IT'S IN THE BONES: AN EXPLORATION OF HUMAN BONE PROTEIN FROM THE 18TH CENTURY FORTRESS OF LOUISBOURG, NOVA SCOTIA AND ITS POTENTIAL APPLICATIONS IN BIOARCHAEOLOGICAL RESEARCH

by

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ABSTRACT

Bioarchaeology has begun to employ biochemical methods as a means to further understand human skeletal remains at a biomolecular level. Specifically, osteocalcin (an abundant, non–collagenous bone protein) is of interest because of its clinically identified relationship with biological factors (i.e., age and sex) and pathological conditions (i.e., trauma and disease) that can be macroscopically observed in archaeological bone. The aim of this study was to extract and quantify osteocalcin from 27 individuals from the Fortress of Louisbourg (1713–1758) skeletal collection to explore whether these clinical trends related to osteocalcin were also visible in archaeological bone. Osteocalcin was successfully extracted from femoral bone samples and interpreted in tandem with sex, age, activity, and evidence of pathology. This study demonstrates the applicability of biochemical analyses as an additional line of evidence when conducting macroscopic skeletal assessments of biological and pathological factors, as well as, represents the first archaeological study of osteocalcin in human skeletal remains from a Canadian context.

DEDICATION

For Kath and Fritz

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CHAPTER 1 – Introduction

1.1 Purpose and Significance

Bioarchaeology studies the relationship between human skeletal remains and the social and cultural practices of past populations (see Agarwal and Glencross, 2011; Buzon, 2012; Larsen, 2003; Martin et al., 2013). Traditionally, bioarchaeological research uses human skeletal remains to explore elements of diet, migration, violence, status, sex, health, etc. and their relationship to the lived experience. Macroscopic observation is the foundation of bioarchaeological studies to interpret the lived experience but this type of analysis can omit important information that may only be accessed as a microscopic level. Recently, there has been a growing interest in analyzing the skeleton at a biochemical level to unlock this invisible information about past lives. As a result, biochemical analyses have come to the forefront of the discipline to further expand our interpretive potential and how we access and understand the lived experience. Specifically, there has been an increased focus on the quantification of bone protein based on its ability to preserve well in archaeological contexts but also its ability to speak to biological and potentially pathological processes that might otherwise be invisible from macroscopic analysis alone. One such bone protein is osteocalcin. Synthesized by the bone–forming osteoblast cells, osteocalcin concentrations reflect the processes of bone metabolism impacted by various biological factors such as sex, age, and activity but also pathological conditions such a disease and trauma. Therefore, the study of osteocalcin can provide a unique insight into the process of skeletal metabolism and variations that may be tied to the biological and/or pathological profile of an individual providing an additional means of analysis in bioarchaeological research.

The goal of this thesis project is to expand upon the methods of bone protein extraction from human skeletal remains. Specifically, establishing a consistent sampling technique and sampling location on the skeleton and validating the quantification methods most commonly employed for osteocalcin analysis. Additionally, this research will attempt to link osteocalcin concentrations in bone with known biological and pathological conditions that impact bone metabolism. This research is relevant as it will further validate the current archaeological protein extraction and quantification methods, provide comparative data for other bioarchaeological studies that have examined bone proteins, and will provide an opportunity to explore whether the clinically–identified relationships between biological and pathological factors can also be captured in archaeological remains. As well, this project will be the first to extract osteocalcin from a Canadian bioarchaeological skeletal collection providing new insight into the early French colonial experiences in Atlantic Canada.

1.2 Objectives and Research Questions

The main objective of this thesis is to further explore the methods of protein extraction and how the data obtained may be transformed into a better understanding of the biological and pathological influences on osteocalcin synthesis. The three primary research questions of this thesis are:

1. Can osteocalcin be successfully extracted from bioarchaeological samples?
2. What biological and/or pathological patterns emerge from the osteocalcin data and how does this correlate with the macroscopic skeletal assessment of these individuals in terms of age and sex patterns, activity level, or the presence/absence of pathological conditions characterized by bone remodeling?

3. Is there a similar level of preservation (diagenesis) across all bioarchaeological samples?

1.3 Organization of the Thesis

This thesis contains six chapters. The first chapter explains the importance of biochemical analysis within bioarchaeology and outlines the objectives of the thesis and the research questions that will be explored. Chapter 2 is a literature review providing detailed background information on bone proteins, specifically osteocalcin, and its known clinical relationship with biological factors (i.e., sex, age, activity) and pathological conditions. The use of osteocalcin in bioarchaeological contexts will also be reviewed. Diagenesis is also explored and how it can affect the survivability of bone protein in archaeological remains. A brief history on the Fortress of Louisbourg is also provided in order to establish context between the individuals in this study and their historical and geographic backgrounds in colonial Canada. Chapter 3 outlines the materials and methods used in this thesis. Specifically, it discusses the demography of the sample population, how bone samples were collected, the steps to quantify both osteocalcin and total bone protein, the assessment of diagenetic change, and the x–ray procedure employed to measure cortical bone thickness. This chapter also outlines the macroscopic pathological assessment completed for this thesis. Chapter 4 summarizes the results of this thesis, including diagenetic analysis, bone protein quantification, mid–shaft cortical bone thickness measurements in relation to biological and pathological considerations, and pathological patterns. Chapter 5 leads an interpretive discussion of the major findings in both the context of the clinical and the bioarchaeological literature. It begins with the exploration osteocalcin in conjunction with biological and pathological considerations

which are compared to clinical standards and archaeological findings. When possible, the biological and pathological osteoclacin patterns of this thesis are also connected to the lived experience at Louisbourg. Next, the diagenesis results are discussed in regard to the Fortress burial conditions that contributed to bone deterioration and how diagenetic change impacts osteocalcin survivability. Lastly, this chapter discusses the literature and methodological limitations of this study. Chapter 6 concludes the thesis by providing a summary of the research in relation to the previously stated research questions, provides suggestions of future research directions, and details how this thesis contributes to the growing importance of biochemical analyses within bioarchaeological research.

CHAPTER 2 – Literature Review

2.1 Introduction

This literature review chapter is divided into two main sections. The first section discusses the non–collagenous protein osteocalcin and its significance in bioarchaeological research. It begins with an overview of bone and its components including osteocalcin. Next, the function of osteocalcin is outlined and how biological factors including sex, age, and activity level influence osteocalcin concentrations over the life course. Following this, the mechanism of the stress response is described, particularly how long periods of stress can negatively impact bone and osteocalcin levels. This section concludes with an examination of previous protein and osteocalcin research within bioarchaeology. The second section of the literature review provides a brief history of the Fortress of Louisbourg providing context for the archaeological individuals used in this research.

2.2 Components of Bone and the Role of Osteocalcin

2.2.1 Components of Bone

Bone is a dynamic connective tissue that is composed of organic and inorganic elements (Brickley and Ives, 2008; Lee and Einhorn, 2001). The inorganic material is made of calcium hydroxyapatite which provides bone with the necessary rigidity for strength and resistance to fracture (Brickley and Ives, 2008). The organic elements consist of collagen, which makes up 98 percent of the total organic material, and non–collagenous protein, the remaining two percent (Brickley and Ives, 2008; Lee and Einhorn, 2001). Collagen and non–collagenous proteins function as the structural component of bone (Lee and Einhorn, 2001). In particular, non–collagenous proteins are

important because they facilitate the mineralization and binding of calcium hydroxyapatite (Brickley and Ives, 2008).

2.2.2 The Role of Osteocalcin

The most abundant non–collagenous protein is osteocalcin (Booth et al., 2015; Collins et al., 2002) which is synthesized and secreted by the osteoblasts (i.e., bone–forming cells) during the late mineralization phase of bone formation. Synthesized osteocalcin is either deposited in the mineralized bone matrix (matrix osteocalcin) (Bailey et al., 2018; Christenson, 1997) or secreted into the blood as serum osteocalcin (Bhadricha et al., 2019; Christenson, 1997; Ingram, 1994). Matrix osteocalcin undergoes carboxylation which is a chemical reaction that adds a carboxyl group to osteocalcin's chemical formula. The synthesis of vitamin K in the osteoblast cell initiates carboxylation of osteocalcin (Rathore et al., 2016) which transforms it into an active y–carboxyglutamate (Gla) osteocalcin protein (Menon et al., 1987; Patti et al., 2013). The active osteocalcin (Gla) is important because it enables the protein to bind with calcium and hydroxyapatite which prompts bone deposition and mineralization (Brickley and Ives, 2008; Calandrelli et al., 2010; Christenson, 1997; Collins et al., 2002; Patti et al., 2013; Thomas, 2017). Matrix osteocalcin remains in bone until it is eventually degraded by osteoclastic resorption as part of normal bone remodeling (Christenson, 1997; Neve et al., 2013; Thomas et al., 2017). When matrix osteocalcin is degraded through this remodeling process, it enters the blood as fragmented serum osteocalcin (Gorski, 2011) and expelled by the kidneys within a few hours (Cundy et al., 2014; Rathore et al., 2016).

2.3 Biological Factors that Influence Osteocalcin Concentrations

2.3.1 Sex and Osteocalcin Levels

Osteocalcin levels vary between biological sex because of differing levels of circulating sex hormones that help regulate bone growth and remodeling (U.S. Department of Health and Human Services, 2004; Manolagas et al., 2002; Mizokami et al., 2017). Estrogen and testosterone are sex hormones that are produced by both sexes. There is a higher abundance of estrogen in females than males (Smy and Straseki, 2018), and a higher abundance of testosterone in males than females (Clifton et al., 2016). Both estrogen and testosterone initiate bone growth and remodeling by stimulating the osteoclasts and osteoblasts (U.S. Department of Health and Human Services, 2004). However, osteocalcin levels fluctuate in adult females who become pregnant. Throughout pregnancy, temporary bone loss occurs (Beauchesne, 2012; Mays et al, 2006) because the growing fetus demands energy from the female that would otherwise be contributing to her homeostatic bone function. Due to bone loss, osteocalcin levels decrease during the first trimester up to 50% lower than normal (Polak–Jonkisz et al., 1998; Wei et al., 2018). However, osteocalcin levels do increase during the third trimester (Polak–Jonkisz et al., 1998; Wei et al., 2018) and throughout the postpartum period as bone begins to replenish the mass that was lost during pregnancy (National Institutes of Health, 2015).

2.3.2 Age and Osteocalcin Levels

The age of an individual can also influence levels of osteocalcin because the rate of bone growth and remodeling changes throughout the lifecourse (Ingram et al., 1994). Rapid growth occurs during the first year of infancy (i.e., the first three years of life) due to a dramatic increase in nutrition from a rich diet of breast milk (Lejarraga, 2002) but

declines after the weaning period (Bogin, 2001; Lejarraga, 2002). During childhood (i.e., three to seven years) growth and osteocalcin levels remain regulated until the mid–growth spurt (Lejarraga, 2002). The mid–growth spurt is caused by an increase in growth hormones between the ages of six and eight years (Bogin, 2001; Lejarraga, 2002). During this growth spurt, there is only a small increase in bone length but a significant increase in bone volume (Lejarraga, 2002; Smith, 2004). Therefore, osteocalcin levels during the mid–growth spurt might increase but will most likely not be significant. After the mid–growth spurt, growth plateaus until adolescence when puberty begins (Bogin, 2001). This period is marked by the secretion of sex and growth hormones that initiate another growth spurt (Ellison, 2002). During this adolescent growth spurt, bone is signaled to increase in size by laying down bone faster than it can be remodeled (U.S. Department of Health and Human Services, 2004; Kirmani et al., 2011; Manolagas et al., 2002; Ortoft and Oxlund, 1996). Therefore, osteocalcin concentrations are elevated because of increased osteoblast activity during this period of rapid development (Cremers et al., 2008; Vanderschueren et al., 1990). Adulthood is reached when reproductive maturity and bone growth is complete (Bogin, 2001). Instead of prioritizing growth and development, the adult human skeleton focuses on maintaining its healthy bone structure. This is achieved by removing bone that has become weakened or damaged over time from use and replacing it with new bone (Seeman, 2008). In general, the adult skeleton will remodel three percent of compact bone each year (Manolagas et al., 2002; Martin et al., 2015). Osteocalcin levels remain relatively stable in adulthood but are differentiated between the sexes based on sex hormone differences, as discussed (Jung, 2016).

Senescence or old age begins when the reproductive years of adulthood end (Bogin, 2011). In females, old age starts at the beginning of menopause which generally occurs around 50 years of age. Whereas in males, old age begins after 70 years when total sperm volume, density, and output declines (Ng et al., 2004). During this time, sex hormones dramatically affect osteocalcin levels, particularly in menopausal females where there is a decrease in estrogen production (U.S. Department of Health and Human Services, 2004). Because estrogen naturally protects against bone loss, the cessation of estrogen production during menopause leads to increased bone remodeling where osteoblast production slows in comparison to osteoclast remodeling (Manolagas et al., 2002; Martin et al., 2015). Therefore, over time, a significant amount of bone mass is lost in elderly females (Ingram et al., 1994) and osteocalcin is decreased. Although bone mass decreases in elderly males, it is not as significant when compared to females (Ingram et al., 1994) because testosterone converts into estrogen which promotes continued bone formation (U.S. Department of Health and Human Services, 2004). Therefore, osteocalcin levels do not significantly change between adult and elderly males (Jung et al., 2016).

2.3.3 Activity and Osteocalcin Levels

There are two types of growth that are involved in long bone development; interstitial and appositional growth. Interstitial growth occurs at the epiphyseal plates which increase the length of long bones and stops when the growth plate closes (Black, 2016; Karaplis, 2008; Weaver et al., 2016). Appositional growth increases bone diameter by stimulating bone cell activity at osteogenic surfaces (i.e., periosteum and endosteum) (Black, 2016; Martin et al., 2015; Weaver et al., 2016). Unlike interstitial growth which

ceases after puberty has ended, appositional growth continues to occur throughout adult life and is stimulated when bone is biomechanically stressed from muscle activity (Mays et al., 2009; Sparacello et al., 2010). When individuals are active, force is exerted on the skeleton via muscles which stimulate the development of new osteoblasts to counteract the stress of the mechanical force (Alghadir et al., 2015). Therefore, active individuals will have denser bone and more osteocalcin compared to those who do not regularly exercise (e.g. Chahla et al., 2015; Kim et al., 2010). Typically, the mid–shaft thickness and density of long bones are commonly used as a proxy for biomechanically induced appositional growth in clinical (see Goldman et al., 2009; Rauch, 2005; Taaffe et al., 2003) and bioarchaeological studies (see Feik et al., 2000; Frisancho, 1970; Mays et al., 2009; Sparacello et al., 2010).

2.4 The Stress Response and How It Affects Osteocalcin Levels

2.4.1 The Stress Response

Stress is defined as an external stimulus (i.e., physical or psychological) which threatens the resting state of body homeostasis (Tsigos et al., 2002). When an external stressor is identified, the hypothalamus and pituitary axis of the brain activates which initiates the stress response to re–establish homeostasis (Tsigos et al., 2002). A central component of the stress response system is the glucocorticoid hormone that is synthesized and secreted by the adrenal glands of the kidneys (Aguilera, 2011; Herman and Cullian, 1997; Tsigos et al., 2002). During homeostasis, a consistent and regulated amount of glucocorticoids (i.e., homeostatic glucocorticoids) are released into the body which promotes bone health (Zhou et al., 2013). Homeostatic glucocorticoids stimulate osteoblast proliferation and differentiation which increases the number of osteoblasts

available to lay down new bone (Hadjidakis et al., 2006; O'Brien, 2004; Rauch et al., 2010). However, during stress response activation, increased levels of glucocorticoids are released into the body prompting physiological changes (i.e., increased heart rate, rapid breathing to increase oxygen intake, release of blood sugar and stored fat for energy) in order to mitigate the stressor and effectively terminate the stress response (Aguilera, 2010; Tsigos et al., 2002). However, if the stressor is chronic, the prolonged and elevated secretion of glucocorticoids is maladaptive and creates negative effects (i.e., increased risk of myocardial infarction, impairment of brain functioning, delayed immune response) which threaten the health of an individual (Aguilera, 2010; Herman and Cullinan, 1997; McEwen 1998).

