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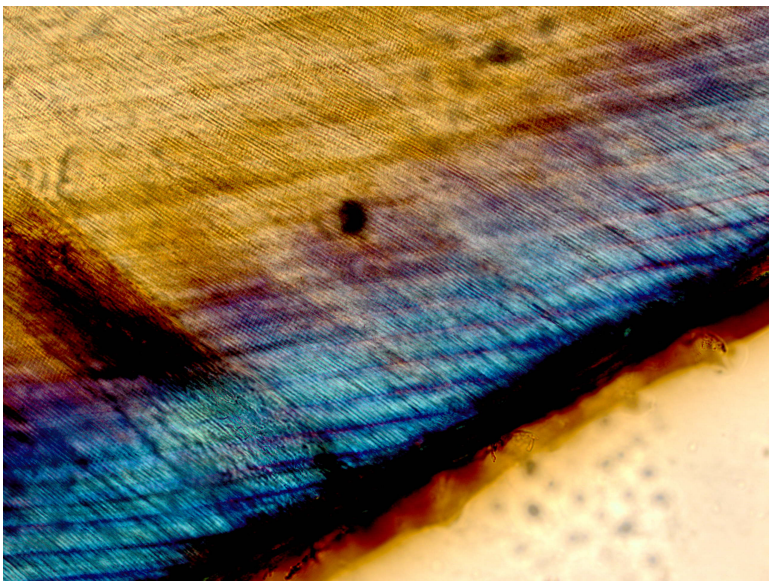
Long period growth lines in enamel and body size in humans: a test of the Havers-Halberg Hypothesis

Simon Chapple



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Long period growth lines in enamel and body size in humans: a test of the Havers-Halberg Hypothesis

Simon Chapple
Student ID - 12957183
sac87@kent.ac.uk

School of Anthropology and Conservation
University of Kent

Project supervisors – Dr. Patrick Mahoney
Dr. Chris Deter

Total word count excluding references – 39,381

Total word count including references – 49,947

Acknowledgments –

I would like to start by thanking Dr. Patrick Mahoney for introducing me to the world of dental histology, for providing detailed instruction in histological techniques, and for allowing the freedom and time to experiment and practice with the methodology to help me really develop and hone the required skills associated with such an challenging but rewarding technique. In addition to this, I would like to thank the Skeletal Biology Research Center at the University of Kent for allowing access to the skeletal collection used in this study. Access to such a fascinating archeological collection is always a privilege.

Thanks also to Dr. Patrick Mahoney and Dr. Chris Deter for their support and guidance during the build up to this year, and also throughout this entire research. In addition to the support, encouragement and experience they provided in relation to this research, both have helped me significantly in developing a stronger academic profile by providing and encouraging opportunities to present work, assist in teaching, or develop useful additional skills.

I would like to thank my parents, Andy and Sue Chapple, for their financial and moral support throughout this year, and throughout my entire educational and academic life so far. None of this would have been possible without their continued help and encouragement.

Finally, I would like to thank Kim for her support and reassurance, and also for putting up with me talking about teeth constantly over the last year.

Abstract -

According to the Havers–Halberg Oscillation hypothesis (HHO), evidence of a metabolic biorhythm retained in enamel as Retzius periodicity (RP) positively correlates with average body mass and the pace of life history across the majority of mammalian species. In humans, RP is highly variable between individuals, but it is unknown if it correlates with body size, as it does across species. Here, stature and body mass was estimated in an archeological sample of modern humans (n=23). Retzius periodicity was reconstructed for permanent teeth from the same individuals. Reduced major axis regression revealed that RP is significantly and negatively correlated with stature and body mass in adult humans. Individuals with higher RPs were of smaller stature and body mass than those with lower RPs. These results support an intraspecific HHO hypothesis, whereby increases in body size within humans are achieved through an accelerated biorhythm, and reflected by a lower RP. Results presented here lay a new foundation for studies of enamel histology and life history *within* modern humans, with potential applications to our fossil ancestors.

