completed using the IBM SPSS statistical software (Version: 29.0.0.0 (241)) to assess the relationship between osteocalcin and cortisol and factors such as diagenesis. This was done by separating the data into two groups based on the top and bottom $50^{th}$ percentile of a given parameter. This was done in light of correlation and regression analysis due to the limited sample size of this study. A sample set of 30 data points is considered the minimum when conducting regressions and correlations to avoid the possibility of a false negative (Bonett and Wright, 2000; Etz and Arroyo, 2015; Frankel et al., 2009).

In addition to traditional statistical tests relying on p-values, effect size was calculated using SPSS. Where p-value statistics rely heavily on sample size to translate findings to a population level, effect size focuses on the practical importance of observations, unaffected by sample size and notably does not utilize a p-value to mark significance (Chen et al., 2019; Etz and Arroyo, 2015; Kargar et al., 2020; Serdar et al., 2021; Sullivan and Feinn, 2012). When there is a strong effect size observed in a given comparison, but that same comparison does not produce a significant p-value ($p<0.05$), the phenomenon has been previously attributed to the sample size being too small to translate to a population level (see Etz and Arroyo, 2015; Serdar et al., 2021; Sullivan and Feinn, 2012). Given the small sample size for this thesis and the specific research questions, these statistical tests were determined to be the most appropriate to measure the relationships between osteocalcin, cortisol, and extraneous factors such as diagenesis.

## 3.9 Conclusion

This chapter focused on providing a contextual background for the Rochefort Point Cemetery and its ongoing excavation as it relates to the samples used in this research. This chapter also provided an overview of the methods used to chemically extract and quantify cortisol (hair and bone) and osteocalcin (bone), including demineralization, chemical aliquoting, total protein quantification, ELISA, and FTIR-ATR. Finally, this chapter outlined the mathematical and statistical methods used to quantify and statistically analyze the collected data.

# Chapter 4: Results

## 4.1 Introduction

This chapter will begin with a brief review of the sample population demographic data and the intra-observer error testing results. Both the cortisol and osteocalcin ELISA tests will then be reviewed, and the nonparametric comparisons and effect size tests between osteocalcin and cortisol (both from hair and bone) will be considered, as well as their relationship to one another. The chapter will also review sample diagenesis, and month-to-month stress concentrations.

## 4.2 Sample Overview

The sampling criteria for this study, outlined in Chapter 3, section 3.4, allowed for 12 individuals to be included. Of the 12 individuals, all but one had cranial fragments present, and all individuals had hair present (see Table 4.1). Due to poor preservation, most individuals (n =8) could not be assigned biological sex through macroscopic analysis, but those who could be assessed (n = 4) were biological males. Definitive age ranges were also difficult to assign due to the poor preservation; however, it was possible to determine that the youngest individual in this sample was at least 14.5 years of age, and the oldest individual was approximately 35 years of age (see Table 4.1).

Table 4.1: Skeletal sample information for the Rochefort Point Cemetery

| Burial # | Provenience | Hair | Skeletal Element | Estimated age (years) | Biological sex |
|---|---|---|---|---|---|
| 3/2017 | 55L34A | ✓ | Cranium | 30-35 | Male |
| 12/2017 | 55L34AK | ✓ | Cranium | 24+ | Male |
| 15/2017 | 55L34B | ✓ | Cranium | Unknown | Unknown |
| 45/2018 | 55L34N | ✓ | Femur | 18 | Unknown |
| 88/2019 | 55L34AK | ✓ | Cranium | 25-29 | Male |
| 120/2021 | 55L34T | ✓ | Cranium | 15.5+ | Unknown |
| 124/2022 | 55L34UX | ✓ | Cranium | Unknown | Unknown |
| 136/2022 | 55L34FQ | ✓ | Cranium | 18+ | Male |
| 154/2022 | 55L34FQ | ✓ | Cranium | 16.5+ | Unknown |
| 172/2023 | 55L34BB | ✓ | Cranium | 14.5+ | Unknown |
| 194/2023 | 55L34BBCC | ✓ | Cranium | 15.5+ | Unknown |
| 211/2023 | 55L34CCDD | ✓ | Cranium | 15.5+ | Unknown |

## 4.3 Intra-observer Error

After completing the cortisol, osteocalcin, and BCA ELISAs (see Appendix A), the CV was calculated for all duplicate samples, as well as the grand mean per assay as described in section 3.8.1 (see Table 4.2). For the cortisol assay, all samples fell well within an acceptable level of variation, and the grand mean was 1.274%. For the BCA assay, variation within the test had a grand mean of 3.09%. Variation was higher for the osteocalcin ELISA with a grand mean CV of 6.451%, likely due to three samples (12/2017, 124/2022, and 211/2023) (see Table 4.2).

Table 4.2: Chart of calculated coefficient of variances (CV) of cortisol, osteocalcin, and BCA ELISA readings. Highlighted in yellow show an elevated CV that warranted a repeated ELISA

| **Cortisol Sample** | **CV (%)** | **Osteocalcin Sample** | **CV (%)** | **BCA Sample** | **CV (%)** |
|---|---|---|---|---|---|
| 3/2017 Month 1 | 0.021 | 3/2017 | 2.473 | 3/2017 | 5.58 |
| 3/2017 Month 2 | 0.257 | 12/2017 Assay 1 | 15.055 | 12/2017 | 2.23 |
| 3/2017 Bone | 0.444 | 15/2017 | 3.005 | 15/2017 | 2.53 |
| 12/2017 Month 1 | 0.759 | 45/2018 | 4.788 | 45/2018 | 2.92 |
| 12/2017 Bone | 1.773 | 88/2019 | 3.173 | 88/2019 | 1.78 |
| 15/2017 Month 1 | 1.790 | 120/2021 | 4.054 | 120/2021 | 2.70 |
| 15/2017 Month 2 | 2.788 | 124/2022 Assay 1 | 15.640 | 124/2022 | 0.427 |
| 15/2017 Month 3 | 0.218 | 136/2022 | 4.215 | 136/2022 | 0.658 |
| 15/2017 Bone | 0.683 | 154/2022 | 0.304 | 154/2022 | 4.84 |
| 45/2018 Month 1 | 1.938 | 172/2023 | 4.635 | 172/2023 | 4.42 |
| 45/2018 Bone | 1.695 | 194/2023 | 1.840 | 194/2023 | 4.17 |
| 88/2019 Month 1 | 0.065 | 211/2023 Assay 1 | 18.227 | 211/2023 | 2.88 |
| 88/2019 Bone | 0.976 | **Average** | 6.451 | **Average** | 3.09 |
| 120/2021 Month 1 | 1.027 | | | | |
| 120/2021 Month 2 | 0.225 | | | | |
| 120/2021 Month 3 | 3.015 | | | | |
| 120/2021 Bone | 0.777 | | | | |
| 124/2022 Month 1 | 0.442 | | | | |
| 124/2022 Bone | 0.971 | | | | |
| 136/2022 Month 1 | 0.453 | | | | |
| 136/2022 Month 2 | 1.563 | | | | |
| 136/2022 Bone | 0.078 | | | | |
| 154/2022 Month 1 | 1.123 | | | | |
| 154/2022 Bone | 0.035 | | | | |
| 172/2023 Month 1 | 3.901 | | | | |
| 172/2023 Month 2 | 0.862 | | | | |
| 172/2023 Bone | 0.701 | | | | |
| 194/2023 Month 1 | 1.622 | | | | |
| 194/2023 Month 2 | 7.020 | | | | |
| 194/2023 Month 3 | 2.151 | | | | |
| 194/2023 Bone | 0.453 | | | | |
| 211/2023 Month 1 | 0.694 | | | | |
| 211/2023 Bone | 1.504 | | | | |
| **Average** | 1.274 | | | | |

These three samples were quantified a second time in a new ELISA as their high levels of intra-assay variance in the original assay were likely due to human error in the

timing of when the stop solution was added to the ELISA colour change reaction (Daly et al., 2005). The CVs of this second assay were lower and brought the grand mean down to 3.910% (Table 4.3). Therefore, the values used in the subsequent statistical analysis and reporting in this study were the osteocalcin concentrations for 12/2017, 124/2022, and 211/2023 from this re-assay.

Table 4.3 Chart of calculated coefficient of variances (CV) of repeated osteocalcin ELISA readings

| **Osteocalcin Sample** | **CV (%)** |
|---|---|
| 12/2017 Assay 2 | 0.215 |
| 124/2022 Assay 2 | 6.154 |
| 211/2023 Assay 2 | 12.064 |
| **New Total Average** | 3.910 |

4.4 Statistical Considerations

Following preliminary Shapiro-Wilk tests of normality and F-tests of equality of variances, it was found that there was not a consistent agreement with the required assumptions of parametric statistical testing (i.e., normal distribution and equal variance) (see Sheskin, 2011). Therefore, nonparametric testing was used for all subsequent statistical comparisons. An additional point of note is regarding Individual 45/2018. As discussed in section 4.2, Individual 45/2018 was the only individual where the bone sample was harvested from the femur instead of the skull. Due to the established differences in bone turnover rates in different regions of the skeleton (see Chapter 2, section 2.3.3) along with other factors that will be discussed in Chapter 5, the data from Individual 45/2019 will be reported separately and were not included in the overall sample comparisons for osteocalcin or cortisol. However, the hair sample from 45/2018 was included in the overall sample analyses for hair cortisol.

## 4.5 Osteocalcin

Of the 11 individuals evaluated for osteocalcin in this study, concentration values ranged between 2.361 and 7.606 nanograms of osteocalcin per microgram of total bone protein (ng/ug). The average osteocalcin concentration across these 11 individuals fell slightly to the left of the center at 5.370±1.82 ng/ug with a median concentration value of 5.778 ng/ug (see Table 4.4). Despite Individual 45/2018 being excluded from the overall sample analysis involving any data derived from bone, their osteocalcin concentration is reported in Table 4.4 as a point of comparison.

Table 4.4: Calculated osteocalcin concentrations per individual

| **Burial #** | **Osteocalcin Concentrations (ng/ug)** |
|---|---|
| 3/2017 | 6.796 |
| 12/2017 | 7.606 |
| 15/2017 | 4.206 |
| 45/2018 | 6.291 |
| 88/2019 | 7.335 |
| 120/2021 | 2.361 |
| 124/2022 | 3.951 |
| 136/2022 | 6.619 |
| 154/2022 | 6.961 |
| 172/2023 | 5.778 |
| 194/2023 | 3.849 |
| 211/2023 | 3.610 |

### 4.5.1 Osteocalcin and Diagenesis

As discussed in Chapter 3, section 3.7.1.2, three metrics of diagenesis were calculated (i.e., IRSF, C/P, and AmI/P). The IRSF values ranged between 3.206 and 3.908, with an average of 3.524±0.25 and a median value of 3.521. The C/P ratios ranged between 0.132 and 0.306, with an average value of 0.207±0.065 and a median value of

0.164. Finally, the AmI/P ratio values ranged between 0.078 and 0.259, with an average value of 0.177±0.065 and a median value of 0.154 (see Table 4.5). Again, Individual 45/2018 was excluded from the overall sample analysis involving any data derived from bone, but their values for each FTIR metric are reported in Table 4.5 as a point of comparison.

Table 4.5: Calculated Infrared Splitting Factor (IRSF), Carbonate-Phosphate ratio (C/P), and Amide I-Phosphate ratio (AmI/P) values per individual

| **Burial #** | **IRSF** | **C/P** | **AmI/P** |
|---|---|---|---|
| 3/2017 | 3.268 | 0.306 | 0.220 |
| 12/2017 | 3.452 | 0.241 | 0.223 |
| 15/2017 | 3.206 | 0.292 | 0.259 |
| 45/2018 | 3.664 | 0.180 | 0.116 |
| 88/2019 | 3.255 | 0.287 | 0.246 |
| 120/2021 | 3.908 | 0.161 | 0.124 |
| 124/2022 | 3.785 | 0.132 | 0.078 |
| 136/2022 | 3.677 | 0.155 | 0.124 |
| 154/2022 | 3.276 | 0.227 | 0.255 |
| 172/2023 | 3.703 | 0.152 | 0.126 |
| 194/2023 | 3.715 | 0.164 | 0.154 |
| 211/2023 | 3.521 | 0.164 | 0.138 |

Diagenesis is a major known influence on concentrations of bone osteocalcin (see Scott et al., 2020; Smith et al., 2005). As a consequence of this phenomenon, evaluating the relationship diagenesis has to the osteocalcin concentrations of this study is crucial to ensure authentic comparisons to other factors going forward. As discussed in Chapter 3, section 3.8.2, at least 30 data points are necessary for statistical correlation tests to avoid a false negative confidently (see Frankel et al., 2009). Since correlation statistics were not appropriate for evaluating any trends in the data, osteocalcin samples were separated into two groups based on the top and bottom 50$^{th}$ percentile of the calculated FTIR metrics. This allowed for the determination of whether a pronounced and statistically significant

spread of osteocalcin concentrations was caused by a given factor (in this case, diagenesis via the FTIR metrics). This analysis was completed using a Mann-Whitney U test, the primary nonparametric test to evaluate the differences between two groups by comparing group medians (Fay and Proschan, 2010).

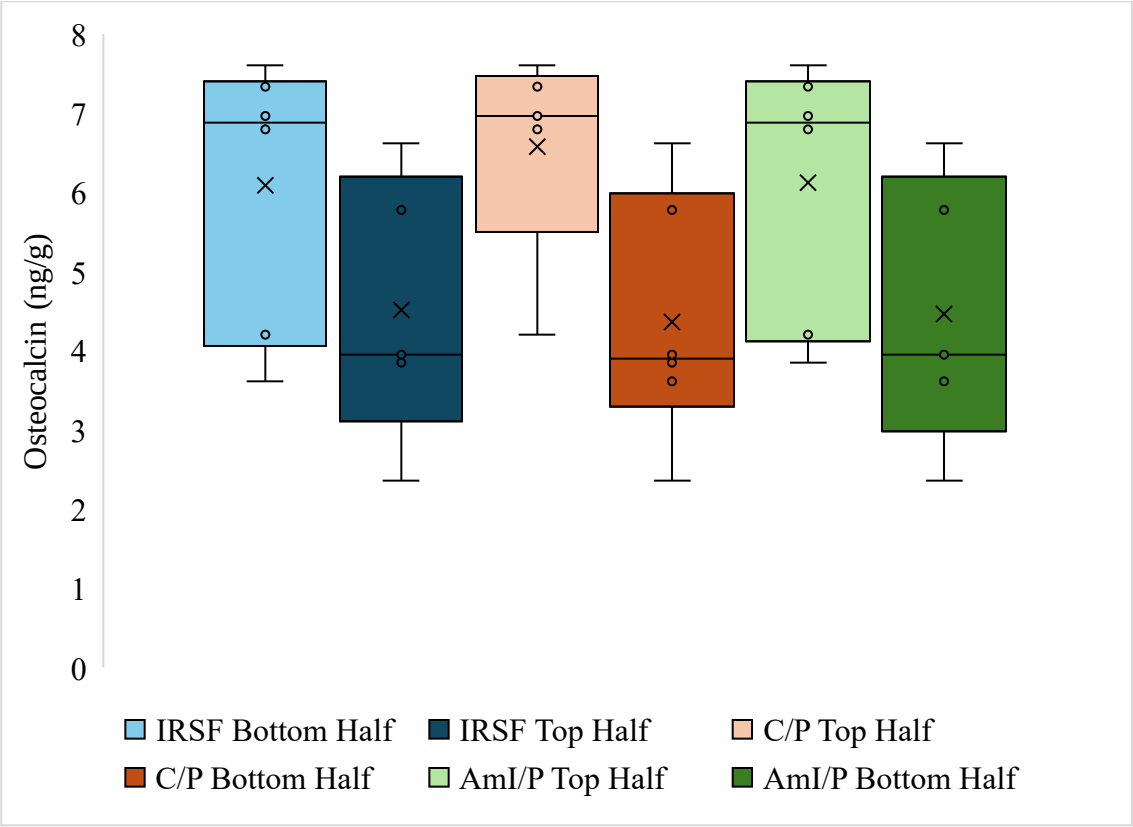


Figure 4.1: A box-and-whisker plot depicting the comparison of osteocalcin concentrations when separated into two groups based on the top and bottom 50th percentile of FTIR calculated values; Individual 45/2018 not included

As shown in Figure 4.1, as diagenesis increases (note that with IRSF, diagenesis worsens as the value increases), there is a visual decrease in osteocalcin concentrations. This is supported by statistically significant Mann-Whitney U tests for osteocalcin with IRSF and C/P, which means a statistically significant trend exists between increasing diagenesis and decreasing osteocalcin (see Table 4.6). Cliff's delta is a nonparametric effect size measurement that tests the probability that a value in a group is higher than

that in another (Marfo and Okyere, 2019). Ranging from negative one to one, Cliff's delta provides a measurement of small, medium, and large practical significance—or how apparent a difference is to the naked eye (Chen et al., 2019; Kargar et al., 2020; Marfo and Okyere, 2019; Sullivan and Feinn, 2012). When Cliff's delta was used in tandem with the Mann-Whitney U test, it presented a noticeable practical significance shown by medium-to-strong Cliff's Delta values for all three comparisons (see Table 4.6).