2.4.2 Glucocorticoids and Their Effects on Bone and Osteocalcin

Elevated glucocorticoid levels also affect skeletal health. Indirectly, high levels of glucocorticoids suppress the secretion of pituitary growth hormone (GH) which is imperative for the healthy growth and activity of both osteoblasts and osteoclasts (Tsigos et al., 2002). Additionally, osteoblasts and osteoclasts have glucocorticoid receptors which are targeted by glucocorticoids (Manelli and Giustina, 2000). The glucocorticoid receptors mediate the effects of glucocorticoids on bone cells (Zou et al., 2017) but can also generate destructive cellular activity when they are over activated by chronically elevated levels of glucocorticoids (Moutsatsou et al., 2012).

There are three main direct effects that glucocorticoids have on bone. First, osteoclast activity increases through the promotion of cell maturation and survival which results in increased bone resorption (Manelli and Giustina, 2000; Rehman and Lane, 2003; Scott et al., 2016). Second, osteoblastic genesis and replication are inhibited

(Manelli and Giustina, 2000). Lastly, osteoblasts undergo premature programmed cell death (i.e., apoptosis) which decreases their activity (Hadjidakis et al., 2006; Manelli and Giustina, 2000; Scott et al., 2016; Tsigos et al., 2002). Overall, elevated glucocorticoids disrupt bone homeostasis (Manelli and Giustina, 2000; Rehman and Lane, 2003) and reduce bone mass through a combination of increased bone resorption and decreased bone formation. Therefore, osteocalcin will begin to degrade and levels will decrease as bone is undergoing resorption resulting from chronic stress.

While the overall long–term effect of elevated glucocorticoid levels is the suppression of bone formation (Schorlemmer et al., 2005), this effect differs between the two types of bone (i.e., cortical versus trabecular) and location within the skeleton (i.e., axial versus appendicular). Cortical bone is the compact, smooth covering found on the outer surfaces of all bones. Glucocorticoids promote cortical bone resorption by targeting osteogenic tissues (i.e., periosteum, endosteum) which leads to a porous bone surface and reduced cortical thickness (Ortoft and Oxlund, 1996; Schorlemmer et al., 2005). Trabecular bone is found in the ends of long bones and compressed inside the flat and irregular bones of the axial skeleton. Glucocorticoids have a more prominent effect on trabecular bone (Rehman and Lane, 2003) because they damage the structure and density of the trabeculae by reducing their thickness and the overall honeycomb structure of this bone type (Augat et al., 2003; Bouvard, 2013; Schorlemmer et al., 2005). With regards to skeletal location, the axial skeleton has greater overall bone loss due to its higher abundance of trabecular bone compared to the appendicular skeleton (Laan et al., 1993). Therefore, since there is variability in the effects of glucocorticoids on different bone types and locations, osteocalcin levels also secondarily affected.

2.5 Protein Research in Bioarchaeology

2.5.1 Ancient Protein Studies

There is a growing interest in osteocalcin in paleoproteomic research, specifically its extraction and viability in ancient bone. Specifically, ancient protein researchers are interested in the relationship between osteocalcin survivability and diagenesis. Diagenesis is the chemical exchange between bone and its surrounding burial environment (Beauchesne, 2012) which will ultimately lead to the partial or complete chemical destruction of bone (Brown, 2011; Kontopoulos et al., 2018). Factors that contribute to diagenesis are temperature, soil pH, and humidity (Buckley et al., 2008; Smith et al., 2005). Considering these diagenetic changes are important as diagenesis affects the mineral structure of bone (hydroxyapatite) where osteocalcin is stored (Collins et al., 2002). As the hydroxyapatite of bone deteriorates over time, so to can osteocalcin. Therefore, researchers have questioned if osteocalcin survivability is more strongly affected by geographical location or temporal period.

Smith and colleagues (2005) tested osteocalcin survivability across different geographic and temporal archaeological sites in Europe and discovered that osteocalcin could survive in remains from older sites (Upper Paleolithic) which indicates that future paleoproteomic studies will not be restricted temporally. The authors note that the age of the sample does not affect osteocalcin concentrations but the harshness of the burial environment does have an effect. Specifically, the oldest sample, a 53,000 year–old bone, had a relatively unaltered mineral state with high levels of osteocalcin compared to a Medieval sample that was poorly preserved. Collins and colleagues (2000) further explored this by testing whether osteocalcin could survive in wet and dry archaeological

contexts. They found that osteocalcin did survive in both conditions but was severely affected by the degree of mineral preservation, where poor mineral bone preservation saw a decrease in the survival of osteocalcin. Since diagenesis and osteocalcin content are intrinsically linked, it is impossible to interpret the quantity of osteocalcin in archaeological material without first knowing the rate of bone decomposition.

2.5.2 Protein Research in Bioarchaeology

Bioarchaeology–specific proteomics is still in its infancy; however, Tuross (1991) and Cattaneo and colleagues (1992) were the first to extract ancient proteins from human remains in a bioarchaeological context. Specifically, Tuross (1991) tested human rib fragments excavated from a 300–year–old burial site in South Dakota and Cattaneo and colleagues (1992) extracted protein from human remains of different temporal periods between 400 BCE and 1644 CE. These studies were significant because they were the first to successfully extract protein from human remains but were also the first to explore the potential link between ancient proteins and skeletal health. For example, Cattaneo and colleagues (1994) focused on protein production as it relates to pathology, specifically multiple myeloma (i.e., bone marrow cancer) finding that it was possible to use ancient proteins extracted from skeletal material to diagnose archaeological disease. Building on this preliminary work, Scott and colleagues (2016) recently extracted osteocalcin from femoral and clavicle samples from a 13th–17th century Danish population to see if fluctuations in protein concentrations may correlate with macroscopic evidence of skeletal stress. Osteocalcin was successfully extracted from all individuals and although patterns between age categories (i.e., osteocalcin decreased in older adults) and sex (i.e., females had decreased osteocalcin concentrations when compared to males) emerged,

their differences were not statistically significant. However, the authors did find a significant decrease in osteocalcin levels for those with chronic levels of degenerative bone changes (i.e., osteoarthritis) where bone remodeling would have been atypical. The significance of this research is that it introduced a new biochemical avenue of research where ancient proteins can potentially be tied to specific pathological processes macrospically visible in bone. Fundamentally, however, to move osteocalcin research forward in the bioarchaeology discipline, more needs to be known about osteocalcin concentrations in bone and how they are influenced naturally by biological factors and to what extent the stress response can truly be measured in these ancient proteins.

2.6 The Fortress of Louisbourg History

The Fortress of Louisbourg was a French colonial settlement located on the coast of Île Royale (Cape Breton Island, Nova Scotia) and continuously occupied for approximately forty–four years between 1713 and 1758 (Donovan, 1995; Johnston, 2004). The French established Louisbourg after their loss of Plaisance (Newfoundland) to the British after the Wars of Spanish Succession (Johnston, 1984). The relocated inhabitants from Plaisance, Newfoundland and other regions across Acadia formed the original population at the Fortress. In 1713, the population of the Plaisance colony relocated to Louisbourg and consisted of 116 men, 10 women, and 23 children (Donovan, 1995). Additionally, 67 Acadian families totaling 500 people re–located to Louisbourg between 1713–1734 (Donovan, 1995). Throughout its short history, Louisbourg operated as a military stronghold but was also the seat of the French cod fishing industry and generated tremendous wealth for the Crown by exploiting cod along the Grand Banks and becoming an important port along the transatlantic trade route (Fortin, 2000; Johnston,

1984). Due to its ideal location and wealth, the Fortress soon became a 18th century metropolis that operated as a major trading and shipment centre (Johnston, 1984). People flocked to Louisbourg from various urban European cities and other established French and New England colonies in North America (Johnston, 1984). During the Fortress's final year, 7,000 people permanently resided at Louisbourg and hundreds of merchants, fisherman, and sailors seasonally visited (Johnston, 1984). The Fortress of Louisbourg was besieged twice by British forces which inevitably resulted in its demise. The first siege occurred in 1745 at the hands of the New Englanders who occupied the Fortress thereafter until 1748 when the treaty of Aix–la–Chapelle was signed and saw the colony returned to the French (Donovan, 1995; Owen, 2001). The second and final siege led by the British was in 1758 which lead to the complete dismantlement of the Fortress of Louisbourg (Johnston, 2007). It is this rich history at Louisbourg that will provide the context for this thesis and the bioarchaeological analysis of bone protein.

2.7 Conclusion

This literature review focused on the bone protein osteocalcin and how biological factors (sex, age, activity) and stress (pathology) can influence its concentration. Although protein research is still within its infancy in bioarchaeology, the potential use of osteocalcin as an indicator of early biochemical stress is becoming more apparent. The complex relationship between osteocalcin and various influencing factors will directly inform this thesis research which will extract osteocalcin from archaeological individuals from the Fortress of Louisbourg skeletal collection.

CHAPTER 3 – Materials and Methods

3.1 Introduction

This chapter provides a contextual overview of the two skeletal collections from the Fortress of Louisbourg (Block 3 and Rochefort Point) used in this analysis. Also outlined are the methods used for osteocalcin analysis in archaeological bone, specifically sample extraction, total bone protein quantification (BCA assay), osteocalcin quantification (ELISA), and bone diagenetic testing (FTIR).

3.2 Permissions and Access to the Skeletal Collection

Permission to harvest bone powder from the Block 3 and Rochefort Point skeletal collections was granted by Dr. Amy Scott, Parks Canada, and the Roman Catholic Diocese of Antigonish, and the Anglican Diocese of Nova Scotia and Prince Edward Island. All steps were taken to minimize destruction of the bone during the sampling process. Bone sampling took place in the UNB Bioarchaeology Research and Teaching (BART) Laboratory with protein extraction and quantification completed at the Palaeoproteomics Laboratory at the Natural History Museum of Denmark, University of Copenhagen, Denmark under the supervision of Dr. Mathew Collins and Dr. Alberto Taurozzi. FTIR–ATR diagenetic testing analysis was completed in the UNB Chemistry Department under the supervision of Dr. Anna Ignaszak and mid–shaft cortical bone thickness x–rays were completed at Brookside Dental under the supervision of Dr. Adrienne Langille.

3.3 The Fortress of Louisbourg Skeletal Collection

3.3.1 The Block 3 Cemetery

Block 3 was the first formal cemetery established at Louisbourg in 1713 and was located next to where the parish church was to eventually be erected (Johnston, 1984). The Block 3 cemetery was used by residents from the original French population that immigrated to Louisbourg from Plaisance and Acadia. In 1723, the Block 3 cemetery was repurposed for residential expansion forcing a relocation of the burials to Block 34 (Johnston, 1984). During the modern reconstruction of the Fortress (1960s – 1970s), it was discovered that not all individuals had been successfully relocated to Block 34 (Harris, 1974). As a result, the remaining burials (n=26) from the original Block 3 cemetery were excavated and stored in the UNB BART lab for further study.

3.3.2. The Rochefort Point Cemetery

The Rochefort Point cemetery was the fourth community cemetery established at Louisbourg and was likely in use by 1738. After the first siege in 1745, New Englanders used this cemetery extensively over their four–year occupation period (Johnston, 1984). When the French returned to the Fortress in 1748, they continued to use Rochefort Point; however, likely just for soldier burials, choosing a different location to bury residents (Johnston, 1984). Excavation of the Rochefort Point cemetery began in 2017 in response to rapid and ongoing coastal erosion. To date, over 100 individuals have been excavated from the site and are currently stored in the UNB BART lab.

3.3.3 The Population Sample

From the Block 3 collection, 19 adult individuals were studied (13 males, 5 females, 1 undetermined sex) ranging between 17.5 and 45 years. From the Rochefort

Point collection, 8 adult male individuals were studied ranging between 16.5 and 35.5 years (see Table 1).

Sex was assessed and recorded based on os coxae morphology including the traits of Phenice (Buikstra and Mielke, 1985; Phenice, 1969), the greater sciatic notch (Milner, 1992), and sexually dimorphic cranial features (Acsadi and Nemeskeri, 1970) as outlined by Buikstra and Ubelaker (1994). Age at death was estimated based on pubic symphysis changes outlined by Todd (1921a, 1921b) and Suchey–Brooks (1990), and auricular surface changes outlined by Lovejoy et al. (1985). For young adult individuals, epiphyseal closure (Schaefer et al., 2009) and third molar eruption (Ubelaker, 1989) were also used for age estimation.

All individuals in this sample were macroscopically analyzed for stress lesions which were recorded as present or absent. Pathological processes (e.g., infection, degeneration) can impact bone health and more specifically, normal bone remodeling (Martin, 1991; Temple and Goodman, 2014). Because of the close association between osteocalcin production and bone metabolism, an assessment of pathological conditions allows for a preliminary exploration of the relationship between osteocalcin concentration and stress at the time of death. For this study, only pathological conditions which manifested in adulthood and were "active" at the time of death were assessed. Because bone is constantly being remodeled, any changes in osteocalcin levels as a result of childhood stress would be absent in adult bone. Therefore, evidence of childhood stress was excluded from this pathological analysis. The following pathological changes were assessed in this study: dental caries and abscesses, alveolar bone resorption, periosteal new bone formation, degenerative joint disease, trauma, and metabolic disease using the

criteria outlined by Brickley and McKinley (2004), Buikstra and Ubelaker (1994), Hillson (1996), Lovell (1997), Ortner and Eriksen (1997), and Roberts and Manchester (2010).

Table 1: Block 3 and Rochefort Point skeletal sample sex and age data

Site Location	Burial #	Provenience	Sex	Average age (years)
Block 3	Burial 1	3L17F6.1	Male	37.5
Block 3	Burial 2	3L5B6–1	Male	22.5
Block 3	Burial 6	3L5A6.2	Female	32.5
Block 3	Burial 7	3L19c5.1	Male	32.5
Block 3	Burial 8	3L6AB.1	Female	40
Block 3	Burial 10	3L5A7–2	Female	40
Block 3	Burial 11	3L5A8.1	Female	40
Block 3	Burial 12	3L17G6.1	Male	30
Block 3	Burial 13	3L17D5.1	Unknown Sex	17.5
Block 3	Burial 15	3L17E6.1	Possible Female	27.5
Block 3	Burial 16	3L6A9.1	Male	27
Block 3	Burial 17	3L17U6.1	Male	34.5
Block 3	Burial 18	3L17V4.1	Male	45
Block 3	Burial 19	3L17V5.1	Male	17.5
Block 3	Burial 20	3L17V6.1	Male	22.5
Block 3	Burial 22	3L20M2.1	Possible Male	30
Block 3	Burial 23	3L6N12.2	Male	37.5
Block 3	Burial 24	3L17H8.1	Possible Male	30
Block 3	Burial 25	3L17H9.1	Possible Male	30
Rochefort Point	Burial 1/2017	55L34A	Male	16.5
Rochefort Point	Burial 3/2017	55L34A	Male	32.5
Rochefort Point	Burial 5/2017	55L34A	Male	32
Rochefort Point	Burial 12/2017	55L34A/K	Male	24
Rochefort Point	Burial 14/2017	55L34B	Male	32
Rochefort Point	Burial 21/2017	55L34C	Male	30
Rochefort Point	Burial 28/2017	55L34D	Male	25.5
Rochefort Point	Burial 52/2018	55L34D	Male	35.5

3.4 Collecting the Bone Samples

Cortical bone was targeted over trabecular bone as it is more abundant, easier to collect, and easier to clean. Although trabecular bone is more sensitive to osteocalcin fluctuations (Rehman and Lane, 2003), accessing trabecular bone is more destructive and

there are further challenges with obtaining a clean trabecular sample. The distal posterior femur was selected for sampling as the site is not a prominent muscle attachment site, is relatively easy to access to sample, and preserves well archaeologically. Individuals were selected for sampling if the overall preservation of the femur (left or right side) was adequate (i.e., cortical bone present) and the sampling site was intact. All individuals from the Rochefort Point collection were sampled on both the left and right side to allow for osteocalcin comparisons between different sides of the body, unfortunately the Block 3 collection was too fragmentary for this left and right side comparison.

Bone powder was collected from a 1cm by 2cm surface area at the designated sampling site. The outer contaminated cortical bone was removed using a Dremel (model 300) and then the sample was collected from the cleaned cortical bone. The Dremel tip was sanitized using an ethanol flame to limit contamination between the initial cleaning phase and sample collection phase. In total, approximately 40μg of bone powder was harvested from each individual; two 10μg samples of bone power was used for protein analysis and 20μg of bone power was used for diagenetic FTIR–ATR testing.

