THESIS

ARE LINES OF ARRESTED GROWTH IN BONE INDICATIVE OF SEASONAL METABOLIC SUPPRESSION IN BEARS?

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ABSTRACT

ARE LINES OF ARRESTED GROWTH IN BONE INDICATIVE OF SEASONAL METABOLIC SUPPRESSION IN BEARS?

Large hibernators such as bears have seasonal metabolic suppression, hibernation (Tøien et al. 2012). During hibernation bear's activity is very low; to the point most other animals would exhibit disuse bone resorption. However bears do not exhibit disuse bone resorption during this time (McGee-Lawrence et al. 2008). Are lines of arrested growth (LAGs) in bone indicative of seasonal metabolic suppression in bears? Through the use of toluidine blue stain light microscopy slides and backscattered scanning electron microscopy images (SEM), LAGs were counted and correlated with age. LAGs have a strong correlation with age. This is indicative of LAGs formation once per year, during set hibernation cycles. LAGs are metabolic markers, in bears with set hibernation cycles. These metabolic markers could be used to identify the specific time in which there is metabolic suppression, in bears. This identification could be used in the future to track blood serum and other chemical markers in an attempt to understand bear's natural resistance to disuse bone resorption. Bears ability to not exhibit disuse bone resorption could be biomimetically studied, in an attempt to adapt this protection to humans. Since humans experience disuse osteoporosis (extended bed rest and spaceflight) and osteoporosis (older population specifically women).

ACKNOWLEDGEMENTS

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DEDICATION

I dedicate my thesis work to my family. The biggest thanks must go to my wife, Mary. She was my biggest critic and supporter. She also lovingly put up with me during the whole process. Also a special thanks to my parents. Thanks for pushing and supporting me all of my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

Problems with Disuse and Age in Bone 1.0

Bone could be best described as a living material. As such it can be predictable and unpredictable in the same sense. Some people may agree that living things are chaotic. Sometimes living things follow observable paths and patterns that humans seek, other times living things are seemingly random and chaotic. This must be taken into account and with a grain of salt. Such an attitude could lead to complacency and as said above living things are often not complacent. Many times people try to simplify the components to understand the whole. Other times people say that looking at the whole is the only way to understand what makes up an object. Both are common approaches in science and engineering. A current problem that is becoming more relevant to humans as average life expectancies increase is how to treat an aging human structure. In this case, bone being a primary physical structure to humans that gives: rigidity, points for muscles to attach, such that humans can move, protects organs and more sensitive parts of the human body, to serve as a chemical and cellular reservoir for the body, and the list could go on. That being said, bones can become broken or fractured. Bone fractures are an increasing problem, especially for an aging population. As with younger people fractures generally represent a mistake and are an inconvenience for them. For an older population, fractures are not always the result of a mistake but could be just a sudden occurrence. A fracture in an aging person could also prove to be life-threatening and\or an overall reduction in the quality of life.

A fracture is just addressing the outcome of an underlying problem. The World Health Organization (WHO) states that this is just a "clinical end result" of osteoporosis (WHO, 1994).

Overall the group of terms coined as the underlying problems, to fracture, is bone mineral density (BMD), osteoporosis, and porosity. The WHO correlates osteoporosis and bone. Patients with a BMD greater than 1 standard deviations of a healthy young adult are considered low in "bone mass" (WHO, 1994). While a patient with a BMD greater than or equal to 2.5 standard deviations of a healthy young adult are considered to be osteoporotic (WHO, 1994). In Table 1, page 3, the WHO shows a general approximation to the amount of white women, in the US as of 1990, that have osteoporosis. There is no readily available reason to believe that osteoporosis would be higher or lower in women of different races. Also it is unexpected that the levels of osteoporosis are lower in other countries. Levels of osteoporosis might actually be higher in other countries, due to the lack of readily available healthcare and\or lack of food quantity and quality. Osteoporosis is of course more problematic in women, than men. This is due to a drastic change in hormone production, menopause. The differences in the porosity\BMD in between sexes are pointed out in several papers (WHO, 1994; McCalden et al., 1993). According to the National Osteoporosis Foundation (NOF), Osteoporosis is the cause of approximately two million fractures, which cost approximately $19 billion, each year. The NOF then goes on to predict that, in 2025, at the current rates in the growth of osteoporosis related fractures, this number could grow to three million fractures, which cost approximately $25.3 billion, each year (NOF.org). This is also assumed that this is only for the United States and would be much larger if the whole would was added into these statistics.

Table 1: Relative percentage of white women, in the US as of 1990, with osteoporosis (WHO, 1994)

Age Range (Years)	Any Site (%)	Hip Alone (%)
30-39	0	0
40-49	0	0
50-59	14.8	3.9
60-69	21.6	8.0
70-79	38.5	24.5
80+	70.0	47.5
≥50	30.3	16.2

There is another way to achieve an "osteoporotic" like state. This is through the lack of use. This is done when there is a lack of physical forces applied to bone. Such in the cases of: extended bed rest, being bound to a wheelchair, astronauts, and other types of circumstances where there is physical unloading of bones. This is shown in an article were these phases of extended unloading of bone caused a reduction in overall BMD, or an increase in porosity (Takata and Yasui, 2001). In such cases there are results similar to those in the aging population, specifically women. Often this form of osteoporosis is called disuse osteoporosis (Takata and Yasui, 2001). This has been scientifically studied on astronauts. A researcher suggests that being in microgravity or a space like environment, an astronaut could lose up to 0.8% - 1.5% of bone volume per month (Lang et al. 2004). This is also a very big problem currently for mankind. Not only are people living longer, and experiencing osteoporotic mishaps, but also astronauts are experiencing similar bone loss to that of an aging person. That be it, currently astronauts are exposed to a set amount of space time that is considered safe. Such that as being a younger individual they should be able to fully recover what was lost. This is an obvious problem with space being the next big frontier for humans to expand to. Bone loss due to disuse osteoporosis is a problem that must be rectified before long space travel is attainable, even in a young population. That is of course if simulated gravity is not used in space travel. Other studies have shown what a lack of physical forces are applied to bone (Jaworski and Uhthoff

1986; Kaneps et al. 1997; Zerwekh et al. 1998; Yonezu et al. 2004; Spector et al. 2009). The bone loss, in osteoporosis and disuse osteoporosis is due to an imbalance. There is more bone resorption than there is bone formation. This is an overall imbalance in a process called bone remodeling (Skedros et al., 2005; Frost, 1990; Parfitt, 1994; Rubin et al., 1996).

Some hibernators exhibit special characteristics over non-hibernators. Hibernators, like bear, are able to drop there metabolic rate significantly over the course of their hibernation. Bears can drop their metabolic rate to only 25% of their base metabolic rate (Tøien et al. 2012). This comes with only a marginal decrease in core body temperature of approximately 5^{o}C (Tøien et al. 2012). Interestingly, during this hibernation there is little to no radial growth, but there is bone turnover but at a much slower rate, approximately 25% (McGee-Lawrence et al. 2008). Tøien's and McGee-Lawrence's research match up nicely. Bear exhibit the opposite of what one would expect to see. They do not experience disuse osteoporosis even though they are not moving a lot for extended periods of time (McGee-Lawrence et al. 2008). One would expect with this prolonged lack of loading on the bones to see disuse osteoporosis. Similar to what one would expect to see in humans if they tried to replicate the bear's hibernation patterns. Smaller hibernators, like ground squirrels, do not have the same bone protection, from lack of loading, during hibernation (Carey et al., 2003). Also, hibernation of small hibernators and larger hibernators differ. Larger hibernators, like bears, have a gradual metabolic depression to a minimum and then a slow rise out of hibernation (positive parabolic shape), once they are done hibernating (Tøien et al., 2012). Small hibernators, ground squirrels, go in to gradual metabolic depression to a minimum, much like bears. Yet small hibernators have a few days where they basically fully wake up. Their metabolic rate sharply increases, to a non-hibernation state, and then drops sharply back to the previous minimum (Carey et al., 2003).

Bone Remodeling Overview 1.1

Bone remodeling involves two major processes. These two processes include: the resorption of bone and the laying down of new bone. This is accomplished by two cell types, osteoclasts and osteoblasts, accordingly with the previous statement (Skedros et al., 2005; Frost, 1990; Parfitt, 1994; Rubin et al., 1996). See Figure 1, below, this shows a general illustration of the remodeling process. During the first process of the resorption, osteoclasts reabsorb bone forming what is called a resorption cavity. This resorption cavity is a physical shape that outlines the space in which new bone will be filled in (Skedros et al., 2005; Sokolof, 1973; Parfitt, 1984; de Ricqle`s et al., 1991; Zhou et al., 1994).

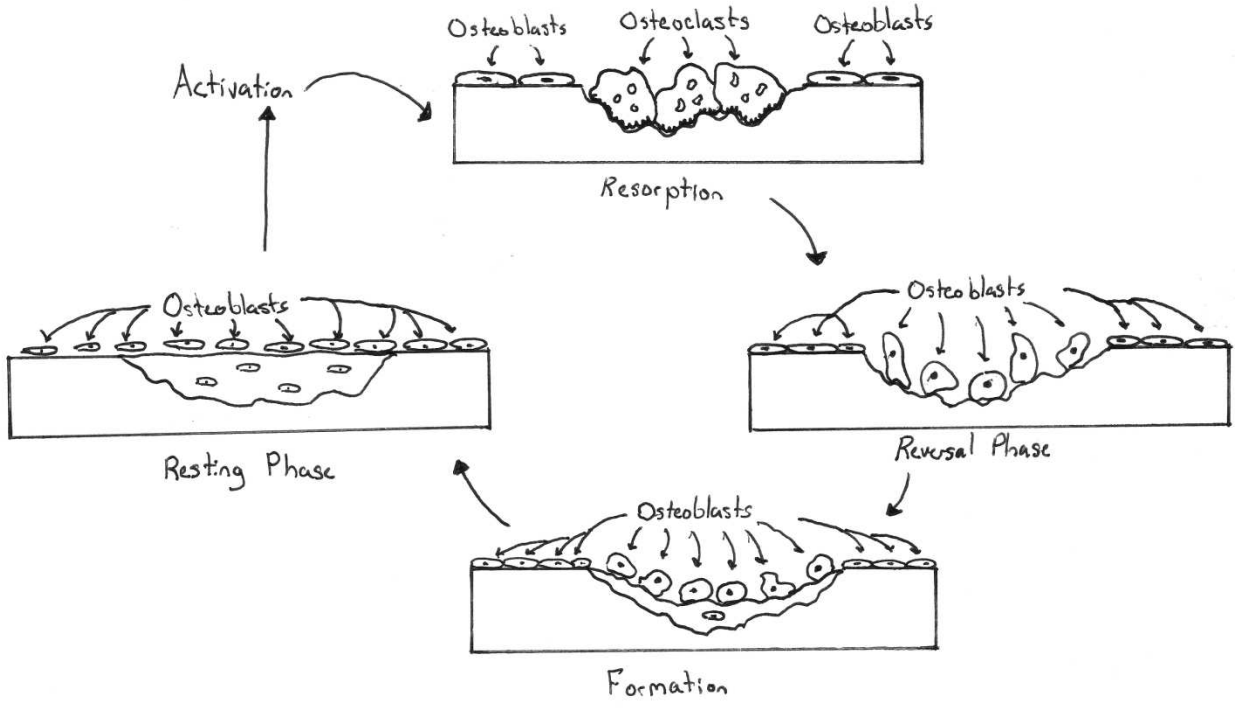


Figure 1: Shows the cycle that is bone remodeling. Activation of the osteoclasts leads to resorption. After the bone has been reabsorbed osteoblasts arrive and begin the reversal surface, to start to lay down new bone. Osteoblasts continue after the reversal surface to lay down even more new bone. Once the resorption cavity is filled there is a resting phase that waits once again to be activated and start the process anew.

New bone will be laid down, in this resorption cavity, by osteoblasts in the form of collagen. This is typically in the form of collagen I (Rubin et al., 1996). Collagen as a whole is not very stiff but allows for deformation. Collagen I is not just in a single stranded form. It is a summation of alpha chains wound into a more complex three stranded structure, called tropocollagen. Tropocollagen is generally considered to be ~300nm in bone (Rubin et al., 1996; Fratzl et al., 2004). See Figure 2, below, this shows a general illustration of formation and hierarchy of collagen molecules.

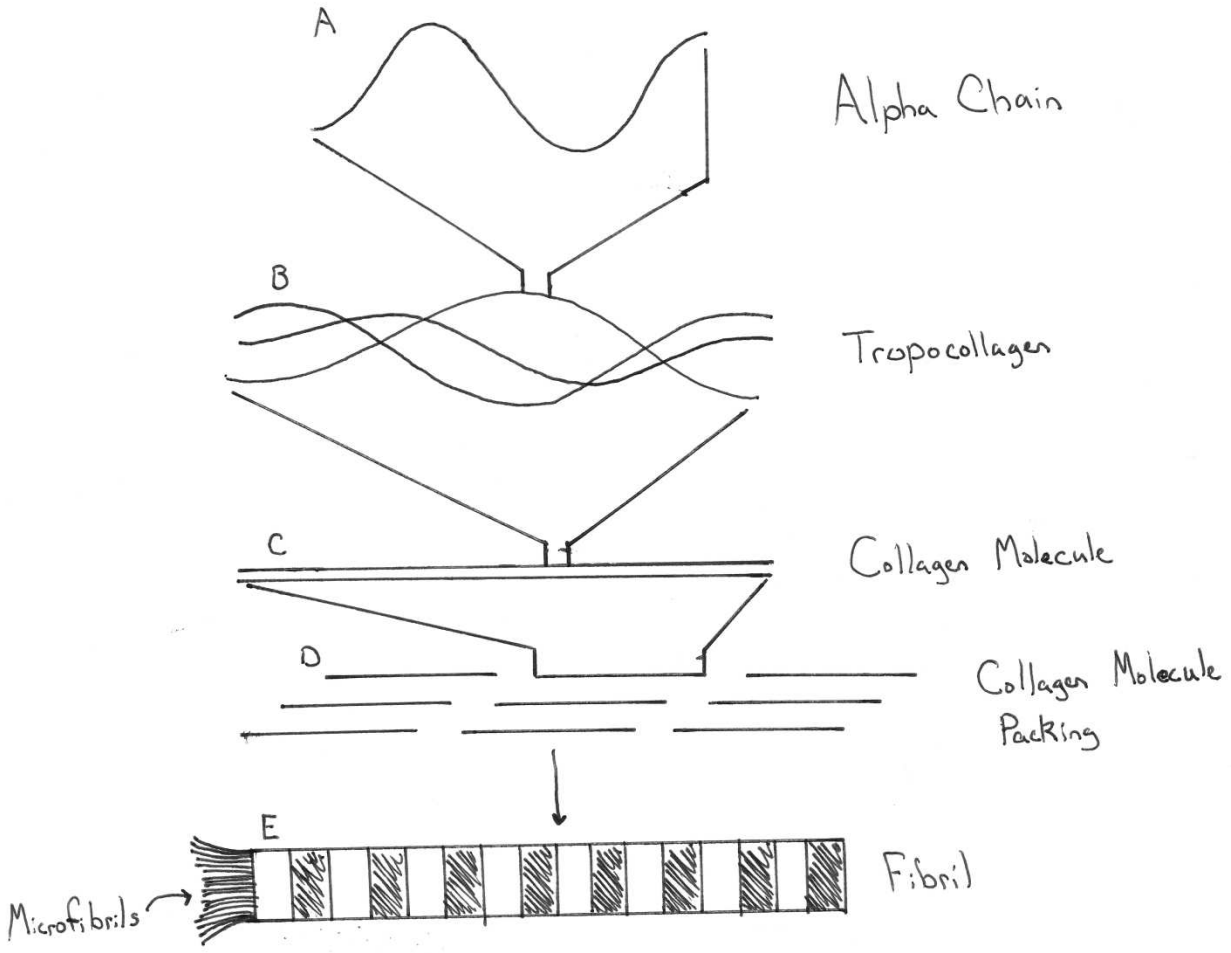


Figure 2: Shows the general hierarchy of collagen with A being a single alpha chain. B shows 3 alpha chains coming together to from tropocallagen. C shows a collagen molecule. D shows the packing of collagen molecules. The spaces show where mineralization can occur. E shows a fibril, which is the summation of many microfibils, which in turn is a long chain of collagen molecules.

The addition of crosslinking among tropocollagen will improve the stiffness and compressive strength of bone. There is a gap between each tropocollagen band, which the mineral hydroxyapatite will reside, see Figure 2 D page 6.

Mineralization also plays a large part of the organization and overall strength of a bone. In this case the primary mineral in bone is hydroxyapatite [$Ca_5(PO_4)_3(OH)$] or known as apatite [$Ca_5(PO_4)_3(F, Cl, OH)$] in nature (Rubin et al., 1996). Yet others have different names for the mineral. In Weiner and Traub's 1992 article they call it dahllite [$Ca_5(PO_4,CO_3)_3(OH)$]. Later, in Weiner and Wagner's 1998 article they call it carbonated apatite [$Ca_5((PO_4)(CO_3))_3$]. This may be due to the lack of hydroxyl groups (OH) in the NMR and FTIR analysis of bone (Loong et al., 2000; Rey et al., 1995). It is more generally called hydroxyapatite though, but there are other names for the primary mineral in bone. It is also known that each mineralized gap structure has two crystals or plates, termed by Robinson, and they stack in a hexagonal crystal structure (R. Robinson 1952; Bone Biology and Mechanics Lab Indiana University-Purdue University, accessed 2015). See Figure 3, below, this shows a general illustration of a hexagonal crystal structure.

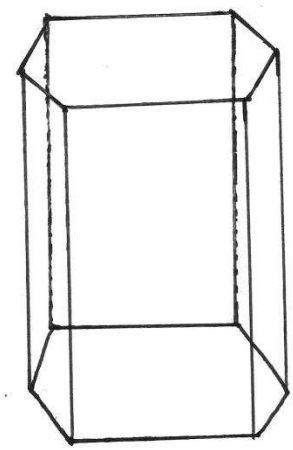


Figure 3: Shows an illustration of a hexagonal crystal structure.

Hydroxyapatite "connects" the tropocollagen bundles that are already crosslinked. Rubin et al. (1996) suggests that these mineralization sites generally have a crystal approximately 20 to 80 nm in length and 2 to 5 nm thick. Other researchers further go on to say the crystal is approximately 50 nm long by 2 to 3 nm thick by 25 nm wide (Fratzl et al., 1992; Landis and Glimcher, 1978; Nyman et al., 2004;Wachtel and Weiner, 1994). See Figure 4, page 9, this shows a general illustration of the mineralization process between tropocollagen bundles. The amount of mineralization sites that are filled will represent the level of mineralization for the bone. The level of mineralization generally gives "stiffness" to the collagen. The appropriate level of mineralization is thus very important biologically, because having hypo-mineralization or hyper-mineralization could be detrimental. It has been suggested by Glimcher, in his 1987 article, that the mineral phase in bone takes up approximately 43% of the total volume of the bone. Glimcher also said that the primary chemicals of bone's mineral phase are calcium (Ca) and phosphate (PO_4). There is also a little carbonate (CO_3) and some impurities (sodium, magnesium, potassium, citrate, fluoride, and phosphite) (Glimcher, 1987).

Once collagen has been laid down, in the bone remodeling process, collagen is aligned and mineralized. The alignment and level of mineralization is dependent on general use and time. The direction of alignment of collagen fibers dictates the mechanical properties of bone. When first laid down, collagen will be very random in orientation. Collagen can then shift its alignment and become more mineralized (Lawson et al., 1998). In cortical bone collagen is laid down into formations. These formations are called osteons. Osteons are a unit of collagen and minerals that make up the structure of cortical bone. In both the Rubin et al. 1996 and Skedros et al. 2005 articles, osteons are shown and described as a structure formed from bands of collagen fibers, i.e. concentric lamellae. An osteon is a product of bone formation, yet there are two

different types of osteons. These two types are primary osteons and secondary osteons. A primary osteon results from the first time bone is laid down, in the case of modeling. A secondary osteon is the result of bone remodeling. See Figure 5, page 10, shows the difference between the primary and secondary osteons.

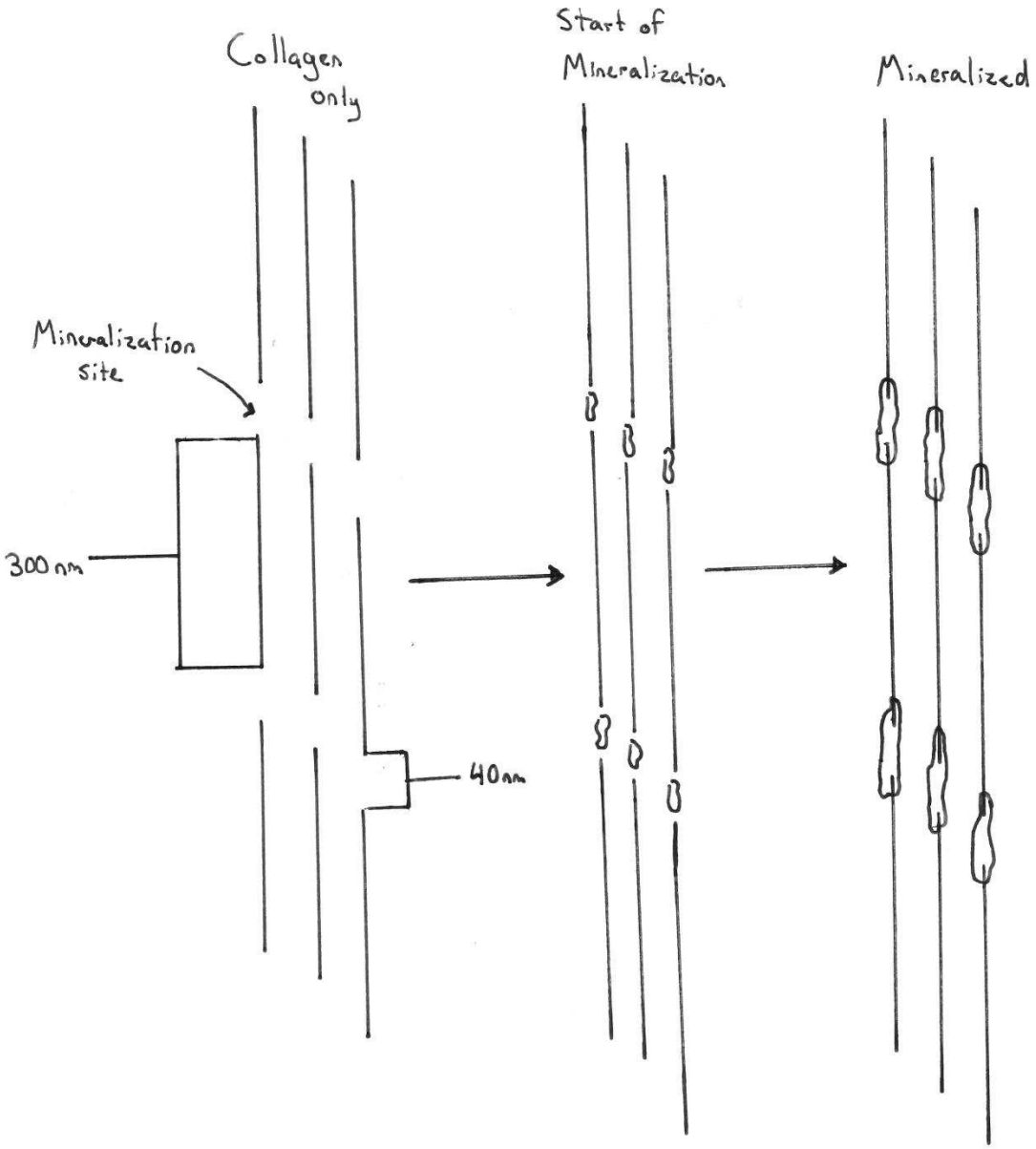


Figure 4: Shows hydroxyapatite crystal growth between tropocollagen bundles.