Table 4.6: Mann-Whitney U test results comparing osteocalcin concentrations when separated into two groups based on the top and bottom 50th percentile of FTIR calculated values along with the Cliff's delta for each comparison

| **Test Comparison** | **Mann-Whitney U** | **P-Value** | **Cliff's δ** |
|---|---|---|---|
| Osteocalcin separated by the top and bottom 50$^{th}$ percentile of IRSF values. | 6 | **0.037** | 0.52$^{**}$ |
| Osteocalcin separated by the top and bottom 50$^{th}$ percentile of C/P values. | 2 | **0.022** | 0.84$^{***}$ |
| Osteocalcin separated by the top and bottom 50$^{th}$ percentile of AmI/P values. | 7 | 0.08 | 0.67$^{***}$ |

Significant p-values are bolded. $^{*}$Indicates small, $^{**}$medium, and $^{***}$strong practical significance (effect size). Individual 45/2018 not included.

Before osteocalcin can be compared to other factors in this chapter, the severity of diagenesis must be established to ensure it does not play a major role in the reported osteocalcin concentrations. To achieve this, the collected FTIR metrics (i.e., IRSF, C/P, and AmI/P) calculated in this study were compared to established diagenetic parameters from previously published literature described in Chapter 3, section 3.7.1.2 (see Fig. 4.2). Individual 45/2018 was also compared to these established parameters of diagenesis severity and is included in Figure 4.2 as a point of comparison. As can be seen in Figure 4.2, none of the individuals included in this study (including Individual 45/2018) exceeded the limits of what is considered well-preserved archaeological bone across all

employed metrics and, therefore, can be confidently used in the remainder of the analyses (Chesson et al., 2021; France et al., 2020; Trueman et al., 2004).

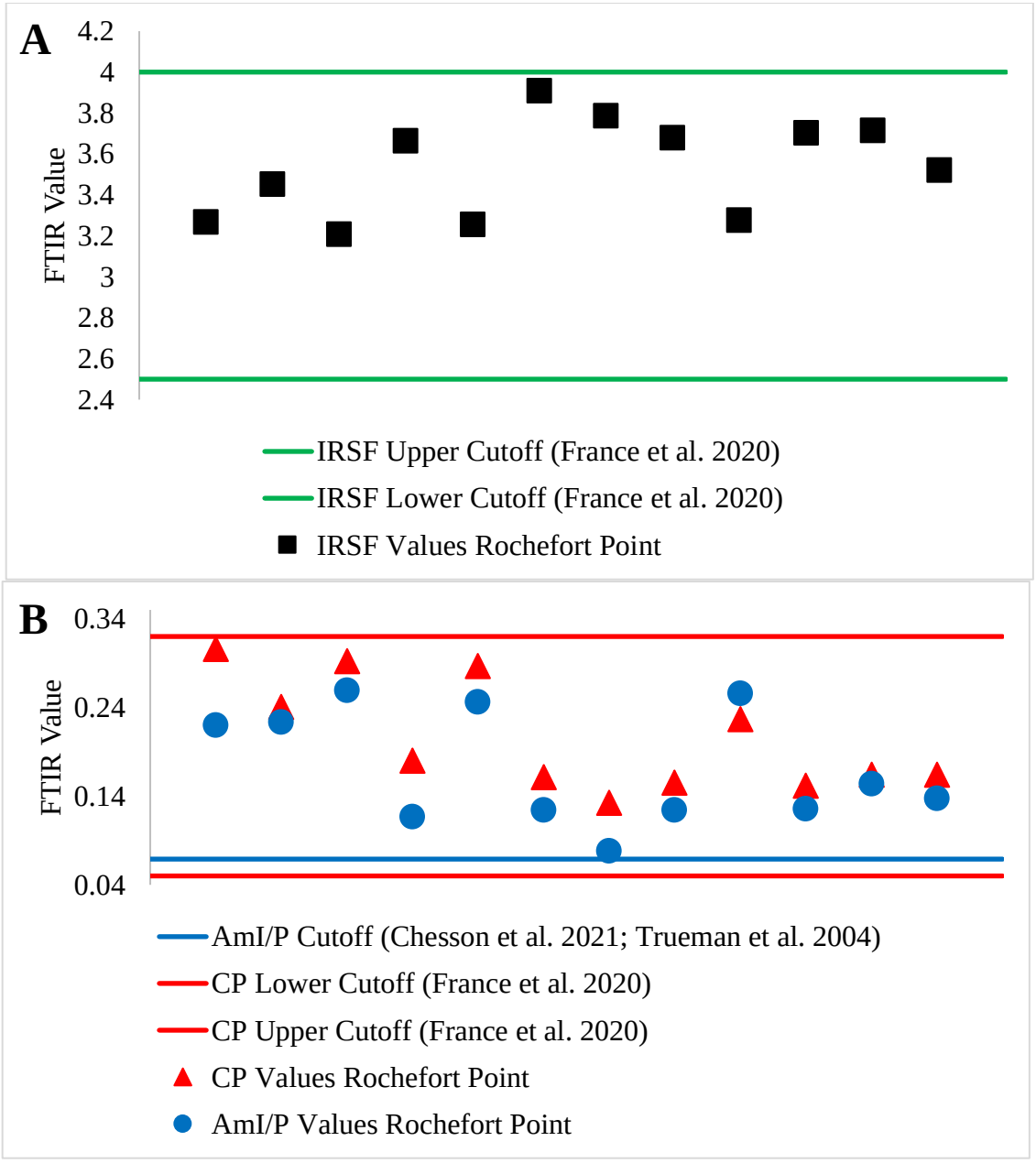


Figure 4.2: A graph of collected IRSF (A), C/P (B), and AmI/P (B) values of samples in relation to their respective cut-offs outlined by France et al. (2020), and Trueman et al. (2004) and Chesson et al. (2021)

## 4.6 Cortisol

There were 21 total hair samples from the 12 individuals in this study, where cortisol was chemically extracted and quantified successfully. Of these 21 hair samples, cortisol concentrations ranged between 3.819 and 31.864 nanograms of cortisol per gram of hair (ng/g) with an average value of 14.712±7.36 ng/g and a median value of 15.438 ng/g (see Table 4.7). Cortisol was successfully extracted and quantified from all 12 bone samples, with bone cortisol from Individual 45/2018 not included in the overall sample analysis but still reported in Table 4.7, as a point of comparison. The remaining 11 concentrations of bone cortisol ranged between 3.339 ng/g and 12.606 ng/g, with an average value of 7.29±2.95 ng/g and a median value of 7.29 ng/g (see Table 4.7).

Table 4.7: Cortisol concentrations from all hair and bone samples

| **Burial #** | **Month 1 (ng/g)** | **Month 2 (ng/g)** | **Month 3 (ng/g)** | **Bone (ng/g)** |
|---|---|---|---|---|
| 3/2017 | 15.785 | 6.651 | NA | 3.926 |
| 12/2017 | 5.101 | NA | NA | 3.339 |
| 15/2017 | 15.438 | 12.019 | 7.372 | 5.632 |
| 45/2018 | 3.819 | NA | NA | 10.078 |
| 88/2019 | 11.218 | NA | NA | 4.422 |
| 120/2021 | 29.567 | 31.864 | 8.200 | 8.175 |
| 124/2022 | 9.722 | NA | NA | 12.606 |
| 136/2022 | 16.453 | 17.254 | NA | 7.286 |
| 154/2022 | 18.296 | NA | NA | 8.093 |
| 172/2023 | 9.936 | 20.833 | NA | 11.011 |
| 194/2023 | 18.697 | 12.126 | 20.326 | 9.438 |
| 211/2023 | 18.376 | NA | NA | 6.207 |

### 4.6.1 Hair Cortisol

Of the 12 individuals in this study, half (n = 6) had enough hair to assess a second month of cortisol concentrations (i.e., at least 2cm of hair total); of those six, half (n = 3)

were able to have a third month of cortisol concentrations assessed (i.e., at least 3cm of hair total) (see Fig. 4.3).

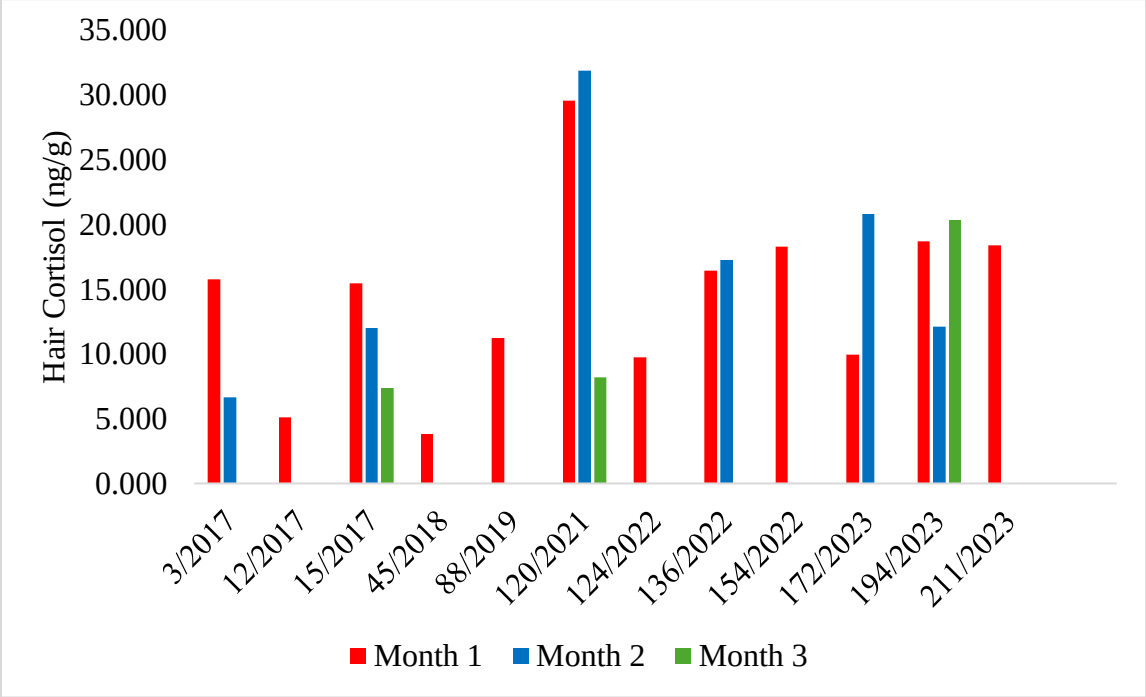


Figure 4.3: A bar chart of hair cortisol concentrations across all samples

To compare the differences between months of cortisol concentrations, a Kruskal-Wallis with Dunn's Post Hoc test was used. These tests are a nonparametric method for comparing differences between two or more groups since they account for the issue of compounding P-values (Bewick et al., 2004). When comparing the cortisol readings per month using these tests, there was no significant difference in concentration values. It is important to note, however, that there was a medium to strong practical significance observed when using a Cliff's delta test between the monthly readings of cortisol, meaning that while not statistically significant, there is a very apparent visual difference between the months of cortisol—meaning that in practical terms they are distinct from one another (see Table 4.8).

Table 4.8: Kruskal-Wallis Tests, Dunn's Post Hoc Tests, and Cliff's Delta comparing multiple months of hair cortisol concentrations with Bonferroni corrected P-Values

| **Burial #** | **Kruskal-Wallis Test** | **H(1)** | **P-Value** | |
|---|---|---|---|---|
| 3/2017 | | 2.4 | 0.12 | |
| | **Dunn's Post Hoc Test** | **Z Score** | **P-Value** | **Cliff's δ** |
| | Months 1 to 2 | 1.55 | 0.12 | 1.0*** |
| 15/2017 | **Kruskal-Wallis Test** | **H(2)** | **P-Value** | |
| | | 3.71 | 0.16 | |
| | **Dunn's Post Hoc Test** | **Z Score** | **P-Value** | **Cliff's δ** |
| | Months 1 to 2 | 0.53 | 1.00 | 0.5** |
| | Months 1 to 3 | 1.87 | 0.18 | 1.0*** |
| | Months 2 to 3 | 1.34 | 0.54 | 1.0*** |
| 120/2021 | **Kruskal-Wallis Test** | **H(2)** | **P-Value** | |
| | | 4.57 | 0.10 | |
| | **Dunn's Post Hoc Test** | **Z Score** | **P-Value** | **Cliff's δ** |
| | Months 1 to 2 | 1.07 | 0.86 | -1.0*** |
| | Months 1 to 3 | 2.14 | 0.10 | 1.0*** |
| | Months 2 to 3 | 1.07 | 0.86 | 1.0*** |
| 136/2022 | **Kruskal-Wallis Test** | **H(1)** | **P-Value** | |
| | | 0.00 | 1.00 | |
| | **Dunn's Post Hoc Test** | **Z Score** | **P-Value** | **Cliff's δ** |
| | Months 1 to 2 | 0.00 | 1.00 | 0.0 |
| 172/2023 | **Kruskal-Wallis Test** | **H(1)** | **P-Value** | |
| | | 2.4 | 0.12 | |
| | **Dunn's Post Hoc Test** | **Z Score** | **P-Value** | **Cliff's δ** |
| | Months 1 to 2 | 2.4 | 0.12 | -1*** |
| 194/2023 | **Kruskal-Wallis Test** | **H(2)** | **P-Value** | |
| | | 1.14 | 0.56 | |
| | **Dunn's Post Hoc Test** | **Z Score** | **P-Value** | **Cliff's δ** |
| | Months 1 to 2 | 0.53 | 1.00 | 0.5** |
| | Months 1 to 3 | 0.53 | 1.00 | -0.5** |
| | Months 2 to 3 | 1.07 | 0.86 | -0.5* |

Significant p-values are bolded. *Indicates small, **medium, and ***strong practical significance (effect size).

Among the individuals with multiple-month cortisol readings, individual 120/2021 had both the highest cortisol concentrations and the most dramatic shifts in cortisol concentration both visually and by effect size (see Fig. 4.8 and Table 4.8).

Comparing the sequential months of cortisol concentrations for hair longer than 1 cm (n = 6), the average first month was 17.646±6.52 ng/g (n = 6), the second month was 16.79±8.8 ng/g (n = 6), and the third was 11.966±7.25 ng/g (n = 3).