3.5 Bone Sample Demineralization

All samples were demineralized producing a liquid substrate and pellet to be used for protein quantification. Ten percent weight/volume of Ethylenediaminetetraacetic acid (EDTA) was added to each bone sample to begin the demineralization process. The amount of EDTA added was proportional to the weight of each sample. Once the EDTA was added, each sample was oscillated on a tube rotator in a fridge (5°C) for 24 hours. After this 24–hour period, each sample was centrifuged for 10 minutes at 14,000g to separate the solid pellet from the liquid substrate.

3.6 Bone Sample Filtering

Each sample was then filtered with 1X phosphate–buffered saline (PBS) to remove the demineralization agent (EDTA) which interferes with the effectiveness of protein quantification. After demineralization, all samples contained 342mM EDTA (see Table 2) which had to be reduced to the acceptable limit of 6.1mM EDTA (see Table 3) as outlined in the ELISA manufacturer's instructions. All samples were filtered using Amicon Ultra–0.5 Centrifugal Filter Devices inside a filtering tube. 400µL of the liquid substrate was pipetted into the filter and centrifuged for 25 minutes at 14,000g reducing the sample volume to 50µL (342mM EDTA). For the first filtering of the samples, 450µL 1XPBS was pipetted into the filter and centrifuged for 25 minutes at 14,000g reducing the sample concentration to approximately 34.3mM EDTA. This was followed by a second dilution of 450µL of 1X PBS which was pipetted into the filter and centrifuged for 10 minutes at 14,000g reducing the samples to a final concentration of 3.43mM EDTA. Final samples were collected by centrifuging each filter upside down in a filter tube for 2 minutes at 1,000g. 1X PBS was added to each filtered sample to bring the total elution volume up from the original 50µL to 200µL. This filtered sample (200µL) was a 2X concentration from the original liquid substrate (400µL) and was used for osteocalcin and total bone protein quantification.

Table 2: moles of 10% w/v EDTA within demineralized sample prior to filtering

Moles of 10% EDTA = 10g / 292.24g/mol = 0.0342moles M = moles / liters of solution = 0.0342m/0.1L = 0.342M M = 0.342M or 342 mM

Table 3: Summary of filtering cycle to obtain samples that contain less than 6.1mM EDTA

Filtering/Dilution	Initial volume and mM	Dilution	Elution volume and mM
First Filtering	400µL of 342mM	-	50µL of 342mM
First Dilution	50µL of 342mM	450µL of 1X PBS	500µL of 34.2mM
Second Filtering	500µL of 34.2mM	-	50µL of 34.2mM
Second Dilution	50µL of 34.2mM	450µL of 1X PBS	500µL of 3.42mM
Third Filtering	500µL of 3.42mM	-	50µL of 3.42mM

3.7 Total Bone Protein Quantification

A total bone protein assay quantifies the concentration of all bone proteins present within a solution. This quantification step is important because the amount of osteocalcin will be understood in relation to the total bone protein of each sample. A preliminary study was conducted to establish the parameters of the protein quantification methods used (see Appendix 1). A bicinchoninic acid assay (BCA) ran standards and samples in triplicate for intra–observer error and to create a standard deviation. 10µL of the BCA standard was pipetted directly into the bottom of the corresponding standard wells. 5µL of each sample was also pipetted directly into the bottom of the corresponding sample wells. Since the samples were 2x concentrated after filtering, halving the amount of tested sample compared to the standard adjusts for the concentration difference. The BCA working reagent was prepared, mixed on a vortex mixer, and 200µL was pipetted into each BCA standard and sample well. The BCA well–plate was incubated on a hotplate at 37°C for 33 minutes covered with tinfoil to protect the samples from light. The BCA well–plate was measured at 560nm in a Multiscan ^{TM} FC Microplate reader.

3.8 Enzyme–Linked Immunosorbant Assay (ELISA)

3.8.1 Preliminary ELISA

The osteocalcin concentration from each bone sample was obtained using an Enzyme–Linked Immunosorbent Assay (ELISA). The ELISA is preferred because it is the standard method within paleoproteomic research, cost effective, requires minimal equipment, is easy to run, and is capable of detecting small amounts of protein (Cattaneo et al., 1992). Running samples neat (i.e., no diluent) would produce unreadable osteocalcin concentrations as these values would be too high and would fall above the standard curve. A preliminary test ELISA ran standards in triplicate and 9 samples in singlet at high (1:10) and low (1:100) concentrations (see Appendix 2) to determine the ideal concentration that all samples should be run at to ensure they would all fall within the standard curve and produce readable osteocalcin values. The 9 bone samples chosen for the preliminary test were based on predicted high and low osteocalcin concentrations determined from the macroscopic preservation of each bone sample. To prepare these dilutions, the filtered substrate was mixed with 1XPBS (diluent). 100µL of RD1–117 diluent was pipetted into each well followed by 50µL of the standard or sample. The well–plate was incubated on a microplate shaker (450 RPM) at room temperature (25°C) for two hours. During this time, any osteocalcin present in the samples bound to the pre–coded antibodies on the bottom of each well. The excess liquid from the well–plate was disposed of and each well was washed with 300µL of a 1X wash buffer four times to get rid of any other proteins and contaminates in the solution. After the last wash, 200µL of the human osteocalcin conjugate was added to each well and incubated on a microplate shaker (400 RPM) for two hours at room temperature (25°C). During this time, the

conjugate bounds to the exposed receptor of any osteocalcin molecules present. After these two hours the plate underwent four rounds of washing as previously discussed. 200µL of a substrate solution was pipetted into each well and placed on a benchtop for 38 minutes protected from light until colour change was complete. The substrate solution signaled osteocalcin presence by acting upon the enzyme which was attached to the conjugate antibody changing the well colour. Similar to the BCA assay, a darker colour corresponded to greater a concentration of osteocalcin. 50µL of the stop solution was pipetted into each well to stop the colour change from continuing. The colour change was quantified by measuring light absorbency using a 450nm–560nm 4 parameter logistic equation and a linear regression equation on a Multiscan ^{TM} FC Microplate reader. Based on the results of this preliminary analysis of nine samples (see Appendix 2), it was determined that a dilution of 1:30 would best capture all remaining samples. However, B3–24L at 1:10 had a low concentration of osteocalcin at 14.0ng/mL and was below the standard curve at the 1:100 dilution; therefore, both B3–24L and B3–24R were run at a 1:10 dilution.

3.8.2 Re–Assay ELISA

Of all the samples tested, only three needed to be re–assayed. B3–13R had a concentration of 3.4ng/mL which fit within the curve but was low and a dilution factor of 1:5 was predicted to give a more accurate reading. B3–24R at a dilution factor of 1:10 had a concentration above the top standard and could not be calculated. A dilution factor of 1:30 for B3–24R was predicted to obtain an osteocalcin concentration within the standard curve. RP–52L had poor agreement between replicates and was re–assayed at dilution factors of 1:10 and 1:30 (see Appendix 2). In addition to these three samples, 14

others were also run in this re–assay ELISA chosen based on their coefficient of variation (CV) percentage determined by the initial ELISA results. Samples with high CV percentages indicated that the precision and repeatability of the sample was poor and were therefore re–assayed. The same procedure outlined in 3.8.1 was used for the re–assay ELISA.

3.9 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared (FTIR) spectroscopy using the attenuated total reflectance (ATR) method was used to detect the amount of diagenetic change in each sample. Samples were run in triplicate for intra–observer error. Approximately 20μg of bone powder was placed on the sampling plate in contact with the diamond crystal of a Bruker Alpha || FTIR spectrometer. The evanescent waves at 565nm, 590nm, and 605nm were read in triplicate to calculate the infrared splitting factor outlined by Beasley and colleagues (2014) which reflects diagenetic change. Sterilization of the sampling plate, diamond crystal, and scoopula used to transfer the bone powder was completed between each sample using a 70% ethanol solution.

3.10 Cortical Bone Thickness Measurements

3.10.1 Mid–Shaft Femoral X–Rays

All 46 femora in the sample population had well–preserved femoral mid–shafts that were x–rayed in order to identify any relationship between osteocalcin and activity. The femora were placed on their posterior surface on a table with a #2 dentistry bite wing film underneath the mid–shaft of the bone, determined from a linear measurement of maximum femoral length. A Heliodent Plus intraoral x–ray unit was positioned above the anterior mid–shaft surface to capture both the medial and lateral periosteal edge and the

endosteal boundaries of the medullary cavity. The x–ray unit was set to 7 kVp (kilovoltage peak) and a 0.08 second exposure, standard settings based on the density of femoral cortical bone. Each x–ray was taken approximately 20cm from the x–ray beam to the anterior bone surface due to the standard cone length of the x–ray unit. The x–ray plate was digitally scanned using a Dentsply Sirona Xios Scan.

3.10.2 Cortical Bone Thickness Measurement

ImageJ (version 1.52) was used to measure the mid–shaft of the cortical bone with the 1.5mm triangle embedded on the x–ray film used as the scale. Both the medial and lateral cortical boundaries from the outer periosteal edge to the inner endosteal border were measured in triplicate. The medial and lateral measurements were averaged to produce a single cortical thickness measurement for each femur.

3.11 Conclusion

This chapter provided information on the Block 3 and Rochefort Point cemeteries to contextualize the sample populations used in this research. Additionally, this chapter provided an overview of the methods used to successfully extract bone protein from femoral bone samples, specifically BCA assay and ELISA methods. The FTIR–ATR method that was used in this research was also outlined as it relates to diagenetic change in bone. Lastly, the procedure for x–raying and measuring the mid–shaft cortical bone was discussed as an indicator of appositional growth.

CHAPTER 4 – Results

4.1 Introduction

The two equations used to generate osteocalcin data were the linear regression formula and the 4–parameter logistics equation. The linear regression formula may exclude osteocalcin concentrations that do not fall within the linear curve used for regression. However, the 4–parameter logistic equation uses a curved line which best fits all osteocalcin concentrations into the equation. Therefore, all the data presented in this chapter was generated from the 4–parameter logistics equation because it was the best fit model and produced the most reliable and accurate osteocalcin results. Further, osteocalcin values are reported as per total bone protein (ng/μg) rather than osteocalcin per total weight of bone sample (ng/mg) because the amount of bone collected for each sample was the same, whereas the surviving protein may be different across samples; therefore, presenting the osteocalcin data as per total bone protein (ng/μg) will better reflect osteocalcin variability. The osteocalcin values were first analyzed in relation to diagenetic change which can impact the survival of protein over time. Next, osteocalcin variability was examined between the left and right femora and in relation to biological factors including sex, age at death, and cortical bone thickness. Lastly, osteocalcin concentrations were analyzed alongside evidence of pathological conditions active at the time of death.

4.2 Intra–observer Error: Osteocalcin, Cortical Bone Thickness, and FTIR

Intra–observer error was calculated for the triplicate osteocalcin (ng/μg) values using a one–way ANOVA which tested for significant differences between the first, second, and third osteocalcin values collected. No significant differences were found,

indicating there was a strong agreement between the three values collected for each sample ($F(2,135) = 0.019$, $p = 0.981$). As a result, the triplicate osteocalcin values (ng/µg) were averaged to create one concentration that represented each femoral sample (see Table 5). When assessing intra–observer error for the medial and lateral cortical thickness measurements using a one–way ANOVA, there was a strong agreement between measurements (see Table 4). As a result, the medial and lateral measurements of each side were averaged to produce a single cortical thickness measurement for the left and right femora. Intra–observer error was also tested on the triplicate IR–SF values using a one–way ANOVA with no significant differences between values, indicating observer consistency ($F(2,135) = 0.001$, $p = 0.999$). As a result, the triplicate IR–SF values were averaged to produce one value to represent each femoral sample.

Table 4: One–way ANOVA test results for intra–observer error of cortical bone thickness measurements

Femora Side	Measurement Location on Mid–Shaft	df Between Groups	df Within Groups	f–value	p–value
Left Femora	Medial	2	60	0.001	0.999
	Lateral	2	60	0.005	0.995
Right Femora	Medial	2	72	0.005	0.995
	Lateral	2	72	0.001	0.999

4.3 Left Versus Right Femoral Osteocalcin Levels

There was similar variability between femoral sides amongst both the Block 3 and Rochefort Point individuals where the right femora (Block 3 = 6.78 ng/µg, Rochefort Point = 6.84ng/µg) had consistently lower osteocalcin values than the left femora (Block 3 = 8.97ng/µg, Rochefort Point = 8.59ng/µg). Despite this however, when using an independent samples t–test, no significant differences were found between the paired left and right osteocalcin values across the entire sample ($t = 0.627$, $df = 36$, $p = 0.535$).

Because femoral sides were not significantly different, the left and right values were averaged to produce a single osteocalcin concentration that represented each individual throughout the remaining analyses (see Table 5).

Table 5: Osteocalcin concentrations determined using the 4–parameters logistic equation output data

Burial # (n=27)	Left Femora (ng/µg)	Right Femora (ng/µg)	Femoral Average (ng/µg)
B3–1	7.15	8.90	8.03
B3–2	7.35	13.47	10.41
B3–6	16.97	11.98	14.47
B3–7	3.66	2.48	3.07
B3–8	4.65		4.65
B3–10	9.89		9.89
B3–11	13.69		13.69
B3–12	19.27	8.21	13.74
B3–13	1.80	0.32	1.06
B3–15	4.18	3.36	3.77
B3–16	19.13		19.13
B3–17	13.80	11.46	12.63
B3–18	7.19	5.26	6.22
B3–19		3.63	3.63
B3–20		2.59	2.59
B3–22	4.05		4.05
B3–23	14.20		14.20
B3–24	2.04	13.51	7.78
B3–25	3.42	3.02	3.22
RP–1	7.72	9.27	8.49
RP–3	17.17	6.93	12.05
RP–5	17.06	9.14	13.10
RP–12	7.19	6.42	6.81
RP–14	5.70	5.86	5.78
RP–21	3.78	8.67	6.22
RP–28	7.80	5.74	6.77
RP–52	2.30	2.68	2.49

4.4 Sex Versus Osteocalcin Levels

The Block 3 females had a higher osteocalcin average (9.30ng/µg) but a smaller range of variation (3.77ng/µg – 14.47ng/µg) compared to the Block 3 male average (8.36ng/µg) and range of variation (2.59ng/µg – 19.13ng/µg). However, when comparing the Block 3 male and female osteocalcin values using an independent samples t–test, there were no statistical differences between osteocalcin values and the sexes ($t = 0.34$, $df = 16$, $p = 0.738$). The Rochefort Point males had an average osteocalcin concentration of 7.72ng/µg and a range of variation between 2.49ng/µg to 13.10ng/µg. Since the Rochefort Point sample was only males, no sex–specific statistical tests could be completed. When the Block 3 and Rochefort Point male osteocalcin values were compared using an independent samples t–test, there were no statistically significant differences in osteocalcin values between the males in these two groups ($t = 0.307$, $df = 19$, $p = 0.762$). As a result, the Block 3 and Rochefort Point males were combined for any further sex–specific analyses throughout the thesis.

4.5 Age at Death Versus Osteocalcin Levels

Since the Block 3 and Rochefort Point sample sizes were small, the age at death ranges were inadequate to perform statistical analysis. When divided by sex, females had a slight trend for osteocalcin to increase with age while male osteocalcin levels remained more stable throughout adulthood (see Figure 1).