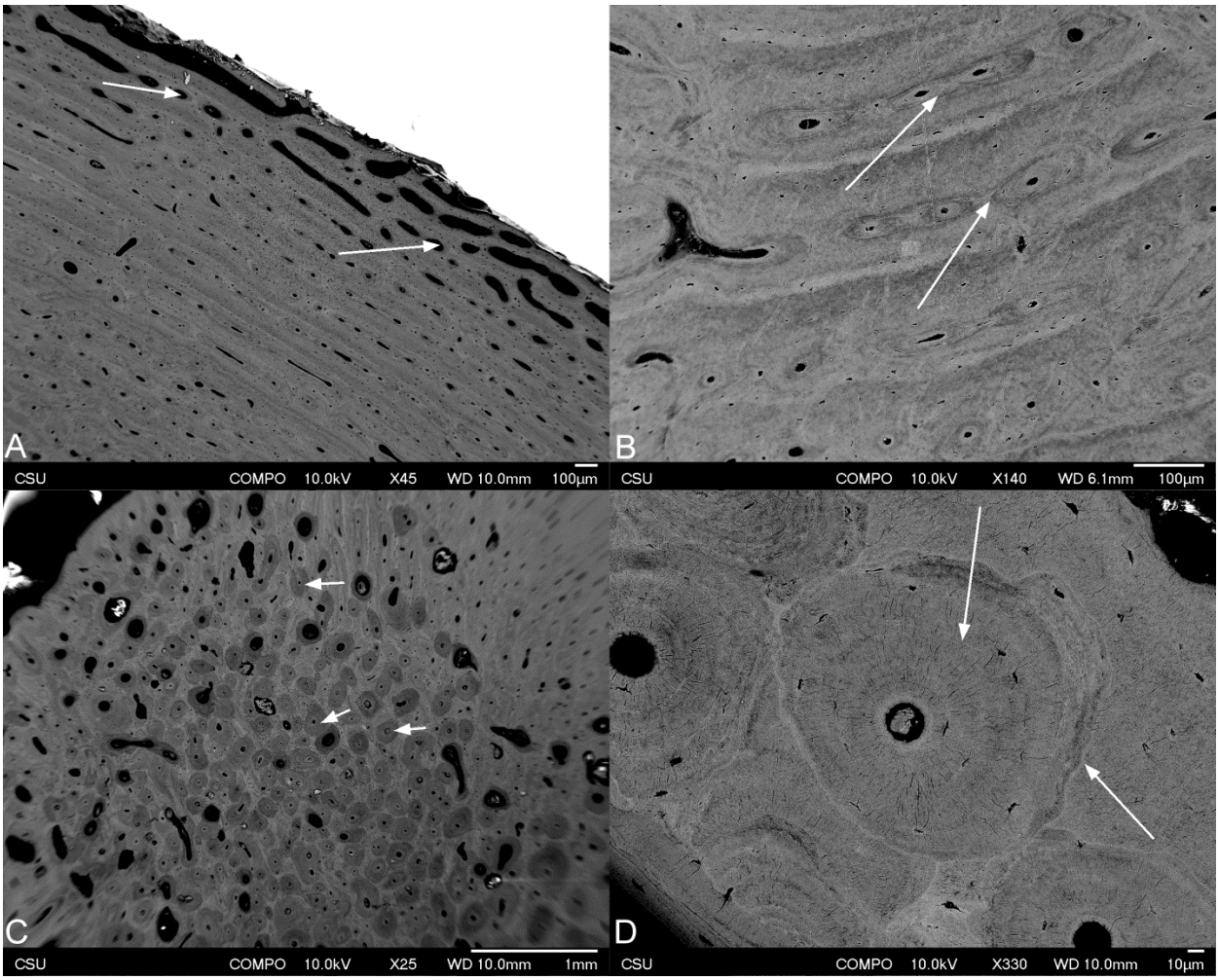


Figure 5: SEM images showing secondary and primary osteons. Image A, in the top left, shows bone modeling and the formation of primary osteons, on the periosteal (outer) surface. Regions of current primary osteon formation are pointed out by arrows. Image B, in the top right, shows a zoomed in image of completed primary osteons. Primary osteons are pointed out by arrows. Primary osteons are identified by the lack of lines encompassing single osteons, i.e. cement line. Also the lack of bone remodeling, and linear osteons stacking signifies primary osteons. Image C, in the bottom left, shows a region high in remodeling. In this region of high remodeling, there are many circular objects that are darker than their surroundings. These darker circular objects are an example of secondary osteons. Several fully filled in secondary osteons are pointed out by arrows. Image D, in the bottom right, shows a zoomed in image of a secondary osteon. The secondary osteon is identified by the thinner line encompassing the osteon, i.e. cement lines. Arrows point to the inside of the secondary osteon and the cement line.

This alignment of the collagen in lamellar bone is generally stress orientated. In a mechanical sense the lamellar bone is much tougher, but with a caveat. This caveat is that this is only in a certain loading direction that the bone is tougher. It could be said that collagen will be orientated into an organization that is strongest in the loading of this specific region of bone. In

an example by Fratzl et al. 1996, reorganization of collagen is shown as the collagen orientation is shown to change from the surface of the bone as one travels deeper into the bone. The surface collagen fibers are almost parallel to the surface. This would imply collagen fibers closer to the surface are in line with the long bone giving a high level of strength in one direction, but also a high level of resistance to surface shear. The fibers turn to approximately 45 degrees as they travel deeper in the bone. This provides strength in multiple directions, but at a lower level than the previous collagen fibers. Also in cases of a habitual loading pattern, the alignment of collagen fibers in long bones, lamellar bone would be tougher, than bones with random collagen alignment. Generally long bones are loaded biologically in bending. In the end though collagen fiber orientation is dependent on how that specific part of the bone is loaded, and can be adaptive. Collagen fiber can re-orientate to changes over time in response to the forces applied on the bone.

To further the idea of the purpose of bone remodeling, one must look at the function and purpose of bone, as it pertains to living beings. Also one must look at the reason that bone is remodeled in the first place. As stated before bones are the primary physical structure to humans that gives: rigidity, points for muscles to attach such that humans can move, protects organs and more sensitive parts of the human body, to serve as a chemical and cellular reservoir for the body, and the list could go on. According to Rubin et al. (1996), bones represent a reservoir for minerals, containing 99 percent calcium, 85 percent phosphorus, and 66 percent magnesium for the entire body. Also elaborated by Rubin et al. (1996), is that bone will adapt to mechanical demands such as exercising and bed rest. Exercising represents a form of mechanical loading which will induce damage to bones in the form of fatigue microcracking. The bone will respond to this by up regulating bone remodeling and bone synthesis. As for the opposite case, bed rest

would show a lack of mechanical demand on the bone structure. While bed rest is helpful after a prolonged time of increased bone loading, this would allow the bone remodeling to catch up with the repairing of the bone. Generally prolonged disuse or unloading of bones will shift the process of bone remodeling into removing more bone than is being laid down. Another point of interest that Rubin et al. (1996) brings up is the metabolic importance of bone. Bone is a material that needs to be balanced in its formation. Too much mineralization will lead to hypercalcemic bone that will be rigid and prevent energy absorbing deformation. Too little mineralization will lead to hypocalcemic bone that will be soft and deform too much (Hall et al., 1993; S. A. Wainwright et al., 1982). Both of the previous cases are not ideal since they provide less overall toughness than a more balanced mineralization would. In the first case the bone would be ridged compared to normal bone and could absorb energy without deforming much. This would lead to an increased modulus of the bone, but fracture much sooner. In the second case the bone would not have enough rigidity and deform fairly easily compared to normal bone. This would yield a relatively soft bone, lack toughness, and have a low modulus when compared to a normal bone; but the bone would be able to go through large deformations (Hall et al., 1993; S. A. Wainwright et al., 1982). These types of metabolic actions could drastically change the ability of the skeletal structure to perform its basic functions. Thus a balance is the best outcome when it comes to the metabolism of bone. While osteoporosis is not a direct outcome of bone metabolism it has to do with the inability of the osteoblasts, lying down of bone, to keep pace with the osteoclasts resorbing bone. This could be considered a metabolic procedure (Rubin et al. 1996). Osteoporotic bone has the same outcome as hypercalcemic or hypocalcemic bone. The bone's overall toughness is reduced, which happens through increased porosity in the bone.

All of these properties of bone must be taken into account when attempting to understand processes and structures that reside in bone.

Cement Lines Overview 1.2

A "reversal surface" is the point where bone resorption stops and bone formation will start (Skedros et al., 2005; Sokolof, 1973; Parfitt, 1984; de Ricqlès et al., 1991; Zhou et al., 1994). This "reversal surface" is of particular importance, since it is the location of structure of interest for the current study. In cortical bone, the border of the resorption cavity is a boundary that separates where new bone and old bone will eventually meet. This line has two different names. Generally it is termed a reversal line during the remodeling process, and a cement line after the remodeling process is done (Skedros et al., 2005). An example of a cement line is shown in Figure 5 image D, on page 10. The brighter white band surrounding the secondary osteon is a cement line. The first documented use of the term cement line was done by Von Ebner. In 1875 Von Ebner described a cement line as a "kittlinien" (putty line or glue line). This reversal line or cement line is approximately less than 5 µm in size (Skedros et al., 2005). Generally this is "a seam" among portions of the new and old bone (Skedros et al., 2005; Sissons, 1962; Castanet, 1981). Once bone is reformed in the resorption cavity, the overall structure is a secondary osteon. As before, an osteon is defined as an integral part of cortical bone, which has concentric layers of lamellar bands, surrounding a central hollow shaft, called a Haversian canal. See Figure 6, page 14, this shows a general illustration of concentric layers of lamellar bands, surrounding a central Haversian canal. It has also been suggested that reversal lines or cement lines are absent in primary bone. They are said to be only present in secondary bone. This is stated to be due to the fact that resorption of primary or secondary bone is needed

to form a resorption cavity in which a reversal surface and consequently a reversal line or cement line can be formed (Skedros et al., 2005; Pritchard, 1972; Sokolof, 1973; Jee, 1983; de Ricqle`s et al., 1991; Zhou et al., 1994).

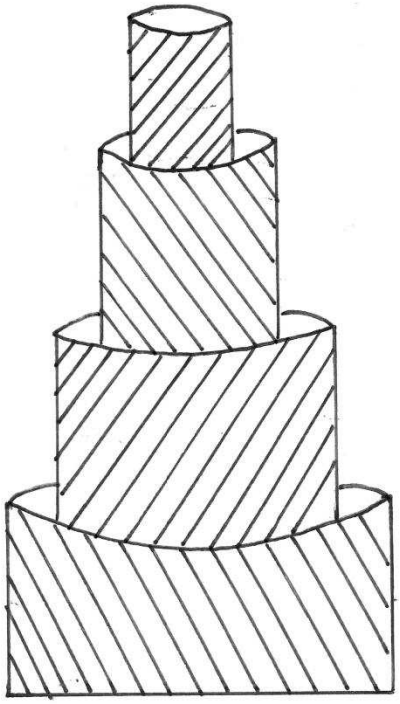


Figure 6: Shows concentric lamellar bands surrounding a central Haversian canal. The cement line would surround the outermost lamellar band.

There are several ways in which researchers have used to analyze the composition of the cement lines. There has also been a history of disagreement in the elemental composition of said cement lines. The elemental composition is more termed as the level of mineralization of the cement line when compared to surrounding bone. Some studies have used microradiographs of bone and then there was information that was extracted from the brightness of the cement line when compared to the surrounding bone. This is called the grayscale levels and is an imaging technique that is used, in this specific case, to determine the mineralization of the regions of interest in bone (Skedros et al., 2005). In the following studies, the researchers determined that

the brighter cement lines, in the microradiographs, were indicative that the mineralization of the cement lines was higher than that of the surrounding bone (Amprino and Engstro¨m, 1952; Jowsey, 1960, 1964, 1966; Smith, 1963; Philipson, 1965; Heuck, 1971; Lacroix, 1971; Yaeger, 1971; Dhem and Robert, 1986; Skedros et al., 2005). Since cement lines have been described as "highly mineralized materials", there have been other studies implying that the interface between new bone and old bone, cement lines, is a brittle interface (Philipson, 1965; Castanet, 1981; Parfitt, 1984). Yet as stated the actual mineralization of the cement line, has a history of disagreement.

The idea that cement lines are "highly mineralized" has been challenged by several others. The studies include not only a biomechanical approach but also a compositional approach (Fawns and Landells, 1953; Lakes and Katz, 1979; Lakes and Saha, 1979; Katz, 1980; Frasca, 1981; Frasca et al., 1981; Lakes, 1995). In the article from Frasca et al., in 1981, it was suggested that in human compact bone, with an increased amount of secondary osteons and thus presumably more cement lines, had different results in shear testing. The bones were tested in shear and compared to other cortical bone with less secondary osteons. The bones with a higher number of secondary osteons had a reduced shear modulus and a larger viscous behavior. It was then deduced that these results from the increase in overall viscosity of the sample, were due to the increase in cement lines of the bone. This would suggest that osteons might be more viscous than formerly assumed. This was not fully supported due to a lack of microscopic study and that this was only performed on a single sample (Frasca et al., 1981). In the article by Katz, in 1980, there is a previous model that used an assumed stiffness of cement lines to be one fourth that of the surrounding bone. Also in the article of Lakes and Saha, in 1979, there is some evidence that there is possibly some fluidity of "cement lines" in well hydrated samples, which have been

induced to extended torsional load. Yet these "cement lines" were found in a primary bone sample of the diaphysis of bovine long bones. As stated above, it is assumed that there can be no "cement lines" without the bone remodeling process since "cement lines" are the fundamental outcome of bone resorption and the following replacement of that bone. This bone being absorbed includes either secondary or primary bone, yet the outcome will always be secondary bone.

This article brings up an interesting point that not only has the idea of the level of mineralization in cement lines has been historically debated, but also the definition of cement line has also been debated or at least possibly confused for other bone based structures. Both in the article by Skedros et al., in 2005, and Davies et al, in 2000, they provide a general review of the argument over the terming of cement lines. In an article by Curry and Zioupos (1994) they argue that the use of the term "cement lines" is misused by some researchers. Curry and Zioupos also point out the errors of some of the articles that conclude that cement lines are regions of not highly mineralized materials. In the 1994 article by Curry and Zioupos, they point out that the "cement lines" that Lakes and Saha described, in their 1979 article, are not cement lines. Cement lines, by their definition, can only be present in secondary bone. Curry and Zioupos align the definition of cement lines with those who present a cement line as high level of mineralization that surrounds a resorption cavity after new bone has been laid down (Amprino and Engstro¨m, 1952; Jowsey, 1960, 1964, 1966; Philipson, 1965; Heuck, 1971; Lacroix, 1971; Yaeger, 1971). Curry and Zioupos apparently strongly disagree with some other researchers that use the term cement lines to describe similar bright lines that appear in primary bone. These other lines can appear in primary or secondary bone without the sign of bone remodeling. Some of these lines are termed resting or arrest lines, that appear just like cement lines in brightness

and assumed mineralization, but the resting or arrest lines are typically larger in width (Skedros et al., 2005; Weinmann and Sicher, 1955; Lacroix, 1971; Zagba-Mongalima et al., 1988; McKee and Nanci, 1995). These resting or arrest lines are also considered to be a result of reduced bone formation or a complete stop in bone formation, not a remodeling process that involves resorption (Skedros et al., 2005; Frost, 1963, 1973; Kornblum and Kelly, 1964; Jaffe, 1972; Pankovich et al., 1974; Parfitt, 1983; de Ricqle`s et al., 1991; Nyssen-Behets et al., 1994; McKee and Nanci, 1995). In a paper by Kohler et al., in 2012, these lines are called lines of arrested growth. These lines will be covered in more depth in the following section. Some of the confusion comes from the interchanging of these two lines in some papers (Zhou et al., 1994; McKee and Nanci, 1995). Also, some researchers term cement lines as the substance or material that is put down in sites of initial bone formation of either primary or secondary bone (Frasca et al., 1981a; McKee and Nanci, 1995; Davies, 1996). The term cement lines seem to have a bit of disagreement and this does not necessarily mean that either party is wrong. There needs to be some clearing up and agreement of the term cement line and what a cement line means. If there is another line that looks similar to a cement line then it should be further studied, and if necessary other biological terms derived for these other bone structures.

The idea of a more rigid and hypermineralized cement lines would add validity and back up some of the previous mechanical and more visible properties of bones, suggested by other researchers. One of these suggested more mechanical and visible properties are crack propagation. Some researchers suggest that cement lines play an important role in: fatigue properties, microcracking, crack propagation and crack blunting (Burr et al., 1985, 1988; Advani et al., 1987; Choi and Goldstein, 1992; Norman et al., 1995; Schaffler et al., 1995; Prendergast and Huiskes, 1996; Braidotti et al., 1997; Norman and Wang, 1997; Boyce et al., 1998; Guo et

al., 1998; Yeni and Norman, 2000; O'Brien et al., 2003; Sobelman et al., 2004). If cement lines were hypermineralized and thus brittle, then it would make sense what some researchers observe. It is observed and suggested that cracks tend to travel along cement lines, around osteons, rather than through osteons. Also it has been suggested that cement lines have low amounts or are devoid of collagen, which as stated before is the primary protein that makes up mature bone, with the mineral hydroxyapatite. These findings were presented by several researchers: Skedros et al., 2005; Weidenreich, 1930; Maj and Toajari, 1937; Dempster and Coleman, 1961; Evans and Bang, 1966; Schaffler et al., 1995; Wang and Norman, 1996; Norman and Wang, 1997; Boyce et al., 1998; Jepsen et al., 1999. This is much similar to the idea of a precipitant in a metal. Generally in mechanical and materials science based approach, one would see cracks that initiate, in fatigue or uses that fall outside of the components specified range of use. Many times this same idea is seen that crack will travel around precipitants, which are tougher than the surrounding material (Hall et al., 1993; S. A. Wainwright et al., 1982). Only if there is sufficiently high levels of energy will the crack actually travel through the precipitant (Hall et al., 1993; S. A. Wainwright et al., 1982). One could consider these examples to be analogous to bone. As in the precipitants are osteons and the surrounding material is the cement line. Yet in this example much of the material would be a sea of precipitants held together by little material. This could of course inhibit the similarity of these comparisons. A final suggestion of some researchers is that cement lines play a role in energy dissipation: through energy absorption, fracture, viscous dampening, and elasticity (Dempster and Coleman, 1961; Piekarski, 1970; Lakes and Katz, 1974; Carter and Hayes, 1977; Saha, 1977; Lakes and Saha, 1979; Gottesman and Hashin, 1980; Katz, 1981; Martin and Burr, 1982; Burr et al., 1985; Choi and Gol stein, 1992; Norman et al., 1995; Guo et al., 1998; Currey, 2002; Les et al., 2004). All of these

suggestions, of course hinge on the relative mineralization of the cement lines. All assumptions are based on if one could consider a cement line hypermineralized. Many of the stated observations above of mechanical properties such as cracks traveling along cement lines would lean more towards the idea that cement line would be a relatively brittle material, when compared with surrounding material.

Generally the level of mineralization of cement lines was determined by the brightness or darkness of the cement lines in micrographs. Generally these micrographs were obtained through the use of a scanning electron microscope (SEM) in backscatter mode. The level of grayscale was used with image processing to determine the relative levels of mineralization, when comparing it with other surrounding bone. Again, this is another point of disagreement among researchers. The bright lines are generally agreed upon by most researchers. Some researchers think that these lines may be optical aberrations when looking at bone. Specifically some researchers analyzed cement lines and came to the conclusion that they "contain significantly less calcium and phosphorus" (Schaffler et al., 1987). Not only did Schaffler et al. use an SEM with energy dispersive X-ray spectroscopy (EDX) as a secondary form of analysis they came to the same conclusion. Along with Schaffler et al., Burr et al. also came to a similar conclusion that cement lines were not considered to be relatively high in mineralization and not optically different than surrounding bone (Burr et al, 1988). In direct contradiction to both Schaffler et al. and Burr et al., there are a larger handful of researchers that agree on the idea that cement lines are brighter than the surrounding bone (Skedros et al., 2005; Boyde et al., 1990; Boyde and Jones, 1996; Boyde and Kingsmill, 1998; Howell and Boyde, 1999; Dorlot et al., 1986; Roschger et al., 1993, 1997; Grynpas et al., 1994; Fratzl et al., 1996; Cool et al., 2002; Bachus and Bloebaum, 1992; Bloebaum et al., 1997; Vajda et al., 1999). Obviously there seems to be more

researchers that agree on the idea that cement lines are brighter lines in bone when compared with the surrounding bone. Combining this with other studies that show brighter features in bone on backscattered electron (BSE) images, represent higher levels of minerals (Reid and Boyde, 1987; Skedros et al., 1993a, 1993b; Roschger et al., 1995; Bloebaum et al., 1997). One could conclude that these, generally agreed upon, brighter cement lines in bone are higher in mineralization than the surrounding bone. Yet historically, there were disagreements on this point. It seems that using BSE grayscale images alone are not enough and more advanced techniques are needed to better understand the mineralization of cement lines.