### 4.6.2 Bone Cortisol

#### 4.6.2.1 Bone Cortisol Compared to Hair Cortisol

To measure the relationship between cortisol extracted from bone and hair, bone cortisol concentrations were separated into two groups based on those who fell between the top and bottom 50$^{th}$ percentile of hair cortisol concentrations. For individuals where multiple months of cortisol were acquired, those concentrations were averaged together (see Table 4.7). As shown in Figure 4.4, there is a direct relationship between increasing bone cortisol and increasing hair cortisol. While this relationship was not statistically significant using a Mann-Whitney U test, there was a strong practical significance seen in the Cliff's delta effect size measurement (U:6; P-Value: 0.1207; δ: 0.6***), meaning that as the hair cortisol concentrations rose, bone cortisol generally trended in the same direction.

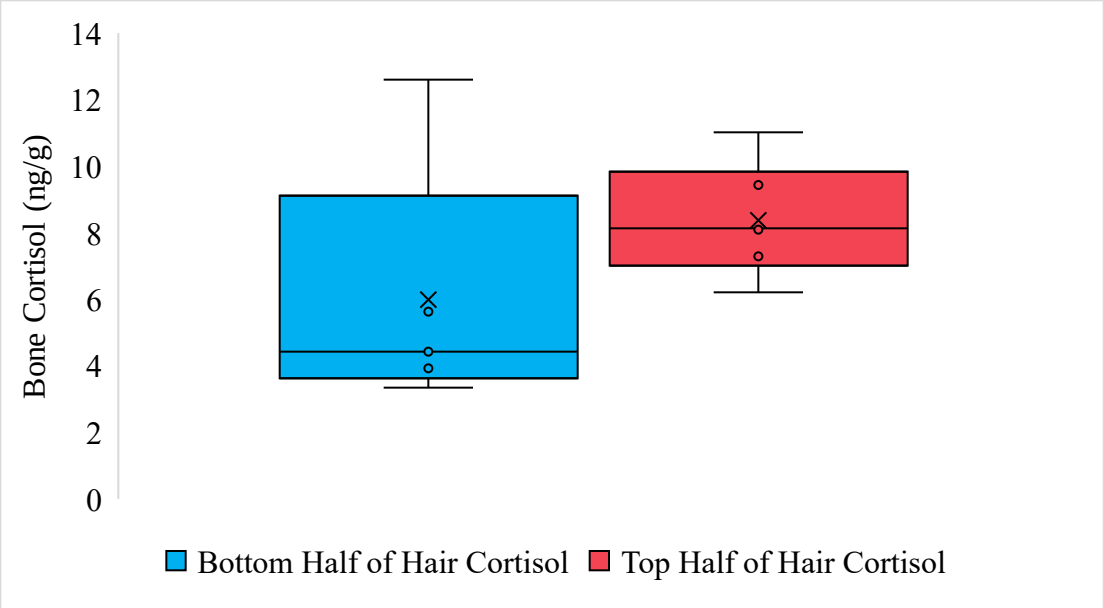


Figure 4.4: A box-and-whisker plot comparing bone cortisol concentrations when separated into two groups based on the top and bottom 50th percentile of hair cortisol values; Individual 45/2018 not included

#### 4.6.2.2 Bone Cortisol and Diagenesis

As discussed in Chapter 2, section 2.3.2, to date no research has been conducted on the impact that diagenesis has on hard tissue cortisol. Therefore, similar to how osteocalcin and diagenesis were compared in section 4.5.1, all bone cortisol readings were separated into groups based on the top and bottom 50$^{th}$ percentile of the FTIR-ATR diagenetic values and compared to one another (see Fig 4.5). As shown in Figure 4.5, as the level of diagenesis increases (note that for IRSF--higher values indicate an increased amount of diagenetic alteration), so does the concentration of cortisol extracted from bone. This is further supported by a Mann-Whitney U statistical test (Table 4.9), which shows a statistical difference in bone cortisol concentrations when separated by calculated IRSF and C/P values. Additionally, there are very strong practical

significances across all three FTIR diagenesis markers when Cliff's delta tests were run (IRSF, C/P, and AmI/P) (see Table 4.9).

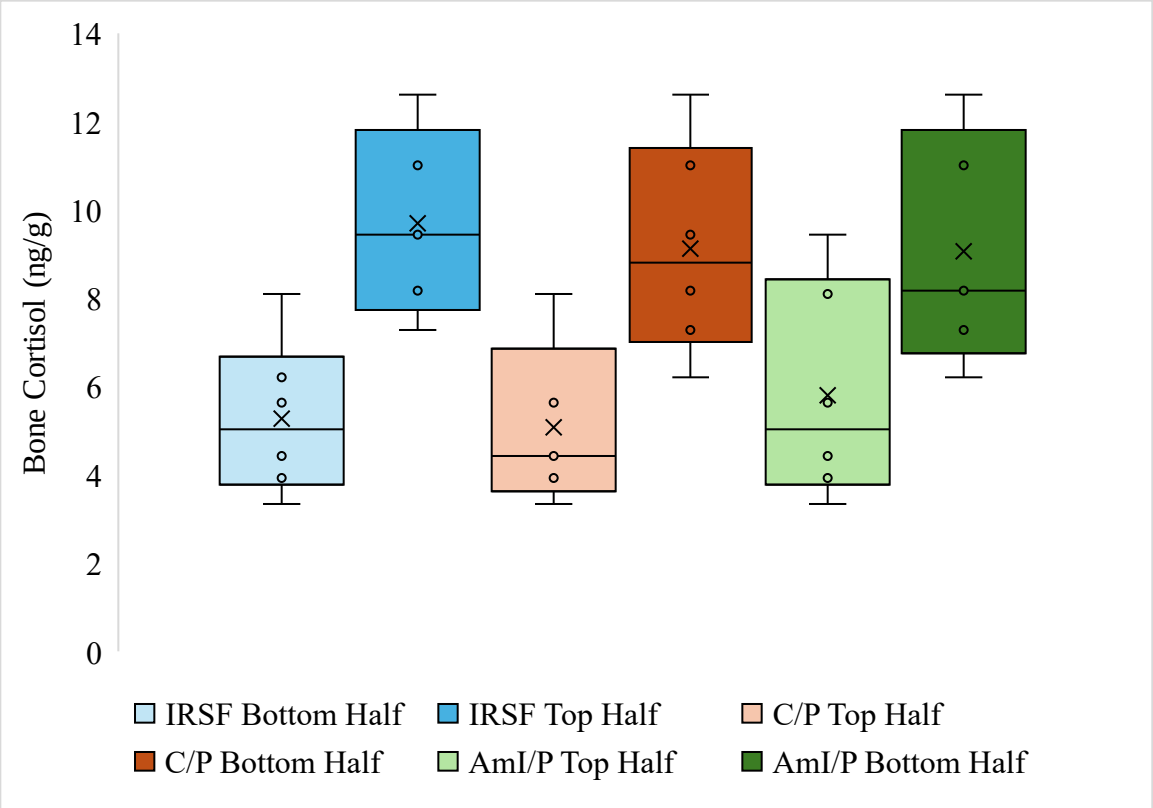


Figure 4.5: A box-and-whisker plot depicting the comparison of bone cortisol concentrations when separated into groups based on the top and bottom 50th percentile of FTIR calculated values; Individual 45/2018 not included

Table 4.9: Mann-Whitney U test comparison of bone cortisol concentrations when separated into groups based on the top and bottom 50th percentile of FTIR calculated values along with the Cliff's Delta value for each comparison

| **Comparison** | **Mann-Whitney U** | **P-Value** | **Cliff's δ** |
|---|---|---|---|
| Bone cortisol separated by the top and bottom 50$^{th}$ percentile of IRSF | 1 | **0.014** | -0.867$^{***}$ |
| Bone cortisol separated by the top and bottom 50$^{th}$ percentile of C/P | 2 | **0.022** | -0.933$^{***}$ |
| Bone cortisol separated by the top and bottom 50$^{th}$ percentile of AmI/P | 5 | 0.083 | -0.667$^{***}$ |

Significant p-values are bolded. $^{*}$Indicates small, $^{**}$medium, and $^{***}$strong practical significance (effect size). Individual 45/2018 not included.

## 4.7 Cortisol's Relationship to Osteocalcin

### 4.7.1 Hair Cortisol and Osteocalcin

To investigate the relationship between hair cortisol and osteocalcin, the concentrations of hair cortisol were separated into two groups by the top and bottom 50$^{th}$ percentile of osteocalcin readings among the tested samples (see Fig 4.6). When observing the pattern of hair cortisol concentrations relative to osteocalcin concentrations in Figure 4.6, cortisol concentrations in hair gradually decrease as osteocalcin increases. While the difference between the groups of hair cortisol based on the top and bottom 50$^{th}$ percentile of osteocalcin was not statistically different from one another, there was a medium-sized practical significance observed between the two groups with a Cliff's delta test (U: 9; P-Value: 0.314; δ: 0.4$^{**}$).

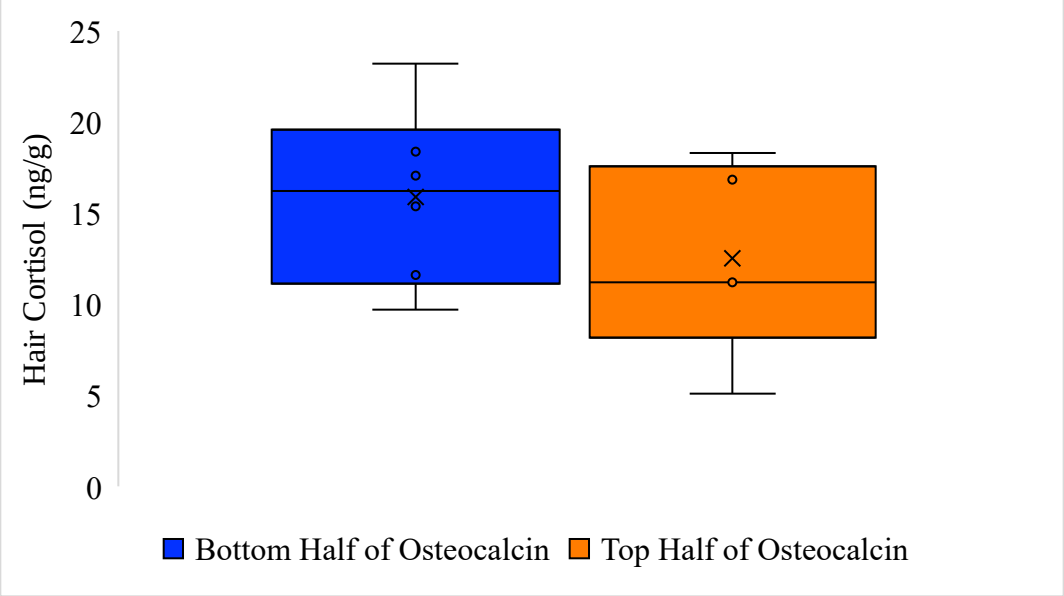


Figure 4.6: A box-and-whisker plot depicting the comparison of hair cortisol concentrations when separated into two groups based on the top and bottom 50th percentile of osteocalcin values; Individual 45/2018 not included

4.7.2 Bone Cortisol and Osteocalcin

Similarly, bone cortisol readings were separated into two groups based on the top and bottom 50$^{th}$ percentiles of collected osteocalcin concentrations to study the relationship that bone cortisol may have to osteocalcin. Similar to Figure 4.6, Figure 4.7 presents a noticeable inverse relationship between osteocalcin concentrations and bone cortisol, meaning that as osteocalcin rises, cortisol concentration decreases. The relationship was statistically significant and had a very strong practical significance when running a Mann-Whitney U test and companion Cliff's delta test (U: 4; P-Value: **0.047**; $\delta$: 0.84$^{***}$).

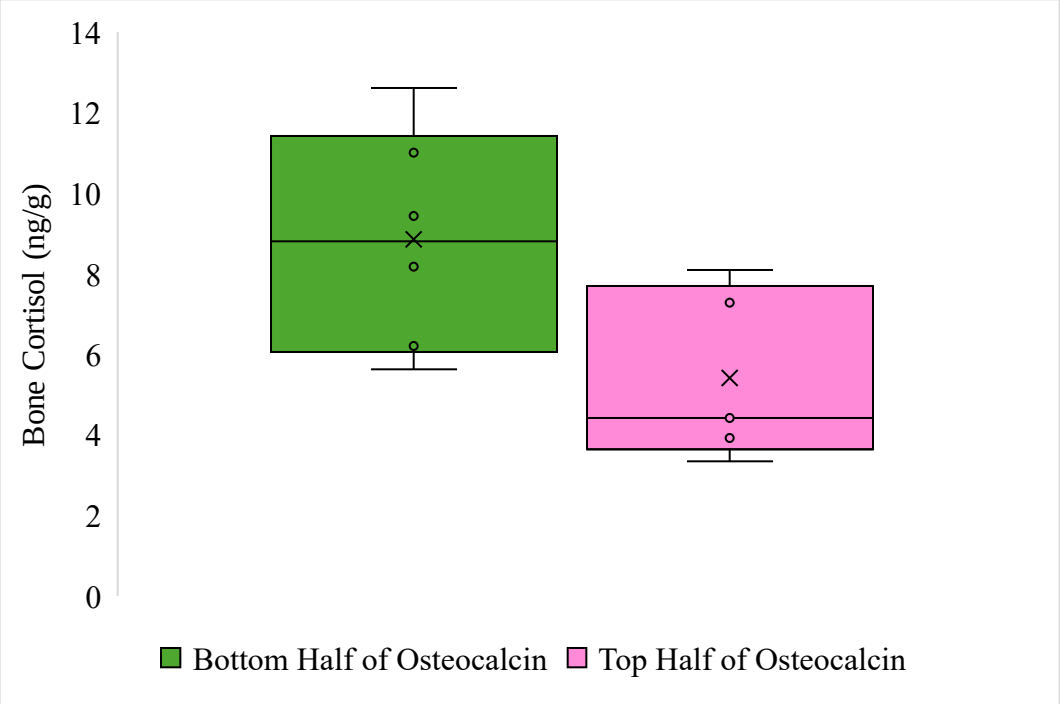


Figure 4.7: A box-and-whisker plot depicting the comparison of bone cortisol concentrations when separated into two groups based on the top and bottom 50th percentile of osteocalcin values; Individual 45/2018 not included

## 4.8 Individual 45/2018

As discussed, Individual 45/2018 was excluded from the majority of this sample analysis, specifically data analyses related to bone samples. This was because the samples for Individual 45/2018 was from the femur instead of the parietal bone. While previous research has suggested no statistical difference between the chemical concentration of hormones based on the skeletal element used for extraction (Scott, 2015; Scott et al., 2016), data from Individual 45/2018 does not align with this. For example, Individual 45/2018 has both the lowest hair cortisol concentration (3.819 ng/g) and bone cortisol concentration (10.078 ng/g) in the upper quartile of all concentrations (see Table 4.7 and Fig. 4.8). While the data from this thesis would suggest this high bone cortisol would be accompanied with a low osteocalcin concentration (see Fig. 4.7), the collected osteocalcin concentration for Individual 45/2018 was also in the upper quartile (see Table 4.4 and Fig. 4.8). While the reason for this pattern in the femur over the parietal will be further discussed in Chapter 5, it is likely due to a difference in skeletal element remodelling and other intrinsic factors.