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1. Introduction

Biological rhythms, defined as the cyclic change in the level of a bodily chemical or function, exert considerable regulatory control over numerous aspects of an organism's physiology, development, and behaviour (Hastings, 1997). In some cases, when these biological rhythms are associated with continually additive modes of growth, and the structure being grown has preservable hard tissue, structural evidence of incremental growth may be observed (Scrutton, 1978). This structural evidence often then provides interesting insights into the temporal nature of the rhythmic processes involved in the formation of the hard tissue. In dental enamel, structural evidence of biological rhythms are retained in form of incremental markings. One of these incremental markings, the cross striation, represents a daily periodic rhythm of enamel secretion that appears to remains consistent in its periodicity both within and across species and aligns with the prominent environmental cycle of night and day (Fitzgerald, 1998). Another incremental marking observable in histological sections of enamel are the longer period Retzius lines. These incremental markings are thought to represent robust, periodic disruptions in enamel formation that remain consistent in periodicity within an individual (Bromage *et al.,* 2009). Unlike the daily enamel increments however, Retzius periodicity has no obvious environmental counterpart and varies in periodicity in multiples of whole days between species and sometimes between individuals of the same species. Subsequently, Retzius periodicity represents a comparatively unusual and poorly understood biological rhythm.

A significant step in the understanding what these long-period markings in enamel may represent was made when studies showed that the periodicity of the Retzius line positively correlated with body mass across species. Since then, more recent studies have shown that Retzius periodicity (RP) for some mammal species mirrors the periodicity of growth increments in bone, and also corresponds with rates of bone growth (Bromage *et al.,* 2009, 2012). This has two important implications. Firstly, it suggests that Retzius lines, and the underlying rhythm causing them, are not exclusively related to processes of

enamel development but appear to be the manifestation of a larger systemic biological rhythm, and secondly, suggests that RP is not just correlated with body mass but may represent a biological rhythm that is actually driving or regulating aspects of mammalian growth and development in some way. Furthermore, RP has also been shown to correlate with many other life history and life history-related variables across species, including age at gestation length, birth weight, and age at first reproduction (Bromage *et al.,* 2012). Based on these correspondence and correlations, a hypothetical metabolic biorhythm termed the Havers-Halberg Oscillation (HHO) was proposed as the coordinating mechanism.

Although currently still in the early stages of understanding the HHO, this newly discovered systemic rhythm has the potential to become an exciting new analytical tool for investigating mammalian life history and evolution in the future. What is not understood is whether the same RP relationships exist within humans. Compared with the other primate species, humans are characterised by particularly high RP variability (Reid and Dean, 2006). Investigating whether a similar relationship exists between RP and body size in humans, a preliminary study showed that RP was lower among individuals of larger height and weight (Bromage *et al.,* 2009, 2015). Unfortunately however, sample sizes were too small for meaningful conclusions. Consequently, it remains to be determined whether the HHO hypothesis holds within humans. This research seeks to explore this and investigate the relationship between RP and body size in an archeological sample of modern humans. If a relationship exists between RP and body size in humans as it does across species, it will lay a new foundation for studies of enamel histology and life history within humans and may even have future applications and implications for the study of fossil hominin ancestors.

1.1 Study aims and hypothesis

The aim of this study is to test the hypothesis postulated by Bromage *et al.* (2009) that Retzius periodicity in dental enamel correlates with adult body size in modern humans. While body size is an abstract concept not a concrete parameter (Hemmer, 2015), the term is used here to refer to both adult body height and body mass.

H1 = Based on the preliminary findings by Bromage *et al*. (2009), hypothesis one is that a negative correlation will exist between Retzius periodicity and body size in humans.

H2 = Based on the observed relationship across species (Bromage *et al.,* 2009), hypothesis two is that a positive correlation will exist between Retzius periodicity and body size in humans.

H0 = The null hypothesis is that no correlation exists between Retzius periodicity and the body size variables in humans.

2. Literature review

This literature review is divided into two main sections. As this study focuses on investigating the human relationship between body size and the periodicity of the long-period Retzius line retained in enamel, the first section begins with a brief description of modern human tooth development and amelogenesis. It is during this latter developmental process that Retzius lines are formed. This is followed by a review and discussion of some of the numerous incremental markings created during this latter stage of enamel development. After this, the interspecific correlations with Retzius lines are described, and the hypothetical HHO is introduced. Collectively, this first section lays the groundwork for understanding the aims and justifications of this research.

In the second section of the literature review, biological rhythms are introduced. For reasons discussed later, examples of biological rhythms provided here remain notably broad and include rhythms of various time interval and function. After this, the basic features, mechanisms and cues that maintain and entrain biological rhythms are briefly described. This second section of the literature review is not essential for understanding the basic aims of this research but highlights the comparatively novel and unusual nature of the Retzius line rhythm, while laying a broad foundation for interpreting and discussing the possible nature and origin of the rhythmic biological processes and mechanisms involved. This review ends with a focus on the complexity of biological rhythms in nature and describes a few of the many ways in which unusual rhythmic expression may occur.