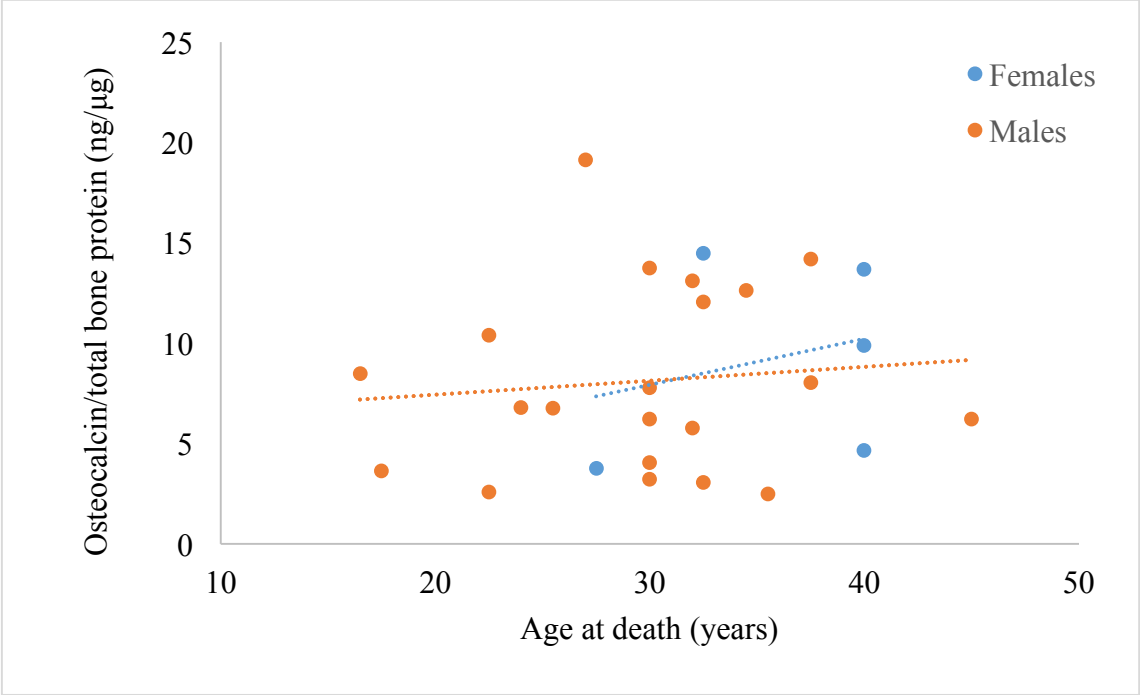

Figure 1: Block 3 and Rochefort Point osteocalcin concentrations by age at death and sex. Both female (R^2 = 0.07) and male (R^2 = 0.01) display weak positive correlations

4.6 Mid–shaft Cortical Bone Thickness Measurements Versus Osteocalcin Levels

The mid–shaft cortical bone thickness measurements for all femora used in this study are provided in Table 6. The left femora cortical thickness average was 6.92mm and ranged between 4.01mm and 9.78mm whereas the right femora cortical thickness average was 6.57mm and ranged between 4.43mm and 9.03mm. When compared using an independent samples t–test, there were no significant differences in cortical bone thickness between the paired left and right sides (t = 0.599, df = 36, p = 0.553). Therefore, the medial and lateral mid–shaft cortical thicknesses measurements of both the left and right were averaged to produce a single measurement to represent each individual throughout the remaining analyses (see Table 6).

Table 6: Block 3 and Rochefort Point mid–shaft cortical bone thickness measurements from the left and right femora

Burial # (n=27)	Left Femora (mm)	Right Femora (mm)	Difference (mm)	Left and Right Averaged Value (mm)
B3–1	4.51	5.93	1.41	5.14
B3–2	4.01	4.43	0.42	5.88
B3–6	5.36	6.40	1.04	5.48
B3–7	6.95	4.95	2.00	8.92
B3–8		5.48		6.87
B3–10		8.92		6.56
B3–11		6.87		5.22
B3–12	6.38	5.74	0.64	4.22
B3–13	4.76	5.52	0.77	5.95
B3–15	6.57	6.56	0.01	6.06
B3–16		6.03		6.03
B3–17	5.62	7.08	1.47	6.35
B3–18	7.04	6.21	0.83	6.63
B3–19		6.62		6.62
B3–20		5.40		5.40
B3–22	6.20			6.20
B3–23	6.18			6.18
B3–24	5.68	5.95	0.27	5.82
B3–25	5.59	7.02	1.43	6.30
RP–1	6.00	6.20	0.19	6.10
RP–3	6.59	7.13	0.53	6.86
RP–5	9.78	8.04	1.74	8.91
RP–12	7.12	7.47	0.35	7.30
RP–14	7.07	7.43	0.36	7.25
RP–21	7.98	9.03	1.05	8.50
RP–28	6.84	7.32	0.49	7.08
RP–52	6.56	6.44	0.12	6.50

Amongst females, the average mid–shaft cortical thickness was 6.74mm and values ranged between 5.48mm to 8.92mm. Males had a lower cortical thickness average of 6.45mm but a larger range in variation between 4.22mm and 8.91mm. When the mid–shaft cortical thickness of males and females were compared using an independent samples t–test, no significant differences were found between the sexes ($t = 0.538$, $df =$

24, p = 0.595). Due to the small age at death range, no statistical analyses could be performed; however, when plotting age against cortical thickness there was a very slight correlation between increased cortical thickness and age (see Figure 2).

Figure 2: Scatterplot graph of the Block 3 and Rochefort Point mid–shaft cortical bone thickness measurements plotted against age at death displaying a weak positive correlation ($R^2 = 0.06$)

When comparing osteocalcin concentrations with cortical thickness, using a Pearson's correlation test, there was a low degree of correlation (r = 0.123, df = 25, p = 0.542). When plotted against one another, there was a slight increase in osteocalcin levels as mid–shaft cortical bone thickness increased as expected (see Figure 3).

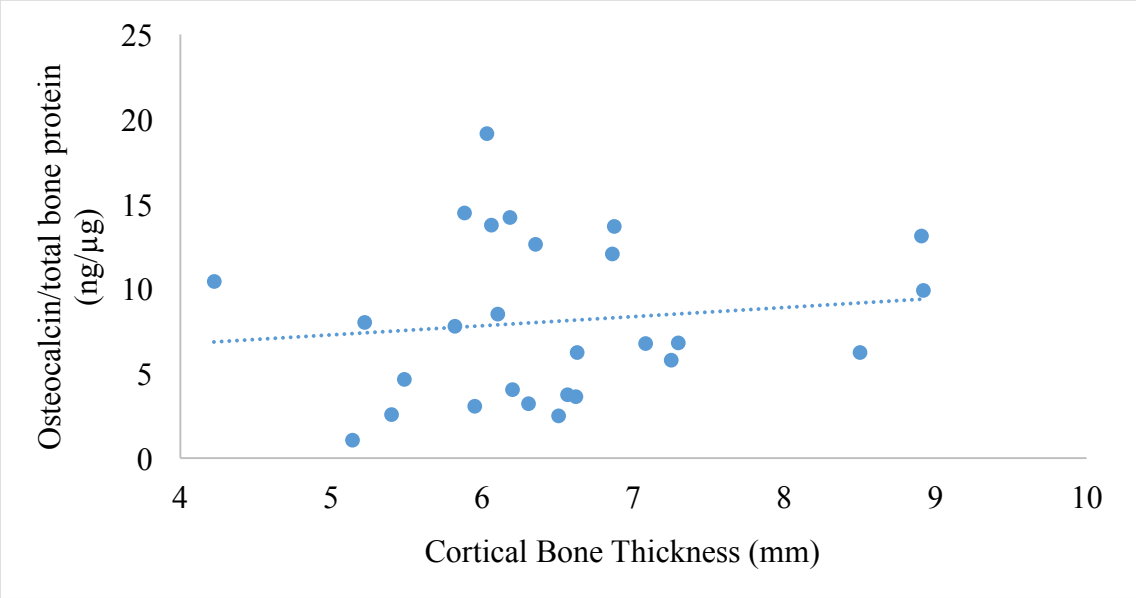

Figure 3: Scatterplot graph of the Block 3 and Rochefort Point mid–shaft cortical bone thickness measurements plotted against osteocalcin concentrations ($R^2 = 0.015$)

4.7 Pathological Condition Versus Osteocalcin Levels

Table 7 summarizes the presence or absence of each pathological condition (outlined in Chapter 3, section 3.3.3) assessed in this study. The most common pathology present was dental caries which were evident among16 individuals, periosteal reaction affected nine individuals, alveolar resorption was present in five individuals, degenerative joint disease was present among four individuals, abscesses were found among three individuals, and two individuals presented evidence of metabolic disease. A series of independent samples t–tests found no significant differences in osteocalcin levels (ng/µg) between individuals with each specific pathological condition and those without (see Table 8). However, there was a significant difference in osteocalcin levels between individuals with and without evidence of metabolic disease ($t = 2.249$, $df = 25$, $p = 0.035$). This difference will be further discussed in Chapter 5, section 5.5.1 but it is most likely a result of the small sample size (n=2) of individuals with metabolic disease and

their combined high osteocalcin average (14.77ng/µg) compared to the osteocalcin average of individuals without metabolic disease (7.53ng/µg).

Table 7: Pathological conditions present amongst the Block 3 and Rochefort Point individuals. 1 indicates present, 0 indicates absent

Burial #	Caries	Alveolar Bone Resorption	Abscess	Periosteal new bone formation	Degenerative Joint Disease	Metabolic Disease
B3–1	1	1	1	1	1	0
B3–2	1	0	0	0	0	1
B3–6	0	0	0	0	0	0
B3–7	1	1	0	1	0	0
B3–8	1	0	0	0	0	0
B3–10	1	0	0	0	0	0
B3–11	0	0	0	0	0	0
B3–12	1	0	1	1	0	0
B3–13	0	0	0	0	0	0
B3–15	0	0	0	0	0	0
B3–16	1	0	0	0	0	1
B3–17	0	0	0	0	0	0
B3–18	1	0	0	1	0	0
B3–19	1	0	0	0	0	0
B3–20	0	0	0	0	0	0
B3–22	0	0	0	0	0	0
B3–23	0	0	0	0	0	0
B3–24	0	0	0	0	0	0
B3–25	0	0	0	0	0	0
RP–1	1	0	0	1	0	0
RP–3	1	0	0	0	1	0
RP–5	1	1	0	0	0	0
RP–12	0	0	0	0	0	0
RP–14	1	0	1	1	0	0
RP–21	1	1	0	1	1	0
RP–28	1	1	0	1	1	0
RP–52	1	0	0	1	0	0
TOTAL	**16**	**5**	**3**	**9**	**4**	**2**

Table 8: The results of the independent samples t–tests that compared osteocalcin values amongst individuals with against those without a specific pathological condition

Pathological Condition	t–value	df	p–value
Caries	0.37	25	0.715
Alveolar Resorption	0.328	25	0.746
Abscess	0.427	25	0.673
Periosteal reaction	1.028	25	0.314
Degenerative Joint Disease	0.088	25	0.93
Metabolic Disease	2.249	25	0.034*

* p–value significant at a 95% confidence interval

Overall, individuals with pathological lesions present at the time of death had an average osteocalcin concentration of 8.36ng/ug, with a wide range of variation between 2.49ng/ug to 19.13ng/ug. Similarly, individuals with no evidence of pathological lesions present at the time of death had an average osteocalcin concentration of 7.66ng/ug, but a smaller range of variation between 1.06ng/ug to 14.47ng/ug. An independent samples t–test showed no significant differences in osteocalcin concentrations between those with and without pathological conditions present at the time of death ($t = 0.37$, $df = 25$, $p = 0.715$).

4.8 FTIR–ART Versus Osteocalcin Levels

When the IR–SF values of the paired femora were compared in using an independent samples t–test, no significant differences were found between the left and right sides ($t = 0.599$, $df = 36$, $p = 0.553$). Therefore, the three left IR–SF values and the three right IR–SF values were averaged together to create a single value to represent each individual throughout the remaining thesis. The averaged IR–SF values from the FTIR–ATR output ranged from 3.30 to 4.33 amongst the Block 3 and Rochefort Point samples. When osteocalcin levels were compared to IR–SF values using a Pearson's correlation, there was a low degree of correlation ($r = 0.337$, $df = 25$, $p = 0.086$). Although diagenetic

change had occurred as values greater than 3.4 are generally considered degraded to some extent (see Beasley et al., 2004; Trueman et al., 2008), most individuals show a similar level of diagenetic change allowing for a cross–comparative analysis of osteocalcin values (see Figure 4). The variability which does exist could be the result of differential diagenetic change or could reflect true differences in osteocalcin levels influenced by intrinsic (i.e., sex, age) or extrinsic factors (i.e., pathology, activity).

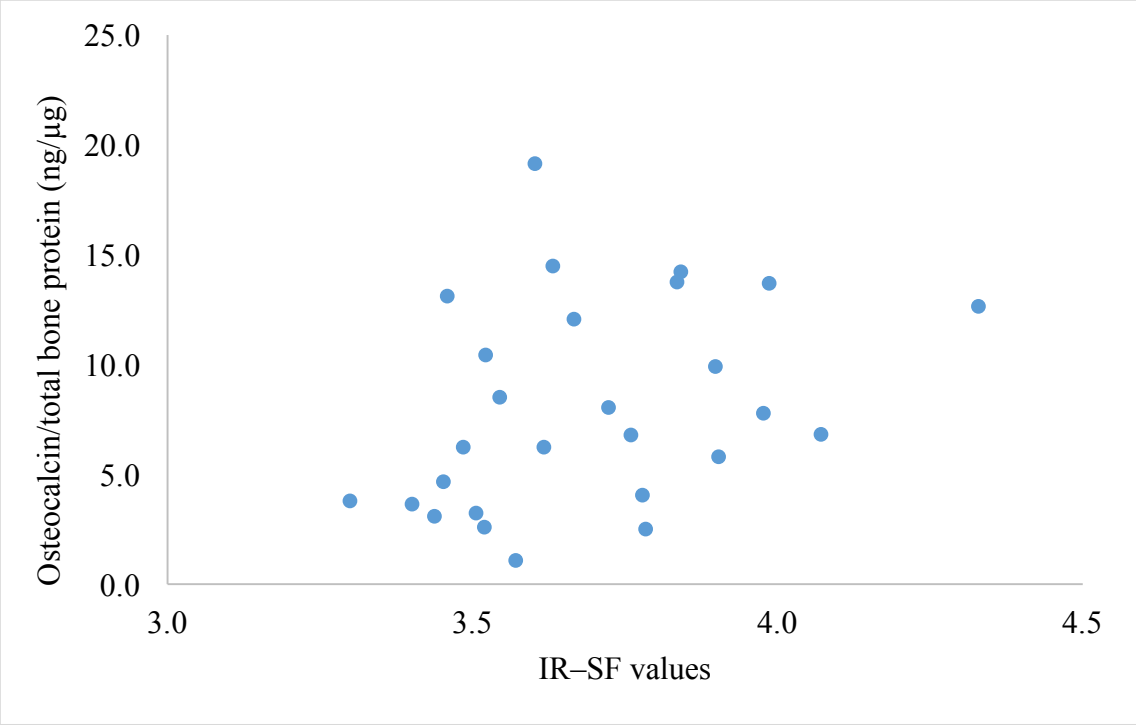

Figure 4: Scatterplot graph of the Block 3 and Rochefort Point IR–SF values plotted against osteocalcin concentrations

4.9 Conclusion

Osteocalcin was successfully extracted from all femoral bone samples from the Block 3 and Rochefort Point individuals. Although diagenetic change had occurred, it was similar across all samples and did not dramatically affect the survival of bone protein. Overall, there were no statistically significant differences in osteocalcin levels when compared across the left and right femora, between the sexes, age at death, or mid–

shaft cortical bone thickness. Similarly, pathological lesions active at the time of death were also not statistically associated with fluctuations in osteocalcin concentrations.

CHAPTER 5 – Discussion

5.1 Introduction

This chapter interprets the bone protein results of this thesis by contextualizing and comparing this quantitative analysis to archaeological and clinical studies. This discussion will begin by evaluating osteocalcin values in tandem with biological factors to discuss any existing patterns within the data and how this research compares to known clinical relationships. Also, osteocalcin will be discussed in relation to evidence of macroscopic skeletal pathology in order to identify any potential relationships with disease processes that have affected the skeletal tissue of those from 18th century Louisbourg. Next, there will be an explanation of the FTIR data as a reflection of diagenetic change and how the preservation of the skeletal remains may have impacted bone protein survival. The chapter concludes with a discussion of the methodological and interpretive limitations of this research.

5.2 Osteocalcin Concentration as it Relates to Footedness

As expected, the bone protein results showed no significant differences in osteocalcin concentrations between the left and right femora. Clinical studies have demonstrated the existence of a dominant and non–dominant leg, where the dominant leg is used primarily in mobilization (i.e., start walking) and the non–dominant leg is used for stabilization and strength tasks (Vaisman et al., 2017; VanMelick et al., 2017; Velotta et al 2011). This is supported by muscle strength studies which have found that the dominant leg has stronger leg extensor muscles and the non–dominant leg has stronger leg flexor muscles (Chhibber and Singh, 1970; Lanshammer and Ribom, 2011). However, clinical studies have tested the bone mineral density between the dominant and

non–dominant lower limb and have found no significant differences (see Faulkner et al., 1993; Nazarian et al., 2010; Rao et al., 2000; Yang et al., 1997). Archaeological studies on footedness remain limited but Macho (1991) found no differences in femoral length between the lower limb sides. Although footedness exists and the dominant and non–dominant lower limb have different roles in mobility, strength, and stability, the femur is biomechanically stressed equally between left and right sides. Therefore, the equivalent osteocalcin concentrations between femoral sides in this research was expected.