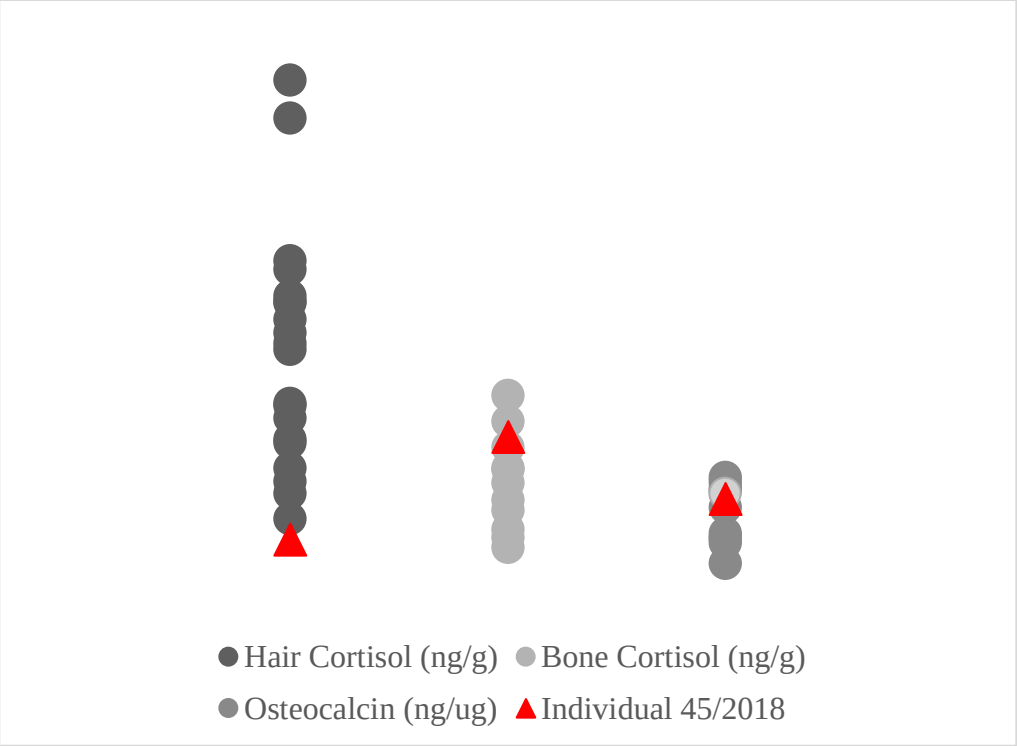


Figure 4.8: A jitter plot comparing hair cortisol, bone cortisol, and osteocalcin concentrations of the 11 individuals sampled from the parietal bone versus Individual 45/2018—sampled from the femur

## 4.9 Conclusion

This chapter has presented the results and statistical analysis of this research, including an overview of all demographic (i.e., sex and age) and sample information, as well as intra-observer error results. More specifically, the concentrations of osteocalcin and cortisol (both hair and bone) were presented for each individual and discussed in relation to diagenesis. The month-to-month concentrations of hair cortisol and the relationships between hair cortisol and bone cortisol were also compared. Cortisol concentrations from hair and bone were also compared to osteocalcin concentrations in bone.

# Chapter 5: Discussion

## 5.1 Introduction

This chapter interprets the results of this research. It will begin by discussing the impact of diagenesis on the osteocalcin and cortisol concentrations extracted from this study. This chapter will then compare the osteocalcin and hair cortisol concentrations with other published studies to evaluate the relative stress levels and factors influencing these concentrations. Cortisol from both hair and bone will be compared, and the implications of their relationship will be explored. A comparison between the two sources of cortisol and osteocalcin will also be conducted, and the biological role that osteocalcin plays in times of high stress will be discussed. Finally, this chapter will conclude by discussing gaps in the published literature, along with sampling and methodological limitations.

## 5.2 Diagenesis

A clear statistical relationship exists between increased diagenesis and depreciating osteocalcin values. This phenomenon has been well-researched in the literature (Ajie et al., 1991; Hughes, 2020; Scott et al., 2020, 2016; Smith et al., 2005) and was expected in this study. Notably, the FTIR metrics directly speaking to bioapatite integrity (i.e., IRSF and C/P) had statistical significance. This is likely because osteocalcin is stored by tightly affixing itself to bone bioapatite, meaning that as bioapatite becomes compromised, so too does osteocalcin (Smith et al., 2005). At the same time, osteocalcin is found in a one-to-one ratio with bone collagen but notably does not directly contribute to its preservation and thus does not have as strong a relationship

to osteocalcin concentrations (Ajie et al., 1991; Dobberstein et al., 2009; Scott et al., 2020). This is likely why the FTIR metric measuring collagen integrity (i.e., AmI/P) did not have statistical significance despite following the same trend.

In contrast to these osteocalcin patterns, increasing diagenesis had a strong statistical and visual relationship with appreciating cortisol concentrations in bone. Cortisol extractions chemically simulate the effects of leaching, and increased diagenesis makes bone more vulnerable to leaching—causing artificial inflation of cortisol concentrations in those more comprised samples (East, 2021; Martin, 2021; Meyer et al., 2014; Nielsen-Marsh et al., 2000; Nielsen-Marsh and Hedges, 2000). These observations mean that if diagenesis were disproportionate within a sample set or between sample sets, the concentrations of osteocalcin and cortisol could be artificially low or high, respectively, and would not represent genuine differences in stress. This emphasizes the necessity for studies focused on molecular analysis of archaeological remains to measure and report the degree of diagenesis within their sample set to ensure proper comparisons between studies (Smith et al., 2023).

The impact of diagenesis is a crucial consideration in paleoproteomic research, and while diagenesis has affected the samples in this study, it has done so relatively consistently. All samples included in this study fall within well-established archaeological parameters of preservation (see Fig.4.2) (Chesson et al., 2021; France et al., 2020; Smith et al., 2023; Trueman et al., 2004). Therefore, based on these diagenesis measurements, meaningful comparisons can be made moving forward without diagenesis playing a significant role in these interpretations.

## 5.3 Osteocalcin

Research into bone osteocalcin is in its infancy; therefore, reference ranges are not particularly robust. That being said, Table 5.1 provides some of the ranges observed from previous bioarchaeological studies to be compared with this research.

Table 5.1: Reference ranges of archaeological osteocalcin concentrations compared to the concentrations from this study

| **Study** | **Sample Size** | **Reference Range (ng/μg)** | **Sampled Element** |
|---|---|---|---|
| Hughes (2020) | 27 | 1.80-19.13 | Femur |
| Scott et al. (2020) | 46 | 26.0-310.0 | Femur |
| Scott et al. (2016) | 20 | 2.92-60.64 | Femur and clavicle |
| This study | 12 | 2.36-7.61 | Parietal and femur |

The ranges of the collected osteocalcin concentrations in this study are considerably lower than those of these previous studies (see Table 5.1 and Fig. 5.1). Based on previous bioarchaeological research on osteocalcin and the data of this project, an argument can be made that the individuals at the Fortress of Louisbourg experienced a greater level of physiological stress compared to these other populations. Both this study and Hughes' (2020) osteocalcin concentrations are noticeably lower than the Scott et al. (2020, 2016) comparative samples (see Fig 5.1). When tested statistically, the compiled osteocalcin concentrations from this study and Hughes' (2020) were statistically lower than those collected from Denmark by Scott et al. (2020, 2016) when tested with a Mann-Whitney U test (2020-U: 10 p-value: 0.00; 2016-U: 485 p-value: 0.01). This difference between the populations tested by Scott et al. (2020, 2016) and those from the Fortress of

Louisbourg lends credence to the fact that these differences between osteocalcin concentrations are likely stress-related.

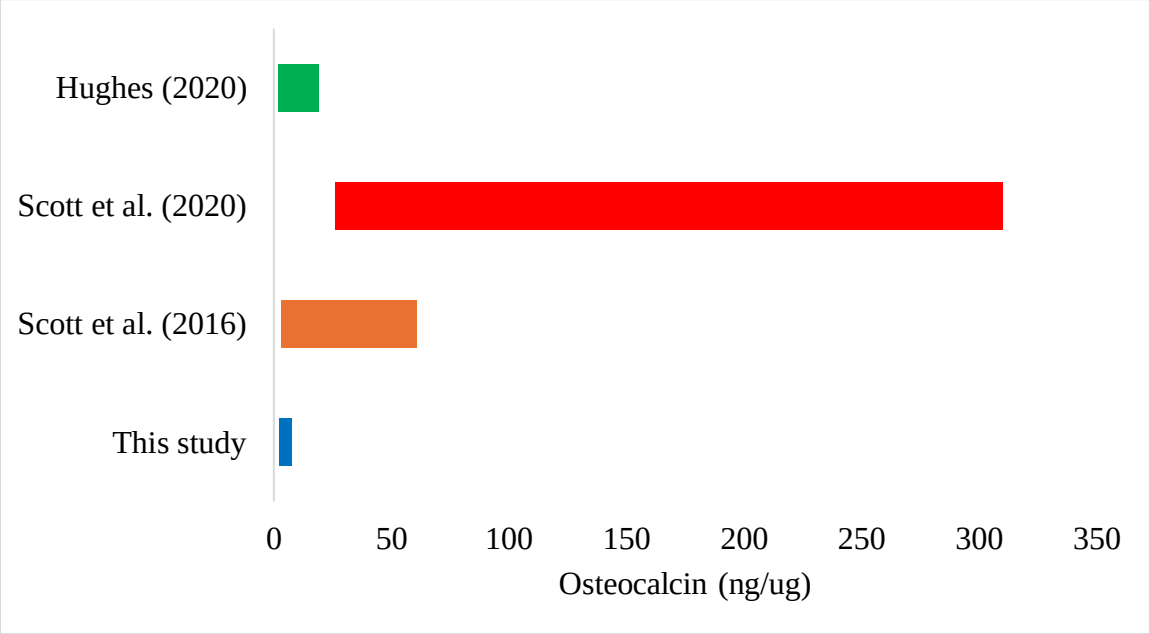


Figure 5.1: A range graph comparing concentrations of previous archaeological osteocalcin studies compared to this study

Scott et al. (2020, 2016) focused on two populations from 13th- 17th century Denmark. In contrast, the Fortress of Louisbourg is an 18th-century population of French and New Englander settlers in modern-day Canada (Scott et al., 2022). One societal factor potentially contributing to elevated stress levels in the Fortress population versus that of these Danish populations is dietary composition and variety. Restricted diet and deficiencies in nutrients and calories encourage the activation of the ASR and trigger a release of nutrients stored in the fat and muscle, resulting in elevated physiological stress (Hill et al., 2022; Lopresti, 2020; Lozupone et al., 2012; Madison and Kiecolt-Glaser, 2019). Additionally, for osteocalcin specifically, when the critical nutrients of bone mineralization are lacking, osteocalcin cannot be carboxylated and stored in the bone—resulting in high concentrations in the blood serum (García-Maldonado et al., 2024;

Prowting et al., 2022; Seibel, 2002). Isotopic data from Denmark contemporaneous to the two Danish samples (see Walser et al., 2020; Yoder, 2012, 2010) found a variable diet that consisted of regular terrestrial proteins, legumes, fish, cereals, and dairy products, even across social strata. In contrast, the diet at the Fortress of Louisbourg, which due to the majority of food being imported, consisted primarily of bread, salted meats, and cod, and lacked the essential vitamins and nutrients necessary for healthy bodily maintenance (Balcom, 1995; Fonzo et al., 2020; Lane-Jonah and Vechambre, 2015; Welker and Quintana Morales, 2022). Signs of vitamin deficiencies, such as rickets, have persisted in the archaeological record at the Fortress of Louisbourg (see Hinton, 2019), which reflects the dietary constraints that could have contributed to the Fortress of Louisbourg population presenting elevated stress levels over that of the comparative Danish populations. This argument is further supported when comparing the Fortress of Louisbourg population to the specific Post-Medieval subsample explored by Scott et al. (2016), which was more food insecure. In this comparison, the osteocalcin concentrations are more statistically similar between the individuals at Louisbourg and those in Post-Medieval Denmark (U: 343 p-value: 0.1), further supporting that osteocalcin variation in this instance may reflect food-specific stressors between these populations.

Considering the synergistic effect that diet has on susceptibility to disease, there is the possibility that a contributing factor to the differences between the Fortress population and those from the Danish population (Alt et al., 2022). Scott et al. (2016) discuss the prevalence of disease, both in the historical record and when analyzing their sample population, in the post-medieval period rather than in the medieval period. This phenomenon, as well as the increased food insecurity of the Post-Medieval Period of

Denmark, can potentially be an additional contributing factor as to why the osteocalcin concentrations amongst this time period's sample population are statistically similar to that of the Fortress of Louisbourg population. This contrasts with the Medieval Period, which had a lower disease load and level of food insecurity, thus, statistically had higher osteocalcin concentrations (Scott et al., 2016). Due to the preservation of this study's sample set, a comprehensive analysis of disease among those sampled was not possible; however, given the known prevalence of diseases associated with poor living conditions and food quality (e.g., cholera and dysentery), disease load, as well as diet, may be contributing factors to the increased stress that the Fortress population experienced compared to that of the comparable Medieval Danish Population (Alt et al., 2022; Tésio, 2008).

Within the Fortress of Louisbourg populations (this study and Hughes 2020), differing osteocalcin concentrations likely represent different time periods of stress. As shown in Figure 5.2, the osteocalcin concentrations from this study are lower than those reported by Hughes (2020). While this pattern is visually apparent, it was not statistically significant (U: 104 p-value: 0.201). While it is surprising that this visual difference is not statistically supported, this is likely because of the time period of systematic stress that femoral versus cranial osteocalcin concentrations represent. Scott et al. (2016) argued osteocalcin concentrations from differing elements reflect different periods of osteocalcin incorporation into the skeletal tissue. Scott et al. (2016) found that there was no statistical difference between osteocalcin concentrations across their tested skeletal elements (i.e., clavicle and femur) (Scott et al., 2016). However, they argued that the clavicle's thinner cortical bone reflects more recent osteocalcin concentrations compared to the thicker

femur cortex, which reflects a longer period of osteocalcin accumulation (Scott et. al 2016). This means testing elements of different cortical thicknesses may represent various stress periods. In this study, the parietal bone, with its much thinner cortical surface (i.e., 1.26 mm on average), likely reflects osteocalcin concentrations closer to the time of death than the thicker femoral cortical bone (i.e., 5.7 mm on average) (Hollensteiner et al., 2018; Kakutani et al., 2023; Scott et al., 2016). This point can be further observed in Table 5.2, where the median osteocalcin concentration of this study most closely aligns with the median osteocalcin concentration of clavicle extractions in the study by Scott et al. (2016). Despite these differences not garnering a statistical difference, osteocalcin concentrations across skeletal elements can create a holistic timeline of lived stress based on cortical thickness and remodelling.