2.1 Early tooth morphogenesis

Almost all mammals, including humans, have deciduous and permanent sets of teeth. In humans and many other primate species, the deciduous set begin to form in utero and erupt soon after birth, while the first permanent teeth start to form around the time of birth and erupt around the time of weaning (Hillson,

2014). In humans, the final stage of dental development is highly variable but tends to correspond with the end of adolescence and early adult stage of life. Consequently, the entire process of human dental development for both sets of teeth begins in utero at around 4 weeks gestation age, and continues until an age of around 18-25 years (Hillson, 2014).

Tooth formation is the result of a series of tissue interactions between ectodermally-derived oral epithelium and neural crest-derived mesenchyme (Jernvall and Thesleff, 2012). Although the formation of the tooth crown is a continuous process, it is commonly divided into five stages; initiation, bud, cap, bell, and the later crown stage associated with amelogenesis and dentinogenesis. The initiation stage involves the formation of two arc-shaped epithelial thickenings, called the dental lamina, in positions corresponding with the future dental arches of the upper and lower jaw. Continued epithelial cell proliferation and swelling then begins along the dental lamina at specific localized regions corresponding to the location of the future teeth (Hillson, 2014). The bud stage is recognised when the epithelial swellings invaginate into the underlying mesenchyme, creating a bud-like structure called the enamel organ. From here, the enamel organ develops an indentation beneath the epithelial ingrowth, signifying the transition into the cap stage (Hillson, 1996). The hollow cap-shaped indentation is filled with ectomesenchyme called the dental papilla, which eventually gives rise to the dentine and pulp of the tooth, and additional mesenchyme outside the hollow forms a bag-like structure, called the dental follicle (or dental sac). Collectively, these structures form the tooth germ (Hillson, 2014). The early bell stage is characterised by the differentiation of the epithelial cells within the dental organ. These are the outer enamel epithelium, inner enamel epithelium, stellate reticulum, and stratum intermedium. The internal and outer enamel epithelium are continuous at the cervical loop, which make the edge of the bell-shaped structure (Hillson, 1996). During this early bell stage, small groups of cells at the apex of the internal enamel epithelium stop dividing. As the cells in between these small groups continue to divide, the epithelium buckles into folds, creating a bell shaped structure (Ten Cate, 1998). For a tooth with multiple cusps, there are several points where these cells stop

dividing. This subsequently causes the internal enamel epithelium to buckle into several folds and take on a shape that loosely corresponds with the shape of the future enamel-dentine junction (EDJ) (Hillson, 2014). Late in the bell and early crown stage, hard tissue formation begins. Beginning at the future cusp tips, the cells of the inner epithelium cease mitotic activity and begin to differentiate into pre-ameloblasts. This induces the outer cells of the dental papilla to increase in size and differentiate into odontoblasts. The odontoblasts then begin dentinogenesis by depositing a collagen-rich predentin matrix directly beneath the epithelial-derived basal lamina. The contact of pre-ameloblasts with this predentin induces their maturation into fully-formed ameloblasts (Thesleff and Aberg, 1997). As the basal lamina begins to disintegrate, direct cytoplasmic finger-like projections from the ameloblasts causes the dentin to mineralize (Katchburian and Burgess, 1977). After an initial layer of 'rodless' aprismatic enamel is formed along the future EDJ, the ameloblasts migrate away from this point and develop Tomes processes on the distal ends of their cell bodies (Kodaka *et al.,* 1989). These pyramid-shaped projections have secretory and nonsecretory regions that provide the architectural structure for organizing enamel into its rod and interrod pattern (Cevc *et al*., 1980; Fejerskov and Thylstrup, 1986). As new odontoblasts differentiate cervically, new ameloblasts respond in similar fashion by reciprocal induction. The cervical limit of the enamel crown is established at the point where the inner and outer enamel epithelia fuse to form Hertwig's epithelial root sheath (HERS) (Simmer *et al.,* 2010). Importantly, the formation of the permanent dentition follows through these same developmental stages.