Although statistically insignificant, there were three outliers that had notable osteocalcin differences between femoral sides (B3–12, B3–24, RP–3). These disparities could have been a result of variable diagenetic change, however, the IR–SF values were within a comparable range (see Table 9). This variability between sides could have been due to different artificial preservation practices that were employed after excavation. For example, the Block 3 individuals were covered in shellac, a chemical varnish, in order to preserve the remains. However, not all of the Block 3 individuals showed this disparity between left and right osteocalcin values despite all being similarly covered in varnish post–excavation. Since osteocalcin can be used as a measure of bone metabolism, these side differences may be the result of underlying biological factors such as biomechanical stress. Although footedness does not affect bone mineral density as previously discussed, there may have been unequal biomechanical loading applied to the femoral sides that induced asymmetrical bone production which disproportionately impacted the deposition of osteocalcin. Similarly, underlying pathological factors that were not macroscopically visible on the sampling site may have impacted bone homeostasis and affected these osteocalcin values. Lastly, depending on the depth of the cortical bone where the sample

was taken, differential protein levels may be representing different timelines of deposited osteocalcin. It is assumed that each cortical bone sample was harvested at a similar depth to represent a consistent timeline of bone turnover across both the left and right femora. However, depending on the depth of the sample and preserved cortical bone thickness, the osteocalcin concentrations captured may be representing different timelines of deposited osteocalcin, thus introducing these drastically different concentrations between the left and right side.

Table 9: Three outliers that have femoral differences in osteocalcin concentrations

Burial # (n=3)	Left Femur (ng/µg)	Right Femur (ng/µg)	Difference (ng/µg)	IR–SF Values
B3–12	19.27	8.21	11.07	3.83
B3–24	2.04	13.51	11.46	3.98
RP–3	17.17	6.93	10.24	3.67

5.3 Osteocalcin Concentration as it Relates to Biological Sex and Age

As presented in Chapter 4 section 4.4 and 4.5, there were no significant differences in osteocalcin concentrations between males and females and osteocalcin values also remained static with increasing age. This lack of a clear relationship between osteocalcin values and age and sex may be due to the small sample size. Individuals with very high or low osteocalcin concentrations may be skewing the osteocalcin trends and could be inhibiting the visibility of any preliminary patterns. Similar statistical insignificance can be seen in other bioarchaeological studies that compare osteocalcin concentrations to sex and age. Vanderschueren and colleagues (1990) extracted osteocalcin from 57 males and 63 females ranging between 19 and 90 years of age and found no significant differences between the sexes. However, the males in their study had higher osteocalcin averages than females and levels declined as age increased amongst both sexes. Arguably, these trends are tied in part to this increased sample size; however,

the wide age distribution across the sample also allowed for age–specific patterns to emerge. Scott and colleagues (2016) extracted osteocalcin from the clavicle and femur of nine females and 11 males. Similar to this thesis, the authors had a small sample that consisted of a limited age range (18 to 55 years) and found no significant differences between the sexes and age groups. However, females had lower osteocalcin values than males and osteocalcin decreased in older adults. The authors suggest that the relationship between estrogen and glucocorticoids affected osteoblastic functioning which resulted in females having lower osteocalcin levels (see Chapter 2, section 2.4.2). Similarly, Scott and colleagues (in review) extracted osteocalcin from the femur from eight adult females and 22 adult males. The authors found no significant differences in osteocalcin concentrations between the sexes and females had a slightly higher osteocalcin average (186ng/µg) than males (181ng/µg). This study is the most alike to the current thesis in terms of sample population (size and demography), sampling site, and sex–related osteocalcin patterns. However, Scott and colleagues (in review) also report a decline in osteocalcin levels amongst older adult females which was not the pattern that emerged in this research.

Clinically, blood serum osteocalcin has been extracted to test for trends between age and sex groups. It is important to understand that increased serum osteocalcin concentrations indicate greater bone resorption and decreased matrix osteocalcin levels. Although serum osteocalcin cannot be extracted from archaeological remains, matrix osteocalcin should be able to be used as a proxy to understand serum osteocalcin trends in archaeological populations. Kasai and colleagues (1993) and Jung and colleagues (2016) extracted serum osteocalcin from participants of a large age range (0 to 80 years)

and did not find any significant differences between adult males and females. Osteocalcin levels remained stable throughout adulthood but began to decline in older adults (>50 years). A common theme across these discussed studies is that osteocalcin levels decrease as age increases which can be attributed to the natural slowing of bone formation with age. Although this pattern did not emerge in the current research, it is likely due to the small age range of the Louisbourg individuals (16.5 to 45 years) and the lack of older adults within the sample population, a common issue in bioarchaeological research (Agarwal, 2016; Cave and Oxenham, 2016; Welinder, 2001). Similarly, a significant difference in osteocalcin values between males and females was expected based on clinical examples of this sex–specific pattern (Hannemann et al, 2013; Rauchezauner et al., 2007; Resch et al., 1993), however, this expected sex–specific patterning does not seem to emerge consistently across all bioarchaeological and clinical studies. This trend may in part be influenced by the difficulty in obtaining osteocalcin values that reflect accurate biological levels in both serum and matrix samples. Within a clinical context, serum osteocalcin levels fluctuate throughout the day and are 15% higher in the morning compared to the afternoon (Cundy et al., 2014). Consequently, serum osteocalcin levels will vary depending on the time of extraction and may not accurately represent overall levels that are clearly associated with biological factors. Similarly, extracted osteocalcin from bioarchaeological remains produces a snap–shot of protein levels at the time of death and factors associated with death and diagenetic change after body deposition may be distorting any relationship between biological factors and matrix osteocalcin values.

Despite this lack of a clear statistical relationship in this study, two interesting patterns emerged when looking at osteocalcin values across age and sex groups: male

osteocalcin levels were stable throughout adulthood and females had a higher osteocalcin average than males. It was expected that the Louisbourg males would have stable osteocalcin levels throughout adulthood because they have reached peak bone mass and skeletal maturity which sees the skeleton focus primarily on bone maintenance and homeostasis (Hannemann et al, 2013). Static osteocalcin levels for males in adulthood have been observed in the previously discussed bioarchaeological (Scott et al., 2016; Scott et al., in review; Vanderschueren et al, 1990) and clinical studies (Hannemann et al., 2013; Jung, 2016; Kasai, 1993). Therefore, the stable osteocalcin results amongst Louisbourg males align well within the previously established literature and importantly demonstrates the comparability between serum and matrix osteocalcin values and how clinical studies are an accurate foundation on which to build emerging bioarchaeological research focused on this protein. Although statistically insignificant, the slightly higher female osteocalcin averages compared to males could be due to the protective role of estrogen on bone. Estrogen affects the activity of both osteoblast and osteoclast cells. Estrogen leads to increased bone formation by stimulating the proliferation and activity of osteoblast cells while also inhibiting the formation and activity of osteoclast cells (Hadjidakis et al., 2016; Kini et al., 2002; Manolagas et al., 2002). However, during menopause there is a loss of estrogen which promotes destructive osteoclastic activity causing a disruption of bone remodeling equilibrium (Christenson, 1997; Manolagas et al., 2002). Amongst the Louisbourg females, the distinction between pre and post–menopause is important to identify in order to suggest that estrogen is influencing osteocalcin levels. The age at which menopause begins has remained relatively consistent over time. Historical written documentation has recorded that menopause begins around

50 years which is supported by bioarchaeological studies that have found age–related cortical bone changes in adult females over 50 years in archaeological populations (see Mays et al., 2000; Singh et al., 2002; Pavelka et al., 1991). This suggests that the menopausal experience is similar amongst females in past and contemporary populations. Since the Louisbourg females within this study are between the ages of 27 and 40 years, they were most likely not experiencing menopausal changes and their estrogen levels would not have been depleted. Therefore, the high osteocalcin levels amongst the Louisbourg females could be a result of protective estrogen. Although no bioarchaeological studies currently exist which support this relationship between estrogen and osteocalcin, there are a number of clinical studies that have found statistically lower serum osteocalcin levels (high matrix osteocalcin) in pre–menopausal females compared to males of the same age group and post–menopausal females (Hannemann et al., 2013; Resch et al., 1993). However, it is important to consider that within these modern contexts, participants may have been on medication (i.e., contraceptives, calcium supplements, and hormone replacement therapy) that could have influenced serum osteocalcin levels that would not have been available to the individuals living in 18th century Louisbourg (Chiu et al, 1999; Hannemann et al., 2013).

5.4 Osteocalcin Concentration as it Relates to Cortical Bone Thickness

When looking at cortical bone thickness as a proxy of activity (see Chapter 2, section 2.3.3), there were no significant differences between the left and right side as expected. While fluctuating asymmetry may reflect periods of stress (see Dare et al., 2019; Doyle et al., 1977) and can sometimes be associated with different limb lengths (Rauch, 2005), cortical thickness does not seem to be affected. As expected, cortical bone

thickness remained stable as age increased. All the Louisbourg individuals were in adulthood and had previously underwent developmental growth spurts which have an effect on the thickness and length of long bones, particularly the mid–growth spurt (six to eight years) which impacts appositional growth (i.e., cortical thickness) (Bogin, 2001; Lejarraga, 2002). Contrarily, it was unexpected that there were no significant differences in cortical bone thickness between males and females. The skeleton is inherently sexually dimorphic, and it has been demonstrated that the femoral mid–shaft diameter and cortical thickness is statistically larger in males than females (e.g., Black, 1978; Feik et al., 2000; Peacock et al., 2009; Wescott, 2006). The lack of difference in cortical thickness amongst Louisbourg males and females could be a consequence of the limited sample size (n=27) compounded by the small number of females (n=5) within the study. In particular, the B3–10 female had the widest cortical measurement (8.92mm) amongst all the samples which dramatically increased the female range and average. As a result, the relationship between cortical bone thickness and sex may be impacted by the sample bias of the study. When considering the combined influence of sex and age on cortical bone thickness, it is important to discuss occupational roles at Louisbourg. When an individual begins a labour–intensive occupation, their skeleton will adjust to the arduous workload and compensate by stimulating bone growth (Mays et al., 2009; Sparacello et al., 2010). Over time, the skeleton will become denser and more robust as it becomes accustomed to daily biomechanical loading (Alghdair et al., 2015). During this time period, the individuals sampled were of working age and may have been employed for some time before death (Donovan, 1985). As a result, the skeletal structure of these individuals would have adjusted to these increased occupational demands over time, likely resulting in this

similar cortical bone thickness despite their age differences.

Further, the roles of men and women living in the French colonies were not as tightly regulated as they were in France because survival in the New World outweighed social and gender roles in regard to occupation. This would be especially true for women where restricted independence in mainland France was never fully enforced in the colonies (Norton, 1984) and women therefore, had the opportunity to engage in roles that were previously unattainable (MacInnes, personal correspondence, 2020; Mays, 2004). At Louisbourg, working women would have predominantly occupied domestic roles including wives, servants, dressmakers, or laundresses (Crowley, 1990). These occupations would have largely used upper body strength and would not have induced enough lower limb biomechanical stress to impact appositional growth of the femoral midshaft. Conversely, Louisbourg males fulfilled a variety of labour–intensive roles including fishing, sailing, and labourer–soldiers (Crowley, 1990; Johnston, 1984; Pitcher 2014). Similar to the females, these arduous occupations would have primarily engaged the upper body and may not have affected the musculature in the lower limbs (see Mirka et al., 2005). The work men and women performed at the Fortress would not have resulted in significant differences in cortical thickness between the sexes because the lower body was not predominately engaged in common place work of either men or women.

Based on the lack of relationship between cortical bone thickness and sex or age, it was also expected that there would be no relationship with osteocalcin (see Chapter 4, section 4.6). Simply put, activity, as determined from cortical bone thickness, is the same amongst Louisbourg males and females and as a result there is no relationship between

activity and osteocalcin levels in this study. Unfortunately, there are currently no bioarchaeological or clinical studies that have explored the relationship between matrix osteocalcin and physical activity. Clinical studies have investigated serum osteocalcin levels in relation to physical activity, but they are generally in the context of overall body health and not skeletal appositional growth. As well, it is important to understand that clinical studies can only evaluate overall serum levels reflecting generalized skeletal turnover and cannot isolate osteocalcin entering the bloodstream from a particular skeletal element. For example, Rochefort and colleagues (2011) extracted serum osteocalcin from 27 participants of whom half underwent a physically activity regime and the other half did not. The authors found elevated serum osteocalcin levels amongst the participants who were physically active compared to the inactive individuals. The authors suggested that the elevated serum levels were a result of greater bone turnover and formation in response to activity. Similarly, Chahla and colleagues (2015) extracted serum osteocalcin from 54 participants of whom engaged in various degrees of daily physical exercise. The authors found elevated levels of serum osteocalcin amongst those who participated in moderate to high levels of physical activity compared to low level physical activity indicating greater bone turnover in response to physical activity. As well, Fernández–Real and colleagues (2009) tested the serum osteocalcin levels of 195 participants in three cross–sectional studies and found that regular physical activity increased serum osteocalcin levels. The authors suggested that exercise acting on the skeleton stimulated bone turnover and resulted in the increased release of osteocalcin into the bloodstream. These clinical studies do suggest that osteocalcin can be affected by physical activity, however, at Louisbourg, the lack of relationship between cortical bone

thickness across sex and age categories suggests that activity is either not an influencing factor to the observed osteocalcin variability at the Fortress, or that cortical bone thickness is not a strong enough indicator of overall activity patterns to affect matrix osteocalcin levels.

5.5 Osteocalcin Concentration as it Relates to Skeletal Pathological Conditions

As previously discussed in Chapter 2, section 2.4.2, chronic glucocorticoid levels secreted in response to an external stressor can negatively impact osteoblastic activity which affects osteocalcin levels (see O'Brien, 2004; Manelli and Giustina, 2000; Rehman and Lane, 2003; Schorlemmer et al., 2005). Within clinical studies, synthetic glucocorticoid treatment is often used to understand the relationship between stress hormones and serum osteocalcin. Although synthetic glucocorticoids are slightly different than endogenous glucocorticoids produced naturally by the body, these studies have demonstrated a clear relationship; increased synthetic glucocorticoids result in decreased serum osteocalcin levels. Cooper and colleagues (2005) studied 135 women and 171 men who had their serum osteocalcin levels tested before and after glucocorticoid treatment. The authors found that serum osteocalcin levels had decreased when glucocorticoid treatment was complete suggesting that chronic glucocorticoid levels suppressed osteoblastic activity which decreased the secretion of serum osteocalcin into the bloodstream. Similarly, Sasaki and colleagues (2001) studied 12 patients that underwent glucocorticoid therapy for the first time as a treatment to renal disease. The authors measured serum osteocalcin levels before and after treatment and found that osteocalcin levels decreased significantly resulting from the negative impact of administered glucocorticoids on bone formation. Clinically, there is a well–reported link

between osteoporosis (a medical condition in which bone mineral density decreases due to hormonal changes with age) and high serum osteocalcin levels (see Aonuma et al., 2009; El–Dorry et al., 2015; Manelli and Giustina, 2000; Sasaki et al., 2001). For example, Singh and colleagues (2015) studied 82 females who were divided into either a control group (i.e., osteoporosis absent) or a case group (i.e., osteoporosis present) and both groups had their bone mineral density and serum osteocalcin levels measured. The authors found that females with osteoporosis had significantly higher serum osteocalcin levels compared to the control group due to increased bone resorption. Throughout these clinical studies, it is widely accepted that osteoblastic functioning is affected by glucocorticoid treatment therapy whereby making osteocalcin a useful proxy to understand changing glucocorticoid levels in the body. It was expected that the Louisbourg individuals with evidence of skeletal pathology would have lower osteocalcin levels compared to those with no evidence of pathology as shown in the clinical literature. However, the opposite was true where individuals with skeletal lesions had an average osteocalcin concentration of 8.36 ng/µg and a wide range of variation between 2.49 ng/µg to 19.13 ng/µg compared to those without evidence of skeletal pathology with an osteocalcin average of 7.66 ng/µg and a smaller range of variation between 1.06 ng/µg to 14.47 ng/µg. Despite these visible trends, they were no statistical differences between those with and without skeletal lesions present; however, this lack of relationship may in part be associated with combining both proliferative and erosional pathological conditions in this assessment.