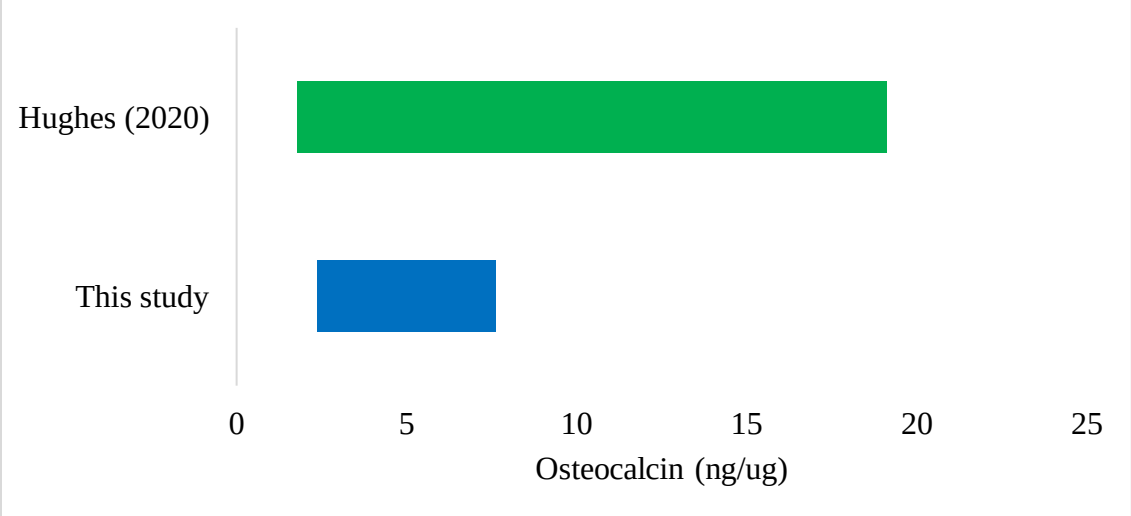


Figure 5.2: A range graph comparing osteocalcin concentrations of the two osteocalcin studies conducted from the Fortress of Louisbourg

Table 5.2: Comparison of median osteocalcin concentrations with respect to sampled element and cortical bone thickness

| **Study** | **Sampled Element** | **Average cortical thickness (mm)*** | **Median osteocalcin concentration (ng/μg)** |
|---|---|---|---|
| Scott et al. (2016) | Femur | 5.7 | 11.1 |
| Scott et al. (2016) | Clavicle | 3.2 | 8.2 |
| This study | Parietal | 1.26 | 5.8 |

*(Hollensteiner et al., 2018; Kakutani et al., 2023; Peebles et al., 2022)

The value of assessing the timeline of osteocalcin concentrations through multiple skeletal elements is best explored through an example. Individual 3/2017 was included in this project as well as in the work of Hughes (2020). The osteocalcin concentration from their femur (12.05 ng/μg) is notably higher than the concentration extracted from the parietal bone (6.796 ng/μg). This would indicate a much higher level of stress closer to death than the time period represented by the femur. Individual 3/2017 was a 30 to 35-year-old male with evidence of significant dental wear along with robust muscle attachments and Schmorl`s nodes in their mid-to-lower back (see Scott, 2019). Both of these skeletal pathological lesions are potential signs of stress (i.e., diet and access to resources, activity patterns, poor hygiene, potential injury, etc.) and may have directly contributed to the activation of their ASR (Benazzi et al., 2013; Dar et al., 2010; Petraru et al., 2022; Pezo-Lanfranco et al., 2020; Robb, 2019; Roberts and Manchester, 2007). Also important to note is, outside of some evidence of enamel hypoplastic lesions on the maxillary dentition, they lack early life indicators of stress (i.e., cribra orbitalia and porotic hyperostosis) (Scott, 2019). More important, though, would be the physical toll that years of labour would have on the body.

Given their age at death, Individual 3/2017 would have been part of the workforce for approximately 15 to 20 years based on the typical age of social adulthood at Louisbourg (i.e., approximately 7-8 years of age) (Johnston, 1984). Given the muscular robustness of Individual 3/2017, they likely participated in a highly physical profession (Johnston, 1984; Pearson and Buikstra, 2017; Scott, 2019). While in the short term, mechanical loading leads to the formation of new bone to accommodate muscular attachment, long-term and constant strain leads to a transition from bone formation to bone destruction such as osteopenia, herniation (i.e., Schmorl's nodes), and, most notably, depleted osteocalcin concentrations in the bone tissue (Barbe and Popoff, 2020; Kunutsor et al., 2018; Moser and van der Eerden, 2019). Therefore, the argument could be made that the difference in skeletal evidence for early-life stress versus later-in-life strain is reflected in the higher osteocalcin concentration recorded in the femur (see Hughes, 2020; Scott, 2019). In contrast, the diminished osteocalcin concentration in the parietal bone matches well with the skeletal evidence of stress towards the end of life, creating more of a timeline approach to the study of stress for this particular individual.

## 5.4 Cortisol

### 5.4.1 Hair

Since there are no diagnostic ranges for hair cortisol (see Wright et al., 2015), reference ranges from previous clinical and archaeological studies are compared with the collected samples from Louisbourg (see Table 5.3). Compared to these previous works, the cortisol readings from this study are generally similar, albeit on the lower end of the range (see Fig. 5.3)

Table 5.3: Clinical and archaeological reference ranges of hair cortisol concentrations compared to the concentrations from this study

| **Study** | **Archaeological or Clinical** | **Sample Size** | **Age Range (years)** | **Reference range (ng/g)** |
|---|---|---|---|---|
| Cieszynski et al., 2018 | Clinical | 44 | 53-73 | 2.0-51.63 |
| Chan et al., 2014 | Clinical | 39 | 20-76 | 27-200 |
| Dettenborn et al., 2012 | Clinical | 360 | 1-91 | 7.2-31.2 |
| Föcker et al., 2016 | Clinical | 20 | 15-18 | 2.86-22.24 |
| Henley et al., 2014 | Clinical | 15 | <20-60+ | 189-400 |
| Henley et al., 2013 | Clinical | 32 | NA | 26-204 |
| Gonzalez et al., 2019 | Clinical | 232 | 30-60 | 40-128 |
| Pereg et al., 2011 | Clinical | 56 | 50-72 | 76.58-949.9 |
| Sauvé et al., 2007 | Clinical | 46 | 20-76 | 17.7-153.2 |
| Smeeth et al., 2023 | Clinical | 923 | 6-19 | 31.6-181.2 |
| Stalder et al., 2012 | Clinical | 155 | 20-30 | 7.1-28.3 |
| Thomson et al., 2010 | Clinical | 32 | 20-51 | 26-204 |
| East, 2021; Kellis 2 Cemetery - Egypt | Archaeological | 119 | >18-69 | 1.16-255.11 |
| East, 2021; Terry Collection - United States | Archaeological* | 38 | 18-91 | 34.66-1190.49 |
| Kellner et al., 2022; Zorropata -Peru | Archaeological | 2 | 20-35 | 3.9-10.3 |
| López-Barrales et al., 2015; San Pedro de Atacama - Chile | Archaeological | 19 | 19-38 | 33.7-152 |
| Schaefer, 2017; Huaca De Los Sacrificios - Peru | Archaeological | 10 | NA | 18.02-247.95 |
| Tisdale et al., 2019; Kellis 2 cemetery - Egypt | Archaeological | 10 | 19-60 | 272.5-467 |
| Webb et al., 2015a; Cahuachi - Peru | Archaeological | 5 | >3-12 | 757-2507 |
| Webb et al., 2015b; Cahuachi and Huaca del Loro - Peru | Archaeological | 14 | NA | 125-2392 |
| Webb et al., 2010; Cajamarquilla, Leymebamba, Puruchuco, Tucume, and Nasca - Peru | Archaeological | 10 | Adult** | 91-707 |
| This study | Archaeological | 12 | <14.5-35 | 3.82-31.86 |

*Historical (early-to-mid twentieth century) **Description of age lacked specificity

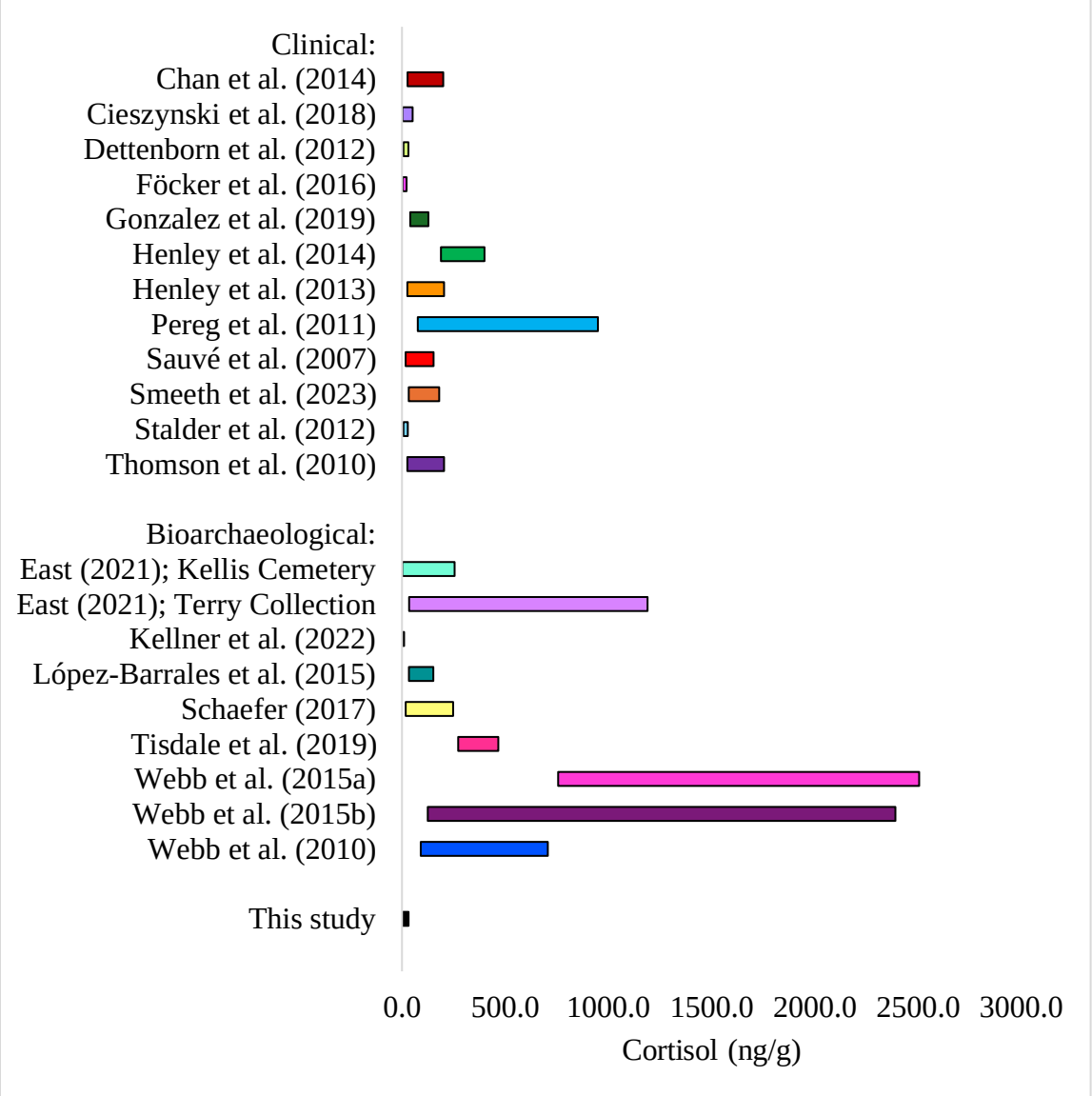


Figure 5.3: A range graph comparing the concentrations of previous archaeological hair cortisol studies compared to this study

The lower cortisol concentrations observed in this study are unlikely to represent lower stress levels in individuals from the Fortress of Louisbourg. Instead, they more likely reflect taphonomic and methodological influences. Of the current bioarchaeological studies focused on hair cortisol, this is the first to use hair samples preserved due to an associated artifact, namely copper pins (see Chapter 2, section 2.3.2).

While copper is a well-known preservative agent due to its antimicrobial properties (see Wilkin et al., 2023), it notably does not protect hair from environmental conditions. As discussed previously (see Chapter 2, section 2.2.2.2), cortisol in hair is vulnerable to being washed out by water. As the Rochefort Point Cemetery is located on a peninsula jutting into the Atlantic Ocean, the site is inundated with water (CBCL Limited, 2010; Dunham, 2014) that could have stripped hair of its cortisol over time. This watery environment is likely a main contributing factor to the overall lower cortisol values observed in this study rather than stress (Hamel et al., 2011). This is further substantiated when comparing the Rochefort Point values to other studies focusing on buried individuals, such as Schaefer (2017) and Webb et al. (2015a, 2015b, 2010). Despite these examples representing those who were also buried, the cortisol values are higher due to other favourable environmental conditions (i.e., better soil drainage and a dry climate) that are not characteristic of Louisbourg.

In addition to the impact of environmental taphonomy, laboratory methods also likely played a critical role in the lower cortisol concentrations observed. Many studies have extracted cortisol from hair by applying heat to the methanol aliquoting process (Meyer and Novak, 2012). Heat notably increases the solubility of a solute (cortisol) in a solvent (methanol), meaning that the reported cortisol concentrations in studies that did and did not apply heat during extraction likely differ due to extraction efficiency as opposed to genuine differences in stress levels (Meyer et al., 2014; Meyer and Novak, 2012; Sánchez Chino et al., 2019; Yamanashi et al., 2016). This is further supported by the round-robin study by Russell et al. (2015), where four independent labs extracted cortisol from the same hair samples, half of whom did not employ heat in their extraction.

While the concentrations between labs correlated strongly, the cortisol concentrations from the two labs using heat were systematically higher than those from the labs that did not (Russell et al., 2015). This means that when studies use comparable methods (i.e., heat versus no-heat extraction), comparisons can be made confidently. However, comparisons between studies with different extraction methods introduce an additional consideration of extraction efficiency and its impact on interpretations of stress. This point is further supported when comparing this study (which did not employ heat in its extraction) to both clinical studies (Dettenborn et al., 2012; Föcker et al., 2016; Smeeth et al., 2023; Stalder et al., 2012) and the most recent bioarchaeological study (Kellner et al., 2022), which also did not employ heat. Unsurprisingly, the reported cortisol concentrations from this study most closely align with these other non-heat studies (see Fig. 5.4), and any differences observed when controlling for the extraction method can be more confidently associated with actual differences in stress levels over methodological variation (see Fig. 5.4).

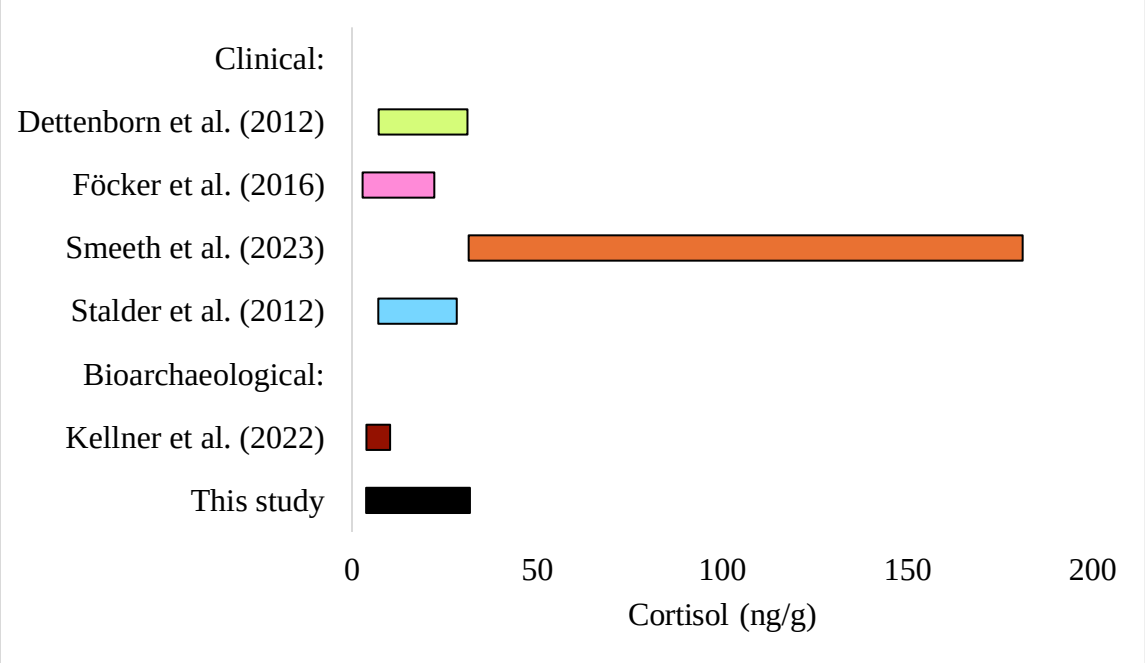


Figure 5.4: A range graph comparison of hair cortisol studies that did not use heat during extraction

The hair cortisol results of this study from all collected hairs can most appropriately be compared to those of Kellner et al. (2022). Kellner et al. (2022) focused their cortisol analysis on two individuals from the Nasca region of modern-day Peru in the Early Intermediate to Middle Horizon Periods (400-1000 CE). When comparing the cortisol concentrations of these two individuals to the concentrations reported in this project, the tested individuals from the Fortress of Louisbourg had statistically higher concentrations (U: 20.5 p-value: 0.04). Weather and the impact climate can have on stress are major differing factors between these populations. Both the Nasca Region and Cape Breton face extreme weather, with the Nasca Region experiencing frequent drought (see Conlee, 2021) and Louisbourg experiencing long and cold winters, heavy fog, and harsh Atlantic winds (see Owen, 2003). The arid environment and frequent droughts of the Nasca Region were well known to those living there, with agriculture and social

organization reflecting widespread adaption to these living conditions (Conlee, 2016; Proulx, 1999). By contrast, most of the French living in Louisbourg were not born in Cape Breton but originated from southwest France and Normandy/Brittany in the northwest; both of these regions experience temperate weather year-round (Johnston, 1995; Laanaia et al., 2016). Even the occupying New Englanders said the weather at Louisbourg was worse than back home.