2.2 Amelogenesis and Mineralization

The life cycle of an ameloblast can be divided into as many as six or seven distinct stages, however it is commonly described within a 3-stage process of cytodifferentiation, matrix secretion, and enamel maturation (Smith, 2004). Cytodifferentiation has already been briefly described above and involves the process of epithelial cell transformation into ameloblasts. During this

transformation, the cell becomes more columnar in shape, the nucleus is re-orientated to the proximal end, and a Tomes' process develops at the secretory end. Additionally, the cell increases the number of organelles needed for protein synthesis and secretion, and junction complexes form between adjacent cells that help the cells to synchronise their secretory activity (Warshawsky, 1978). In the secretory stage, the ameloblasts move away from the EDJ and enamel ribbons elongate by adding successive increments of amorphous calcium phosphate (ACP) directly on the tips of existing mineral ribbons (Beniash *et al*., 2009).

Enamel matrix proteins and proteases are secreted at the mineralization front of the ameloblast and are necessary for the formation of enamel ribbons. These include amelogenin, enamelin, tuftelin, ameloblastin (Robinson *et al.,* 1998), and the secretory protease metalloproteinase-20 (MMP20) (Sierant and Bartlett, 2012). The precise functions of all these proteins remain unclear, however human mutations in genes coding for these products result in either a complete lack of enamel formation, the deposition of only a small layer of aprismatic enamel along the EDJ, or characteristic hypoplastic or hypomineralized enamel (Zeichner-David, 2001; Simmer and Hu, 2002; Stephanopoulos *et al.,* 2005). The ACP ribbons then convert into calcium hydroxyapatite (HAP) a short time after their secretion. Of the various enamel proteins secreted, amelogenin comprises over 90% of the matrix and is thought to influence the conversion of ACP into HAP (Kwak *et al*., 2009). Mineral crystallites forming the rods grow progressively along the *c*-axis, parallel to one another as ameloblasts move away from the dentin surface.

As described, the nature of the Tomes' process establishes a two-compartmental system whereby matrix of the interrod enamel exits near the base of the Tomes' process, and the matrix for the enamel prism exits from the tip of the process. The crystallites of the interprismatic enamel are said to have the same composition as the prismatic enamel, but differ in their arrangement and orientation (Maas and Durmont, 1999). Therefore, TEM-visible boundaries between prisms and interprismatic enamel represent sudden changes in crystallite orientation, not compositional differences (Smith, 2004). In addition

to this, some also recognise a narrow space in between the interprismatic and prismatic enamel, called the prism sheath (Maas and Durmond, 1999; Yoon *et al.,* 2015). The prism sheath is described as a narrow space created where crystallites of prismatic and interprimatic enamel meet at sharp angles, and where protein and water accumulates. This accumulation of protein and water results in a distinct structure of hypomineralized tissue around the prism boundaries. While some recognise the existence of the prism sheath as a distinct feature of enamel ultrastructure (Maas and Durmond, 1999), many authors do not differentiate between prism sheath and interprismatic enamel and consider sheaths to be a product of microscopy techniques (Lynch *et al.,* 2010).

At the end of this secretory stage, once the ameloblasts have completed their principal secretion by achieving full enamel thickness a certain distance from the EDJ, the Tomes' processes are lost and the cell reduces in size (Smith, 2004). This reduction in size also coincides with a degradation of many of the secretory organelles in the ameloblast, and another alteration of form, demonstrating that the ameloblasts have changed in function, and therein marking the beginning of the enamel maturation stage. At the beginning of the maturation stage, the enamel has reached its full thickness but is composed of what Sierant and Bartlett (2012) call "a soft, cheese-like substance" (p.632), due to the high levels of secreted protein. However, in what Nanci and Smith (1992) have described as an unusual cellular event, the next stage of amelogenesis involves the bulk destruction of almost all the proteins previously laid down in the secretory phase. Having been initially cleaved and degraded by proteinases soon after their secretion (Scully *et al.,* 1998), the various protein fragments are drastically degraded further by the secretion of the serine protease kallikrein 4 (KLK4), facilitating the removal of the organic matrix from the extracellular compartment (Simmer and Hu, 2002). These changes stop the growth of the hydroxyapatite crystallites along the c-axis, and accelerate their growth in thickness and width, filling the spaces once occupied by the degraded proteins (Smith, 2004).