In the Skedros et al. 2005 article, the use of SEM EDS is used to show that there is more evidence to support the idea that brighter regions in BSE images are of higher mineralization than darker regions. In effect Skedros et al., 2005 article, uses quantitative SEM EDS to show that there is a significantly higher level ($p < 0.05$) of % Ca in the cement lines (25.13±0.46) vs. in the osteon (23.82±0.49) of humans' femurs and radii. Yet in the Skedros et al., 2005 article, there was no significant difference between interstitial bone and cement lines. The understanding and use of SEM and EDS will be covered more in a dedicated section, 1.4, on electron microscopy. From the supported idea of BSE grayscale images, any region in bone that is significantly brighter than the surrounding should contain higher levels of mineralization. This is true since calcium is generally considered to be the densest element, in bone, that is not trace. From the research on cement lines and how BSE brightness signifies hypermineralization in regular bone, the theory can be applied to other regions of interest, in the bone. One such region of interest is Lines of Arrested Growth (LAGs).

Lines of Arrested Growth (LAGs) Overview 1.3

As stated above in the cement line section, sometimes similar lines to cement lines appear in bone. Both Curry and Zioupos stated that some researchers confuse cement lines and these other lines, which appear in bone, and called such line cement lines. This then adds confusion to the idea of what a cement line is. It was also said that these new lines can appear in primary or secondary bone without any sign of remodeling. These new lines were termed as resting or arrest lines, that appear just like cement lines in brightness and assumed mineralization, but the resting or arrest lines are typically larger in width (Skedros et al., 2005; Weinmann and Sicher, 1955; Lacroix, 1971; Zagba-Mongalima et al., 1988; McKee and Nanci, 1995). It was said that these resting or arrest lines are also considered to be a result of reduced bone formation or a complete stop in bone formation, not a remodeling process that involves resorption (Skedros et al., 2005; Frost, 1963, 1973; Kornblum and Kelly, 1964; Jaffe, 1972; Pankovich et al., 1974; Parfitt, 1983; de Ricqle`s et al., 1991; Nyssen-Behets et al., 1994; McKee and Nanci, 1995). See Figure 7, page 22, it shows a comparison of LAGs vs. a cement line. By definition a line of arrested growth implies a complete cessation of bone growth. Unlike in cement lines LAGs are found in the modeling of bone, not the remodeling (Hall et al., 1993; Chinsamy-Turan, 2005; Carter et al., 1991; Ray et al., 2009; Sander et al., 2006). Modeling of the long bone can occur radially on the periosteal surface. This radial growth allows for LAGs to form and to be seen in cortical cross sections. Annuli are a line that is similar to LAGs. Annuli can be confused for LAGs, in some cases. They are a region of bone growth that, unlike LAGs, are characterized by slowed and not a complete cessation of growth (S. Ray et al., 2009; P. M. Sander et al., 2006; Hall et al., 1993). Annuli are lines similar to LAGs in shape, but generally are larger. Annuli can also precede and\or follow LAGs.

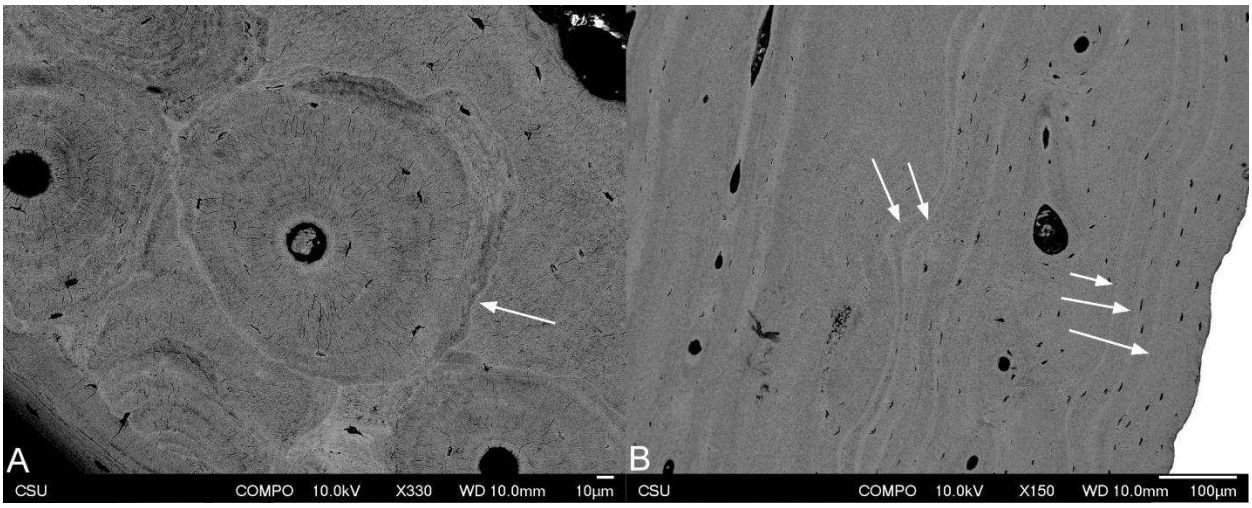


Figure 7: Shows an example of a cement line vs. LAGs. Image A, left, shows a cement line (arrow). Image B, right shows an example of LAGs (arrows). LAGs generally run all the way around a cross section, circumferentially, were cement lines on encompass only an osteon.

LAGs have been initially studied in dinosaurs' fossils (Chinsamy-Turan, 2005; Sander et al., 2006). Dinosaurs are said to have many of the similar biological abilities and function of present reptiles. One of the important biological features was cold blooded. Since the dinosaurs were cold blooded they could not generate their own internal body temperature. They were dependent on the environment for their temperature, they are thus called ectothermic. Due to this lack of internal body heat it was suggested that ectotherms are sensitive to colder temperatures. A colder temperature could cause the animal's metabolism to drop and this forms a LAG. The LAG would form due to adverse environment factors to bone growth. Many LAGs have been identified in well preserved fossils (Chinsamy-Turan, 2005; Sander et al., 2006; Woodward et al., 2011). Also modern day alligators, which share some common ancestors with dinosaurs and are reptiles that have many of the same biological features, show present of LAGs (Woodward et al. 2014). This idea of LAGs being thermally regulated works in ectotherms. This would mean than endotherms, or animals that do maintain internal temperature, could not have LAGs. An article by Köhler et al., in 2012, suggests that LAGs being are present in

ungulates, which are endotherms. This contradicts the historical thoughts, by dinosaur researchers, that LAGs are based off of thermal regulation. Fastovsky et al. (2012) and Weishampel (2004) suggested that endotherms are able to control their internal temperature then, due to this consistency, they would be less inclined to have LAGs or devoid. Understanding such LAGs could provide a great deal of insights into climates and living situation of extinct vertebrates if the LAG were indeed a result of thermal regulation. Yet it seems more than that is at play. Köhler et al. (2012) suggests that the LAGs are formed due to a lack resource and environmental pressure, on the organism. Specifically Köhler et al. (2012) suggests that LAG formation is a process of metabolic downturn, meaning that metabolism is the primary driving factor to LAG formation. Köhler et al. (2012) suggests that water and food are primarily high during a certain season and then dip off in another season, i.e. summer and winter. During the season of plentiful water\food, metabolism is up and bone formation is in fully swing. During the season with less water\food, metabolism is down and bone formation slows and\or stops. Bennett et al. (1981, 1987) agree that only an elevated metabolism would be able to have bone formation. Yet Bennett et al. (1981, 1987) also state that bone formation is tied to the overall ability to maintain body temperature. Köhler et al. agrees that it is generally accepted that ectotherms have LAGs present due to the change in the environment yet uses it in the term of metabolism. If an ectotherm was subjected to a colder environment, like winter, then metabolism would decrease along with bone formation, and LAGs could form. Also if the same ectotherm was subjected to a warmer environment, like summer, then metabolism would increase along with bone formation.

In an article by Castanet et al. (2003), it was also shown that LAGs are present in endotherms. The focus of this article was not to debate the formation of LAGs in endotherms,

compared to ectotherms. Castanet et al. (2003) were trying to find out the number of LAGs and how many formed. In this study they used grey mouse lemurs, which are small nocturnal primates. They tried to relate LAG formation to circannual light cycles. In the study Castanet et al. (2003) stated they expected to see one plus or minus one LAG form per circannual light cycle. This was not exactly backed up by their graphs since they were not as close to a slope of one as one would expect. Their definition of cycle changed for two separate groups. In one group the term cycle meant a whole year. In the other group there were 1.3 cycles per year. This second group had an accelerated cycle rate. During these accelerated cycles Castanet et al. (2003), simulated light patterns of a normal year but in an accelerated fashion. The grey mouse lemurs in the accelerated group had the same percent of light for both a "winter and summer" season effects, but at an accelerated rate. From this Castanet et al. (2003) showed that they could match the yearly normal cycle grey mouse lemurs, with their accelerated grey mouse lemurs, for LAG formation. Castanet et al. (2003) also stated that there could be no more than seven LAGs for grey mouse lemurs. After the age of seven then, in a normal setting, grey mouse lemurs could not be aged through the counting of LAGs (skeletochronology). While this number may not be the same for other animals it does show one interesting feature of LAGs, and that is that as the animal ages into maturity radial growth\bone modeling slow down. This causes LAGs to pile up on the periosteal surface of older animals. By pile up, it is meant that the separation between each LAG in older is less and less to the point they look like, double, triple or even several look like just one LAG. Seven is a nice number in the case of Castanet et al. (2003). This is due to that seven years old is generally a fairly old grey mouse lemur. In the wild they generally do not live this long due to predation\natural causes. This was only an issue in captivity since grey mouse lemurs could live to ten to twelve years old, when these natural pressures were removed.

Double LAGs are another oddity of LAG formation. Since there is currently not a lot of information on LAGs there is even less on double LAGs. The idea of environmental cues playing a role in LAG formation is still present. Most of the info present on double LAGs is in amphibians, an ectotherm. In one case, M. Iturra-Cid et al. (2010) study double LAGs in Chilean frogs. In another case, F. M. Guarino et al. (2008) studied double LAGs in Rana holtzi, another frog indigenous to Turkey. A double LAG signifies two complete cessations in bone growth, generally during one year. Double LAGs are hard to differentiate in older animals. As stated above in Castanet et al. (2003) LAGs to pile up on the periosteal surface of older animals causing the separation between each LAG in older is less and less to the point they look like, double, triple or even several look like just one LAG. Yet double LAGs in younger animals or even ones in older animals that are not near the periosteal surface, show evidence of true double LAGs. Both M. Iturra-Cid et al. (2010) and F. M. Guarino et al. (2008) have many suggestions of what contribute to double LAG formation. One of these is sex, it is suggested that males may be more prone to double LAGs. Another one that seems to have a larger impact is altitude. Both studies suggested this could play a larger role in double LAG formation. Yet this would be due to temperature and access to resources. Double LAGs were attributed to one in the summer, due to aestivation, and one in the winter, due to periods of hibernation. This also forgoes extreme environment events such as severe drought, extreme heat waves, extended winter, and\or bimodal summers\winters (having almost two separate seasons with an oddly mild time separating the two). Any of these kinds of extreme events could cause multiple LAGs, sometimes more than two. This is of course in ectotherms and not endotherms. It is unknown if these trends carry over or not but one could speculate that double LAGs could be found in endotherms and could be due to any of the above reasons. It seems it is important that generally

double LAGs form due to two adverse time periods for the animal. Seems that these two points may have a stronger correlation with winter and summer, but that is not for sure. Also double LAGs are a term and there can be triple LAGs and more depending on the situation. Finally double LAGs may be hard to determine at the periosteal surface of older animals.

Köhler et al. (2012) and Castanet et al. (2003) have shown that LAGs have been found in mammals. Yet the understanding of a LAG function is still not completely known (P. M. Sander et al., 2006). It is a curious thing to see LAGs in mammals since it is generally understood that mammals are endotherms and regulate their own internal balance. This can be described under the term of metabolic down turn that Köhler et al. (2012) describes. If LAGs form due to metabolic downturn then all the environmental cues (temperature, water, food\minerals, light, and other environmental pressures) fit under that term. Then metabolism would be the overarching term since it is influenced by all of those, in some way. Also size might play a role since Castanet et al. (2003) saw them in grey mouse lemurs before Köhler et al. (2012) saw them in ungulates. Body mass and relative size in mammals may also play an issue. Another question could be is this the same in mammals that are non-ruminants, for example bears that hibernate? Hibernation is a drastic change in the animal's metabolism, so something interesting with LAGs would be highly likely. It would also be likely from M. Iturra-Cid et al. (2010) and F. M. Guarino et al. (2008) that double LAGs could be present in bears, due to hibernation length and or altitude of the hibernator. It would prove valuable if these lines truly showed a relative map of the animal's metabolism. It also seems that LAGs could be altered or wiped out by osteon formation, after bone remodeling. This could hide or interfere with the understanding of LAGs. If LAGs are dependent on metabolism this could be used to show age of an animal (skeletochronology) that is of course if one has access to their bones. Comparing LAG's mineral

content to that of cement lines may provide some in site into that material properties and functions. While comparing the number of LAGs to known age may provide insight into age, climate, food, and other possibilities.

Scanning Electron Microscopy (SEM) Energy Dispersive X-ray Spectroscopy (EDS) Overview 1.4

A SEM is an instrument that uses a beam of electrons that are focused at a sample's surface (Boyde, 2012; Vermeij et al., 2012). The electrons then interact with the atoms of the sample. These interactions can result in a variety of outcomes which specific detector can pick up on, and provide analysis of such interactions. The variety of types of resultant interactions include: secondary electrons (SE), backscattered electrons (BSE), X-rays more commonly called characteristic X-rays, and possibly light in the form of luminescence (Boyde, 2012; Vermeij et al., 2012; Friel, J 2003). Luminescence is a lower energy wavelength than X-rays. These lower energy waves could fall anywhere between the ultraviolet, visible, infrared, and even long wavelengths such as radio-like waves. Generally the SEM is used more for the general understanding of the topography of a sample. This is achieved by the summation of the resulting information collected after a beam of electrons have been passed over the sample. The beam of electrons is passed over the sample to a single point. Information is collected from that point and the point of the beam is then moved to a new point. All of the data from the collector from each of these points is then added up to make a digital picture (Boyde, 2012; Vermeij et al., 2012). The image can have a very high level of resolution when compared to light based microscopes. This is due to the fact that electrons have a “higher level of energy” resulting in a smaller wavelength when compared to visible light spectrum. This is easily shown and calculated by the

de Broglie wavelength equation (Engel et al., 2010). Shorter wavelengths have a higher level of energy, thus improving the resolution of an image. There is a caveat though, with shorter wavelengths. Since the shorter wavelengths have a higher level of energy imparted in each wave packet, there is a point where the energy becomes a destructive process. By a destructive process, one means that the sample will be sacrificed in the process of analysis. Generally this is not a problem with SEM since the energy needed to view bone is not high enough to cause damage, and bone is generally more robust to beam degradation than other tissues samples. That being said, it is still possible. SEM is generally considered a non-destructive analytical method, though. Generally destruction is only a concern when more delicate or very small samples are used.

The EDS is an attachment to the SEM instrument. The EDS is a detector that X-rays of all energies are detected (Friel, 2003). As before, the SEM is using an electron beam to excite characteristic X-rays from the material being analyzed. Friel goes on to say that X-rays are absorbed in the active region of a crystal that is inside the detector. As these X-rays are absorbed they generate charged pulses that can be used to determine the X-ray's energy that caused the pulse. This is due to the fact that the energy is proportional to the charged pulse in the crystal itself. This allows for all of the X-rays that hit the detecting crystal to be analyzed basically simultaneously due to the ability of the crystal to act in such a way there is a limitation that this imposes (Friel, 2003). Since the X-rays come into the crystal and all are analyzed, there is no need for focusing the X-rays, according to Friel. This is nice in the fact is makes it easier and a challenge at the same time. This is due to the fact that the detector can only intake the X-rays that are given to it. An analogy would be a person looking out a window in a house. If a person was to stand ten feet away from the window and look out, the picture that they would see is a

smaller portion than if they were one foot away from the window looking out. This analogy to the field of view of a person relates to an EDS and its solid angle. Friel gives an equation for the solid angle. He says that the solid angle is equal to the active area of the detector divided by the distance of the detector to the sample, squared. Generally the active area of the detector is a given and is also a constant, unless multiple detectors are used. Moving the detector closer to the sample could be a way to improve the solid angle, yet this also poses an issue since there is only so close the detector can get before one would ram the detector into the central column or stage of the SEM. All of this must be taken into account in order to help one understand the findings that they are getting from an SEM EDS.

Interaction volume of the sample also plays an important role in the actual results that are obtained. Interaction volume can be defined as the volume or penetration that electrons are able to obtain when bombarding a sample. This volume is roughly tear drop shaped (Skredos et al., 2005; Friel et al., 2003; JEOL, 2015). See Figure 8, page 29, it shows an illustration of the interaction volume and its tear drop shape. See Figure 9, page 31, shows a CASINO simulation of the interaction volume, for two different accelerating voltages.

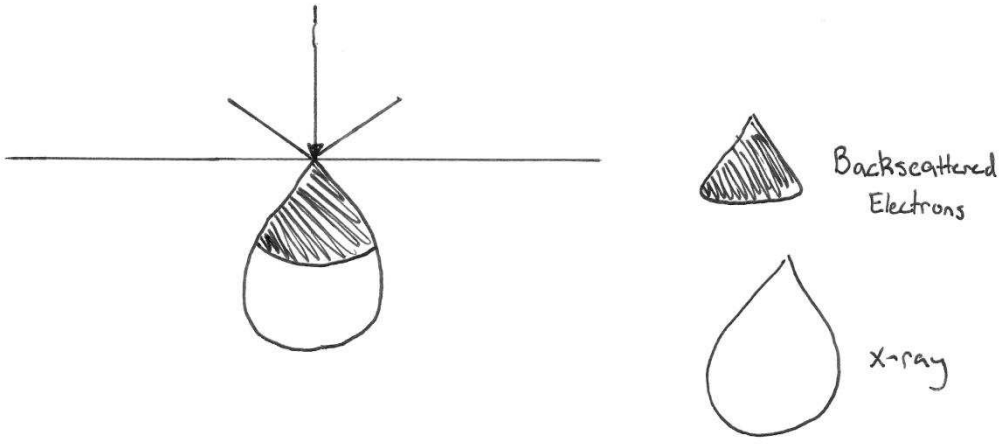


Figure 8: Shows an illustration of an interaction volume. This volume is when a sample is bombarded with electrons, secondary electrons and x-rays are emitted.

In the Skredos et al. 2005 article, this point of accelerating voltage is brought up. It is pointed out that in the Burr et al. 1988 article, an improper accelerating voltage was used. Accelerating voltage is important in SEM and to SEM EDS. Accelerating voltage could be defined as the potential energy used to project the electrons towards the sample. When using the SEM, it is important to go with as small of an accelerating voltage as possible. This lower accelerating voltage will prevent damage and charging of the sample. Burr et al., 1987 and 1988, were trying to look at a pseudo-flat surface and trying to extrapolate grayscale values. From the previous grayscale data Burr et al. 1987 and 1988 thought that cement lines would be more mineralized. The 60 kilo electron volts (KeV) accelerating voltage Burr et al. 1987 and 1988 used, was significantly larger than one needed or expected. This higher accelerating voltage would cause a larger interaction volume and would make it harder to truly understand what one was analyzing. Higher accelerating voltages will give better resolution at high magnification, but can cause damage and artifacts (JEOL, 2015). A higher accelerating voltage could be used for high resolution images, at high magnification. Burr et al., was only at 4000x which even on an early 2000's SEM is considered lower magnification, and as such would not need such a high accelerating voltage. Burr et al. 1987 and 1988 did use a JEOL 1OOCX TEMSCAN SEM. The exact specifications of the instrument are unknown, so it is possible that 4000x was high magnification for that instrument. If this is true then the higher accelerating voltage of 60 KeV could be used to obtain more resolution. Either way, accelerating voltage plays a key role in interaction volume, resolution, and potential sample damage. See Figure 10, page 31, shows another CASINO simulation of the interaction volume, but this time tries to predict the penetrations volume in the X, Y, and Z planes.

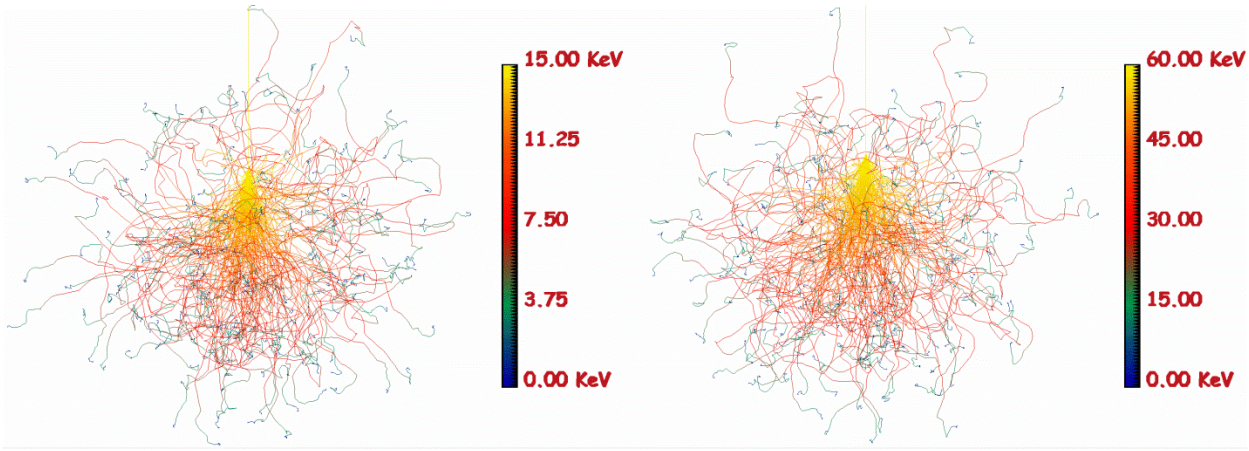


Figure 9: Shows the tear drop shape of the interaction volume. It traces the simulation of electrons impacting on the surface of a sample made of the same elements as bone. Each continuous line represents a simulated electron. On the left is 15KeV and on the right is 60 KeV.