*"worse than any place in the known world Nothing but fogs [sic] rains fevers Death & everything that's bad & Disagreeable"*

-William Pepperell, 1746

Exposure to new inclement weather can be a significant shock to the body where there is an initial strong activation of the ASR to preserve homeostasis (Hayes et al., 2018; LaDage, 2015). However, as the weather becomes more predictable for the individual and they become more acclimated, the same environmental stressor will elicit a more blunted ASR (Hayes et al., 2018; LaDage, 2015). Isotopic data suggests that the Nasca individuals studied by Kellner et al. (2022) were long-time inhabitants of this arid environment and thus had time to acclimate to these drought conditions. By comparison, those buried at Louisbourg were likely only exposed to the harsh living environment relatively briefly before their death and likely did not have the same opportunity to acclimate, which may be why elevated cortisol concentrations were noted in the Louisburg population.

While Louisbourg's cortisol concentrations were higher than those of other archaeological studies, the concentrations were lower than those of some clinical samples

(see Table 5.3). A possible cause for this could be societal factors the clinical study participants faced that those at Louisbourg did not. For example, Smeeth et al. (2023) studied nearly 1,000 adolescent refugees from the 2011 Syrian civil war, in which hair cortisol levels correlated with trauma and post-traumatic stress disorder (Smeeth et al., 2023). When faced with violence or psychological trauma, the HPA axis recognizes the stressor and initiates the ASR in an attempt to prepare the body for the perceived threat (Aiyer et al., 2014; Lynch et al., 2022). Based on historical records and the known location of specific siege camps (i.e., Freshwater Brook – see Johnston, 1984; Moore, 1974), it is unlikely that the individuals interred in the Rochefort Point Cemetery represent those who died in the 1745 siege at Louisbourg. Additionally, the military recruits brought to Louisbourg were predominately from the urban poor (Greer, 1976; Pitcher, 2014; Rawlyk, 1999), making it unlikely that the soldiers at Louisbourg had been previously traumatized by warfare since the Fortress of Louisbourg was commonly their very first deployment. It is a logical conclusion that the differences in these macro-societal shifts are a major contributing factor as to why these Syrian adolescents experienced such a dramatically higher level of stress than those from the Fortress of Louisbourg.

The average cortisol concentrations of the more proximal hair samples (i.e., cortisol concentrations closer to the time of death) were generally higher when compared to earlier months further from the time of death (see Chapter 4, section 4.6.1). This result is unsurprising when comparing these findings to previous archaeological studies, which show a similar pattern of higher cortisol concentrations closer to the scalp (see Webb et al., 2015a, 2015b, 2010). While factors such as hair washing, sun bleaching, and/or

sweating could influence cortisol concentrations, especially as these factors affect different regions of the hair differently, it seems unlikely they greatly influenced the result of this study. For example, washing hair with water was not a common practice for those living at Louisbourg (Markiewicz, 2014), and head coverings, typical for soldiers and civilians as a part of everyday dress and a sign of modesty (Doda, 2023, 2019), would have protected the hair from sun damage and perhaps helped to absorb sweat, especially items made of wool which has natural sweat-wicking properties (Kicklighter et al., 2011). Based on this, it is likely that the differences in cortisol concentrations between different hair segments actually reflect an increase in stress closer to the time of death.

Recent research on historical and modern decedents in the United States has found that individuals who experienced a prolonged dying process generally have higher cortisol concentrations in the last months of life than those who experience a more sudden death (East, 2021), which makes sense based on how long the ASR is activated. Feelings of suffering, pain, and mental distress are common among individuals facing death (Cherny, 2015; Kellehear, 2009), and mental distress is a well-established factor that is known to activate the ASR, leading to elevated cortisol concentrations (Cerda-De la O et al., 2022; Herane-Vives et al., 2020; Psarraki et al., 2021). This is in addition to the already known homeostatic disturbance and activation of the ASR that accompanies the dying process (Erkut et al., 2004; Lathers and Schraeder, 2006; Novoseltsev et al., 2000). Acute diseases such as dysentery were common at the Fortress of Louisbourg (Johnston, 1984), with discomforting symptoms such as fever, pain, chills, and bloody diarrhea (Lampel et al., 2018; Shanks, 2016). From the onset of symptoms, death

typically occurs within one to three weeks, enough time for a stress event to affect cortisol concentrations in hair (Kapoor et al., 2018; Lampel et al., 2018; Shanks, 2016). Therefore, while prolonged periods of dying would certainly affect cortisol concentrations, even diseases more acute in nature, such as dysentery, could prompt the activation of the ASR and affect cortisol concentrations. With this in mind, the dying process of those who lived and died at the Fortress of Louisbourg, whether acute or prolonged, almost certainly would have contributed to elevated cortisol concentrations closer to death that were observed in this study.

Furthermore, East (2021) also noted that individuals admitted into hospice and presumably received palliative care had a slight downtick in their month-to-month cortisol concentrations. This pattern was also observed in three of the six individuals in this study (Individuals 120/2021, 136/2022, and 172/2023), which may indicate that these individuals received some kind of end-of-life/palliative care in their dying processes that reduced their stress load. There were several hospitals founded at the Fortress of Louisbourg in its brief history, including the *Frères de la Charité* (Brothers of Charity) and the larger *Hôpital du Roi* (King's Hospital), which staffed surgeons and dispensers (pharmacists) to treat the wounded and sick (Hoad, 1972; Johnston, 1981). Palliative care in the 18$^{th}$-century included the use of infusions, liquors or syrups derived from plants and herbs with medicinal properties (Hoad, 1972; Johnston, 1991; Mackay, 1985; Thomson, 1996). From a recovered supply order, infusions of note included red poppy syrup and juniper extract (Hoad, 1972; Monier, 1751). Red poppy and juniper act as analgesics and anti-inflammatories, respectively (Bais et al., 2014; Policola et al., 2014; Thomson, 1996). Both infusions would dull the sympathetic nervous system, the key

player in the HPA axis, and would curb the release of cortisol, explaining the downtick observed in more proximal hair segments (Bais et al., 2014; East, 2021; Policola et al., 2014; Tennant and Hermann, 2002). It is possible that the three individuals in this study with slightly decreased cortisol concentrations in their final month received some kind of medical treatment that could have eased their suffering based on this contextual evidence. While the cause of the death for these individuals is unknown, studying the cortisol concentrations can provide crucial insight into what their final weeks to months may have looked like.

### 5.4.2 Bone

Cortisol was successfully extracted from all bone samples in this study; however, it can be safely assumed that cortisol embeds itself more readily into hair as opposed to cortical bone, given the similar ranges found in this study (3.819-31.864 ng/g for hair and 3.339-12.606 ng/g for bone). This is despite bone cortisol requiring nearly 25 times the amount of sample in weight to achieve those concentrations. This difference is likely due to the variance in lipid content between hair and cortical bone. Cortisol, as a cholesterol-based hormone, is incredibly fat-soluble, and the mechanism by which cortisol embeds itself into tissues is likely through dissolving into the lipid structure of the particular tissue (Abdulateef, 2021; Ilias et al., 2023; Moody et al., 2022). On average, hair is 2-6% fat by weight, contributing to its flexibility, strength, and shine (Marsh et al., 2018; Song et al., 2019). While bone does have a notable fat content, most of it is found in the bone marrow, with only small amounts accumulating in the cortical tissue during bone mineralization (During et al., 2015; Tintut and Demer, 2014). What this lower fat content in the cortical bone means is that there is less opportunity for cortisol to embed itself in

the bone matrix, reflected in the higher amount of bone needed to extract the same amount of cortisol as hair (Abdulateef, 2021; Charapata et al., 2018).

The influence of lipid content on cortisol concentrations can even be seen across skeletal elements. As discussed in Chapter 4, section 4.6.2.1, cortisol concentrations extracted from hair and bone visually followed the same pattern—with Individual 45/2018 being the only true outlier. Individual 45/2018 had the lowest hair cortisol concentration (3.819 ng/g), so presumably would have relatively low bone cortisol; however, this individual had one of the highest bone cortisol concentrations (10.078 ng/g). Interestingly, this individual was the only example in this study where cortisol was extracted from the femur, not the parietal bone. In life, the femur, in contrast to the parietal bone, has a sheathing of adipose tissue, which naturally contributes to a higher overall lipid content when compared to the skull, which lacks this adipose layer (Bukhari et al., 2004; Gondim Teixeira et al., 2019; Vogel et al., 2019). Therefore, the natural differences in where adipose tissue is found in the body can influence how much cortisol can be captured in the surrounding skeletal tissues as they form (During et al., 2015). For Individual 45/2019, this is reflected by higher bone cortisol concentrations despite low hair cortisol concentrations, again emphasizing the need for consistency in which skeletal elements are sampled when assessing cortisol concentrations in bone.

#### 5.4.2.1 Comparison Between Bone Cortisol and Hair Cortisol

As discussed in Chapter 4, section 4.6.2.1, the hair and bone cortisol concentrations, while not statistically correlated, visually trended in the same direction. In part, the limited sample size could be the cause of the inability to establish a statistical connection. Still, the lack of statistical correlation is more likely because bone cortisol

and hair cortisol reflect different timeframes of lived stress (Charapata et al., 2018; Kapoor et al., 2018; Serdar et al., 2021). It has been hypothesized that cortisol extracted from cortical bone represents an average of the last 10-20 years of life depending on remodelling rates compared to hair cortisol, which represents stress weeks to months before death (Charapata et al., 2018; Webb et al., 2010). The co-directionality observed between long-term stress (bone cortisol) and short-term stress (hair cortisol) suggests that the stress levels in the final months of these individuals fell largely in line with the typical levels of stress experienced throughout life. This pattern likely reflects the demographics of the individuals buried at the Rochefort Point Cemetery. As previously discussed, the individuals buried on Rochefort Point were not those who died during the siege of Louisbourg (Burke, 1989; Moore, 1974). Instead, the Rochefort Point Cemetery was mainly used by New Englander military personnel during their occupation and then later by the French military and by civilians (Burke, 1989; Moore, 1974). Additionally, those who succumbed to major infectious disease events were also likely omitted from the Rochefort Point Cemetery. For example, those who succumbed to smallpox were buried in a separate cemetery from the general populous (Moore, 1974). Further, individuals with communicable and more chronic conditions (e.g., treponemal disease, tuberculosis) were often sent back to France in an attempt to mitigate transmission (Forbes, 2021; Scott et al., 2023). This means that the individuals buried in the Rochefort Point Cemetery were likely individuals who lived fairly typical lives and died fairly typical deaths (Burke, 1989; Johnston, 1984). While there are cortisol fluctuations among the individuals included in this study, these differences between each month were not statistically different (see Table 4.8). This contextual information about those buried in

the Rochefort Point Cemetery support the notion that the short-term stress of those included in this study fell broadly in line with their long-term stress levels.

## 5.5 The Relationship Between Cortisol and Osteocalcin

The comparison of cortisol to osteocalcin presented a clear inverse relationship, meaning as one rose, the other fell (see Chapter 4, section 4.7). This strongly supports the third hypothesis of this study, which argues that depleted osteocalcin concentrations in bone reflect elevated stress levels and that osteocalcin is a viable biomarker for stress. These findings are unsurprising as they agree with the hypotheses outlined in previous bioarchaeological research (Hughes, 2020; Rich et al., 2022; Scott et al., 2020, 2016) and align with the clinical understanding of osteocalcin. Further supporting this point is an acknowledgement of the vital biological role that osteocalcin's regulation (see Fig. 2. 4) plays in the ASR and how cortisol impacts that . As discussed previously (see Chapter 2, section 2.2.3), cortisol can block osteocalcin from fulfilling its primary goal of promoting insulin production by out-competing it in the pancreas (Adam et al., 2010; Bilotta et al., 2018; Kanazawa, 2015; Scheske et al., 2023). This lack of insulin production ensures the free circulation of chemical energy, an essential process in ASR activation (Adam et al., 2010; Schernthaner-Reiter et al., 2021). However, the biochemical pathways regulating osteocalcin concentrations still indicate a demand for insulin production. Thus, osteoclasts continue to destroy bone tissue and release osteocalcin into the bloodstream to compensate for this (see Fig. 5.5) (Berger et al., 2019; Berger and Karsenty, 2022).

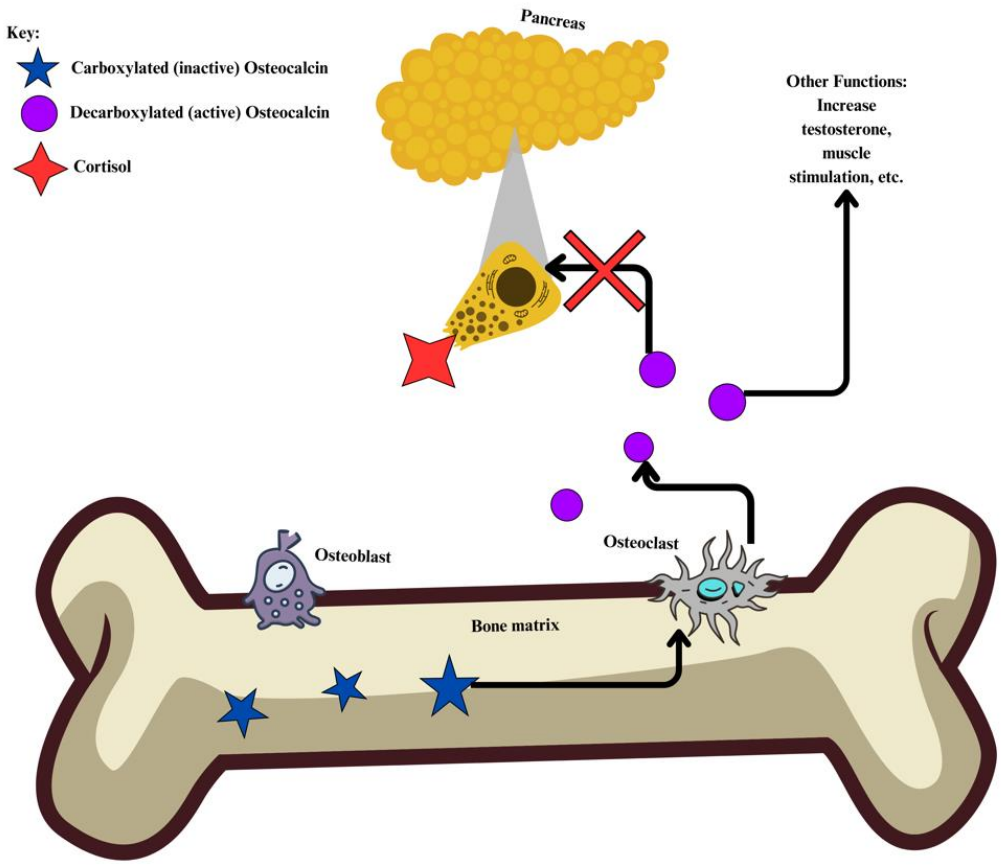


Figure 5.5: A representation of the competitive inhibition of insulin production by cortisol and osteocalcin carboxylation in times of elevated stress

While osteocalcin is kept from being stored in the bone, it can fulfill other roles (e.g., increase muscle stimulation, breakdown of fat, and mental alertness), which are also priorities of the ASR (see Berger et al., 2019; Berger and Karsenty, 2022; Mera et al., 2016; Moser and van der Eerden, 2019; Obri et al., 2018; Otani et al., 2020). These effects are so essential to the goal of threat aversion in the ASR that researchers have suggested that the skeleton serves as a crucial organ of the endocrine system as a reservoir for osteocalcin (Berger et al., 2019; Berger and Karsenty, 2022; Sobel and Berger, 1995).