The removal of these growth-inhibiting enamel proteins exposes the long sides of the crystals to ion deposition. In addition to the degradation and removal of

the organic matrix, an important process associated with the maturation stage is the movement of ions, with ameloblasts moving calcium, phosphate, and bicarbonate into the matrix, and removing water (Robinson *et al.,* 1998). In particular, bicarbonate influx is essential for neutralizing the hydrogen ion acidity generated by hydroxyapatite formation (Smith, 1998; Bronckers, *et al.,* 2015). The pace of mineralization accelerates and the crystallites increase in thickness until they press against one another (Smith, 1998). These processes are necessary to harden the enamel, and appear to be directed by modulating ameloblasts that undergo cyclic oscillations between ruffle-ended and smooth-ended morphological forms (Robinson *et al.,* 1995). Studies of rat ameloblasts have shown that one complete cyclic oscillation between ruffle and smooth-ended form lasts roughly 8 hours, therefore in rat incisors, cyclic transitions occur about 3 times per day (Smith *et al.,* 1987). Within these 8 hours, approximately 4 hours are spent in the ruffle-ended form, while the other half of the cycle is equally divided between smooth-ended form and transitions between the two (Smith *et al.,* 1987). At present, no data exists for cyclic oscillation timings in human ameloblasts, so it is unknown whether these timings differ dramatically or at all. As expected, ameloblasts function differently depending on what form they are taking. It has been suggested that the more prevalent ruffle-ended ameloblasts are responsible for the movement of the ions into the matrix, while the smooth-ended cells are responsible for the removal of protein and water (Ten Cate, 1998). Other studies have suggested that the ruffle-ended ameloblasts are responsible for almost all maturation processes (Smith *et al.*, 1979; Salama *et al.,* 1989), while ameloblasts in smooth-ended form provide the cell with a resting point to process the degraded matrix and prepare for another ruffle-ended phase (Smith, 1998). By the end of the maturation stage, which for the human permanent dentition takes between 3–6 years, the enamel crystals are long, thin, tightly packed hexagonal or rhomboidal rods of hydroxyapatite, with cross-sectional dimensions of 50nm-25nm and can measure up to 1mm in length. This latter process transforms the previously soft enamel into one of the most durable tissues produced biologically (Smith, 1998).

This section outlines the processes involved in the formation of the highly

orientated structure of enamel. However, this picture of enamel is notably generic and simplistic. Due to the differences in the timing of certain events and transitions, the movement of ameloblasts during the secretory phase, and the structural patterns of enamel matrix deposition, there are several other characteristic features of mineralized enamel that cannot be described in detail here. For example, there is variation in how prisms are packed together, as described in detail by Boyde (1964), variation in crystal orientation in some enamel types (Helmcke, 1967), and notable variation in the thickness of aprismatic enamel between individuals and between species (Whittaker, 1982). As many of these features vary from region to region even within a single tooth, cross-species comparisons are difficult, and functional explanations equally challenging (Jiang *et al.,* 2003). Importantly though, none of these features appear to represent a methodological limitation or influence on the formation or recording of incremental dental structures in prismatic enamel.

One aspect that does deserve brief mention in this regard however is enamel rod decussation. While it was previously stated that prisms extend from the EDJ to the crown surface, they do not run perfectly straight through the enamel. Building on earlier work that described prism paths as spiral-shaped and wavey, Boyde (1989) described the course of a prism through the enamel as sinusoidal or helicoidal. More recent work has suggested that a simple sinusoidal or helicoidal pattern does not explain how the wavelength in a longitudinal plane differed from the wavelength of a prism in a transverse plane (Jiang *et al.,* 2003; Macho *et al.,* 2003), however the helicoidal description seems accurate enough to have persisted in the literature. Ultimately, the difficulty in defining a completely accurate single description of a prism path is that the course of a prism likely varies significantly between different areas of the enamel. Under the cusps of the tooth, prisms take on a spiral pattern, resulting in a type of enamel called 'gnarled enamel' (Smith, 2004). In other areas of the tooth, deviations from a straight path are less dramatic and are described more as "parallel sinusoidal undulations" (Antoine *et al.,* 2009, p.51). Importantly, these structures have various implications for dental histologists studying incremental markings. The first of these relates to the gnarled enamel. Recognised as a tight spiral of prisms