5.5.1 Osteocalcin Concentration as it Relates to Specific Pathological Conditions

Data pertaining to the relationship between specific pathological conditions that manifest in bone and corresponding matrix osteocalcin levels is scarce as most clinical studies use serum osteocalcin as a marker of bone health. Hauscka and colleagues (1989) provided a comprehensive clinical overview detailing how serum osteocalcin levels relate to pathological conditions which affect bone. Compared to healthy standards, serum osteocalcin levels were decreased amongst individuals whom had hormonal deficiencies (i.e., hypothyroidism), liver disease, and some cancers (see Lee et al., 2000) which Hauscka and colleagues attributed to low bone formation. For the most part however, serum osteocalcin levels were elevated among individuals with bone diseases including; oversaturation of hormones (i.e., hyperthyroidism), Paget's disease (see Lee et al., 2000; Polak–Jonkisz et al., 1998; Price et al., 1980), fractures (see Lee et al., 2000), rickets and osteomalacia (see Demiaux et al., 1992; Lee et al., 2000; Nagata et al., 2011), cancer, and osteoporosis. Although osteoporosis is associated with high serum levels because of accelerated bone loss, in general, the authors suggest the elevated serum levels were a result of increased bone turnover. The only pathological condition that appeared to have no effect on serum levels was arthritis. Unfortunately, the relationship between serum osteocalcin and dental disease (i.e., caries, alveolar bone resorption, abscesses) and periosteal reaction were not discussed by Hauscka and colleagues (1989).

Bioarchaeological research has also begun exploring the relationship between osteocalcin and glucocorticoid secretion as it relates to periods of stress. Scott and colleagues (2016) quantified the osteocalcin levels from twenty adult individuals with

evidence of various skeletal pathological conditions (i.e., infection, degenerative bone and joint disease). The authors found a significant decrease in osteocalcin levels amongst individuals with degenerative spine disease. Scott and colleagues found no other significant relationships of osteocalcin levels between individuals with and without pathological conditions present which is similar to the results of this thesis. The authors indicate that the lack of relationship may be due to constant bone turnover, disease duration, or the type of skeletal change associated with pathological conditions. Scott and colleagues (in review) extracted osteocalcin from 46 individuals from a Danish archaeological population amongst whom bone infection, degenerative joint disease, and trauma were present. The authors found a significant difference in osteocalcin concentrations between males with and without evidence of trauma present but suggested this result was likely skewed since only one male with the highest osteocalcin value fit into this category. Similarly, there were significant differences in osteocalcin levels between females with and without evidence of leprosy and dental abscesses; however, one female that had both conditions present had a high osteocalcin value which likely influenced this significant result. No other significant differences were found within this study which is also similar to the results of this research.

For this thesis, the various pathological conditions were grouped into three categories; destructive reactions (caries, abscesses, alveolar resorption, metabolic disorder), proliferative reactions (periosteal reaction), and a combination of reactions (degenerative joint disease). The Louisbourg individuals with caries had a higher average osteocalcin concentration of 8.35 ng/µg compared to individuals without caries (7.66 ng/µg), however this difference was not statistically significant. It is unknown whether

the presence of cavities affect osteocalcin synthesis in bone or if chronic glucocorticoids are detrimental to enamel. However, abscesses can form as a result of severe caries which has the potential to decrease osteocalcin levels as bone is being actively destroyed by infection. Yet individuals with abscesses had a higher osteocalcin average of 9.18 ng/µg compared to individuals without abscesses (7.93 ng/µg), although the difference was not statistically significant. Interestingly, individuals with evidence of alveolar bone resorption had an average osteocalcin concentration of 7.43 ng/µg which was lower than those without alveolar changes (8.21 ng/µg). Although this difference is not statistically significant, it may suggest that the resorption of alveolar bone has caused a reduction in osteocalcin where other destructive dental diseases have not.

Since metabolic disease manifests as destructive porotic lesions, it was expected that individuals with metabolic–related pathology would have lower osteocalcin averages. However, these individuals had a statistically higher osteocalcin average (14.77 ng/µg) compared to those without evidence of metabolic disease (7.53 ng/µg). This significant difference was likely influenced by the high osteocalcin concentrations (10.41 ng/µg and 19.13 ng/µg) in the two individuals within this metabolic pathology category. The relationship between metabolic disease and serum osteocalcin levels is still unclear. Hauscka and colleagues (1989) report elevated serum levels amongst those with metabolic bone disease (i.e., rickets, hyperthyroidism, hyperparathryroidism). However, the authors also acknowledge that most studies do not report a correlation between metabolic deficiency and serum levels (see Daniels et al., 2000) and there is also evidence to suggest that decreased serum levels are associated with metabolic deficiency (Aghajanian et al., 2015). Due to the prolific nature of periosteal reaction, it was expected

that individuals with evidence of this pathology would have increased osteocalcin levels compared to those without bone inflammation. In fact, individuals with periosteal reaction had decreased osteocalcin levels (6.75 ng/µg) compared to individuals without periosteal reaction (8.72 ng/µg), although not significantly. Likely, periosteal bone formation was not significant enough to induce overall changes to the biomolecular structure of bone and therefore, osteocalcin was unaffected.

Because bone is being simultaneously created and destroyed during degenerative joint disease, it was expected that these combined boney reactions would balance out and individuals with and without joint disease would have similar osteocalcin levels. In fact, individuals with degenerative joint disease had an average osteocalcin value of 8.26 ng/µg which was similar to individuals without the disease (8.03 ng/µg). The slightly higher osteocalin values (but not significantly) among individuals with joint disease could be attributed to a higher instance of prolific bone reactions occurring where osteocalcin is reflecting increased bone formation. Hauscka and colleagues (1989) report slightly increased serum osteocalcin levels amongst individuals with degenerative skeletal changes (i.e., osteoarthritis) compared to healthy standards and suggest that the elevated levels are associated with high bone turnover. However, as previously mentioned, Scott and colleagues (2016) reported significantly lower osteocalcin values among individuals with degenerative skeletal changes but attribute this result to the compounding effect of old age amongst these individuals as they would have naturally decreased osteocalcin levels. Unfortunately, the relationship between osteocalcin levels and specific pathological conditions remains unclear and is likely the result of variable pathological manifestations.

5.5.2 Osteocalcin Variability as it Relates to Pathological Manifestations

Although no significant differences were found in osteocalcin levels amongst the Louisbourg individuals in conjunction with pathology, there may be methodological or social factors contributing to this result. First, studying the relationship between osteocalcin concentrations and disease prevalence is difficult when using a small sample size and may not sufficiently detect differences between individuals which does not accurately reflect the once living population (Nayak, 2010). Similarly, pathological lesions that were once present may be unobservable due to poor preservation or missing skeletal elements. Thus, individuals in the 'no pathology' group may have been incorrectly categorized if any skeletal lesions that were present at the time of death are now unobservable. As a result, misdiagnosis of skeletal pathology may be contributing to the insufficient detection of differences in osteocalcin concentrations between groups. As previously mentioned, Scott and colleagues (2016; in review) suggest that detecting osteocalcin concentrations associated with pathology is difficult due to the type of bone reaction that occurs in response to disease. Although bone reacts to stress in a limited way (i.e., prolific reaction, destructive reaction, or a combination of the two), the type of reaction will dictate the concentrations of osteocalcin in bone. For example, a prolific reaction with new bone growth should induce higher osteocalcin concentrations, whereas a destructive reaction with bone resorption should result in lower osteocalcin levels. However, the mechanism behind a boney change may not be straightforward and a combination of bone growth and bone destruction could cause variable osteocalcin levels. Likewise, the state of the lesion (i.e., active, healing, or healed) and number of lesions (i.e., singular or multiple) may also be contributing to osteocalcin variability where

osteocalcin levels are reflecting pathological conditions that are differentially manifesting amongst individuals. As well, bone samples were taken from a standardized sampling site and not taken directly from a lesion site which could have provided information about osteocalcin levels in direct relation to pathological conditions.

Particular social factors at the Fortress, including average age at death and health care practices, may have also had an effect on pathology as its relates to osteocalcin concentrations. The average age at death for the individuals within the Louisbourg collection was approximately 25 years (Scott, 2018; 2019). Because individuals were dying young, long term chronic changes to the skeleton were unlikely to develop. Historical records suggest that acute diseases including dysentery and smallpox was prevalent at the Fortress (Johnston, 1984). However, these diseases would have resulted in a quick and early death and the infection would not have had time to affect skeletal tissues or osteocalcin concentrations. Additionally, the Fortress had a hospital which cared for the residents at Louisbourg as well as sailors, soldiers, and prisoners (Johnston, 1984). For the conditions of the 18th century, the Fortress hospital was well equipped with 100 beds, an apothecary, bakery, kitchen, laundry, latrines, and a morgue (Johnston, 1984). In theory, Louisbourg residents may have had a disease that could have led to a skeletal manifestation with time; however, if they were admitted to the hospital and provided with treatment, the disease may have healed before any skeletal changes could occur, including osteocalcin concentrations captured within the bone. Thus, the low average age at death and healthcare system at Louisbourg certainly could have impacted disease prevalence in this sample population and may have contributed to the

insignificant relationship between osteocalcin values and those with and without obvious skeletal manifestations of disease.

5.6 Osteocalcin Concentration as it Relates to Diagenetic Change

As bone deteriorates from the burial environment, the mineral bone structure (i.e., hydroxyapatite) will begin to change and is referred to as the degree of crystallinity. Living bone has low crystallinity and is reflected by low IR–SF values (i.e., 2.0 IR–SF) whereas diagenetically altered bone has high crystallinity and is reflected by high IR–SF values (Surovell and Stiner, 2001). Amongst the Louisbourg samples, it was expected that there would be some diagenetic change, but that these values would fall within a similar range since based on the standardized sampling of macroscopically well–preserved cortical bone. In fact, Louisbourg IR–SF values did fall within a conservative range (3.3 – 4.33) suggesting some diagenetic alternation but the degradation of the hydroxyapatite structure had occurred at relatively the same rate across all samples with minimal biochemical information lost over time. These results are similar to other archaeological studies which commonly report narrow IR–SF value ranges regardless of differential cortical preservation in geographically and temporally variable human and faunal bone samples. For example, Beasley and colleagues (2014) sampled prehistoric shells and compared their IR–SF values to modern human and faunal remains using the FTIR–ATR method. The prehistoric shells had a mean IR–SF of 3.90 with samples ranging between 2.58 to 4.45 while modern bone had a mean value of 3.07 and a range from 2.54 to 4.03. Although the material tested was different (i.e., shells and bone), both the prehistoric and modern samples had similarly narrow IR–SF ranges that overlapped one another. Based on the deterioration of the organic content of bone, the authors

suggest an IR–SF cut off point between unaltered and altered bone where values of 3.3 and below represents no diagenetic alteration while values greater than 3.4 represent at least some diagenetic alteration. Similarly, Trueman and colleagues (2008) quantified the organic content of bone compared to IR–SF values of prehistoric and modern faunal bones. The authors found that the modern samples ranged between 2.8 and 3.4 and displayed high amounts of organic content while the prehistoric bones ranged between 3.4 to 4.0 and contained lower amounts of organic content. Although organic material was lost in the prehistoric samples, the remaining quantity was similar across increasing IR–SF values which the authors considered normal due to the natural loss of material through bone deterioration. When comparing the IR–SF values of the Louisbourg individuals to the 3.4 cut–off point outlined by Beasley and colleagues (2014) and Trueman and colleagues (2008), all samples but one (B3–15) were at or beyond an IR–SF of 3.4 (see Figure 5). In general, broad–range variable IR–SF values would make samples less comparable to one another but the Louisbourg IR–SF range is relatively narrow, demonstrating the high survivability of osteocalcin amongst those with some diagenetic alteration (>3.4); therefore, the osteocalcin data from this study likely reflects true variability within the population and is not the result of differential diagenetic change. When considering other bioarchaeological studies, Scott and colleagues (2016) found an average IR–SF value of 3.7 and a range between 3.370 and 4.184 for medieval Danish samples. When compared to the osteocalcin data, the authors note that although diagenetic change had occurred, the narrow range for their IR–SF values did not affect the extraction of osteocalcin and protein levels were comparable across all samples similar to the results in this thesis. As well, Scott and colleagues (in review) tested the

osteoaclcin concentrations from archaeological human remains in relation to IR–SF values and found a lack of correlation between crystallinity and osteocalcin. However, the authors noted that osteocalcin levels were significantly lower in samples that had IR–SF values >4.0 and suggest that, although osteocalcin can be successfully recovered from bone with high crystallinity, protein survivability is reliant on the burial environment. Since the Louisbourg samples with high IR–SF values still produced high osteocalcin concentrations, the burial environment at the Fortress may be more favourable than the burial environment in the Scott and colleagues (in review) study.

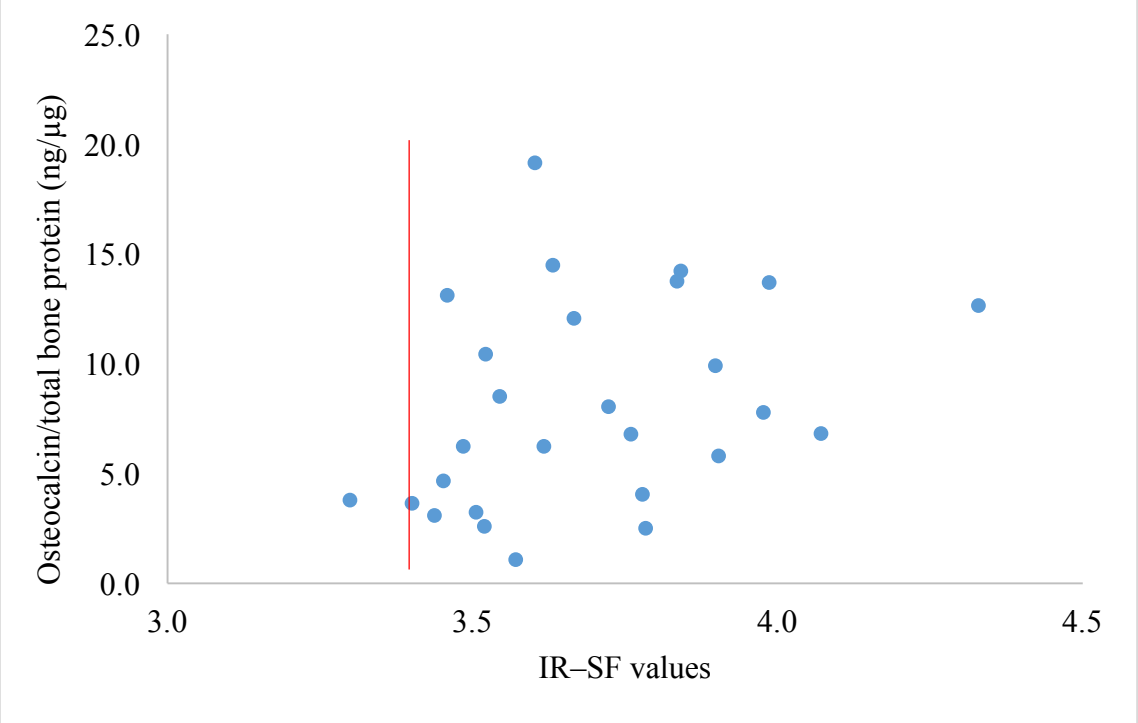

Figure 5: Louisbourg osteocalcin/total bone protein compared to IR–SF values. The red line indicates the 3.4 cut off value established by Beasley et al (2014) and Trueman et al (2008)

5.6.1 Diagenetic Variability as it Relates to the Burial Environment

Although the Louisbourg IR–SF values fall within a narrow range, similar to other bioarchaeological studies, the variability that is present is likely a result of the inconsistent burial environment at the Fortress. For example, soil pH is known to

drastically impact bone crystallinity, where acidic soil conditions (pH 3.5 – 4.5) are more destructive and detrimental to human remains than basic soil conditions (pH 7.5 – 8.0) (Baxter, 2004; Gordon and Buikstra, 1981; Kendall et al., 2018). Specifically, the mineral component of bone is most stable in basic soil conditions but begins to dissolve in environments below 6.0 pH (High et al., 2015; Kendall et al., 2018; Nielsen–Marsh et al., 2007; Scott and Fonzo, 2019; White and Hannus, 1983). As the hydroxyapatite dissolves, bone protein is exposed to the acidic soil and will begin to degrade. For this study, when the recorded burial pH of six individuals was compared to their IR–SF values using a Pearson Correlation test, there was a strong negative correlation ($r = –0.99$) (see Figure 6). This indicates that soil pH is likely the strongest influencing factor on bone diagenesis at Louisbourg where acidic soil is more detrimental to the crystalline structure compared to alkaline soil. Soil hydrology can also impact diagenesis when water in and around human remains will chemically react with the mineral structure of bone and degrade it to be more similar to the burial environment (Collins et al., 2002; Kendall et al., 2018; Nielsen–Marsh et al., 2000). The natural water table on Cape Breton Island is five meters below the ground surface (Government of Nova Scotia, 2017). When comparing the recorded burial depth of the 24 individuals in this study to their IR–SF values using a Pearson correlation test, there was a weak positive correlation in both cemetery groups (Block 3 $r = 0.20$, Rochefort Point $r = 0.43$) (see Figures 7 and 8). This suggests that deeper burials were more consistently waterlogged which did impact the preservation of cortical bone over time and consequently the IR–SF results; however, it was not as strong a predictor as burial soil pH. As well, certain burial practices can also influence the decomposition of the body after death, including the use of coffins and/or shrouds, which

may differentially affect the underlying skeleton and its preservation. It was expected that the Louisbourg individuals buried in coffins would have poorer cortical preservation and have higher IR–SF values compared to those buried in shrouds. This is because coffins have poor water drainage and excess amounts of air within the container which rapidly decompose the body (Nawrocki, 1995; Mant, 1987). However, amongst the Louisbourg individuals buried in coffins (n=5) the average IR–SF value was 3.62 and ranged between 3.44 and 3.83, whereas those buried in shrouds (n=21) had an average IR–SF value of 3.70 and a larger range of variation between 3.30 and 4.33. The assumption that coffin presence would negatively affect skeletal preservation was not upheld in this study as those buried in coffins had better cortical bone preservation which could be the direct result of the coffin walls acting as a shield to the external environment (Nawrocki, 1995), but more likely reflects the influence of burial depth on skeletal preservation regardless of coffin presence.