While both sources of cortisol (hair and bone) show an indirect relationship to bone osteocalcin, we see this relationship far more strongly (both statistically and visually) when comparing osteocalcin to bone cortisol. This is likely due to the time frame that each cortisol source represents. Hughes (2020) and Scott et al. (2020, 2016) argue that the osteocalcin extracted from cortical bone does not represent the time period immediately before death; instead, it is an average of osteocalcin concentrations over several years based on skeletal remodelling rates. Similarly, the cortisol concentrations from bone samples also likely represent a deeper time depth based on the accumulation of cortisol in the cortical tissue over many years (Charapata et al., 2021, 2018; Webb et al., 2010). The similarity in time periods represented by both bone cortisol and osteocalcin is likely why these comparisons garnered statistical significance. By contrast, when comparing osteocalcin with hair cortisol, a significant relationship was absent. This observable relationship between bone cortisol and osteocalcin concentrations supports the confident use of osteocalcin as a proxy biomarker for systematic stress, where low osteocalcin concentrations indicate increased stress.

## 5.6 Limitations

### 5.6.1 Literature Limitations

The breadth of the interpretations that can be made from this study is limited by knowledge gaps within the literature. When quantified from blood, saliva, and urine, cortisol has reference ranges extensively researched and accepted by major medical organizations and can be used diagnostically (e.g., 50-2500 ng/g) (Guber and Farag, 2011). Considering the relative newness of extracting and quantifying cortisol from hair,

diagnostic ranges have not been universally established, with the current practice being to compare results to previously published research (see East et al., 2022; East, 2021; Meyer and Novak, 2012; Russell et al., 2015; Schaefer, 2017; Slominski et al., 2015). This ultimately limits the ability of researchers to have an objective benchmark for the severity of stress in their sample set. By solely relying on comparative analysis, conclusions are subject to inter-laboratory method variation that can influence these comparative datasets and whether real stress differences are being captured.

In the same vein, cortisol extraction from skeletal tissues remains very much in its nascency, with only five publications currently available (three of those using human samples) (Charapata et al., 2021, 2018; Nejad et al., 2016; Quade et al., 2023, 2021). Consequently, there has yet to be an agreed-upon method of reporting cortisol concentrations, which was a limiting factor for this study when interpreting the data. Without the ability to compare and contrast cortisol concentrations, it was impossible to determine the severity of stress across populations based on bone cortisol alone (Quade et al., 2023, 2021). Additionally, inter-laboratory variance and the impact that may have on how cortisol is extracted and ultimately quantified can only be assessed when there is consistency in reporting language. While it will be further discussed in section 6.2.3, either utilizing the reporting language of hair cortisol (nanograms of cortisol per gram of substrate) or normalizing bone cortisol concentrations with total lipid content.

### 5.6.2 Sampling Limitations

The most notable limitation of this study was the limited sample size and the physical preservation of these samples. As discussed in Chapter 3, section 3.8.2, statistical testing relies heavily on a large sample set to make statistical conclusions, and

studies with small sample sizes are severely limited in their statistical power (Dumas-Mallet et al., 2017). Due to how hair has been preserved at the Fortress of Louisbourg, the hair roots were largely absent; therefore, hair segments could only be chronologically ordered and lacked specificity in how close to death the cortisol concentrations acquired actually represented. As such, any conclusions made about the dying processes of these individuals were based on the assumption that the most proximal hair sample was genuinely reflective of the time of death. Without being able to definitively state that these proximal hair samples were immediately close to the scalp, the strength of the analysis for these final months of life is limited.

Based on the inclusion criteria of hair preservation for this study, it was impossible to control for skeletal preservation beyond the required sampling site (i.e., parietal bone). Unfortunately, without this physical preservation, certain intrinsic factors such as age and sex could not be controlled for as much as would be preferred. Additionally, because of these preservation issues, cortisol or osteocalcin could not be studied in terms of age or sex patterns despite age and sex being key influencing factors on circulating cortisol and osteocalcin concentrations (Henley et al., 2013; Noppe et al., 2014; Thomson et al., 2010).

Regarding the second hypothesis of this thesis, data from this thesis suggest that cortisol found in bone depends on the element selected for extraction, as the lipid content of a particular element is an influencing factor. If the lipid content of a sample has been compromised, the cortisol content in a bone sample may be deflated. This means those cortisol concentrations may not accurately represent that individual's long-term stress. While there is a noticeable lipid peak on the FTIR spectrum for bone (Mata-Miranda et

al., 2019), a mathematical metric for quantification or preservation has not been developed. Without this FTIR metric, the only way to measure lipid content is through advanced chemical processing and quantification (Hewavitharana et al., 2020). This limits this study by making it impossible to determine if cortisol differences are genuinely due to stress levels or other extraneous factors.

Taphonomic change is also a limiting factor of this study for the cortisol concentrations extracted from the hair samples. Unlike bone, there is no archaeological metric to measure hair preservation (Cole, 2017; Webb et al., 2010). While hair diagenesis can be holistically assessed through histology and scanning electron microscopy (Petraru et al., 2020), it is highly vulnerable to inter-observer error (Man et al., 2021; Smith and Linch, 1999). This is a limitation because, without an objective scale of hair diagenesis, it is impossible to ensure that diagenesis uniformly affected the hairs in this sample where they can be accurately compared to one another (East et al., 2022; Webb et al., 2010). For example, cortisol could have been systematically washed out of a specific hair sample, impacting the conclusions about a particular individual when comparing these results to an entire sample. Lipids do have an identifiable peak in hair's IR spectrum generated with FTIR (Barba et al., 2022), which means that a scale to measure lipid preservation in hair could in theory be developed, but until the inability to measure hair diagenesis remains a limitation in hair cortisol research.

Finally, previous research has associated fluctuations in osteocalcin concentrations with specific pathological conditions that result in bone resorption (e.g., metabolic disease, rickets, and degenerative spine disease) (Hughes, 2020; Rich et al., 2022; Scott et al., 2016). Macroscopic signs of pathology on the skeleton could not be

assessed due to poor preservation and consequently may have influenced the recorded osteocalcin concentrations. Physical activity can also influence osteocalcin concentrations, where sampling osteocalcin from regions with increased muscle attachment can produce higher values than regions without (see Hughes, 2020). While this is an important consideration, it was controlled for as much as possible by sampling from the ectocranial posterior parietal—a region of the skull with minimal muscle attachment. Being able to control some of these factors (sampling site) and not others (assessment of pathology) complicates the results of this analysis and cross-population comparisons.

## 5.7 Conclusion

This chapter interpreted the results of this study while also discussing the various factors that contributed to these results. While both bone cortisol and osteocalcin concentrations were affected by diagenesis, they were impacted uniformly. Osteocalcin concentrations from the Fortress of Louisbourg were found to be statistically lower than that of other populations in comparative studies, likely due to differences in stress given previous research arguing that lower concentrations of bone cortisol align with elevated levels of stress. Comparisons of hair cortisol studies using similar methods showed that the Fortress population had higher stress than some comparative groups and lower concentrations than others likely due to stressors such as climate and conflict. Month-to-month analyses of hair cortisol saw an elevation of stress as death approached, likely due to the stress of the dying process. The month-to-month analysis also suggested possible medical care in the month before death for three individuals in the sample. However, the

lack of follicle roots make both of these analyses more tenuous. While the relationship was not statistically significant, cortisol concentrations from hair and bone were found to trend broadly in the same direction. This led to the conclusion that short-term stress, while increasing near death, still fell in line with the stress experienced long-term. Finally, when osteocalcin concentrations were compared to bone cortisol, they were found to have an inverse relationship. In practice, this means that osteocalcin is confirmed to be a viable method to measure the stress of archaeological remains. While hair cortisol followed the same pattern as bone cortisol in regard to osteocalcin, it was not statistically significant likely due to differences in the time period of stress captured.

# Chapter 6: Conclusion

## 6.1 Revisiting the Research Questions and Hypotheses

This thesis has aimed to advance the study of biomolecular stress in archaeological populations. Specifically, it focused on cortisol within two archaeological tissues (i.e., bone and hair) in conjunction with osteocalcin to validate the latter's use as a biomarker for physiological stress. This final chapter will revisit the research questions and hypotheses presented in Chapter One, discuss potential avenues for future research, and review the significance of this thesis research.

1. *Can osteocalcin be extracted from 18$^{th}$-century human cortical bone samples from the Fortress of Louisbourg?*

Osteocalcin was successfully extracted from all 12 individuals from the Fortress of Louisbourg. This is unsurprising, considering these methods have been well-established and proven successful when extracting osteocalcin from archaeological bone and aligns with the first hypothesis of this study (Hughes, 2020; Rich et al., 2022; Scott et al., 2016, 2020). Not only were these methods previously successful, but osteocalcin was also previously extracted from individuals at the Fortress of Louisbourg (Hughes, 2020). Therefore, this research further supports the efficacy of these methods.

2. *Can cortisol be extracted from 18$^{th}$-century hair samples from the Fortress of Louisbourg?*

Cortisol was successfully extracted from all 21 hair samples in this study—again, aligning well with the first hypothesis of this thesis. This is the first study that has accomplished this archaeologically in North America, as other studies have primarily focused on populations from dry, arid climates (e.g., Peru and Egypt) (East, 2021;

Kellner et al., 2022; Schaefer, 2017; Tisdale et al., 2019; Webb et al., 2015a, 2015b, 2010). The success of this signifies that quantifiable concentrations of cortisol can be extracted from hair regardless of the time period or geographic location, and the preservation and presence of hair is the most significant driving factor of its ability to be studied.

In addition to extracting cortisol to assess periods of stress, this research has also highlighted an important methodological consideration for these types of studies. The results from this study have shown that there was a notable difference between the cortisol concentrations from studies with varying extraction methods (i.e., heat vs. no-heat extraction); this is because extraction efficiency when aliquoting from a substrate can significantly influence the concentrations of the hormone once quantified (Meyer et al., 2014; Slominski et al., 2015). While this will be discussed more in length in section 6.2, this study confirms that comparative analysis should only be conducted between studies with comparable methods.

3. *Can cortisol be extracted from 18$^{th}$-century human cortical bone samples from the Fortress of Louisbourg?*

Confirming the second hypothesis of this study, cortisol was successfully extracted from human cortical bone for the first time by adapting procedures used in animal studies (see Charapata et al., 2018). This was a significant yet unsurprising result as cortisol has a similar chemical structure and function across mammals (Charapata et al., 2018; Fokidis et al., 2023; Jewgenow et al., 2020; Sperou et al., 2023). Given that this is the first time that cortisol has been extracted from human cortical bone, confirming that

this procedure works allows for the continued advancement of biomolecular stress research in bioarchaeological populations.

Given the novelty of this study, a crucial intrinsic factor was discovered when extracting cortisol from hard tissues. As a fat-soluble hormone, cortisol concentrations in hard tissues are likely heavily influenced by the lipid content of the sampled element (Ilias et al., 2023; Thau et al., 2021). While these results agree with the second half of this thesis' second hypothesis that the selection of skeletal element impacts cortisol concentrations, however, further research needs to be conducted regarding this result and will be discussed (see section 6.2). Briefly though, the direct quantification of lipid content or only sampling from elements of similar lipid concentrations is necessary for accurate comparisons of genuine stress levels between individuals.

4. *Is there a measurable relationship between bone osteocalcin and cortisol (extracted from hair and bone)?*

The concentrations of bone cortisol and osteocalcin were found to have a notable inverse relationship; while hair cortisol and osteocalcin did not have a statistically significant relationship, they followed the same pattern. This trend confirms that elevated stress levels correspond with decreased osteocalcin concentrations in the bone. These findings are heavily supported by both clinical (Berger et al., 2019; Berger and Karsenty, 2022; Bilotta et al., 2018; Karsenty, 2023; Lee et al., 2007; Wei and Karsenty, 2015) and bioarchaeological (Hughes, 2020; Rich et al., 2022; Scott et al., 2020, 2016) studies that have argued that low concentrations of bone osteocalcin indicate heightened levels of physiological stress; however, a direct comparison had yet to be made. Therefore, the actual observed relationship between osteocalcin and cortisol in this study further

confirms the viability of osteocalcin as a biomarker for studying bioarchaeological stress and confirms this study's third hypothesis as correct.

## 6.2 Future Research

### 6.2.1 Osteocalcin as a Stress Biomarker

Osteocalcin remains a novel biomolecule in bioarchaeological research, providing many future study opportunities. While previous research investigated the differences in osteocalcin concentrations between varying skeletal elements (Hughes, 2020; Scott et al., 2016), and areas within the same element (Hughes, 2020), they have all solely focused on sampling from cortical bone. Therefore, future work should examine other types of bone (i.e., trabecular bone) that may be more reactive to periods of stress and metabolic fluctuations within the body (Rehman and Lane, 2003; Vs et al., 2013). Osteocalcin in the trabecular bone may represent a more acute response to external stimuli than cortical bone, where there would be less of an "averaging effect." As such, trabecular bone may be a better proxy for understanding osteocalcin concentrations at the time of death (Hughes, 2020). Additionally, studying the osteocalcin concentrations across the layers of cortical bone (i.e., periosteum, intracortical, and endosteum) may help determine the rate at which osteocalcin embeds itself into cortical bone to create a more precise timeline of stress.

Furthermore, studying osteocalcin alongside cortisol in a larger sample size would be helpful as it would expand the statistical power of any comparison made. Additionally, there is a need to further incorporate macroscopic stress indicators (e.g., cribriotic lesions, enamel hypoplastic lesions, etc.) into these biomolecular analyses. To date, there has only

been one study involving cortisol (Kellner et al., 2022) and four studies focusing on osteocalcin (Hughes, 2020; Rich et al., 2022; Scott et al., 2020, 2016) that have investigated the relationship between archaeological hormones and macroscopic indicators of stress. Incorporating osteocalcin and cortisol with these more established methods would allow for a more nuanced application of stress research and lived experiences in bioarchaeological populations.

### 6.2.2 Variation in Cortisol Methodology

As discussed (see Chapter 5, section 5.4), the extraction method is a vital consideration in cortisol quantification. Since cortisol extraction relies on the solubility of cortisol in a solvent (i.e., methanol), conditions that can influence that solubility (e.g., temperature, surface area, etc.) will impact the amount of cortisol that is extracted (Berna et al., 2004; Schmid and Voigt, 1954; Thakur et al., 2011; Zia et al., 1991). This variability can cause inaccurate conclusions when comparing cortisol concentrations across different studies (Meyer and Novak, 2012; Russell et al., 2015). While studies have highlighted the impact of varying quantification methods (Russell et al., 2015), the effect of extraction methods on cortisol concentrations has not yet been explored directly. Knowing the impact of various methods on the quantification of cortisol and being able to account for these differences could increase the confidence of researcher interpretations and allow for better reproducibility.

### 6.2.3 Bone Cortisol

Similar to the study of bone osteocalcin, studying the variation among different skeletal elements (e.g., femur, skull, scapula, etc.) and sampling sites (e.g., trabeculae,

endosteum, areas of increased muscle attachment, etc.) and the amount of quantifiable cortisol is important in establishing the viability of using bone cortisol to study stress in the past. Unfortunately, a high amount of powdered bone is required for cortisol extraction (250 mg); therefore, further research focused on minimizing the amount of bone needed for this type of analysis is paramount to limit skeletal destruction (Alpaslan-Roodenberg et al., 2021; Charrié-Duhaut et al., 2021; DeWitte, 2015; Smith et al., 2023).