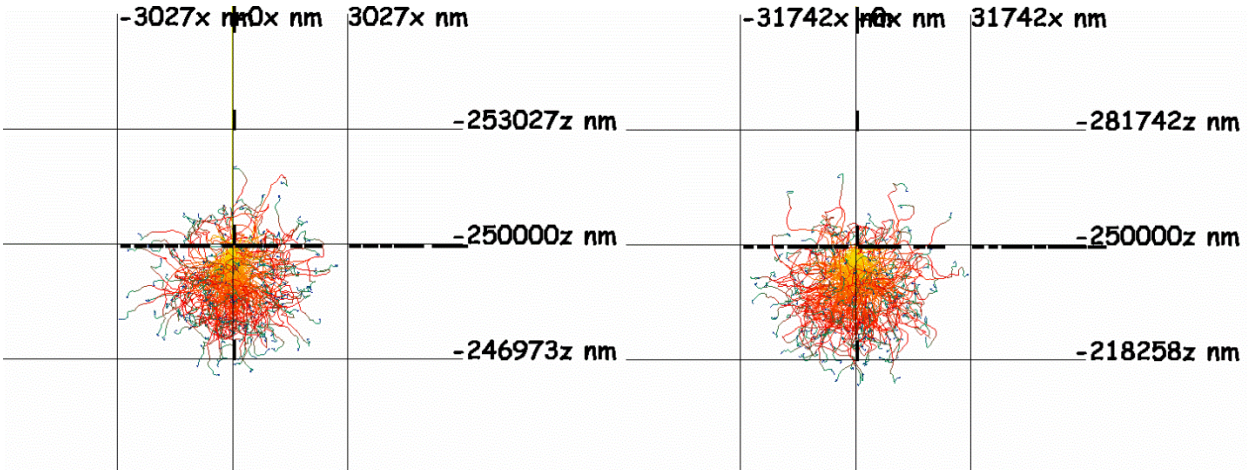


Figure 10: Shows a prediction, in depth and width, of the interaction volume for both 15 KeV and 60KeV. The width is the same in the X and Y directions.

In SEM EDS the accelerating voltage is much more important than in only SEM images. The accelerating voltage must be larger than the energy needed to excite the X-rays of the element in question. Generally it is better to know what elements you think there are vs. blindly looking in a sample and tuning the accelerating voltage to see what one finds. If there is not enough energy then there are certain characteristic X-rays for elements that will not be excited. There is a table that is generally used with any SEM EDS that tells the user the proper

accelerating voltage for the elements that they are interested in. Generally also a rule of thumb is to make the accelerating voltage approximately 1.5–2 times the energy of the element with the highest excitation energy. Too high of an accelerating voltage could cause artifacts or unintended excitations (JEOL, 2015).

The Skredos et al. 2005 article tries to show that when ones uses grayscale to determine the characteristic such as high or low mineralization, interaction volume plays a role. Interaction volume is important since when looking at cement lines in the case, one does not know what is below the surface. Assumptions that cement lines are straight like how they look on the surface may lead to improper conclusions. It is important to remember that cement lines are 3D structures that one cannot predict how they act below the surface. So it is important in the SEM back scattering to get as small of an interaction volume as possible, since as the deeper one penetrates the sample, the less one actually knows what is being detected. Since Burr et al. (1987 and 1988) used 60KeV, which is a larger than one would expect, then their findings might be somewhat inconclusive. This is due to the larger interaction volume and the unknown nature of what is below the surface. Skredos et al. 2005, hypothesizes that the Burr et al. (1987 and 1988) might be getting as much as 35µm of penetration below the surface. CASINO simulations back up this claim. Skredos et al. (2005) used 20 KeV, which is better but still seems a little high for back scattered electron detection. 20 KeV is a more acceptable acceleration voltage for SEM EDS. Skredos et al. (2005) predicts that they are getting less than 10µm of surface penetration, at 20 KeV. CASINO simulations back up this claim.

Accuracy of EDS, will give a relative concentration of minerals in both the cement lines and LAGs. Relative being that with both the cement lines and LAGs, there will be a percent concentration of a specific element (calcium, phosphorous, etc). Even though this is not

quantitative, it will suffice to compare the relative mineral compositions, and should provide enough evidence to relatively compare the elemental make up of cement lines and LAGs. Since standard EDS is not really quantitative but qualitative, it is hard to directly compare between samples. It could be possible to relatively compare LAGs and cement lines in the same samples though. As pointed out by Skredos et al. (2005) the smoothness of the surface plays a key role in EDS. Skredos et al. (2005) showed that any surface features could change the interaction of the electrons, thus changing also how the X-rays are released. This means that surfaces that are not "flat" could provide misinterpreted results. These misinterpreted results could be false positive or positive false for elements. Since all bone by its very nature is porous this already makes it difficult to analysis. Adding on top of that the processes needed to get the sample to the SEM EDS, could further complicate the analysis. Polishing and flat cutting play an important role in the preparation of the sample. With cortical there is at least a lot less porosity to deal with than say tubercular bone. It could also be pointed out for all of the points that Skredos et al. (2005) brings up about Burr et al. (1987 and 1988), there are some limitations that they do not bring up about their own study. Since bone is porous by nature one might say that there is no level of sample preparation that could make it "flat". Skredos et al. (2005), tries to battle the qualitative nature of EDS by using quantitative EDS. Quantitative EDS is the use of highly pure standards. Since the concentration of these highly pure standards is known, one could extrapolate, with math and isolation of key variables, the concentrations of specific elements in the sample. This method should not really be called quantitative EDS, but more it is a way to reduce variance and provide less error than standard EDS. While in Skredos et al. (2005) tried to isolate some of these variables specifically: use of quantitative over qualitative EDS, monitoring of beam current to make sure that was held "constant", and also that take off angle should not change if the

working distance is not changed. Bone is by its nature porous, that being said Skredos et al. (2005) had an impressive polishing procedure, but would that be enough for quantitative EDS? How are holes (from cells, freezing, Haversian canal, and\or etc) missed? The limitation of the sample is something one would have expected Skredos et al. (2005) to bring up.

As discussed in section 1.3 on cement lines, Skredos et al. (2005) article seems to encompass much of the history on the SEM and bones. Specifically Skredos et al. (2005), addresses what happened in the past with SEM and what is generally accepted. In the past only SEM images, specifically in backscatter mode, were used to determine mineralization. This was done through the use of grayscale and brightness of regions. It was argued that lighter regions were more mineralized, and this was debated. It seems that brighter regions in bone are now generally accepted as regions of higher mineralization. SEM EDS was used to determine if the grayscale conclusions were supported or not. Skredos et al. (2005) article, while not while conclusive did show significantly higher levels of Ca in cement lines than the osteon. The part that was not conclusive in the Skredos et al. (2005) article was that there was no significant difference between cement lines and interstitial bone. With the inquiry into LAGs, it is thought that the same principles the show cement lines to be hypermineralized could be applied to LAGs. LAGs are similar in shape to cement lines. As of now, it is unknown if anyone has used SEM EDS on LAGs in the same fashion as was performed on cement lines. Instead of encompassing an osteon, LAGs encompass an entire circumference of a bone at the time they are formed. Also LAGs are brighter than the surrounding bone, similar to cement lines and their surroundings. LAGs are not as well studied as cement lines, and their definition is more fluid as of now. This is shown that in the past, Lakes and Saha, in their 1979 article called LAGs cement lines. Also LAGs are well documented in ectotherms, specifically reptiles\dinosaurs, but endotherms pose

more questions than answers, currently. There is a current need for more study of LAGs, specifically in endotherms, and more study of bone in SEM\SEM EDS in general.

Light Microscopy 1.5

Cement lines, LAGs, and annuli can all be counted in light microscopy. Toluidine blue has been used to stain cross sections of bone (Osborne et al., 2005; Hall et al., 1993). It stains cement lines and LAGs, but not annuli. Cement lines and LAGs will be blue, and annuli will show up white (Hall et al., 1993). Also if a polarizer is used LAGs show "birefringent narrow lines" and annuli show anisotropic pattern (Hall et al., 1993).

Hypothesis and Specific Aims 1.6

Health related issues are on the precipice of becoming a new monster in the future of mankind. The next great frontier is space. It seems now more than ever that it is well within the lifetime of many living humans. As being a new frontier, it will bring about a slew of new problems humans must face. Many of which will be how to adapt humans to outer space or how to adapt outer space to humans. Specifically, one major health problem will be bone health in a micro-gravity or zero gravity setting. Nature has spent many years evolving mankind to the environment that is earth. To inject out species into a whole new environment and expect no issues is foolish. Bones have evolved to respond to certain stimuli, such as gravity. The removal of cyclic loading through gravity would cause the bone's natural processes to reduce overall bone mass. Even though this is somewhat far off problem for most people, it has an analog that is currently a large issue that is or will be for most people. Both osteoporosis and disuse osteoporosis are outcomes that simulate what would happen to bone in a micro-gravity or zero

gravity setting. This gives current issues that can be addressed now, and hopefully applied in the future. Biomemetics have proven to give humans many jumps, in technology, throughout time. Hibernators provide an example that is different, and possibly the answer to both osteoporosis and disuses osteoporosis. Understanding hibernation in other animals can give insight into solutions. Many of the current solutions, for osteoporosis and disuse osteoporosis, show little benefits. Other solutions are "band-aid" like patches that wear out and can cause even worse failures. Large hibernators, like bears, have shown in previous studies the ability to drastically decrease their metabolic rate. Also, bear hibernators have a more continuous hibernation pattern much like an extended sleep. By this is it means they go into a hibernation state and come out only when they are done hibernating. This differs from previous studies on small hibernators that hibernate also, but have brief periods during their hibernation patterns where they wake up. In large hibernators, like bear, they have shown the ability to actually protect bone from resorption, due to disuse. Normally this much disuse would cause disuse osteoporosis, similar to if humans tried this. Humans may have to try something like hibernation for extended space flights, which may take many years to arrive at their destination. Studies on bears have shown the ability to even have some bone turnover in a hibernating state. Understanding hibernating states could go a long way into understanding how bears are able to not lose bone mass. This brings up states of slowed growth and cessation, in bone. Cement lines and LAGs are those regions or boundaries of slowed growth and cessation. In LAGs this is indicative of reduced metabolism, and the complete cessation of bone growth. With a better understanding of LAGs, these lines could be used not only to age, like in skeletalchronology, but to be markers of points when there is no radial bone growth. Comparing LAGs to the better understood cement lines could provide some insight into the makeup and mineralization of LAGs. Understanding how,

when, and how many LAGs form in a population could provide a time set of when LAGs form. A better understanding of LAGs could provide future researchers the “time stamp” to extrapolate the chemical, biological, and mechanical forces that allows bears, during hibernation, to avoid overactive resorption due to disuses.

Central Hypothesis 1 1.7

LAGs' composition will be similar in composition and size to that of cement lines, both being more mineralized than surrounding bone. This is due to the formation of both lines, which are regions or boundaries of slowed growth, in cement lines, and slowed growth followed by cessation, in LAGs.

Aims 1 1.8

Assess LAGs and cement lines, to determine a similarity between the two’s relative chemical composition.

Task 1: Chemically fix, embed, and polish; samples making SEM EDS is possible.

Task 2: Run SEM EDS to determine the chemical composition among LAGs, cement lines, and surrounding bone.

Task 3: Draw conclusions and perform an analysis on the LAGs to further the understanding of LAGs.

Central Hypothesis 2 1.9

LAGs are suggested to form during a period of metabolic suppression. Since bears experience extended region of metabolic suppression, hibernation, there should be one LAG for every year the bear is alive.

Aims 2 1.10

Compare LAGs size and number in both SEM and light microscopy.

Task 1: Compare measurements on LAGs in both light and electron microscopy.

Task 2: Count the number of LAGs in both light and electron microscopy.

Task 3: Plot graphs to draw conclusions and an analysis on the number of LAGs, when there is a known age for each sample.

CHAPTER TWO: METHODS AND MATERIALS

Samples 2.0

Six Utah black bear femurs were used for the mineral comparison of cement line and LAGs. Samples had already been frozen in a -20^{o}C freezer and most all the flesh had been removed. Previous sample care is personally unknown. All black bear samples were from hunted animals that were killed in Utah. Samples were put on ice and delivered to the lab. At the lab they were processed and frozen in a -20^{o}C freezer. During sectioning and times before chemical fixation, none of the samples were outside of the -20^{o}C freezer for more than fifteen minutes. The black bear bones were grouped by age and randomly selected from these groups. This was done by grouping scheme: 1-2, 3-4, 5-6, 7-8, 9-10, and 11-12. As said before from each one of these groups a sample was randomly selected. All samples were males. All animals' ages were given to the lab from the sample collectors. All animals were aged through the counting of lines, in their dentin.

Four grizzly bear femurs were used to investigate if there was visible proof of a LAG forming on the periosteal surface during hibernation. Samples had already been frozen in a -20^{o}C freezer and most all the flesh had been removed. Previous sample care is personally unknown. All grizzly bear samples were from humanly sacrificed animals at Washington State University. Samples were frozen and delivered to the lab. At the lab they were processed and put into a -20^{o}C freezer. During sectioning and times before chemical fixation, none of the samples were outside of the -20^{o}C freezer for more than fifteen minutes. Of these grizzly bear samples there were two sets of two. This means that two of the samples were of the same age and sex. The other two were also of the same age and sex, but different in age compared to the

first set of grizzly bear samples. The first set of grizzly bear samples were one. The second sets were twenty. Within each set one grizzly bear was sacrificed in the summer and the other was sacrificed during hibernation. This was done to have "relatively similar" bears but one hibernating and the other not.

General Sectioning 2.1

General sectioning was achieved through the use of a small vice and hand hacksaw. The sample was placed into the vice. Foam was used to protect the bone from the teeth of the vice and distribute the force of the vice. A fine tooth hand hacksaw was used to cut the samples. If the sample showed signs of getting warm due to friction, the sample was removed and cooled again. This was done to prevent the sample from heating up and rotting\enzymatically degrading. One and a half inch sections were cut from each sample. Since the samples had previously had some of the mid diaphysis removed for other experiments in the lab, another region had to be selected. Even though the mid diaphysis is ideal, other regions close to the mid diaphysis will work. To keep this region similar among all samples the data for the length of all bones before being sectioned was used. Previous data was retrieved on the overall length of each bone before section. From this a percent length of the bone was determined, for which all bone had sample to remove. This turned out to be 38% of the overall bone length from the femoral head. From this 38% point, 0.75 of an inch was measured from each side of this mark. This gave the overall one and a half inch section that was desired. Figure 11, page 41, shows an example of this section. This section was then removed with the hacksaw and vice as described above.

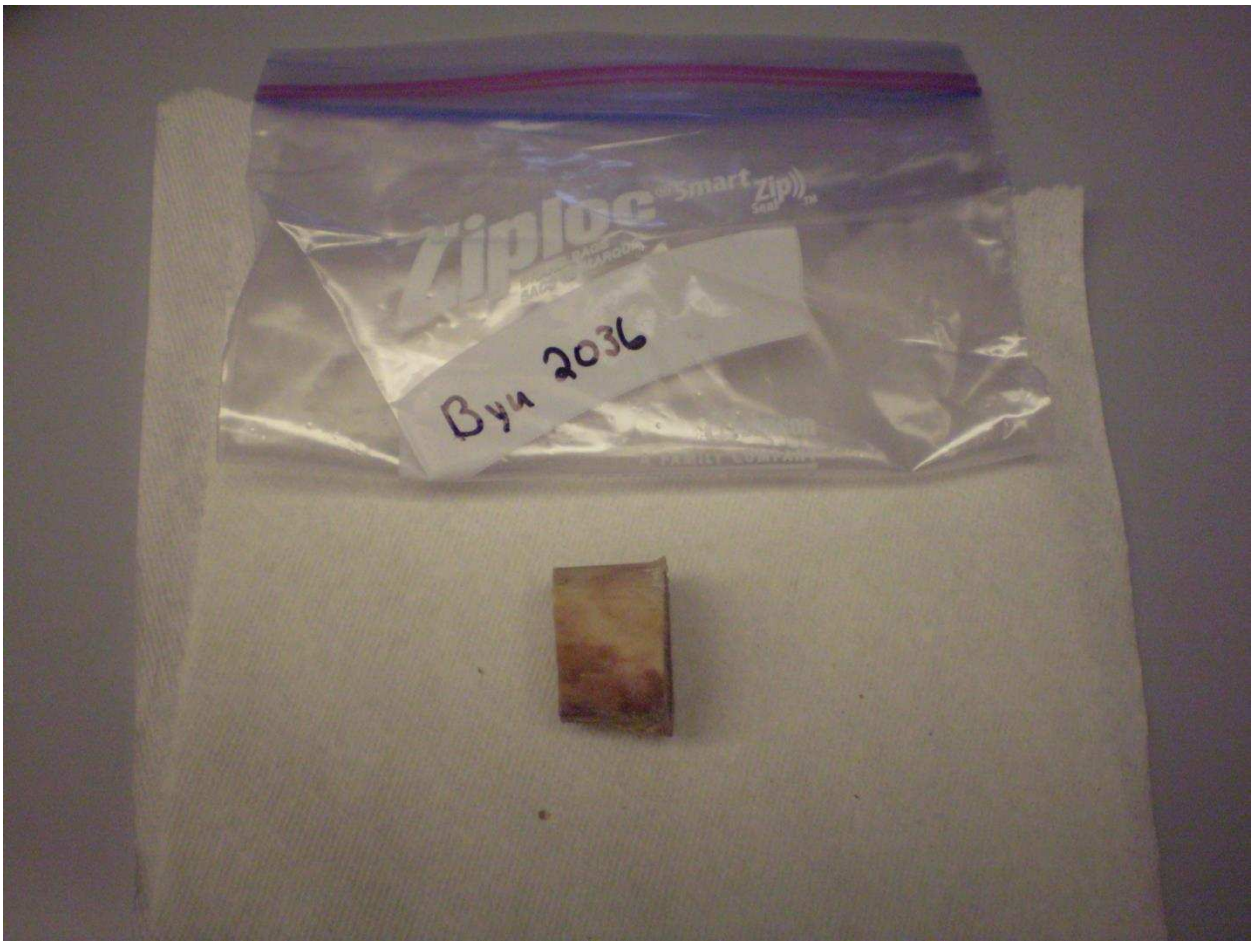


Figure 11: Shows a sample of the sectioned bone after cutting.

Chemical Fixation 2.2

Chemical fixation was accomplished by following Boyde's 2012 procedure for fixing bone, in SEM. In *Bone Research Protocols* (2012), Boyde describes his protocol for fixing bone, in preparation for SEM. Boyde's SEM fixative is as follows: 2.5% glutaraldehyde, 1% formaldehyde, and 0.15 M sodium cacodyalte, at a pH of 7.3. This solution was specifically used due to the speed at which it fixed the sample. With both the two fixing ends of glutaraldehyde and one fixing end of formaldehyde, the chemical fixation process was achieved in approximately 48 hours. The speed of fixation was important due to extended enzymatic degeneration could have been visible at the level of the SEM. This was done to prevent as little

structural damage as possible to the sample, during fixation. In this case specifically 600mL was made to fix the black bear bones and 400mL was used to fix the grizzly bear bones. For the 600mL 7.5g of paraformaldehyde (500g Alfa Aesar CAS:30525-89-4) was weighed out on an electric balance (goes to ten-thousandths). This was then added to 75mL deionized (DI) water. The mixture was stirred on a stirring hot plate, with the use of a magnetic stir bar. The solution was heated to approximately 70^{o}C. When this temperature was reached NaOH (500g Fisher Scientific CAS:1310-73-2) was added dropwise, until the precipitant dissolved back into solution. The heat was turned off. The solution should now be a 1% formaldehyde solution. In another beaker, 19.2618g of sodium cacodylate trihydrate (25g Electron Microscopy Sciences CAS:12300-25) was added to 450mL of DI. This solution was also mixed via a stirring hot plate, with the use of a magnetic stir bar. In this case no heat was used to mix the sodium cacodylate trihydrate solution. To the sodium cacodylate trihydrate solution, 60mL of the total 75mL 1% formaldehyde solution was added. Next, 60mL of 25% glutaraldehyde was added to the sodium cacodylate trihydrate solution. Then, 30mL of DI is added to reach 600mL total solution volume. This solution was a bit basic and did not match the pH of 7.3 that Boyde initially stated in *Bone Research Protocols* (2012). To the solution HCl (500mL Fischer Chemical ACS certified LOT:116239) was added dropwise. After each drop the pH was tested, with pH strips, until the pH approximately reached seven. Then 100mL of the solution was added to small 150mL beakers, with the black bear bone sections sitting in the bottom. These small beakers were used due to the fact they were narrow as possible to still fit the black bear samples, but tall enough fit the 100mL of fixative solution. In a larger beaker more fixative solution would have to be used to cover the samples. Multiple bones would have to be put in the larger beaker to make up for the waste of the excess solution needed to completely cover the

sample. In this case smaller beakers were used, such that each sample had its own beaker and approximately 0.5 inches of fixative solution above the sample. All samples were left for 48 hours and then removed from the fixative solution and placed in 70% EtOH, for storing. The grizzly bear samples were fixed the exact same way as the black bear samples. The only thing that was different was only 400mL of fixative solution was made not 600mL. This was done since there was only four grizzly bear samples compared to the six black bear samples.

General Drying and Embedding 2.3

All samples were stored in 70% EtOH. In preparation for embedding, the samples needed to be dried. Atmospheric H_2O and H_2O left over in the sample needed to be removed before embedding. The concentration of EtOH is gradually increased over several days to remove H_2O. The samples were left in fresh 70% EtOH for one day. Then every 24 hours the solution was replaced with a 10% greater concentration of EtOH, until 100% EtOH is reached. After 24 hours in 100% EtOH, the sample is ready for embedding.

All samples were embedded in Buehler EpoThin, two part epoxy. See Figure 12, page 45, it shows both the hardener and the resin bottles. An Epoxy was used over the traditional Polymethyl methacrylate (PMMA). This was done due to the approximate shrinkage of each resin, during hardening. It was approximated that PMMA, which is in the acrylic family, had a +\- 10% change in size during curing. It was also approximated that Epothin, which is in the epoxy family, had a +\- 3% change in size during curing. The smaller change in volume during curing would cause less damage to the micro-structure, and should provide a better image under electron microscopy. EpoThin was mixed in a 2:1 ratio of resin to hardener. This 2:1 ratio was by volume and not by weight. The 2:1 by volumes cure was good enough and it did not seem

that a 2:1 by weight was needed, to be that accurate. The two parts were added together in a plastic disposable container. The mixture was then hand mixed for five minutes with a wooden tongue depressor\popsicle stick. Approximately 50mL of mixed EpoThin was needed for each sample, with our container size. Plastic 100mL beakers were used to embed the samples. The plastic beakers allowed flex which helped in removing the puck. When the resin was added, it was added to have approximately 0.75 inches, of resin, above the sample. This extra resin on top allowed for the IsoMet to have most all if not all the bone to be finely sectioned, if needed.