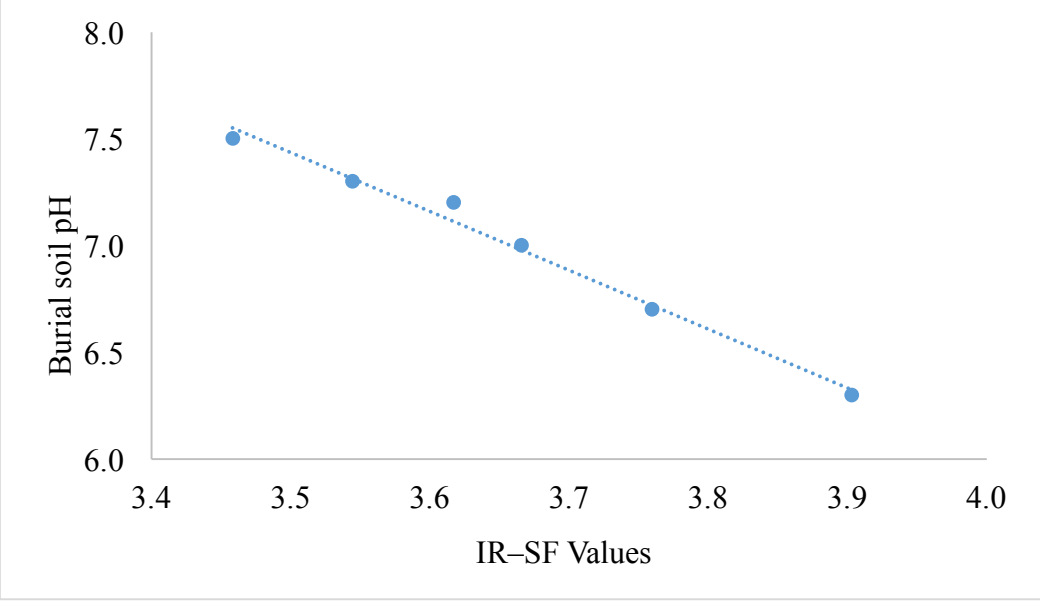

Figure 6: IR–SF values plotted against burial soil pH of 6 individuals from the Rochefort Point sample (Burials 1, 3, 5, 14, 21, 28) (R^2 = 0.987)

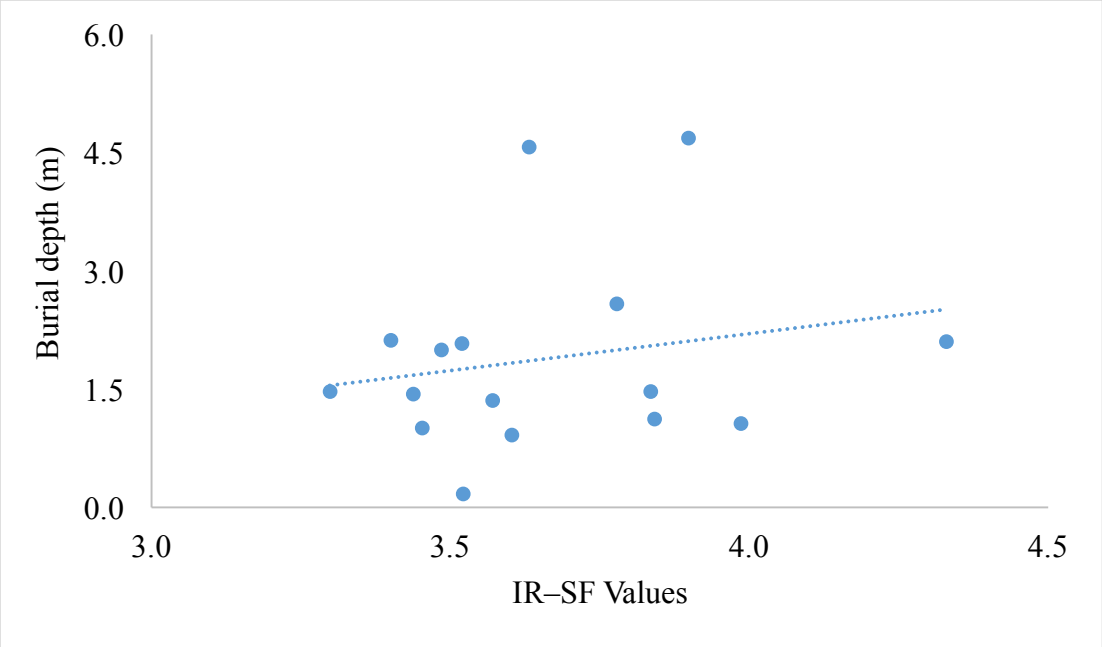

Figure 7: Block 3 IR–SF values plotted against burial depth (R^2 = 0.419)

Note: The Block 3 burial depths presented are not meters below the ground surface and instead represent the depth of each burial in relation to the height of the dumpy level used at the time of excavation. However, the height of the instrument was inconsistently recorded making it impossible to calculate the accurate depth below the ground surface for each burial. As such, the Block 3 and Rochefort Point burial depths could not be compared to one another.

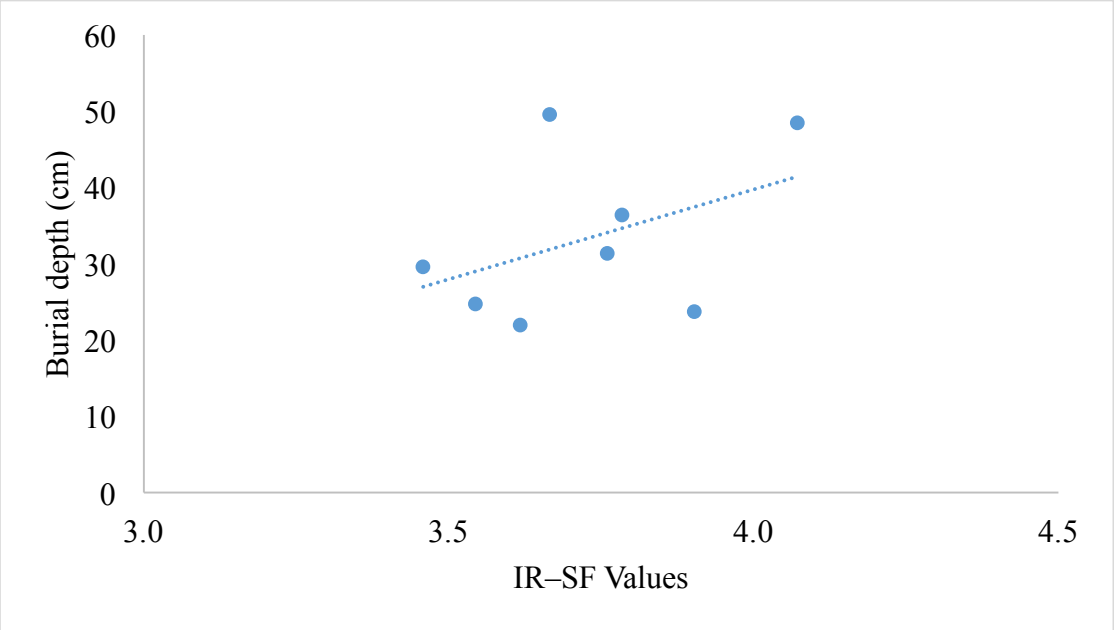

Figure 8: Rochefort Point IR–SF values plotted against burial depth (cm) (R^2 = 0.186)

5.7 Limitations

5.7.1 Literature Restrictions

There are only a few bioarchaeological studies that have extracted human bone protein because the application of biochemical analysis on archaeological material is still in its infancy (see Cattaneo et al., 1992; Cattaneo et al., 1994; Scott et al., 2016; Scott et al., in review; Tuross, 1991). Yet, clinical studies have extensively researched blood serum osteocalcin which has formed an excellent foundation for bioarchaeological research. However, these studies obtain serum osteocalcin data from living modern patients which may not fit what bioarchaeologists observe from matrix osteocalcin data derived from deceased populations. As well, archaeological studies have the added complication of diagenetic change which affects the survivability of bone protein over time. Consequently, there may be disparities amongst cross–discipline comparisons between serum and matrix osteocalcin due to the added challenges of extracting matrix osteocalcin from archaeological bone.

5.7.2 Skeletal Collection Restrictions: Sample Size and Population Demography

Due to the sampling criteria, only 27 of the Block 3 and Rochefort Point individuals were studied from the Louisbourg collection. The sample size was small, which is not uncommon in bioarchaeological research, but poses a problem since the data collected does not necessarily reflect osteocalcin trends across the entire population. As well, small sample sizes more often result in large, inaccurate standard deviations and high data variability which may lead to sample bias. Furthermore, while there were no statistical differences between osteocalcin values and biological or pathological factors in

this study, a larger sample would have increased statistical power and may have detected significant differences generating alternative interpretations.

The small sample size also creates a representative disparity of the original population that existed at the Fortress of Louisbourg. There were always more males than females within the Fortress's population during occupation (Johnston, 2004; Johnston, 1984) and this is reflected in the sample (males =21, females=5). However, these individuals do not accurately represent the individuals that made up the Fortress' transient population during the height of its occupation (Johnston, 1984). Similarly, Fortress residents varied in age with a large child population numbering into the hundreds (Johnston, 1984). Yet, the sample population is relatively conservative and only includes individuals between 16.5 and 45 years with no representation of infants, children, or older adults. Therefore, any connections between bone protein values with sex and age at death within this study are preliminary.

5.7.3 Methodological Restrictions: Sampling Error, Quantikine ELISA Kit, FTIR–ATR

There are three layers that make up cortical bone; endosteum, intracortical, and periosteum. The endosteum (i.e., inner cortical envelope) and periosteum (i.e., outer cortical envelope) are active osteogeneic surfaces that are relatively thin but high in bone turnover (Parfitt, 2002). The intracortical layer, found between the endosteum and periosteum, is the thickest cortical layer but has slow bone turnover (Parfitt, 2002). For this research, it was assumed that the same depth of periosteal layer was sampled capturing a similar timeline of osteocalcin incorporation into the bone. However, differential preservation may have damaged the outer cortical bone causing variable

periosteal thickness amongst individuals where inconsistent sampling depth may have caused sampling error. While attempts were made to minimize sampling inconsistency (i.e., only well–preserved outer cortical bone was sampled and bone powder was harvested from a fixed 1cm by 2 cm sampling area), it is possible that some of the osteocalcin captured in these samples represent slightly different timelines of incorporation, potentially influencing the statistical analyses and interpretations.

Quantikine ELISA kits are commonly employed clinically to detect serum osteocalcin from blood samples of living patients. Using these clinical kits on archaeological material may appear inherently incompatible, but the demineralization and filtering process converts the bone powder into a serum which reacts to the ELISA kit the same as blood from a living patient would. This procedure was successfully adapted for ancient protein studies to detect matrix osteocalcin from archaeological animal bone (see Collins et al., 2000; Ritz et al., 1996; Smith et al., 2005) and later used by bioarchaeologists on human skeletal remains (see Cattaneo et al., 1992; Cattaneo et al., 1994; Scott et al., 2016; Scott et al., in review). However, ELISA kits have been known to produce false positives. Collins and colleagues (2000) explain that "assays are pushed to their limit (concentrated extracts, tested with antibodies at low dilutions) and are therefore prone to yield false positive results" (p. 1139). Within this research, a false positive would indicate osteocalcin present in a sample when in reality it does not contain the bone protein. Although the possibility of false positives should be considered, ELISA kits are reportedly 99.5% accurate (Rao et al., 1997) and the results of this thesis are likely accurate as well. Also, all samples in this research produced positive osteocalcin results and concentrations were often high indicating that the protein was abundant within

the Louisbourg samples. As well, a mock sample with no bone powder was treated the same as all samples from demineralization to assay and consistently generated zero concentrations of either protein and osteocalcin indicating no contamination or false positives.

Although the FTIR–ATR method is widely used within archaeological studies to detect diagenetic change, the accuracy of the splitting factor has been challenged because unaltered bone samples (i.e., well–preserved cortical bone) have produced high IR–SF values indicating significant diagenetic change (see Beasley et al., 2014; Lee–Thorp and Sealy, 2008). In other words, the relationship between mineral crystallinity and bone tissue is still unclear. Because of this, a short range or tight cluster of IR–SF values are more reliable and represent comparable diagenetic change across samples instead of a large range and widely dispersed values which indicates variable diagenetic change. Additionally, ancient protein studies that have explored the relationship between osteocalcin and bone mineral diagenesis do not provide a cut–off threshold that identifies between little and significant crystalline alteration (Kendall et al., 2018; Kontopoulos et al., 2018; Surovell and Stiner, 2001; Turunen et al., 2014). This is an important relationship to distinguish in order to identify osteocalcin levels that have depleted overtime due to bone deterioration and values that have remained stable since death with minimal protein loss. As a result, a standard threshold characterized by lower and upper IR–SF limits currently does not exist. Beasley and colleagues (2014) state that archaeological bone samples with IR–SF values less than 3.3 have "measureable collagen" and samples greater than 3.4 "exhibit alteration" (p. 19). However, the authors do not provide an explanation of how they determined their cut–off point. It is important

to note that the authors based the cut–off point on collagen deterioration which may operate differently than osteocalcin under diagenetic conditions. Currently, no diagenetic cut–off point has been identified for the mineral component of bone that houses osteocalcin. Since IR–SF values are relatively small and are often within a short range, samples may be incorrectly categorized into minimal or significant alteration that does not accurately reflect their crystalline structure. This can lead to inaccurate comparisons between samples with significantly different crystalline alteration. As well, creating an arbitrary cut–off point suggests that cortical bone can generate either quantifiable or unquantifiable osteocalcin concentrations, where unquantifiable values represent inaccurate biochemical signatures. Samples with high IR–SF values of 3.4 or above should not be disregarded and can still be used for comparative analysis but only with other samples with similar diagenetic change. Additionally, the arbitrary cut–off point distinguishes between minimal and significant crystalline structure alteration, but it is unknown whether high and low values within those set categories (i.e., minimal and significant) can be accurately compared. For example, a recently deceased modern human (IR–SF 2.90) (Trueman et al., 2008; Yoder 2010) and archaeological remains (minimum IR–SF 2.58) (Beasley et al., 2014) can both be within the 'minimal alteration' category but may have significantly different cortical preservation. In this study, most IR–SF values were above the 3.4 cut–off point. As well, despite these limitations, FTIR is the most appropriate method to continue exploring bone crystallinity because it is cost effective, requires minimal sample preparation, the spectra variation from particle size is low, it generates accurate IR–SF wavelengths, and is considered a valid method to distinguish between unaltered and altered bone (Beasley et al., 2014).