The overall lipid content of skeletal elements should also be considered when assessing cortisol. Charapata et al. (2018) suggest that total lipid content in a particular individual is a better tool to normalize the extracted concentrations of cortisol in bone as opposed to total bone mass sampled, similar to total protein for bone osteocalcin (Hughes, 2020; Scott et al., 2020). Data from this thesis suggest that total lipid content in a skeletal element influences total cortisol. Still, a more extensive study on the relationship between lipid content and cortisol concentrations in bone is necessary. Additionally, because preservation plays a major role in the efficiency of cortisol extraction, further study of cortisol preservation using multiple methods of analysis (e.g., C/N ratios, collagen percentage, etc.) (Brock et al., 2012; Chesson et al., 2021) is warranted.

## 6.3 Research Significance

Using biochemical methods within bioarchaeology is a quickly growing field of research, to which this thesis directly contributes. Specifically, this is the first bioarchaeological study of its kind to study hair cortisol in an archaeological North American population and the very first to test and extract cortisol from human cortical

bone tissue. This thesis was also the first bioarchaeological study to integrate and assess cortisol and osteocalcin extracted from the same skeletal tissue. The benefit of this comparison was to firmly establish osteocalcin as a biomarker of chronic stress in the skeleton. Aligning with clinical research, this thesis showed that bone osteocalcin has a measurable relationship with cortisol and the stress response system. Additionally, as cortisol research in the past has only been possible using hair and teeth (see Quade et al., 2021, 2023; Webb et al., 2010), the successful extraction of cortisol from bone allows bioarchaeologists to assess stress in regions where not previously possible (i.e., where hair does not preserve). As biochemical applications within bioarchaeology continue to grow, the use of biomolecules like cortisol and osteocalcin will allow us to evaluate stress and its impact on the lived experience of individuals in a way that was not previously possible.

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## APPENDIX A

Table A.1: Raw Osteocalcin ELISA data

| ID | Well(s) | Raw data (OD) | Average | SD |
|---|---|---|---|---|
| 3 | A3, A4 | 0.71, 0.745 | 0.728 | 0.018 |
| 12 | B3, B4 | 2.285, 1.688 | 1.986 | 0.299 |
| 15 | C3, C4 | 0.754, 0.711 | 0.732 | 0.022 |
| 45 | D3, D4 | 0.941, 0.855 | 0.898 | 0.043 |
| 88 | E3, E4 | 0.946, 1.008 | 0.977 | 0.031 |
| 120 | F3, F4 | 0.143, 0.154 | 0.148 | 0.006 |
| 124 | H3, H4 | 0.489, 0.356 | 0.422 | 0.066 |
| 136 | G3, G4 | 0.908, 0.989 | 0.949 | 0.04 |
| 154 | A5, B5 | 1.639, 1.649 | 1.644 | 0.005 |
| 172 | C5, D5 | 0.588, 0.535 | 0.561 | 0.026 |
| 194 | E5, F5 | 0.481, 0.498 | 0.489 | 0.009 |
| 211 | G5, H5 | 0.481, 0.332 | 0.406 | 0.074 |
| Blank | A6, A7 | 0.005, -0.002 | 0.053 | 0.005 |
| C1 (9ng/ml) | G1, G2 | 0.135, 0.127 | 0.131 | 0.004 |
| C2 (30ng/ml) | H1, H2 | 0.932, 1.053 | 0.992 | 0.06 |
| S1 (0ng/ml) | A1, A2 | 0.052, 0.055 | 0.0535 | 0.002 |
| S2 (4ng/ml) | B1, B2 | 0.049, 0.048 | 0.049 | 0.001 |
| S3 (8ng/ml) | C1, C2 | 0.13, 0.108 | 0.119 | 0.011 |
| S4 (16ng/ml) | D1, D2 | 0.517, 0.653 | 0.585 | 0.068 |
| S5 (32ng/ml) | E1, E2 | 0.89, 1.265 | 1.077 | 0.187 |
| S6 (64ng/ml) | F1, F2 | 2.608, 3.185 | 2.897 | 0.288 |
| Repeat | | | | |
| **ID** | **Well(s)** | **Raw data (OD)** | **Average** | **SD** |
| 12 | A3, B3 | 1.393, 1.399 | 1.396 | 0.003 |
| 124 | C3, D3 | 0.244, 0.276 | 0.26 | 0.016 |
| 211 | E3, F3 | 0.328, 0.419 | 0.373 | 0.045 |
| Blank | G3,G4 | 0.001, 0.002 | 0.0015 | 0.001 |
| C1 (9ng/ml) | G1, G2 | 0.105, 0.134 | 0.119 | 0.015 |
| C2 (30ng/ml) | H1, H2 | 0.758, 0.888 | 0.823 | 0.065 |
| S1 (0ng/ml) | A1, A2 | 0.001, -0.001 | 0 | 0.001 |
| S2 (4ng/ml) | B1, B2 | 0.045, 0.051 | 0.048 | 0.003 |
| S3 (8ng/ml) | C1, C2 | 0.192, 0.183 | 0.188 | 0.005 |
| S4 (16ng/ml) | D1, D2 | 0.466, 0.492 | 0.479 | 0.013 |
| S5 (32ng/ml) | E1, E2 | 1.052, 1.093 | 1.073 | 0.02 |
| S6 (64ng/ml) | F1, F2 | 2.128, 2.308 | 2.218 | 0.09 |

Table A.2: Raw BCA ELISA Data

| ID | Well(s) | Raw data (OD) | Average | SD |
|---|---|---|---|---|
| 3 | C3, C4 | 0.237, 0.264 | 0.251 | 0.014 |
| 12 | D3, D4 | 0.352, 0.367 | 0.359 | 0.008 |
| 15 | E3, E4 | 0.405, 0.386 | 0.396 | 0.01 |
| 45 | F3, F4 | 0.3, 0.317 | 0.308 | 0.009 |
| 88 | G3, G4 | 0.276, 0.287 | 0.281 | 0.005 |
| 120 | H3, H4 | 0.265, 0.252 | 0.259 | 0.007 |
| 124 | A5, A6 | 0.236, 0.233 | 0.234 | 0.001 |
| 136 | B5, B6 | 0.306, 0.302 | 0.304 | 0.002 |
| 154 | C5, C6 | 0.393, 0.434 | 0.413 | 0.02 |
| 172 | D5, D6 | 0.239, 0.26 | 0.249 | 0.011 |
| 194 | E5, E6 | 0.321, 0.35 | 0.336 | 0.014 |
| 211 | F5, F6 | 0.304, 0.321 | 0.312 | 0.009 |
| S1 (2000ug/ml) | A1, A2 | 2.86, 3.167 | 3.013 | 0.153 |
| S2 (1500ug/ml) | B1, B2 | 2.443, 2.599 | 2.521 | 0.078 |
| S3 (1000ug/ml) | C1, C2 | 1.678, 1.799 | 1.738 | 0.06 |
| S4 (750ug/ml) | D1, D2 | 1.368, 1.359 | 1.364 | 0.005 |
| S5 (500ug/ml) | E1, E2 | 0.977, 0.994 | 0.986 | 0.009 |
| S6 (250ug/ml) | F1, F2 | 0.533, 0.533 | 0.533 | 0 |
| S7 (125ug/ml) | G1, G2 | 0.278, 0.28 | 0.279 | 0.001 |
| S8 (25ug/ml) | H1, H2 | 0.053, 0.054 | 0.053 | 0.001 |
| S9 (5ug/ml) | A3, A4 | 0.011, 0.01 | 0.01 | 0 |
| S10 (0ug/ml) | B3, B4 | 0.001, -0.001 | 0 | 0.001 |

Table A.3: Raw Cortisol ELISA Data

| ID | Well(s) | Raw data (OD) | Average | SD |
|---|---|---|---|---|
| 3 H0 | A3, A4 | 0.945, 0.946 | 0.946 | 0.001 |
| 3 H1 | B3, B4 | 1.071, 1.066 | 1.069 | 0.002 |
| 3 B | C3, C4 | 0.988, 0.996 | 0.992 | 0.004 |
| 12 H0 | D3, D4 | 1.108, 1.091 | 1.099 | 0.009 |
| 12 B | E3, E4 | 0.994, 1.03 | 1.012 | 0.018 |
| 15 H0 | F3, F4 | 0.932, 0.966 | 0.949 | 0.017 |
| 15 H1 | G3, G4 | 0.96, 1.015 | 0.987 | 0.027 |
| 15 H2 | H3, H4 | 1.058, 1.053 | 1.055 | 0.003 |
| 15 B | A5, A6 | 0.937, 0.924 | 0.931 | 0.007 |
| 45 H0 | B5, B6 | 1.109, 1.153 | 1.131 | 0.022 |
| 45 B | C5, C6 | 0.818, 0.846 | 0.832 | 0.014 |
| 88 H0 | D5, D6 | 0.999, 0.997 | 0.998 | 0.001 |
| 88 B | E5, E6 | 0.959, 0.978 | 0.968 | 0.01 |
| 120 H0 | F5, F6 | 0.829, 0.847 | 0.838 | 0.009 |
| 120 H1 | G5, G6 | 0.826, 0.822 | 0.824 | 0.002 |
| 120 H2 | H5, H6 | 1.01, 1.073 | 1.042 | 0.031 |
| 120 B | A7, A8 | 0.862, 0.876 | 0.869 | 0.007 |
| 124 H0 | B7, B8 | 1.014, 1.023 | 1.018 | 0.004 |
| 124 B | C7, C8 | 0.785, 0.801 | 0.793 | 0.008 |
| 136 H0 | D7, D8 | 0.943, 0.935 | 0.939 | 0.004 |
| 136 H1 | E7, E8 | 0.946, 0.917 | 0.931 | 0.014 |
| 136 B | F7, F8 | 0.892, 0.891 | 0.891 | 0.001 |
| 154 H0 | G7, H7 | 0.911, 0.932 | 0.921 | 0.011 |
| 154 B | G8, H8 | 0.875, 0.876 | 0.875 | 0.001 |
| 172 H0 | A9, A10 | 1.055, 0.976 | 1.015 | 0.039 |
| 172 H1 | B9, B10 | 0.892, 0.907 | 0.899 | 0.008 |
| 172 B | C9, C10 | 0.807, 0.819 | 0.813 | 0.006 |
| 194 H0 | D9, D10 | 0.903, 0.933 | 0.918 | 0.015 |
| 194 H1 | E9, E10 | 0.917, 1.056 | 0.987 | 0.07 |
| 194 H2 | F9, F10 | 0.923, 0.884 | 0.903 | 0.02 |
| 194 B | G9, G10 | 0.853, 0.845 | 0.849 | 0.004 |
| 211 H0 | H9, H10 | 0.927, 0.914 | 0.921 | 0.007 |
| 211 B | A11, B11 | 0.935, 0.907 | 0.921 | 0.014 |
| High Control (40ng/ml) | G1, G2 | 0.212, 0.234 | 0.223 | 0.011 |
| Low Control (10ng/ml) | H1, H2 | 0.424, 0.426 | 0.42 | 0.001 |
| Blank | C11, D11 | 1.423, 1.456 | 1.44 | 0.023 |
| S1 (0 ng/ml) | A1, A2 | 1.33, 1.334 | 1.332 | 0.002 |
| S2 (1 ng/ml) | B1, B2 | 0.856, 0.846 | 0.851 | 0.005 |
| S3 (3 ng/ml) | C1, C2 | 0.671, 0.634 | 0.653 | 0.019 |
| S4 (10ng/ml) | D1, D2 | 0.433, 0.424 | 0.428 | 0.005 |
| S5 (30ng/ml) | E1, E2 | 0.264, 0.258 | 0.261 | 0.003 |

| | | | | |
|---|---|---|---|---|
| S6 (100ng/ml) | F1, F2 | 0.14, 0.142 | 0.141 | 0.001 |

# Curriculum Vitae

## Education

| | |
|---|---|
| 2022—Present | Master of Arts, Anthropology, University of New Brunswick, Fredericton, NB, CA |
| 2017—2021 | Bachelor of Arts, Anthropology: Anthropological Methods and Practice, University of Central Florida**,** Orlando, FL, USA |
| 2017—2021 | Bachelor of Science, Forensic Science: Biochemistry, Minor in Chemistry, University of Central Florida**,** Orlando, FL, USA |
| 2019—2021 | Undergraduate Certificate, Judaic Studies, University of Central Florida**,** Orlando, FL, USA |
| 2017—2021 | Undergraduate Certificate, Crime Scene Investigation, University of Central Florida**,** Orlando, FL, USA |

## Publications

**In Progress** Benjamin L. Kaufman, Nicole Hughes, Kelsey E. Kane, and Amy B. Scott *Operation FTIR Rescue: Assessing bioarchaeological bioapatite preservation at the 18$^{th}$ century Fortress of Louisbourg using FTIR-ATR to aid in rescue archaeology in coastal regions.* Manuscript in progress for submission to Journal of Archaeological Sciences: Reports.

**2023** Dylan Smith, Erin Martin, Benjamin L. Kaufman, and J. Marla Toyne *The Bottom Line: Exploring analytical methods for assessing preservation in archaeological human remains*. https://doi.org/10.1016/j.jasrep.2023.104014

**Research Presentations** (*Indicates presenter)

**2024** Benjamin Kaufman*, Amy B. Scott

*Stress through the centuries: The application of stress hormones to study the health of individuals from the 18th Century Fortress of Louisbourg.* Podium presentation. UNB Skeletal Stories: Bioarchaeological Research From the 18th Century Fortress of Louisbourg, University of New Brunswick**,** Fredericton, NB

**2023** Benjamin Kaufman*, Nicole Hughes, Kelsey E. Kane, Amy B. Scott

*Operation FTIR Rescue: Assessing bioarchaeological bioapatite preservation at the 18th century Fortress of Louisbourg using FTIR-ATR to aid in rescue archaeology in coastal regions.* Poster presentation. UNB Graduate Research Conference, University of New Brunswick**,** Fredericton, NB

**2021** Benjamin Kaufman*, Dylan Smith, Erin Martin, J.Marla Toyne

*Identifying unwanted transformations: The analytical chemistry of FTIR to improve stable isotope research in archaeology.* Podium presentation. Integrative Anthropology Conference, University of Central Florida**,** Orlando, FL

**2019** Benjamin Kaufman*, Lidar Sapir-Hen

*Tel Megiddo: Speciation and slaughter techniques of Late-Middle-Bronze Age Israel.* Podium presentation. History Department Research Showcase, University of Central Florida**,** Orlando, FL

**Awards**

**2023** Dr. Wu Yee-sun and Mrs. Wu Ho Man-yuen Memorial Graduate Bursary: $500 CAD

Faculty of Arts Graduate Assistantship: $16,000 CAD

UNB International Differential Award: $6,000 CAD per year

American Jewish Federation: Gerald Ward Graduate Award: $1,000 USD

**2022** Dr. Wu Yee-sun and Mrs. Wu Ho Man-yuen Memorial Graduate Bursary: $500 CAD

Board of Governors Merit Award: $3,000 CAD

Faculty of Arts Graduate Assistantship: $16,000 CAD

UNB International Differential Award: $6,000 CAD per year

American Jewish Federation: Gerald Ward Graduate Award: $1,000 USD

**2021** American Jewish Federation: Undergraduate College Award: $1,000 USD

**2020** American Jewish Federation: Undergraduate College Award: $1,000 USD

UCF Intelligence Community Center for Academic Excellence Merit Award: $2,000 USD

UCF Orion Grant: $1,400 USD

UCF Scholars Award: $2,000 USD

**2018** UCF Pegasus Bronze Award: $6,000 USD