The samples, now covered in EpoThin, were placed inside a desiccator. It was placed inside the desiccator not for its desiccation\drying ability, but it was the only container on hand which a vacuum could be drawn on. A pump was hooked up to the desiccator to draw a vacuum. Once the desiccator was pumped down ten minute timer was started. These ten minutes was used to degas the resin. After the ten minutes were up, the samples were slowly returned to atmospheric pressure. They were left in the desiccator with the lid off to cure, for 48 hours. Desiccant was removed before this, in order to not waste the desiccant. This turned out to be a very important step that was learned the hard way, but luckily it was on a practice bone. Without degassing there will be a lot of bubbles that make it hard to cut, since the blade can jump around when it hits a pocket. Also one could get unlucky and get a bubble next to the bone, which makes that section weak at holding on to the bone. Degassing then pouring resulted in a lot of bubbles. Leaving the samples to cure under vacuum resulted in large pockets in the resin. The best outcome was to do as stated: pour the resin over the samples, then pull a vacuum on the samples, once a steady vacuum was reached then a ten minute timer was started, after ten minutes the samples were brought back to atmospheric pressure, and left undisturbed to cure for 48 hours. After the 48 hours the samples were pried from the plastic beakers with a scoopula

and a bit of tapping the plastic beakers onto the table. The samples were now encased in EpoThin, which is generally called the puck. See Figure 12, below, it shows an image of a puck, with a bone embedded.

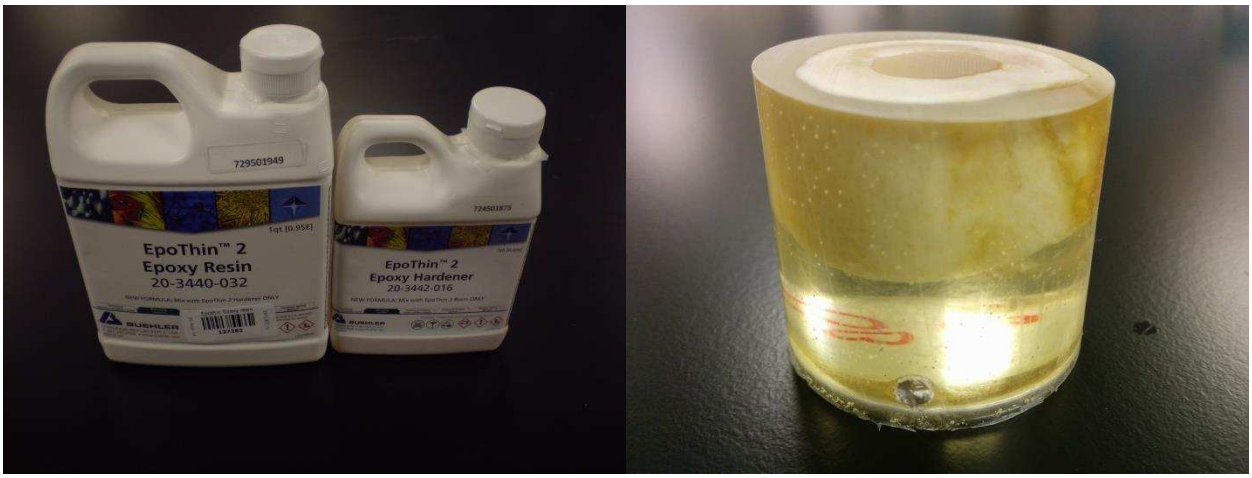


Figure 12: Left shows both the resin and hardener used in the embedding process. Right shows an embedded sample.

Fine Sectioning and Polishing 2.4

Now that the samples are embedded, fine sectioning can begin. Fine sectioning was achieved through the use of a Buehler IsoMet 1000. See Figure 13, page 46, it shows an image of the Buehler IsoMet 1000. The IsoMet is a diamond tipped saw used to cut thin sections of the bone. The samples had six 300 micron thick sections cut off. Originally these sections were cut much thinner, on the order of 100 microns. The 100 micron thick sections would warp on drying. This was after polishing which would remove approximately 10 – 40 microns. This was too thin to keep the sample semi-flat. Even if the samples were dried under a weight, in an attempt to flatten the samples. In the end slightly thicker samples were used to eliminate most of this warping, after drying. The IsoMet is a lubricated saw. In this case only water was used, as a lubricant. It is suggest that methylated spirits could be used to add rigidity to the sample. The sample was rigid enough with the addition of the epoxy. Samples were cut and dried under an

aluminum block. This was used so the samples would dry flat. Sections were then labeled and the inner side was selected for polishing. The outer side was labeled with permanent marker. The quadrants and animal number were written on this outer side.

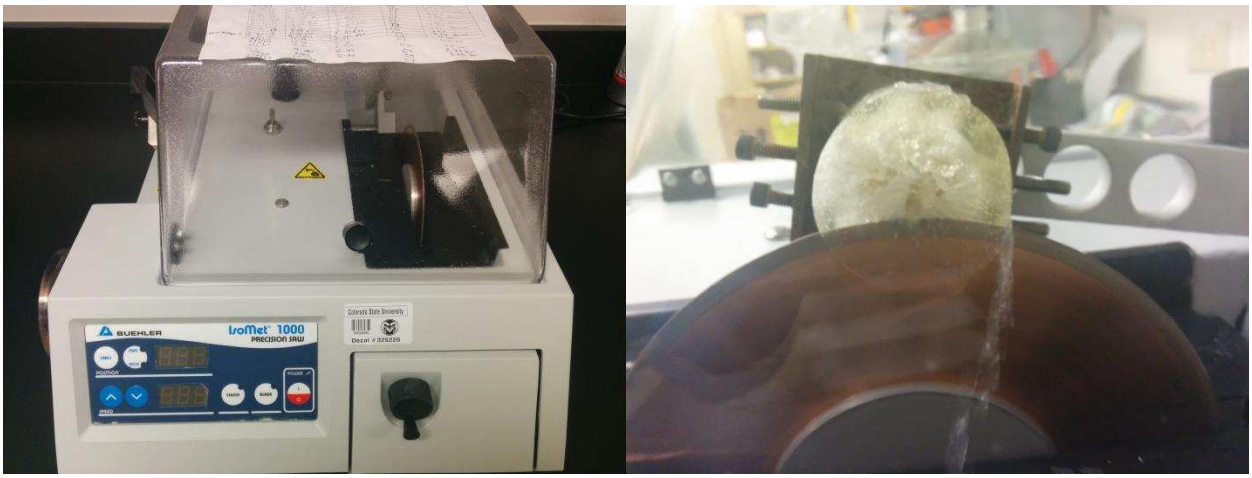


Figure 13: Left shows the Buehler IsoMet 1000 that was used to finely section the bones into 300 micron slices. Right shows the IsoMet 1000 cutting a sample.

With the samples finely sectioned, polishing could begin. Polishing was achieved through the use of a Buehler MetaServ 250. See Figure 14, page 47, it shows an image of the Buehler MetaServ 250. The MetaServ is a motorized wheel that circular disks of sandpaper can be attached to. The MetaServ provides consistent speed and a timer in which to polish the sections. Sections were hand polished since the lab did not own the automated arm that can be attached to the MetaServ 250. Hand polishing is by its nature less reliable and reproducible, as an automated arm that can provide consistent pressure and rotational speed. That being said all attempts were made to improve hand polishing.

Figure 14: Shows the Buehler MetaServ 250 that was used to polish sections of bone.

Initially 600, 800, and 1200 grit sandpapers were used to polish the sections. When these sections were analyzed under the SEM scratches and deep groves could be seen. It was determined that the 600 and 800 grit sandpapers were too low of a grit sandpaper to use. In this case lower grit sandpaper implies the larger the average size of the silicon carbide crystals. In turn, higher grit sandpaper will have smaller on average sized silicon carbide crystals. The smaller the abrasive the finer polish, but the less the material is removed. While the larger the abrasive the less fine the polish, but the more the material is removed. In the end it was determined that 1200 grit sandpaper was best when used alone. This method provided: a reduced polishing time, less materials used, and good enough polishing for the SEM.

In an ideal set up 600, 800, and 1200 grit sanding would be done. It would take a lot of 800 grit sandpaper to smooth out the 600 grit sanding. Also it would take a lot of 1200 grit sand paper to smooth out the 800 grit sanding. After this, one could move on to progressively smaller diamond suspensions, to further polish the section. This ideal polishing clearly takes a lot of materials and a significant amount of time. This could be done for a few extremely high quality samples. Adding many samples to this could lead to a significant amount of time. Again this

really only applies for hand polishing. If an automated arm was used then the time issue is less of an issue. Since there can be a significant decrease in attention and an increase in general exhaustion when hand polishing, limiting the time polishing proved beneficial. Otherwise many breaks would be needed or multiple researchers would be needed. However, multiple researchers could introduce multiple styles of hand polishing. This could be another variable that is less controlled if multiple researchers are used to polish samples. The material usage would still most likely be the same. This is due to the fact that the sanding disks are not supposed to be used for more than five minutes of total polishing time. As the sand paper polishes, material gets stuck on the paper and silicon carbide crystals get dislodged from the paper. In order to keep a pristine polishing surface it is not recommended to polish on the same sandpaper disk for more than five minutes of total polishing time.

All sections were polished with the same method. The original inner side selected in the previous fine sectioning section is selected as the side that will get polished. A clean 1200 grit piece of sand paper is attached to the spinning wheel. The speed of the wheel is set at 250 RPMs. The coolant used is again just water. Again methylated spirits could be used to add rigidity to the sample. The sample was rigid enough with the addition of the epoxy. A one minute timer is set and the wheel is started. The inner surface of the section is lowered onto the spinning wheel. Four fingers, the left and right pointer and middle fingers, are used to stabilize the section. The section is then moved counter-clockwise around the wheel. The wheel spins clockwise. The counter-clockwise rotation is used to actively change the angle of the silicon carbide crystals on the section. This is an attempt to prevent large grooves from forming if the section was held in one place for the full time. Previously the section was held in one place and rotated 45 degrees every thirty seconds. This did not prevent large grooves as much as the

counter-clockwise rotation. After a minute of this the wheel stopped and the section was turned over. The surface was thoroughly cleaned under the lubricant sprayer. The sample was turned back over and this above process was repeated, but for two minutes of counter-clockwise rotation. The wheel stopped after two minutes and again the inner surface was thoroughly cleaned under the lubricant sprayer. The whole two minute process was repeated again. This totaled five minutes of polishing. After this the inner surface was again cleaned under the lubricant sprayer. The 1200 grit sandpaper disk was removed and a new one was placed on. The whole five minute process was repeated for a total of twenty minutes on the timer or four pieces of 1200 grit sandpaper. This process of twenty minutes of total polishing was done for one section from each sample. The sections were dried again under an aluminum block, to prevent warping. The sections were stored in a case with desiccant for two to three days before moving on.

Fine Drying and Sample Transport 2.5

The fine drying and sample transport was only done when it was known that SEM was coming up. Fine drying included putting sections into a glass desiccator and pumping it down. The sections were left under vacuum for a day before moving to the sample transporting system. The sample transporting system included a self-made set up. This was done in order to move the samples to the SEM, while keeping them under vacuum, but not having to carry around a large desiccator. The large desiccator was hard to transport over the distance needed to get to the SEM. An empty micropipette tips box was used to house the sections. Desiccant was placed in the bottom of the tips box. The plastic tip holding section was then placed back and the sections lay on top of this. See figure 15, page 50, it shows the tips box. Toothpicks were used to hold

the sections in place, at least until they were scanned in the SEM. After the SEM, copper tape held the sections in place and the toothpicks were no longer needed. The top of the tips box was then closed. Four holes were drilled in the outside of the box to allow air to move in and out. Ziploc vacuum seal bags were then used to provide enough a vacuum to transport the samples to the SEM. Air had to be removed from the Ziploc vacuum seal bags every time the bag was opened.

Figure 15: Left shows the modified tips box and the general use of copper tape. Note: the copper tape may not be in the right orientation. It is extremely sticky and sometimes pulls off in the removal of the sample from the grounding plate. Since these samples were done the copper tape was put back on to hold them in the box, orientation was not taken into account. Right shows the vacuum system to transport the samples to the SEM, on the day of scanning.

Carbon Coating 2.6

Samples were transported to the SEM building under vacuum. This was done through the use of Ziploc vacuum seal bags as stated in the Fine Drying and Sample Transport section above. Samples were removed from the vacuum and put into the carbon depositor. The carbon depositor was a Kinney model KDTG-3P. The samples are put on a platform below two rods of carbon. One rod is sharpened while the other is flattened. Then tip of the one rod is pressed against the flat surface of the rod. A glass bell fits over the platform and the carbon rods. Both a mechanical and diffusion pump is used to generate a high vacuum, in the bell. The samples are

pumped down 10^{-3} to 10^{-4} torr. The pumps require water cooling and also liquid nitrogen. Liquid nitrogen is used as a coolant also and as a trap. The trap is trying to catch any that could be bad from going into the air. Once the sample is pumped down the power is turned on to those rods. The voltage is slowly raised to a desired point. In effect the current is also raised since the resistance of the material is not changing. Welding glasses are needed to look at the samples and rods now. The carbon is then heated, due to the resistance generated from the use of larger amounts of current going through the carbon rods. The carbon is then evaporated onto the sample(s) below. This voltage is held until: one is done, the rods break, or excessive sparking. Sparking is a bad sign, showing the rods are not contacting each other well. If the rods break before desired level of coating, then one must wait for the rods to cool. After cooling, the rods must be sharpened\flattened and pressed together again. Many times multiple coating attempts were needed to get the desired levels of carbon coating. This was due to the fact that the rods break fairly easily. See figure 16, below, it shows the carbon depositor, power controls, and platform\rod area.

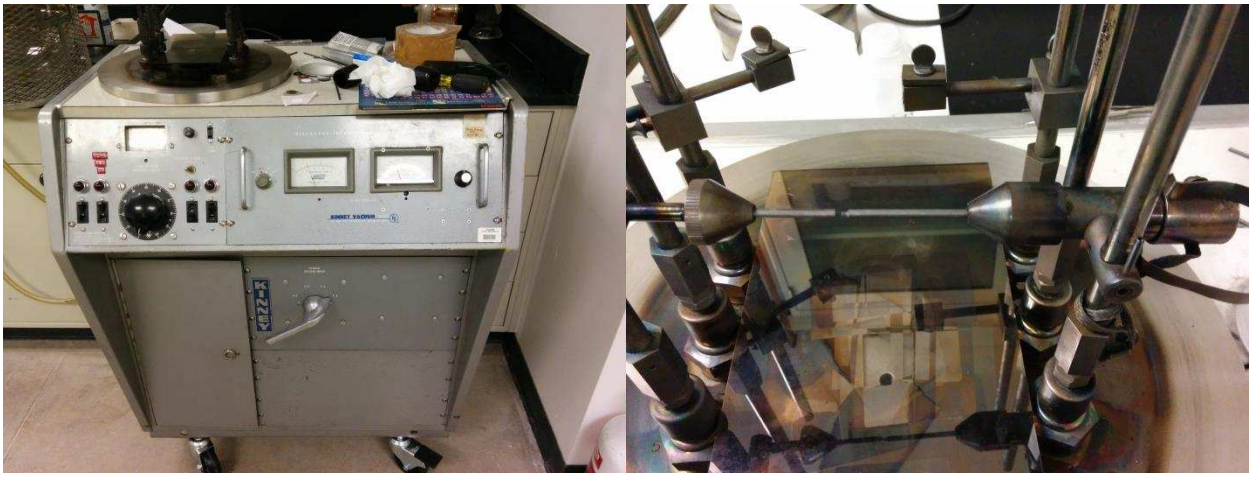


Figure 16: Shows the carbon depositor. Left is a picture of the entire instrument, minus the glass bell. Right, is a close up of the platform and the carbon rods, being pressed together.

Carbon was chosen over gold in this case because of carbon's superior qualities in EDS. A carbon coating provides better data in the EDS phase. It does this by having fewer peaks in regions that interfere with elements of interest. That being said, since it is carbon by its very nature, one could not analyze the carbon in the sample. Carbon being a staple of all organic molecules could be an issue, if one is looking at an organic sample, and for information on relative carbon concentrations. Coating a sample in carbon would also completely obscure any regions of higher carbon concentration. In short, it would not be advised to use a carbon coating if one is trying to look for carbon in a sample, whether that being carbon alone or part of some molecule. Besides the inability to look for carbon with a carbon coating, another drawback of a carbon coating is a reduction of resolution in SEM images. This is only an issue once you go over 10,000x (high resolution). Gold is far superior in this case. It provides better images and does not have the issues of lowered resolution at high magnification. A gold coating was tested but it had a peak in the EDS that overlapped with the phosphorous peak and phosphorous is a key element to look at in bone. In this case no more than 2500x was needed, so the carbon coating is the best choice.

SEM 2.7

The SEM in the Central Instrument Facility at Colorado State University was used. At this time, the Central Instrument Facility had a JEOL JSM-6500F. The samples were taped down to a 2.5 inch diameter round holder. See Figure 17, page 54, it shows the SEM used and a holder. One piece of copper tape was laid down over the middle of the sample; splitting it into two half circles. The tape was carefully placed in the middle of the medial and lateral locations. This was done such that the anterior and posterior regions of the cross section were not obscured.

The anterior region was of particular interest, since that was the region that all cement lines and LAGs that were analyzed, came from. This was in an attempt to isolate the regional variables down to one region. This is due to the fact that different regions of long bones are loaded in different ways. For example, in a human femur, medial regions are loaded more in compression where lateral regions are loaded more in tension. Going back to the tape, copper tape was used, instead of carbon tape, due to its better grounding and less outgassing. The type of copper tape is unknown, as it was what the Central Instrument Facility provided for the SEM. One side of the copper tape was adhesive, while the other side was just plain copper. See Figure 15, page 50, it shows copper tape on several samples prior to removing them from the SEM. This tape was used to ground the sample to prevent charging. While it did a fairly good job at this, higher levels of accelerating voltage seemed to be too much for the tape and coating to dissipate. This is one of the reasons why the accelerating voltage was moved down to as low as possible. Another reason was to reduce the interaction volume specifically for the EDS, but that will be elaborated on more in the EDS section. Samples were loaded into the SEM and put under high vacuum. The stage was centered before the sample was put in. A vacuum of 3.64E-4 torr was established. The back scatter detector is introduced into the chamber, once the vacuum settles, the gun valve is opened. The secondary electron imaging (SEI) detector is then turned on. This allows for the pictures to be taken. The SEI mode is more for orientation and topography, in this case. Back scatter imaging (BSI) is the more important imaging mode in this case. The BSI mode allows for the denser elemental regions to show up brighter (white) and less dense elemental regions to show up darker (black). This elemental distribution is not possible in SEI mode. The BSI mode is used for all the images and EDS work for this project. The SEI mode

was used to: find regions of interest, allow for probe current calibration, focusing the scope, and auto contrast brightness (ACB).

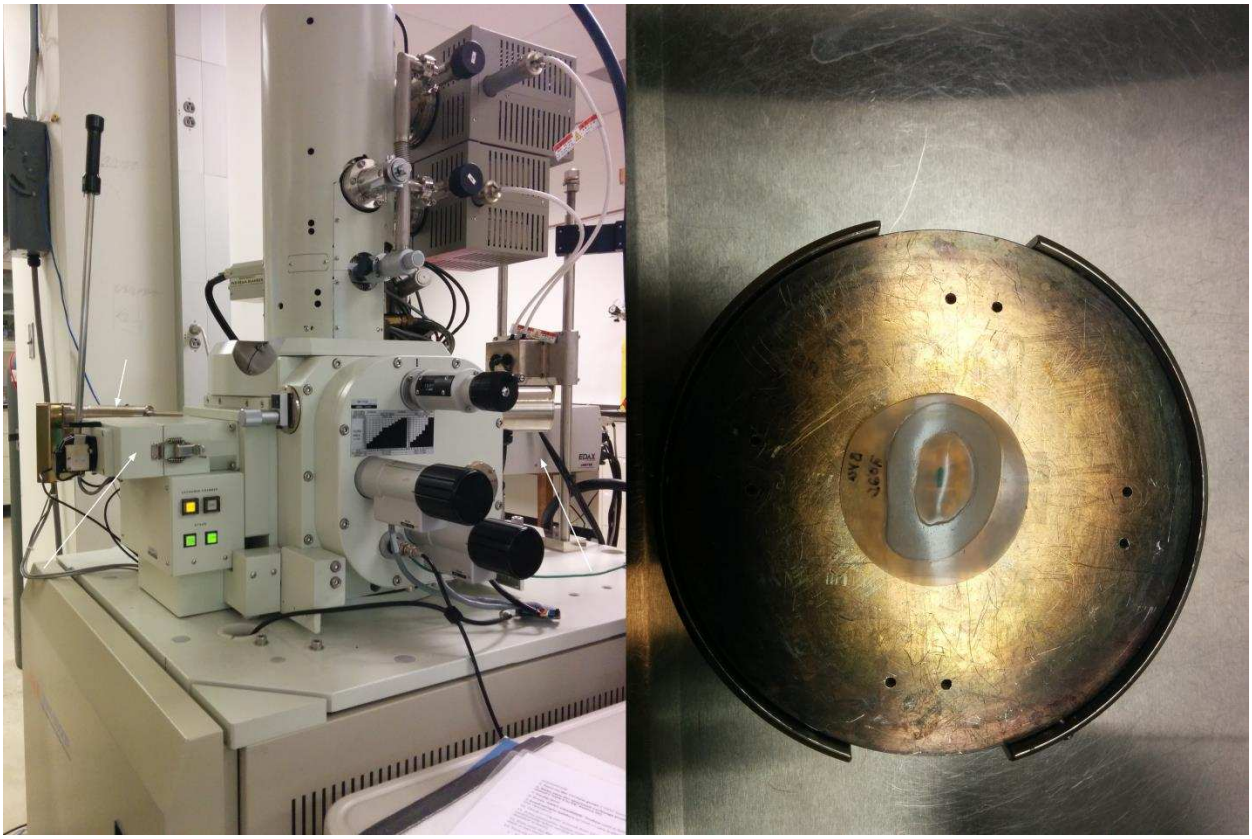


Figure 17: Left shows the JEOL JSM-6500F used in this study. The top left arrow points out the backscatter detector. The bottom left arrow points out the sample input chamber. The right arrow points out the EDS. Right shows a sample holder for the SEM.