5.8 Summary

This chapter explored the biological factors (i.e., sex, age at death, cortical bone thickness) and pathological conditions that may have influenced the osteocalcin concentrations reported for the Louisbourg individuals within this study. Although biological factors have been proven to heavily influence osteocalcin levels within the clinical literature, this does not appear to be true within this study, likely as a result of the small sample size that was demographically restrictive. Despite this, it appears that estrogen may have protected against bone loss which caused higher osteocalcin values amongst females and that activity was not an influencing factor on osteocalcin levels between biological sex. Osteocalcin levels and evidence of skeletal pathology was analyzed in this study but there were no significant differences between those with and without skeletal lesions present, most likely a result of the small sample size and pathological variability (i.e., bone reaction, active vs. healed, and single or multiple lesions). As well, the IR–SF values that represent diagenetic change fell into a narrow range suggesting that bone deterioration was comparable amongst all samples and any variability was most likely a result of an inconsistent burial environment affected by soil pH, soil hydrology, and/or burial practices.

CHAPTER 6 – Conclusion

6.1 Revisiting the Research Questions

This research was meant to expand the use of biochemical analyses in bioarchaeological contexts by exploring current protein extraction methods and the influence of biological and/or pathological factors on osteocalcin concentrations in human skeletal remains The three research questions posed at the outset of the thesis will now be revisited and discussed

1. Can osteocalcin be successfully extracted from bioarchaeological samples?

   Osteocalcin was successfully extracted from all samples using established protein extraction methods (see Scott et al., 2016, Scott et al., in review). In fact, osteocalcin was quantified from all samples indicating that the protein preserved and was abundantly available within the bone samples. This finding is not surprising since concentrated protein levels are commonly reported in archaeological studies from differing geographical locations and temporal periods (see Beasley et al., 2014; Collins et al., 2000; Smith et al., 2005; Trueman et al., 2008). Therefore, it is reasonable to suggest that protein extraction and biochemical analysis will continue to be successful in most bioarchaeological contexts using the outlined methods.

2. What biological and/or pathological patterns emerge from the osteocalcin data and how does this correlate with the macroscopic skeletal assessment of these individuals in terms of age and sex patterns or the presence/absence of pathological conditions characterized by bone remodeling?

Although clinical studies have demonstrated strong relationships between biological factors and osteocalcin concentrations, the results of this study do not reflect these clinical trends. While not statistically significant, females had higher osteocalcin values likely a result of higher estrogen levels which protect and promote bone growth. Males had lower osteocalcin averages than females, but levels remained stable throughout adulthood indicating healthy bone maintenance which is reflected in the clinical literature. This research used cortical bone thickness as a proxy for activity and found no significant differences between the sexes suggesting that the lower body was not predominately engaged in high–stress activities which would have seen an increase in cortical bone diameter (see Alghadir et al., 2015; Chahla et al., 2015; Kim et al., 2010). As well, there was no correlation between osteocalcin concentrations and mid–shaft cortical bone thickness indicating that activity was likely not a primary influencing factor on osteocalcin variability within this study. However, this result could also indicate that mid–shaft cortical thickness is not the most accurate skeletal index to capture true activity levels within the Louisbourg population. Similarly, there were no significant differences in osteocalcin concentrations between individuals with and without evidence of pathological conditions suggesting that disease processes may not have been disruptive enough to affect bone homeostasis. However, this lack of correlation could have also been influenced by a variety of factors such as: the degree of bone reaction, the active or healed state of the lesions, the number of lesions, and/or how long these processes had been affecting in the skeleton. Additionally, demographic (i.e., age at death) and

social factors (i.e., health care) at Louisbourg may have also influenced how/if osteocalcin concentrations can be compared to pathological skeletal changes.

3. Is there a similar level of preservation (diagenesis) across all bioarchaeological samples?

The IR–SF values from this research had a narrow range of variability indicating that while diagenetic change had occurred, it was similar across all samples. Although most samples (n=26) were above the 3.4 cut off point outlined by Beasley and colleagues (2014) and Trueman and colleagues (2008), the conservative range in IR–SF values and high osteocalcin concentrations indicate that osteocalcin values are more likely reflecting population variability and not differential diagenetic change. The slight variation in these IR–SF values however, likely reflect disparities in cortical preservation due to the differential conditions of the burial environment including soil pH, burial depth, and coffin presence.

6.2 Future Research

6.2.1 Sampling Locations

This thesis used the posterior distal third of the femur as a sampling site based on a preliminary study that tested four different femoral sites amongst two individuals (see Appendix 3). However, because only two individuals were used in the preliminary study, these results do not definitively suggest that the sampling site chosen produces an osteocalcin signature that best represents an individual free of other influencing factors. Future research should test multiple sites on the femur to establish a standardized sampling site that can be cross–comparatively used for future studies. Testing different skeletal elements should also be considered to see if osteocalcin values are comparable

across elements. As well, it would be interesting to test osteocalcin levels from the appendicular and axial skeleton to see if protein levels are comparable between major regions of the skeleton. Furthermore, testing the osteocalcin concentrations within the different layers of cortical bone (i.e., periosteum, intracortical, endosteum) using microscopy and thin section analysis could provide insights into the relationship between osteocalcin levels and bone remodeling. Specifically, this type of research could potentially determine the rate at which osteocalcin is deposited and resorbed in each cortical layer and if osteocalcin is being incorporated into the hydroxyapatite of bone differently between these distinct cortical layers. Testing osteocalcin levels between the two types of bone (i.e., cortical and cancellous bone) may reveal variable osteocalcin concentrations within the same element. Although cancellous bone is less dense compared to cortical bone, it has an increased surface area due to its honeycomb appearance which results in a higher bone remodeling rate (Christenson, 1997). Because of this, cancellous bone may be closely affected by minute changes to bone homeostasis as a result of influencing factors such as activity and pathology. Subsequently, cancellous osteocalcin may be more sensitive to these factors and reflect early manifestations of these changes compared to cortical osteocalcin. Although the femora displayed no osteocalcin differences between right and left sides, further research should be done on multiple paired bones to test if bilateral symmetry is systematic. For example, because dominance occurs in the upper limb and variable robusticity represents different levels of activity (see Ubelaker and Zarenko, 2012), it would be interesting to further test for bone protein comparability between the left and right upper limbs. For the Louisbourg

population in particular, handedness may be evident due to differing occupations focused on upper limb strength and robusticity (i.e., fishing, soldiering, manual labour).

6.2.2 Pathological Considerations

Another area of focus for future bioarchaeological protein studies is better defining the relationship between osteocalcin concentrations and pathological conditions which manifest in bone. Fundamentally, it is unknown whether osteocalcin levels are affected locally or throughout the entire skeletal system during periods of stress. In order to better understand this, osteocalcin levels should be tested at specific (i.e., lesion site) and general (i.e., non–lesion site) skeletal regions to explore the similarity of osteocalcin values across larger sections of the skeleton. In particular, testing osteocalcin levels from a lesion and non–lesion site on the same skeletal element will examine if osteocalcin levels fluctuate locally. Additionally, more information needs to be collected about how osteocalcin is impacted by variable bone reactions in response to pathology (i.e., bone proliferation or destruction). Understanding how much osteocalcin levels deviate from standard levels as a result of prolific or destructive bone reactions can help bioarchaeologists understand the severity of pathological impact on bone metabolism. However, testing purely prolific or destructive lesion sites would be difficult since skeletal lesions often present as a combination of these reactions. The state of a pathological skeletal lesion (i.e., active, healing, healed) should also be considered in future studies. Establishing known osteocalcin levels for different stages of a skeletal lesion can potentially lead to the identification of metabolic shifts occurring at a biochemical level from active to healing to healed. However, testing the osteocalcin levels amongst different lesions states can be complicated. Pathological changes in the

skeleton can be variable between individuals even when the causative agent is the same disease. These differences can be related to the severity of the disease, the time elapsed since the disease was acquired, individual immune response to the disease, etc. and as such, needs to be considered further when assessing variability in osteocalcin values captured in bone.

6.3 Research Contributions

This thesis directly contributes to the growing integration of biochemical methods to answer bioarchaeological research questions about the human past. Specifically, this thesis validated and expanded previously established protein extraction methods and is the first study to extract human bone protein from an archaeological site in Canada. By demonstrating how easy, cost–effective, and beneficial protein extraction methods are, this thesis encourages bioarchaeologists to explore new biochemical techniques in order to further develop new research questions beyond the boundaries of macroscopic analysis alone. This thesis is also significant in that it is one of three bioarchaeological studies that specifically looks at osteocalcin extracted from archaeological human remains. Quantifying osteocalcin as an indicator of bone metabolism is still novel within bioarchaeology and can lead to a deeper understanding of how biochemistry can push the boundaries of bioarchaeological research design. As this sub–field expands, bioarchaeologists can build upon the clinically established relationships between osteocalcin, biological factors, and pathological conditions which may be used as an additional line of evidence during the macroscopic assessment of sex, age, activity, and health. This thesis looked at osteocalcin in direct relation to influencing factors specific to

the Louisbourg population and discovered otherwise hidden aspects of these individuals lives that would otherwise be invisible through macroscopic assessment alone.

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APPENDIX 1

There are two main total protein assay types; Bradford and bicinchoninic acid (BCA) assays. The Bradford method is commonly used in laboratories because it is easy and convenient, however, it does not detect bone protein as well as the BCA assay and its effectiveness can be influenced by other chemicals (Walker, 2002). The BCA assay is a simple two step procedure, is sensitive to the presence of protein, more tolerant of compounds that are not protein, and can be compared to known protein standards to quantify unknown samples spectrophotometrically (Kruger, 2002). Both the Bradford and BCA assay involve exposing the sample to an active reagent which creates a reaction and changes the sample colour. The amount of colour saturation reflects the amount of protein present in the sample and is measured using a microplate photometer. This microplate reader exposes the well–plate to a light source and measures how much light successfully passes through each well reflecting the concentration of the sample. Darker colours will have less light pass through the well which corresponds to a higher concentration of protein. Similarly, lighter colours will have more light pass through the well which corresponds to a lower concentration of protein.

A total protein quantification preliminary test of 7 samples was conducted using both a Bradford and BCA assay to determine if the samples would fall within the standard curve and which assay is best at detecting human bone protein. The samples chosen (B3–1L, B3–6L, B3–7L, B3–24L, RP–1L, RP–5L, RP–28L) were based on predicted high and low protein quantities due to differential preservation. Standards were run in triplicate and samples were run in duplicate. The same standard curve was used for the Bradford and BCA assays and 10μL of each standard was pipetted directly into the

bottom of the corresponding standard wells. 5µL of each sample was pipetted directly into the bottom of the corresponding sample wells. Since the samples were 2x concentrated after filtering, halving the amount of tested sample compared to the neat standard adjusts for the concentration difference. 200µL of the Bradford reagent was backwards pipetted into each Bradford standard and sample well. The BCA well–plate was incubated on a hotplate at 37°C for 33 minutes covered with tinfoil to protect samples from sunlight. The Bradford assay was read at 595nm and the BCA assay was read at 560nm in a Multiscan ^{TM} FC Microplate reader. The Bradford samples did not detect as much bone protein as the BCA assay. Therefore, a BCA assay will be used for the total bone protein quantification of all the samples.

B3–7L had duplicate concentrations of 567µg/mL and 598µg/mL, respectively, from the preliminary BCA assay test which were above the top standard of the curve (500µg/mL). To conduct the all samples total bone protein quantification, a new standard curve will be used to ensure results fall within the curve. However, the new standard curve needs to be tested to ensure that it would read properly in a microplate reader. The new standards were as follows; 1000µg/mL, 700µg/mL, 400µg/mL, 200µg/mL, 100µg/mL, 50µg/mL.

APPENDIX 2

Table 10: Dilution factors of samples during ELISA tests

Burial #	Pre–ELISA dilution	ELISA dilution	Re–Assay ELISA dilution
B3–1L	1:10 and 1:100	1:30	
B3–1R		1:30	
B3–2L		1:30	
B3–2R		1:30	1:30
B3–6L	1:10 and 1:100	1:30	
B3–6R		1:30	1:30
B3–7L	1:10 and 1:100	1:30	1:30
B3–7R		1:30	
B3–8		1:30	
B3–10		1:30	1:30
B3–11		1:30	
B3–12L		1:30	
B3–12R	1:10 and 1:100	1:30	
B3–13L		1:30	
B3–13R		1:30	1:5
B3–15L		1:30	
B3–15R		1:30	
B3–16		1:30	1:30
B3–17L		1:30	1:30
B3–17R	1:10 and 1:100	1:30	1:30
B3–18L		1:30	
B3–18R		1:30	
B3–19		1:30	
B3–20		1:30	
B3–22		1:30	
B3–23L		1:30	
B3–24L	1:10 and 1:100	1:10	
B3–24R		1:10	1:30
B3–25L		1:30	
B3–25R		1:30	
RP–1L	1:10 and 1:100	1:30	1:30
RP–1R		1:30	1:30
RP–3L		1:30	
RP–3R		1:30	
RP–5L		1:30	
RP–5R	1:10 and 1:100	1:30	

RP–12L		1:30	
RP–12R		1:30	1:30
RP–14L		1:30	1:30
RP–14R		1:30	
RP–21L		1:30	1:30
RP–21R		1:30	
RP–28L	1:10 and 1:100	1:30	
RP–28R		1:30	1:30
RP–52L		1:30	1:30 and 1:10
RP–52R		1:30	

APPENDIX 3

To determine the best sampling location, a preliminary test was completed on two sex and age matched individuals that tested four different sampling locations on the left femur to identify if there was any variation in osteocalcin concentrations near muscle attachment sites (high biomechanical loading) or on non–attachment surfaces (less biomechanical loading) (see Figure 9). Sampling site D located on a non–muscle attachment surface had the lowest concentration of osteocalcin in both individuals. Whereas, sampling sites A through C were located on or near muscle attachment surfaces and had higher osteocalcin levels than site D. These concentration differences indicate that osteocalcin was least influenced by biomechanical loading at sampling site D which is ideal for best capturing osteocalcin signatures.

Figure 9: The four sampling sites labeled A, B, C, D from the preliminary sampling site test

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Hughes N, Scott AB. 2019. Proteins in Play: Testing osteocalcin variability between different skeletal sampling sites. Canadian Association for Physical Anthropologists Annual Conference, Banff, Alberta.

Scott A, Danforth M, MacInnes S, **Hughes N**, Fonzo M. 2019. Colonial urbanism: A comparative exploration of skeletal stress in two 18th century North American French colonies. American Association for Physical Anthropology Annual Conference, Cleveland, Ohio.

Hughes N. 2019. Understanding the Biochemical Stress Response: A preliminary method to explore variation in osteocalcin protein concentration in the human femur. Association of Professional Archaeologists of New Brunswick Annual Conference. Fredericton, New Brunswick.

Hughes N. 2019. Understanding the Biochemical Stress Response: A preliminary method to explore variation in osteocalcin protein concentration in the human femur. University of New Brunswick Graduate Research Conference. Fredericton, New Brunswick.

Academic Awards:

2019 Parks Canada CEO Award of Excellence for the UNB Bioarchaeology Field School

Canadian Graduate Scholarships – Michael Smith Foreign Study Supplements Program, University of New Brunswick – $3,800.00
Magee – Third Century Postgraduate Merit Award, University of New Brunswick – $1,500.00
New Brunswick Innovation Foundation Award, University of New Brunswick – $3,500.00

2018 Magee – Third Century Postgraduate Merit Award, University of New Brunswick – $1,500.00
New Brunswick Innovation Foundation Award, University of New Brunswick – $3,500.00
Social Sciences and Humanities Research Council of Canada (SSHRC) Canadian Graduate Scholarship, University of New Brunswick – $17,500.00

2017 Anthropology Silver Medal, Brandon University
Deans' Award for Undergraduate Research Excellence, Award of Distinction, Brandon University – $500.00
Crescam Serviendo Award, Brandon University – $120.00
John & Catherine Robbins Graduate Scholarship, Brandon University – $2,870.00