An accelerating voltage of 10 KeV was used for the black bears samples, while an accelerating voltage of 11 KeV was used for the grizzly bears. Both of these accelerating voltages are as low as possible in this situation. Any lower than 10 KeV, there is a possibility that calcium would not be able to be excited. If calcium is not excited, then there would be no elemental data for calcium. So in this case 10 KeV was determined as an absolute minimum accelerating voltage that could be used for EDS and still get information on the elements needed. As for probe current, that was varied. This was done for the EDS. It was varied between 6 and

16 microamps. More about this will be covered in the EDS section. The zoom of the scope and subsequent images were maintained at certain levels. All osteons were imaged at 330x. Cement lines were imaged and point selection, for the EDS, was done at 2000x. LAGs were imaged at 35x or 45x to give an overview. LAGs were also imaged and point selection, for the EDS, was done at 300x. Since the cement lines were much smaller than the LAGs a higher magnification was necessary to view them. This is why there is a difference in magnification between LAGs and cement lines.

EDS 2.8

As stated before an accelerating voltage of 10 KeV was used for the black bears samples, while an accelerating voltage of 11 KeV was used for the grizzly bears. Both of these accelerating voltages are as low as possible in this situation. Any lower than 10 KeV, there is a possibility that calcium would not be able to be excited. If calcium is not excited, then there would be no elemental data for calcium. The accelerating voltage is generally decided by the elements. If there are certain known elements of interest this makes determining the accelerating voltage simpler. There is a table provided at the SEM that shows the energy needed, in KeV, to excite that element. If the elements are unknown, then this is harder, since one does not know what they are trying to excite. A large accelerating voltage is then used which is generally not favorable. The rule of thumb, given by the SEM professors, is that you need 1.5 - 2 times the accelerating voltage, of the element, to get good excitation. A more conservative amount would be 2.5 times the accelerating voltage, of the element. Since the excitation of Ca is ~4.4 KeV, 10 KeV was determined as an absolute minimum accelerating voltage that could be used for EDS and still get good information on the elements needed.

As before, the probe current was varied. This was done for the EDS. Since the surface of the bone samples were not perfectly smooth the probe current was varied between 6 and 16 microamps. In the EDS there is a counts per second (CPS) and a dead time. To get good data the CPS should be 10000 or greater. More CPS means more data per pulse (Hz), of the EDS. Since there are multiple pluses (Hz) per scan, even for just a point, the CPS can have a significant impact if it is too low. All of those counts, per scan, are averaged out to get the overall data. Increasing the probe current will increase the CPS but it generally also increases the dead time. The dead time is a number that is reflective of data that is not being detected. This can be due to: the detector, surface of the sample, type of sample, probe current, accelerating voltage, magnification, and possible others unknowns. This means that for a line scan and point scan the CPS and dead time can change. This is due to not all points being in the same place. The initial CPS and dead time are averaged over the whole sample. Generally the accelerating voltage gets set first. The magnification is the set. Finally, the probe current was varied to get the CPS high enough, but the dead time not too high. In the present study the dead time was kept at 30 or lower. It was suggested that this dead time is a good number for the detector by the SEM professors. For both the dead time and the CPS, they are not static numbers and vary quite a bit, The rules of thumb of > 10000 and < 30, are based on the does the number generally average out to be acceptable for those rules. For example if the dead time is below 30 for none of the ten points but for one point it was 40 that were deemed as acceptable.

In the present study the accelerating voltage gets set first, according to Ca excitation. The magnification is the set, according to the rules in section 2.20 of methods and materials. An image was then taken of the region on the EDS computer. Finally, the probe current was varied, between 6 and 16 microamps, to get the CPS high enough but the dead time not too high. From

this point any scan (point, line, or map) could be done. In the present study point, line, and map scans were performed. The point scans were the most important to this study. Line scans were interesting to report, but ultimately could not be compared statistically. Map scans fell in to the same pool as line scans. For point selection there was 20 points selected for LAGs and 30 points selected for cement lines. For LAGs: ten points were selected on the LAG and ten points were selected outside the LAGs (more periostally). For cement lines: ten points were selected inside the osteon, ten points were selected on the cement line, and ten points were selected outside the osteon, in the surrounding bone matrix. See Figure 18, page 58, for a graphic of how points were selected. Three osteon sets and three LAG sets were scanned per black bear sample. Three LAG sets and three periosteal regions were scanned per grizzly bear sample. With six black bear samples, it makes 540 data point for osteon sets and 360 data points for LAG sets. With four grizzly bear samples, it makes 240 data points for LAG sets and 120 data points for periosteal surface. Line scans were selected arbitrary to cross all sections, stated in the point selection, for: LAGs, cement lines, or periosteal surface. Map scans use the whole image, so no sectioning is needed. All point, line, and map scans were done in the anterior quadrant of all cross sections, in all bears.

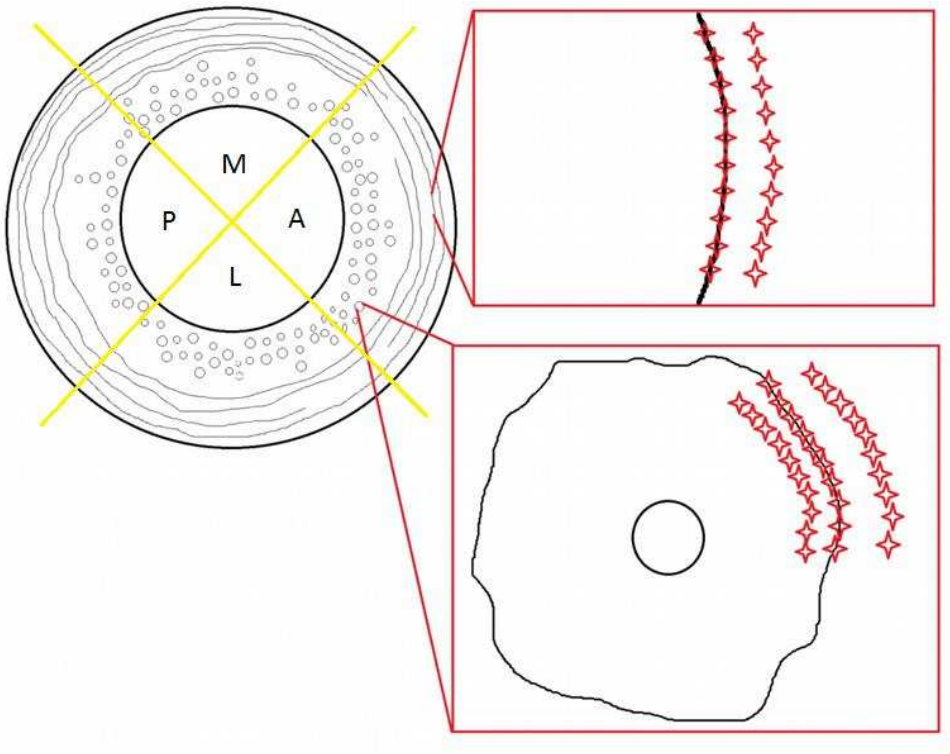


Figure 18: Shows a graphic of how the points were selected for each sample.

Light Microscope 2.9

Slides were stained before using an internal SOP that was adapted from Osborne et al. (2005). The slides were stained with toluidine blue and from a previous study. All six black bears from the SEM EDS data were selected. Another six black bears were selected from the remaining slides in the same way as was stated in 2.0 above. It was a choice to not expand the age range but to fill in the age range with more animals that were within this range. Again they were randomly selected from the age groups, in an attempt to prevent bias. Twelve samples of Florida bears slides, from a previous study, were also imaged. Florida bears slides were stained with toluidine blue and the same methods for the previous black bears slides. Again they were randomly selected from the age groups, in an attempt to prevent bias. Light microscopy images were taken of each sample. Images of both 4x and 40x were taken. See Figure 25, page 70, for

an example of the light microscopy images that were taken. From all the images LAGs were counted and measured, but cement lines were just measured. During the counting, endosteal LAGs were not accounted for. Only LAGs that were not near or on the endosteal surface could be counted. The decision behind this is that endosteal LAGs were formed due to modeling of the bone, in older animals. Endosteal LAGs were not present on younger animals. They were only present on older animals. It was as if they were their own separate set of LAGs. Since it is unknown when the endosteal LAGs started, they could not provide an accurate number of LAGs formed in the animals over its title life. From this it was decided that LAGs not in direct contact with the endosteal surface would provide a better approximation to the total number of LAGs formed in the animal, over the course of its life. All LAGs were counted as separate LAGs**.** After this count, double LAGs were taken into account. This was done by counting double LAGs as one LAG instead of two separate LAGs. Double LAGs could only be counted if they were not on the periosteal surface or younger animals. In older animals specifically, LAGs will stack up on the periosteal surface. With this happening it is hard to determine if there are double LAGs are present. LAGs stack on the periosteal surface due to the radial growth slowing down with age. So the determination of double LAGs were very conservative, meaning some double LAGs could be missed, due to the LAGs stacking on the periosteal surface. Measurement of LAGs and cement lines were done through a program called Bio-Quant. With Bio-Quant, the images could be measure through calibration slides present with the light microscope. With the calibration LAGs and cement lines were measured. Approximately ten to fifteen point measurements were taken per LAG and cement line. The points were then averaged to get a distance value. Multiple cement lines and LAGs were then measured in the same way.

Statistics 2.10

SAS free university edition was used for all statistics. All point scan data was imported into Excel, and out of PDF format such that statistics could be ran on the data. SAS code was written to perform a one way ANOVA, with repeated measures, for each element. The ten points for each element in each section were averaged together. The averages then gave six points for each element in each section of the bear. Averages were put into a box and whisker plot for each section and each element. See Figure 18, page 58, to see each section of ten points that were averaged together. Comparisons were made between: LAG vs. cement lines, LAG vs. outside of LAG, and inside osteon vs. cement line vs. outside of osteon, in the bone matrix. Normal data assumptions applied. These assumptions were: random selection, normal, and Gaussian distribution.

CHAPTER THREE: RESULTS

SEM BSE Images 3.0

BSE images showed that both cement lines and LAGs showed up brighter than surrounding bone matrix. This is true for both black and grizzly bears. Also there is a presence of brighter lines that do not seem to be LAGs. Endosteal LAGs are also present. These were not counted due to reasons stated in *Method and Materials Section 2.9*. See Figure 19, below, it shows examples of all bright lines found during SEM BSI.

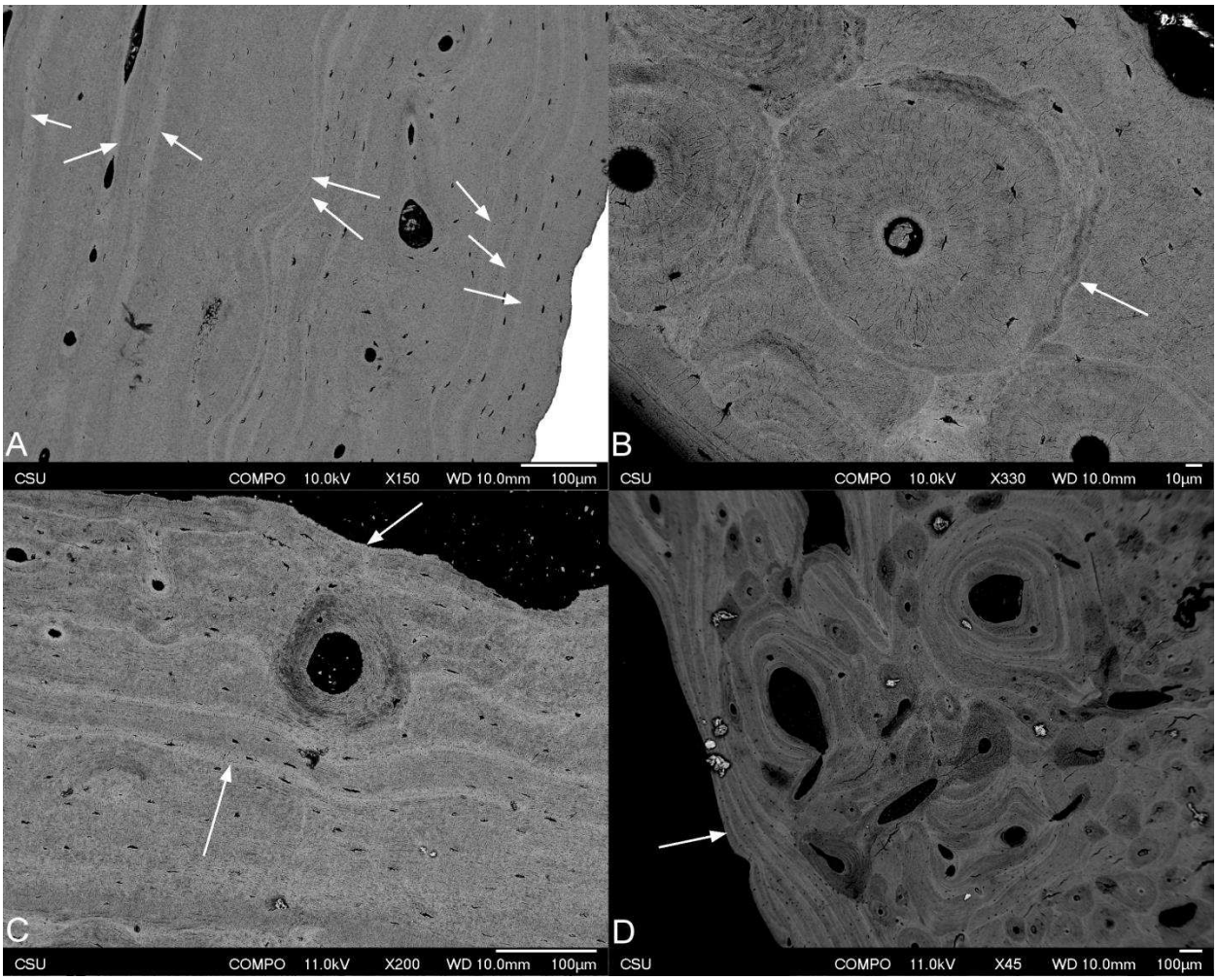


Figure 19: On the top left, image A, there is an example of LAGs and other lines, black bears. The three arrows on the left, point out an example of lines that do not seem to be LAGs, but still show up brighter than the surrounding bone matrix. On the top right, image B, there is an example of an osteon with a surrounding cement line. On the

bottom left, image C, there is another example of the same lines that do not seem to be LAGs, but still show up brighter than the surrounding bone matrix. In this case this is in grizzly bears. The bottom arrow represents these lines. The upper arrow is showing the periosteal surface. Even though visibly there seems to be a LAG on the surface both point and lines scans did not find any significant difference, compared to surrounding bone matrix. On the bottom right, image D, there is an example of endosteal LAGs, in an old grizzly bear.

SEM EDS Data 3.1

This section is to show how data came out of the SEM EDS before statistics were applied. Spectra and tables were outputted after points were selected. Point selection was described in *Method and Materials Section 2.8*. See Figure 20, below, it shows point and line scans for both cement lines and LAGs.

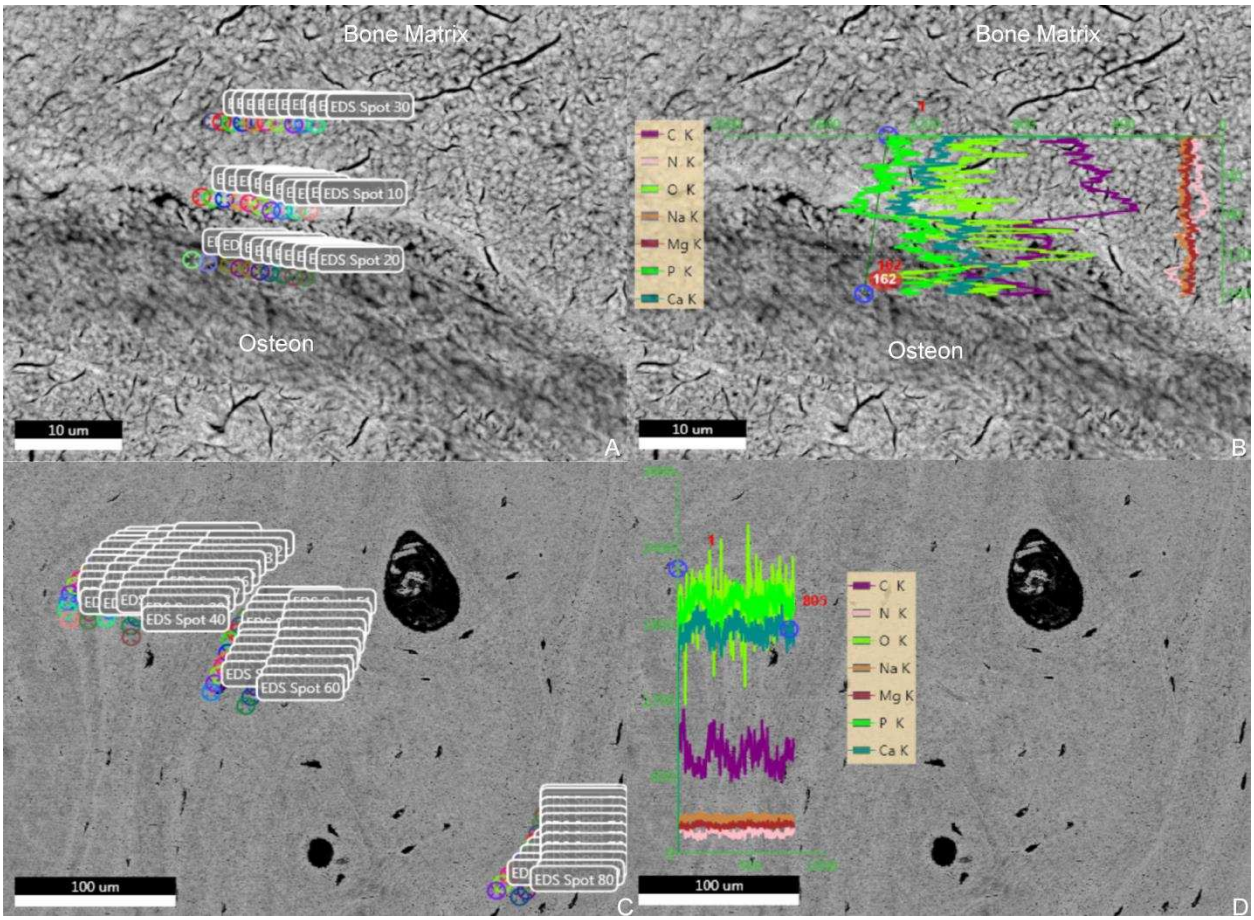
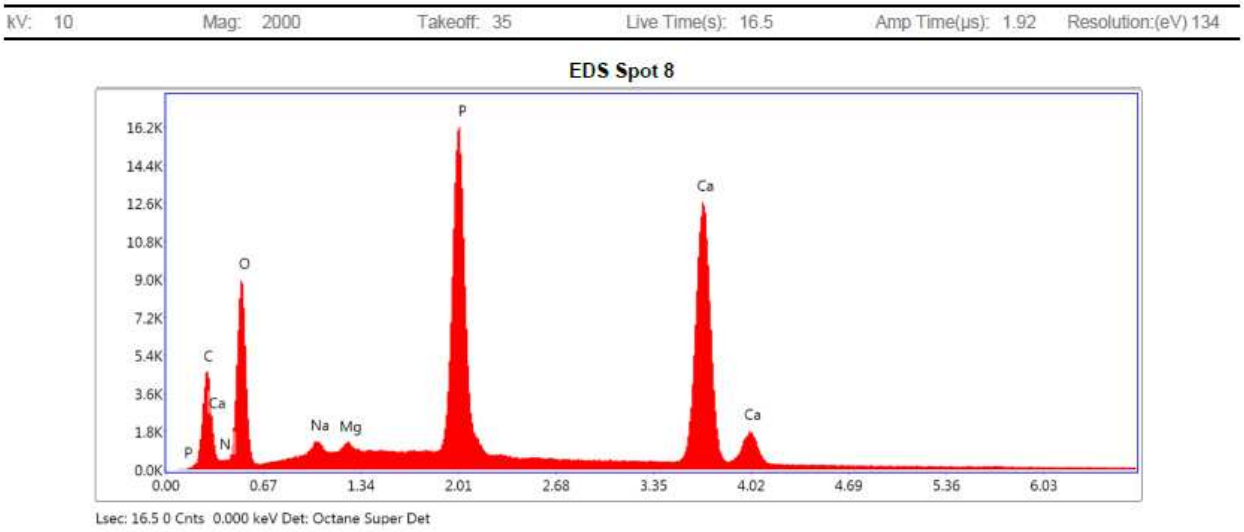


Figure 20: Both images on top are of the same area. In the top left, image A, there is an example of point scan on a cement line. In the top right, image B, there is an example of line scan on a cement line. In the bottom left, image C, there is an example of point scan on LAGs. In the bottom right, image D, there is an example of line scan on LAGs.

See Figure 21, below, for an example of how a point scan's data is outputted.



eZAF Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	R	A	F
C K	8.56	18.14	1366.15	9.56	0.0331	1.1841	0.9229	0.3261	1.0000
N K	0.00	0.00	0.06	99.99	0.0000	1.1508	0.9353	0.1431	1.0000
O K	20.69	32.90	3642.41	9.82	0.0573	1.1219	0.9461	0.2470	1.0000
NaK	0.26	0.29	111.91	16.58	0.0017	1.0077	0.9733	0.6470	1.0043
MgK	0.18	0.19	111.26	18.94	0.0015	1.0218	0.9811	0.7733	1.0080
P K	20.77	17.06	9483.35	3.02	0.1932	0.9580	1.0012	0.9534	1.0183
CaK	49.52	31.43	9048.95	3.31	0.4594	0.9290	1.0240	0.9890	1.0097

Figure 21: Point data as it is given to the user. The top part is a spectra shown what elements the EDS detected and at what levels. Some elements will have multiple peaks. For example Ca has three peaks. This is due to Ca having multiple excitation phases. When bombarded with electrons these multiple phases can cause different wavelengths to be given off in a relaxation phase. Na is most likely contaminant from handling. Skin oils and skin cells have a lot of Na on them, from sweat. This contamination is generally an issue in most spectra, unless one is being very careful not to allow skin contact, either through touch or skin cells. Weight percent was used for statistics, from each of these tables. The K that follows each element is EDS jargon for x-ray emission bands. For all intents and purposes they can be ignored, since only the element is important here.

SEM EDS Statistics 3.2

Comparisons were made between: LAG vs. cement lines, LAG vs. outside of LAG, and inside osteon vs. cement line vs. outside of osteon, in the bone matrix; for black bears. Lines

were visually present at the periosteal surface of the grizzly bear samples. Once the statistics were run on these samples, from the point analysis, it was deemed that this data was not useful qualitatively.

LAGs 3.2.1

In the case of LAGs, Ca, P, and O satisfied the assumptions of the ANOVA. Meaning the data had: variance with no obvious football or horn shapes, distributions that looks mostly Gaussian and not bimodal, and normality that is fairly linear. The Tukey-Kramer method was used to get an adjusted p-value. There are only two groups, in this case, so the normal and Tukey-Kramer p-value are the same. Ca showed no significant difference ($p = 0.1555$) in LAGs (62.40% ± 2.79%) vs. outside LAGs (periosteally) (61.02% ± 2.79%). P showed no significant difference ($p = 0.3538$) in LAGs (19.47% ± 0.85%) vs. outside LAGs (periosteally) (19.69% ± 0.85%). O showed no significant difference ($p = 0.5424$) in LAGs (12.22% ± 1.86%) vs. outside LAGs (periosteally) (11.82% ± 1.86%). See Figure 22, page 65, for box and whisker plots for each element. For C, the sample was coated in C. This made any data obtained not helpful, since carbon was added to the sample to run the sample in the SEM. The coating process is not accurate enough to determine how much C was added to each sample. Also the limitations of SEM EDS would make it hard if not impossible to remove the amount from a percent measure even if the initial amount of carbon added was a known. For: N, Na, and Mg; there data was not good statistically. The data for these elements contained some if not all of the things that were being avoided such as: variance with football or horn shapes, distributions that did not look mostly Gaussian and normality that was not fairly linear.

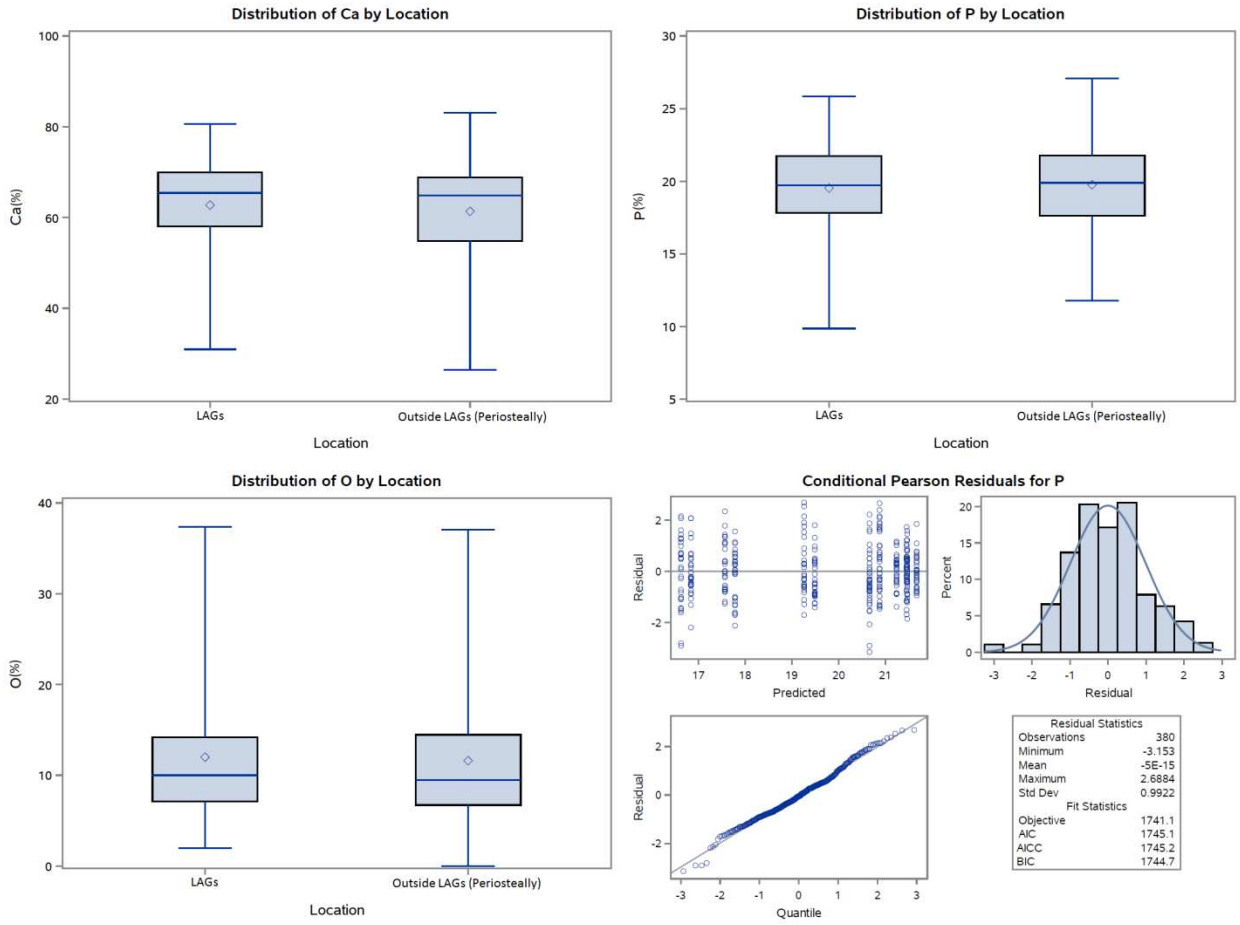


Figure 22: Box and whisker plots for Ca, P, and O. These plots are comparing LAGs. The bottom right image show: variance, distribution, and normality; of the data.

Osteons 3.2.2

In the case of osteons, Ca, P, and O satisfied the assumptions of the ANOVA. Meaning the data had: variance with no obvious football or horn shapes, distributions that looks mostly Gaussian and not bimodal, and normality that is fairly linear. The Tukey-Kramer method was used to get an adjusted p-value. There are three groups, in this case, so the normal and Tukey-Kramer p-value are now different. In this case the Tukey-Kramer p-value was used since it is the more conservative estimation.

Ca

Ca showed no significant difference ($p = 0.0987$) in the osteon (51.04% ± 2.11%) vs. in the cement lines (53.53% ± 2.11%). Ca showed no significant difference ($p = 0.9988$) in the osteon (51.04% ± 2.11%) vs. outside the osteon (bone matrix) (50.99% ± 2.11%). Ca showed no significant difference ($p = 0.0889$) in the cement line (53.53% ± 2.11%) vs. outside the osteon (bone matrix) (50.99% ± 2.11%).

P

P showed no significant difference ($p = 0.0638$) in the osteon (18.52% ± 0.43%) vs. in the cement lines (18.99% ± 0.43%). P showed no significant difference ($p = 0.9988$) in the osteon (18.52% ± 0.43%) vs. outside the osteon (bone matrix) (18.88% ± 0.43%). P showed no significant difference ($p = 0.0889$) in the cement line (18.99% ± 0.43%) vs. outside the osteon (bone matrix) (18.88% ± 0.43%).

O

O showed no significant difference ($p = 0.6388$) in the osteon (17.65% ± 1.13%) vs. in the cement lines (18.37% ± 1.13%). O less ($p = 0.0356$) in the osteon (17.65% ± 1.13%) vs. outside the osteon (bone matrix) (19.64% ± 1.13%). O showed no significant difference ($p = 0.2556$) in the cement line (18.37% ± 1.13%) vs. outside the osteon (bone matrix) (19.64% ± 1.13%).

See Figure 23, page 67, for box and whisker plots for each element. For C, the sample was coated in C. This made any data obtained not helpful, since carbon was added to the sample to run the sample in the SEM. The coating process is not accurate enough to determine how much C was added to each sample. Also the limitations of SEM EDS would make it hard if not impossible to remove the amount from a percent measure even if the initial amount of carbon

added was a known. For: N, Na, and Mg; there data was not good statistically. The data for these elements contained some if not all of the things that were being avoided such as: variance with football or horn shapes, distributions that did not look mostly Gaussian and normality that was not fairly linear.

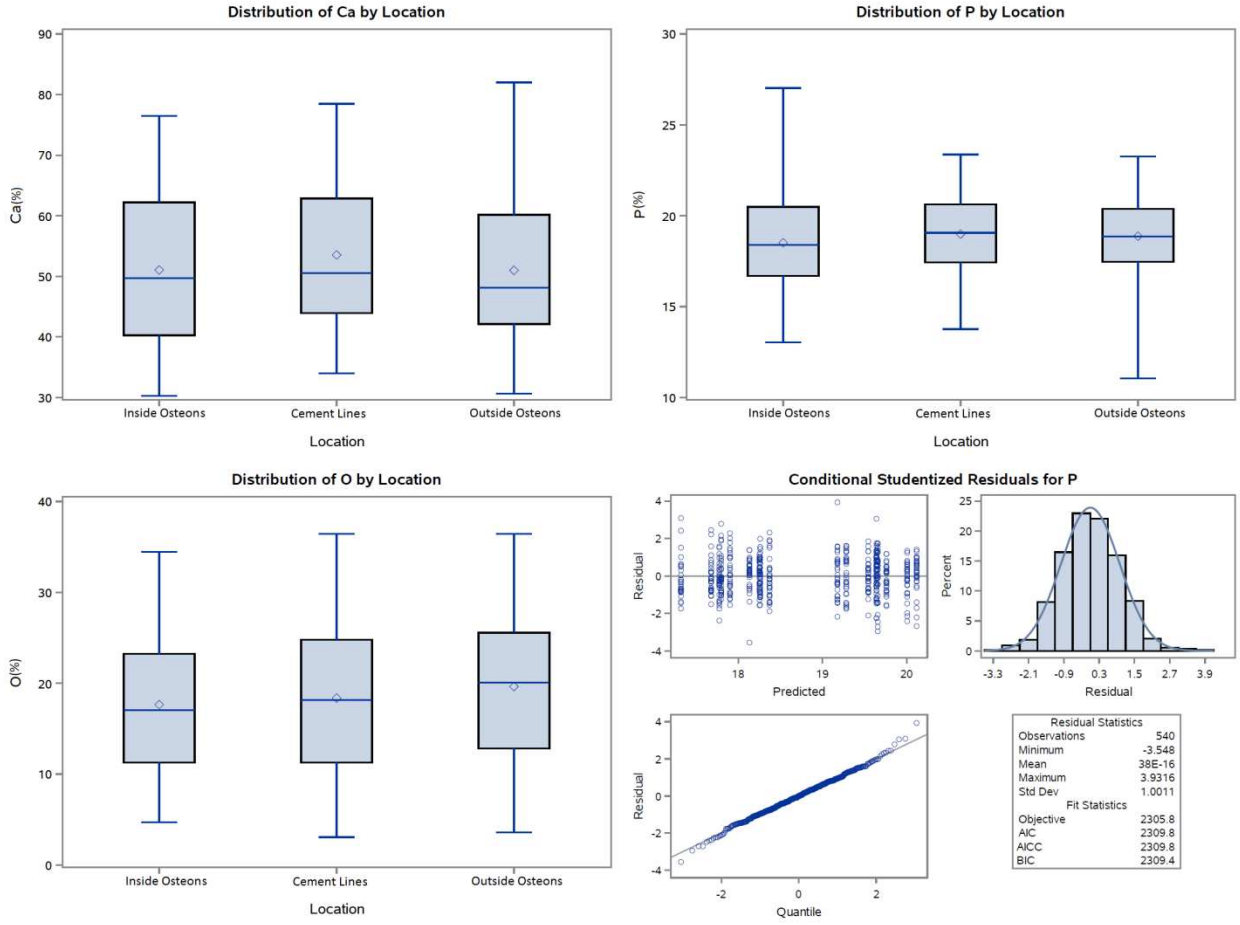


Figure 23: Box and whisker plots for Ca, P, and O. These plots are comparing osteons. The bottom right image show: variance, distribution, and normality; of the data.

LAGs vs. Cement Lines 3.2.3

In the case of LAGs vs. cement lines, data for calcium (Ca), phosphorus (P), and oxygen (O) satisfied the assumptions of the ANOVA. Meaning the data had: variance with no obvious football or horn shapes, distributions that looks mostly Gaussian and not bimodal, and normality

that is fairly linear. The Tukey-Kramer method was used to get an adjusted p-value. There are only two groups, in this case, so the normal and Tukey-Kramer p-value are the same. Ca is greater ($p < 0.0001$) in LAGs (62.47% ± 2.17%) vs. cement lines (53.53% ± 2.17%). P is greater ($p = 0.0389$) in LAGs (19.49% ± 0.53%) vs. cement lines (18.99% ± 0.53%). O is greater ($p < 0.0001$) in cement lines (18.37% ± 1.44%) vs. LAGs (12.15% ± 1.44%). See Figure 24, page 69, for box and whisker plots for each element. For C, the sample was coated in C. This made any data obtained not helpful, since carbon was added to the sample to run the sample in the SEM. The coating process is not accurate enough to determine how much C was added to each sample. Also the limitations of SEM EDS would make it hard if not impossible to remove the amount from a percent measure even if the initial amount of carbon added was a known. For: N, Na, and Mg; there data was not good statistically. The data for these elements contained some if not all of the things that were being avoided such as: variance with football or horn shapes, distributions that did not look mostly Gaussian and normality that was not fairly linear.

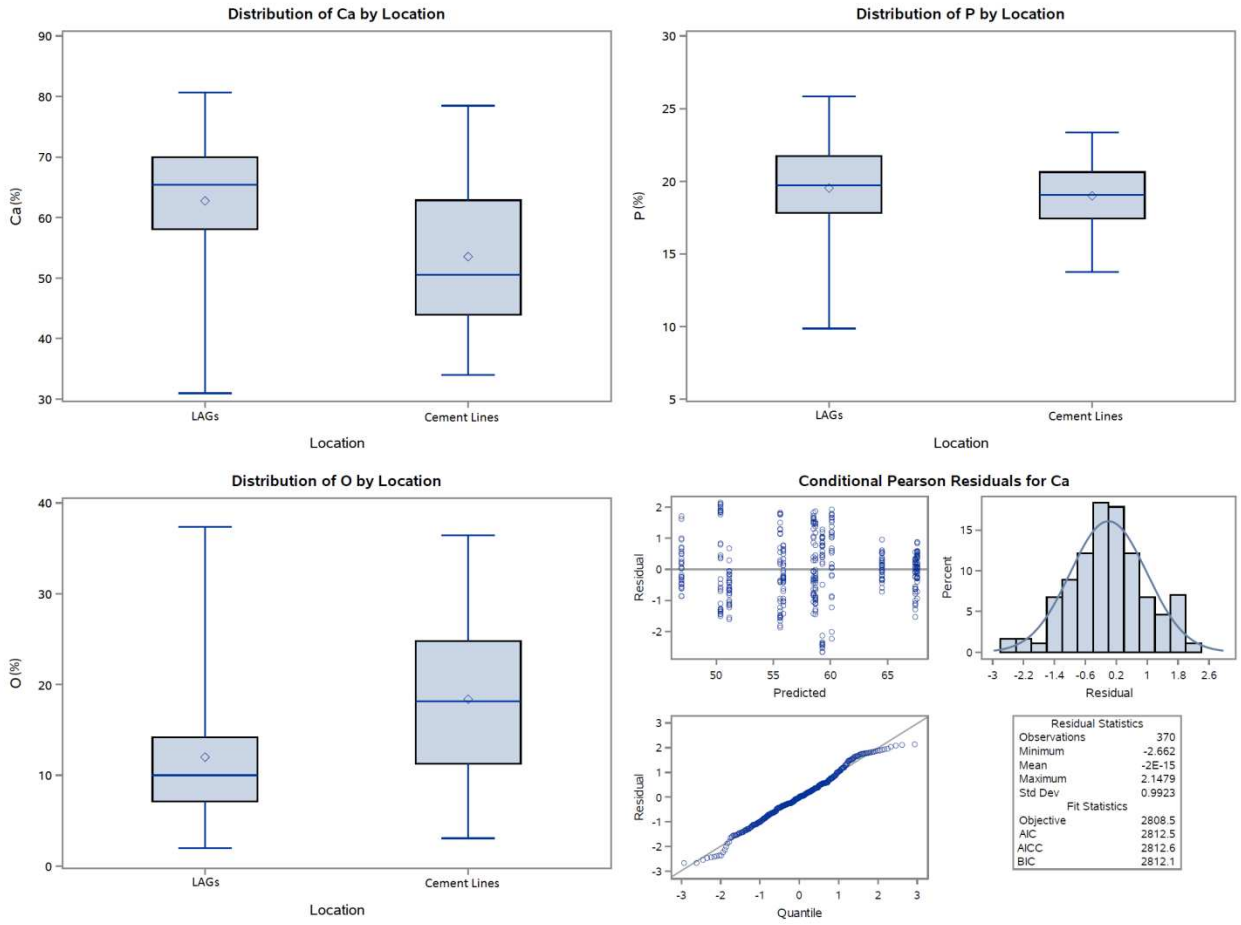


Figure 24: Box and whisker plots for Ca, P, and O. These plots are comparing LAGs vs. cement lines. The bottom right image show: variance, distribution, and normality; of the data.

Light Microscopy Images 3.3

Toluidine blue stained light microscope images, showed both cement lines and LAGs as dark blue compared to the surrounding bone. This is true for both Utah and Florida bears. Double LAGs are present. See Figure 25, page 70, it shows a zoomed in image of a double LAG. Also the double LAG is visible in the zoomed out top image, of Figure 25. Endosteal LAGs are also present in the top image. These were not counted due to reasons stated in *Method and Materials Section 2.9.*

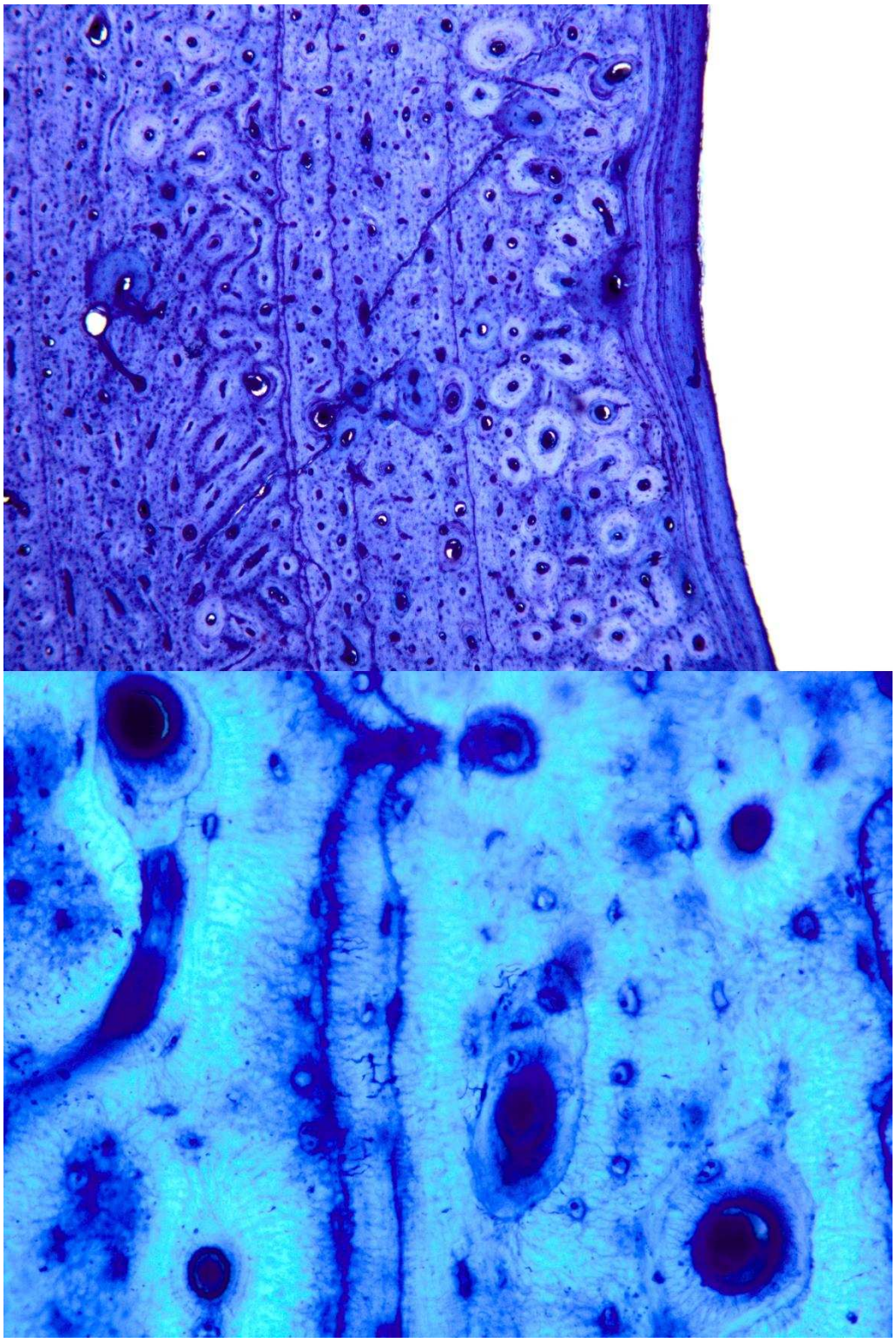


Figure 25: In the top, a 4x image of black bear cortical sample. Dark blue vertical lines are LAGs. More specifically, this is from the anterior region in a femur. The bottom image is the same sample, but zoomed in at 40x. It shows two LAGs close together, or a "double LAG".

Cement and LAG Size Comparisons 3.4

Size comparisons were done for cement lines and LAGs in both light microscopy and electron microscopy (SEM). SEM size analysis procedures were outlined in section 2.1 of the methods and materials. It is unknown were light microscope samples were pulled from on the bear bone. Cement lines in light microscopy averaged out to approximately 1.6 microns ± 0.34 microns (averaged over 17 cement lines). Cement lines in SEM averaged out to approximately 1.3 microns ± 0.58 microns (averaged over 19 cement lines). LAGs in light microscopy averaged out to approximately 3.5 microns ± 1.02 microns (averaged over 53 LAGs). LAGs in SEM averaged out to approximately 4.57 microns ± 1.58 microns (averaged over 16 LAGs). It was unknown if some of these LAGs and\or cement lines were the same ones being compared in both SEM and light microscopy. The light microscope data was pulled from twelve Utah black bear samples. The SEM data was bulled from six black bear samples. The six samples in SEM were six of the twelve samples used in light microscopy.

Radial Growth 3.5

Radial growth measurements were used from previous work. Radial growth measurements were done to see if there was any insight into LAGs due to the age of the bear and the radial growth across a cross section. Data came from 47 hunter killed black bears, all males. See Figure 26, page 72, for a plot of Age vs. Radial Growth.

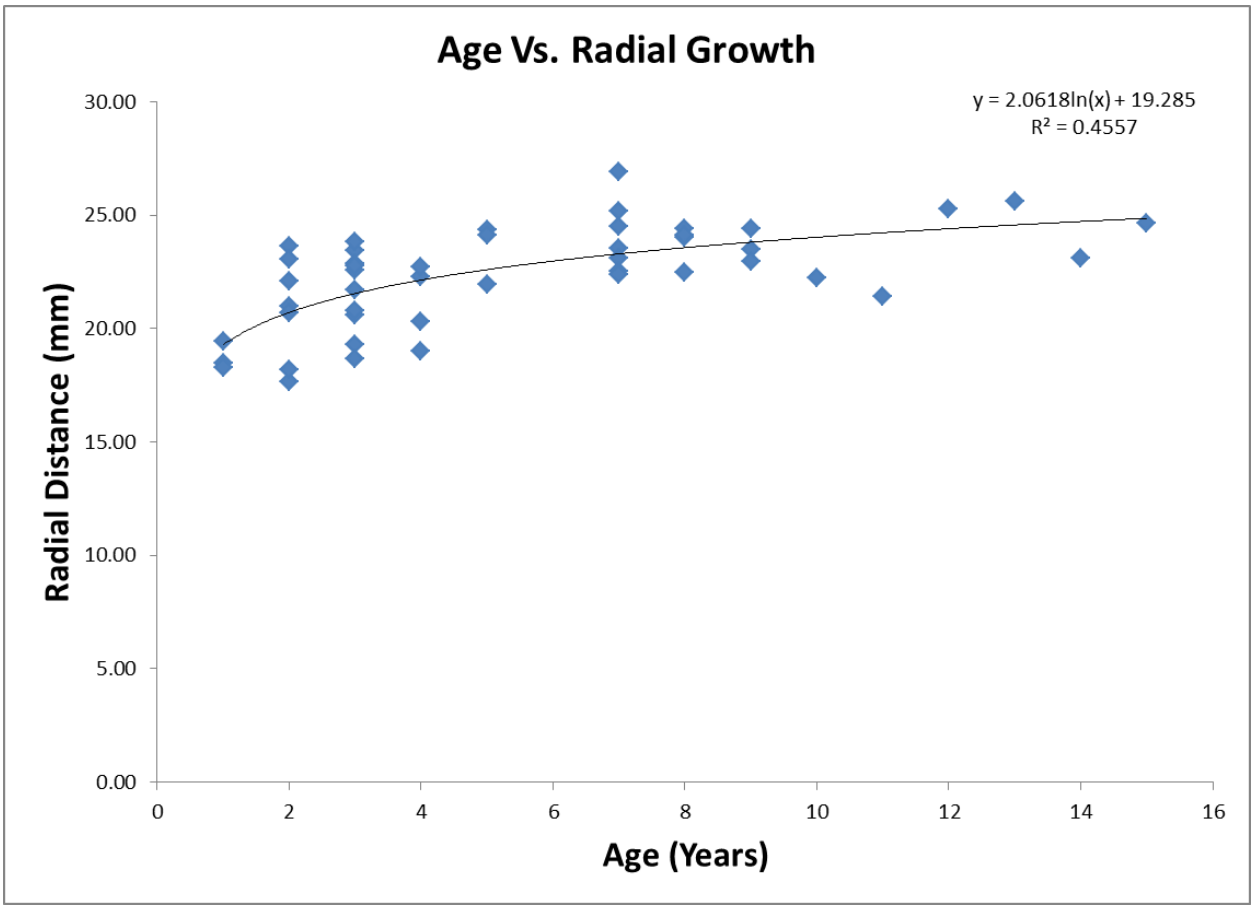


Figure 26: Shows a graph of Age vs. the diameter of the mid diaphysis. The data is from 47 hunter killed black bears, all males.

LAG Counting 3.6

Counted LAGs in black bears showed a slope of 1.24 for non-adjusted black bears, and a slope of 1.14 for adjusted black bears. Counted LAGs in Florida bears showed a slope of 0.72 for non-adjusted Florida bears, and a slope of 0.65 for adjusted Florida bears. See Figure 27, page 73, for plots of counted LAGs.

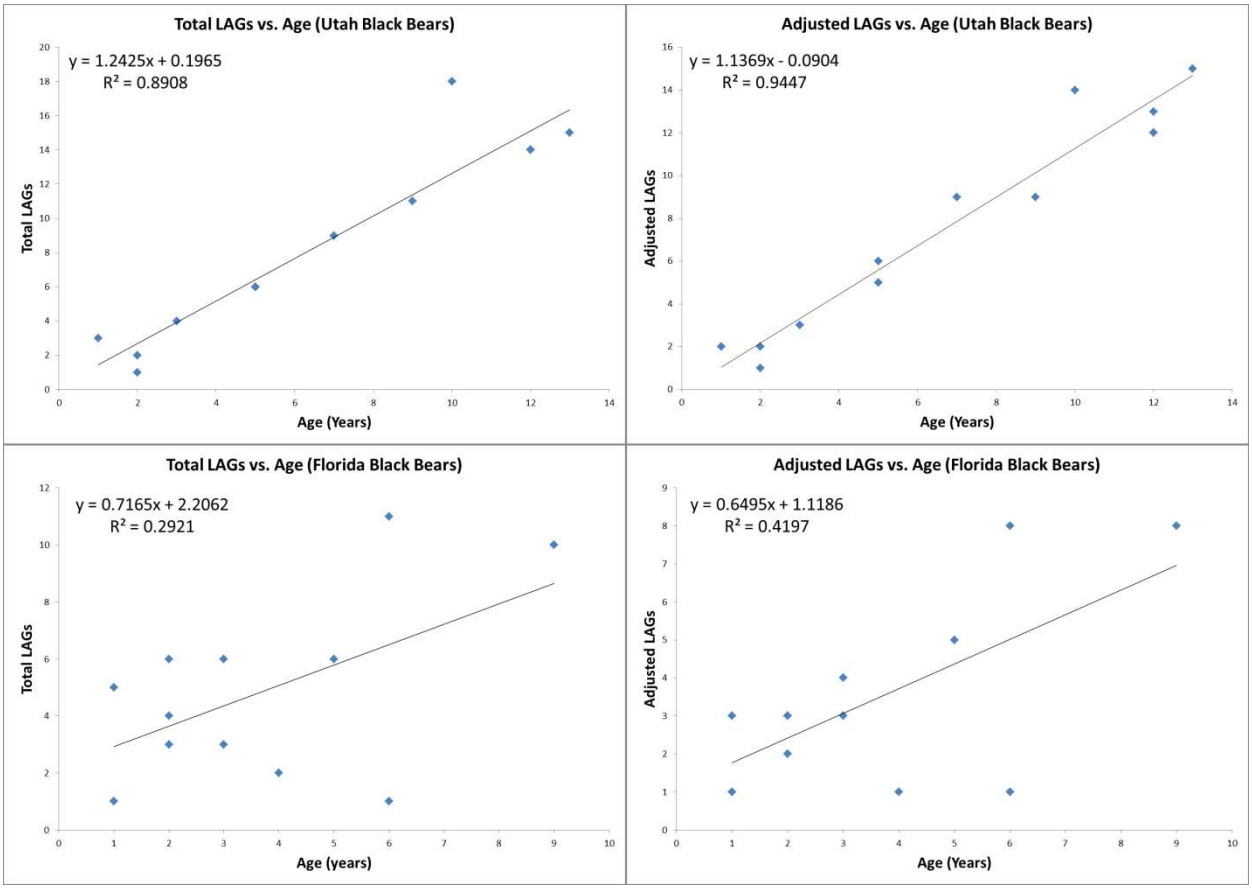


Figure 27: The top two graphs are for black bears. The bottom two graphs are for Florida bears. Both the top left and the top right graph have a p-value of <0.0001. The bottom left graph has a p-value of 0.0697. The bottom right graph has a p-value of 0.0227. All p-values were done through a simple linear regression.

CHAPTER FOUR: DISCUSSION

SEM Images 4.0

In the 2005 article by Skedros, cement lines were described as small bright lines that surrounded secondary osteons. The definition of brighter, in this case, was whiter relative to the surrounding features. These images were cross sections of human cortical bone, and specifically showed up when the SEM was imaging, in the SEI mode. This was confirmed by the present study. Bright lines showed up surrounding secondary osteons, in cross sections of black bear cortical bone. There was that same definition of brighter, whiter relative to the surround features. Again this was specifically when the SEM was imaging, in the SEI mode. It was concluded from Skedros (2005) that these brighter regions were more mineralized. In this case more mineralized, implies a region with higher levels of dense elements. In the case of bone the densest element is Ca, due to hydroxyapatite being the major mineral of bone. The assumption of most dense element is not including trace elements. By this assumption all regions in bone that have brighter regions, could be considered to have higher concentrations of Ca. Many other lines and regions, that appear to be more white relative to the surround features, could be found. These features were more predominant at the outer 1/3 of the bone, as one would travel radially toward the periostium. There is little literature currently on these lines in SEM imaging, specifically dealing with elemental composition and definitions of said features. The original assumption, in the beginning, was that these brighter lines were LAGs. Observation and insights showed that there were too many and the sizes of the lines were not consistent. While it seems now that not all these brighter lines are LAGs, it does seem that some of these brighter lines are LAGs. While not all the other features were defined, it was suggested that they could be annuli

or growth features left over after modeling. As for these unconfirmed features and LAGs, it shows an obvious region of possible growth in knowledge, for future studies.

SEM EDS Point and Line Analysis 4.1

The statistics, of the point analysis, of cement lines and LAGs showed some significant differences. There was a significant difference between LAGs and cement lines. There was a significant difference in Ca, P, and O. Ca is greater ($p < 0.0001$) in LAGs (62.47% ± 2.17%) vs. cement lines (53.53% ± 2.17%). P is greater ($p = 0.0389$) in LAGs (19.49% ± 0.53%) vs. cement lines (18.99% ± 0.53%). O is greater ($p < 0.0001$) in cement lines (18.37% ± 1.44%) vs. LAGs (12.15% ± 1.44%). A possible explanation for this could be the age of the LAGs and cement lines. Generally older structures in bone are more mineralized than younger structures. As for differences in: LAGs vs. outside LAGs (periostially), cement line vs. inside the osteon, cement lines vs. outside the osteon (in surrounding bone matrix), and inside the osteon vs. outside the osteon (in surrounding bone matrix), there was no significant differences. This is the opposite of what was expected. It was expected to see a significant difference between LAGs vs. outside LAGs (periostially), cement line vs. inside the osteon, and cement lines vs. outside the osteon (in surrounding bone matrix). There were no expectations to see a significant difference between: LAGs and cement lines. The present study was done with standard SEM EDS. In the present study, SEM EDS is accurate but not precise enough. Skedros (2005) used quantitative EDS. He was able to show a significant difference between cement lines and bone inside the osteon. This difference was 25.13% ± 0.46% Ca in cement lines vs. 23.82 % ± 0.49% Ca in the osteon. Standard SEM EDS, in the current study, was not able to show a significant difference in these regions. Yet in Skedros (2005) he was not able to show a significant difference in Ca

content between a cement line and intestinal bone outside of the osteon. This shows that even quantitative EDS is not picking up the visible difference seen in BSI. There is something in BSI images, were both LAGs and cement lines show up visibly brighter than the surrounding bone. Yet both normal and quantitative EDS cannot pick up on this visual difference. In both the current study and Skedros (2005) study, the same elements were found. In future studies this shows that standard SEM EDS is acceptable for accuracy of elements, but not as precise as quantitative EDS. Some other method or and improvement quantitative EDS is required to elementally prove this visual phenomenon, in bone.

Light Microscope Images 4.2

Images from the light microscope were stained in toluidine blue. The dye stains GAGs and positively charged species (Hall et al., 1993). Toluidine blue also stains cement lines and LAGs (Hall et al., 1993). This allowed for the separation of LAGs and cement lines from other features. For example there were many lines in SEM BSI. It was hard to differentiate LAGs from other structures (annuli and other features). In the microscope slides LAGs could be differentiated from other structures. One example being LAGs from annuli. Annuli do not stain with toluidine blue, they are white in nature (Hall et al., 1993).This also allowed insight into the size of LAGs. This allowed for a size approximation of LAGs, in SEM. Even though LAGs and cement lines are stained, Toluidine blue stains GAGs and positively charged species. It is unknown what exactly is being stained in LAGs.

Cement Lines and LAGs Size Comparisons in Both SME and Light Microscopy 4.3

Schaffler (1987) described cement lines as a structure greater than 1 µm in size. Skedros (2005) described cement lines as less than 5 µm. From these two it can be concluded that cement lines are between 1 and 5 µm in size. Cement lines in light microscopy averaged out to approximately 1.6 microns ± 0.34 microns (averaged over 17 cement lines). Cement lines in SEM averaged out to approximately 1.3 microns ± 0.58 microns (averaged over 19 cement lines). The present study agrees with Skedros (2005) and Schaffler (1987). As for LAGs, they were described as larger than cement lines (S. Ray et al., 2009; P. M. Sander et al., 2006; B. K. Hall et al., 1993). Not as much size information was given for LAGs as cement lines. LAGs in light microscopy averaged out to approximately 3.5 microns ± 1.02 microns (averaged over 53 LAGs). LAGs in SEM averaged out to approximately 4.57 microns ± 1.58 microns (averaged over 16 LAGs). Both of the present studies showed on average that LAGs are approximately two to three times the size of cement lines. This backs up the previous study stating that LAGs are larger than cement lines.

LAGs Counting 4.4

Counting LAGs could lead to aging of species, like J. Castanet et al. (2003). J. Castanet et al. (2003) used skeletochronology to approximate age. The given ages use dentin to age animals. Dentin is used since it grows at a certain rate. The dentin is not very susceptible to environmental or nutritional changes. That is how the animals were aged in the present study. J. Castanet et al. (2003) suggested that LAGs would be laid down, one per seasonal light cycle. In J. Castanet et al. (2003) case, the cycle was either annually or artificially set. J. Castanet et al. (2003) LAGs vs. age graphs had a slope that was near to one-half rather than one. This is not as

close as one would want. A slope of one would be ideal, since this signifies one LAG is formed per year old the animal is. J. Castanet et al. (2003) also said that in his gray mouse lemurs, they could only have up to seven LAGs. This could be problematic since gray mouse lemurs can live longer than seven years. Gray mouse lemurs only live this long in captivity. In the wild, the life expectancy is five years old. So this approximation that J. Castanet et al. (2003) works for wild animals, due to predation\life expectancy. In the present case LAGs were counted and compared to the known age. In black bears it shows that LAGs are closer to one LAG per year, or a slope of one. The present study's graph, showed that a slope of approximately 1.25 when double LAGs are not taken into account. The present study's graph, also showed that a slope of approximately 1.14 when multi LAGs are taken into account. In both J. Castanet et al. (2003) and the present study is can be concluded that LAGs appear around one per seasonal cycle or year.

Counting LAGs could also lead to markers that show periods of suppressed metabolism. In the J. Castanet et al. (2003) study it was suggested that LAGs most likely showed up during the less light phase when the gray mouse lemurs were lethargic. Köhler et al. (2012) suggests that a change in water, specifically a lack of rain fall, is when LAGs most likely showed up. Both of these are times that represent a change, or possible decrease, in metabolism. In the present study black bear and grizzly bear data suggests that the LAGs show up during hibernation. This is due to all of the black bear samples showing no LAGs forming on the periosteal surface. None of the black bears (Utah or Florida) analyzed were currently in hibernation, when they were sacrificed. In the case of grizzly bears one set of samples were in hibernation and the other set were not in hibernation. One of the grizzly bear samples, in hibernation, show what looked like a small line forming at the periosteal surface. This line was

brighter than the surrounding bone. Visually it looked like a LAG, but EDS was not elementally conclusive. Also if the line was forming it is unknown what it might form into. There were other bright lines present in the bear samples that were not LAGs. Still, all of the factors seemed favorable for a LAG to form, knowing the circumstances behind the sample. The grizzly bear images and the Utah black bear data suggest a link to metabolic suppression, like Köhler (2012) suggests. Radial growth data from the present study did not help with trying to show a direct correlation, with radial growth and LAGs. It would have been helpful to find a certain age in which LAGs started to pile up. This would have given a good age predictor for when to be more careful on what was getting described as a double or multi LAG. It did show a slowdown in radial growth around five to seven years of age. This has some correlation with J. Castanet et al. (2003) and his LAGs stopping, or just piling up too close to differentiate. The radial growth gave a reason why LAGs to pile up at the periosteal surface in older animals. This pile up of LAGs at the periosteal surface was shown in both the present study and J. Castanet et al. (2003). Unlike J. Castanet et al. (2003) there was still more than seven LAGs possible in the present study, also bear bone cross sections are significantly larger then grey mouse lemur cross sections. It was hard to differentiate LAGs in older animals at the periosteal surface, in the current study. The LAG pile up on the periosteal surface, in older animals, and the size discrepancy could show why J. Castanet et al. (2003) said there is a seven LAG limit.

Future Work 4.5

Future work could include: working with quantitative EDS, looking into the other line structures in the SEM EDS, finding bear samples from several time points during hibernation, providing more understanding to double LAGs in bears, and looking at the de-calcified LAGs and cement lines in the SEM. Quantitative EDS could provide better chemical data on LAGs vs. cement lines and the other cases that were compared. In Skedros's (2005) case, quantitative EDS, did not provide that much more insight. Also quantitative EDS is a large project on its own if one's SEM EDS is not set up for it already. Looking into the other structures in the bone that show up during BSI in the SEM could be quite insightful. The other structures could have been annuli. Also a lot of other structures were present around primary osteons. This could be some sort of mineralization left over after modeling, yet it is still inconclusive and could prove useful to look into this. Double LAGs were present in both the longer hibernating\higher elevation Utah black bears, and the shorter hibernating\lower elevation Florida black bears. Insight into reasons for double or multiple LAGs could be helpful. This is due to the fact that most of the data is in amphibians, ectotherms, and little is known in endotherms. Finding samples of same age bears, with the same sex, at multiple time points during hibernation would be ideal. This is most likely not going to happen since that would be a lot of animals to sacrifice, as well as expensive too. Finally an article by Pazzaglia et al. (2012) had an interesting idea. Pazzaglia et al. (2012) de-calcified secondary osteons. In this study they looked at the collagen density and orientation, in lamellar bands and cement lines. This could be done to compare LAGs and cement lines, to see if they have relatively similar collagen density and orientation.

CHAPTER FIVE: CONCLUSION

In the current study it was shown that cement lines and LAGs show up under BSI with similar brightness. This suggests LAGs and cement lines contain higher levels of calcium than surrounding bone. If these regions have higher levels of Ca, then it could be deduced that this Ca comes from hydroxyapatite. From that it could be deduced that cement and LAGs are more mineralized than surrounding bone. It was shown that if one is interested in elemental composition and a general level of each element, in bone, SEM EDS is good. However, in the case of osteons, quantitative EDS was not even enough. In previous works even quantitative EDS failed to extrapolate the elemental difference between cement line and interstitial bone. Yet backscatter images still showed a visual phenomenon, cement lines were brighter than the surrounding bone. It seems that a new analysis technique is needed, when comparing structures in bone. There are many lines that appear more mineralized, than the surrounding bone, when BSI is used. This is based on the idea that brighter regions contain denser elements. In bone Ca is the densest element that is not trace. Not all of these lines appear to be LAGs, so further investigation is needed. These lines could be annuli or some level of mineralization left over from modeling, since some of these regions are found surrounding primary osteons.

The picture seems clearer in light microscopy, due to the wealth of previous knowledge on the subject and the ability to stain. Toluidine blue staining allows for separation of LAGs and cement lines from the bone. It also has a way to show general regions of annuli by the lack of staining. The presence of LAGs in endotherms shows that their formation has to do with something more than just temperature. While temperature may be one of the signs of LAG formation, especially in ectotherms, it is not the overarching term, to describe this process.

Metabolism is that term that is able to explain both why endotherms and ectotherms have LAGs present. This is due to metabolism being an umbrella like term which many other factors fall under and contribute to. Such terms as: temperature, access to water, access to food\minerals, light cycles\circadian rhythms, etc.; could fit under the term metabolism. All of these previous terms could have an effect on metabolism and thus cause LAGs to form. Currently it seems more appropriate to say LAGs are formed due to a change in metabolism.

As for the numbers of LAGs and the ability to count them, it seems they could be helpful in some cases. Especially in younger animals LAGs could be an appropriate measure on which to perform skeletochronology. It is still dependent on the animal since assuming this for all animals could be problematic, but it is not something that should be shelved at first thought. One must be careful when it comes to older animals due to the piling up of LAGs on the periosteal surface. This is due to the slowing of radial growth, in this case in long bones. Also double\multiple LAGs do provide possibly some more insight or more problems, sort of a double edged sword. Double LAGs are a sign of possible environmental extremes that the animal experienced. This could possibly provide some insight into the animal. Again, in older animals these could pile up at the periosteal surface and cause even more confusion. This confusion is due to even more added lines that may not represent one year's worth of growth since they are so close together. Also counting LAGs could provide markers that show periods of suppressed metabolism. The formation of LAGs seems to deal with a change in metabolism. When LAGs form it is important, since it shows a time when the system, of the animal's body, determines conditions are not favorable for bone growth. In bears, this is important since they are hibernators. One should expect LAGs to form in bear due to extended periods of reduced metabolism, i.e. hibernation. Understanding when LAGS form for bears, in hibernation, could

be a key determining factor to understanding their ability to forgo bone loss, due to disuse. If a time point could be determined, LAGs could possibly then be artificially induced. In the study with the grey mouse lemurs, it was shown that one could change the time when LAGs normally formed in animals. When a LAG was formed the serum and other biological factors could be measured. This would be done in order to determine any link with bears and their protection from disuse osteoporosis, during hibernation. A possible unknown biological factor or level of known biological factor could contribute to this protection. LAGs seem to have an important role not only for skeletochronology, and insights in to living conditions, but also for the ability to show metabolic maps and provide key points to test for in an attempt to circumvent disuse osteoporosis.

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