arranged in adjacent groups of opposite orientation, this structural pattern is thought to increase the strength of the enamel in this area (Chatterjee, 2006). Unfortunately however, gnarled enamel often obscures and distorts visual observations of individual prisms, making examinations or counts of incremental features in cuspal enamel either difficult, impossible, or unadvisable. Additionally, this causes problems for studies attempting to determine the rate and duration of enamel secretion from lengths of prisms in cuspal enamel. Early work by Massler and Schour (1946) showed that enamel formation times could be estimated by dividing normal prism length by the characteristic rate of apposition. However, in highly decussated enamel such as gnarled enamel, the lengths of the prisms are significantly longer than the thickness of the cusp due to their spiraling path. To address this problem, Risnes (1986) devised an equation based on a three-dimensional model of spiraling prism path. Many studies now use the 1.15 correction factor produced from this to account for the actual length of the prisms relative to the enamel thickness. As well as the dramatic movement and spiraling of prisms in the gnarled enamel, less pronounced decussation may also have an effect on certain incremental counts. As discussed later, cross-striation counts are commonly utilised by dental histologists as an important tool for reconstructing dental development in both human and non-human primates (Bromage and Dean, 1984; Kelley and Schwartz, 2010; Mahoney, 2011). This process involves counting striations along a prism from a two-dimensional thin section of enamel. However, while prisms appear to be continuous and straight along this two-dimensional section, the phenomenon of decussation means that a visually continuous prism is actually likely to be several prisms that are weaving in and out of the plane of focus (Smith, 2004). While some authors have in the past argued that this may have a notable effect on cross-striation counts (Risnes, 1986; Macho *et al.,* 2003), others have stated that the counting error is unlikely to be significant as long as the cross-striations maintain a regular spacing and the majority of the prisms are parallel to the section plane (Antoine *et al.,* 2009).

2.3 Incremental Structures in enamel

As stated by Scrutton (1978), any organism with preservable hard parts formed by a continually additive mode of growth may provide evidence of incremental growth. As both enamel and dentin form by additive modes of growth, and the tissue is not remodeled by cycles of resorption and deposition, a record of this additive growth is retained in the morphology of the enamel rods. Histological sections of enamel display four types of incremental feature. These are cross-striations, ultradian lines (sometimes referred to as intradian lines), Retzius lines and laminations. While the Retzius lines are of primarily interest in this study, the short-period lines are significantly important for methodological reasons. This section discusses the periodic and structural nature of these four incremental features of enamel, and introduces the recently observed correlation between Retzius periodicity and body mass in primates that is essential to an understanding of this research.

2.3.1 Cross-striations

The first of the incremental structures in enamel to be mentioned are the short-period cross-striations. These regularly spaced light and dark bands, visible under light microscopy, cross perpendicularly along the course of a prism (Hillson, 2014). As overwhelming evidence now suggests that cross-striations have a periodicity of 24-hours, the distance between adjacent cross-striations along a prism represents the amount of enamel deposited by ameloblasts during one full day (FitzGerald, 1998). Measuring the distances between cross-striations can therefore be used to calculate daily rates of enamel deposition, or daily secretion rates (DSR). In humans, average rates are about 4μm/day (Risnes, 1986), however most primates, including humans, show a slight increase in cross-striation distance from the EDJ to the enamel surface (Lacruz and Bromage, 2006). While varying in distance throughout different sections of enamel, cross striations are also more strongly marked and visible in certain regions, making it difficult to follow and count them along the entire length of a

prism (Hillson, 2014).

The majority of early work on the nature of cross-striations comes from Boyde (1964, 1989). Initially he reviewed evidence suggesting that the enamel crystallites centers had notably high levels of carbonate, compared with the surrounding tissue. He then showed that variation in carbonate composition could be seen through differences in the refractive index, which subsequently creates alternating light and dark bands. Believing therefore that the appearance of the cross-striations could be explained by carbonate composition, Boyde theorized that if ameloblasts experience daily rhythms in metabolic activity, then pCO_2 may also vary with the same periodicity and this may translate into differences in carbonate along the prisms. Boyde (1989) also found that high levels of magnesium are found in carbonate-rich regions, and suggested from this that the light and dark bands may correspond with different concentrations of magnesium and carbonate. Due to this larger concentration of carbonate and magnesium, the mean atomic number of the tissue in that region of the enamel prism would decrease, and this would generate a visible dark band under backscattered electron microscopy (Lloyd, 1987). Another theory that has been proposed for why cross-striations exist is based on periodic variations in crystallite orientation. As deviations in the long axis of the crystallites against the long axis of the prisms appear to increase with greater distance from the prism center, the crystals in the outer regions of a thick section of prism will deviate more than crystallites in the outer regions of a thin section of prism (Smith, 2004). Helmcke *et al.* (1967) suggested that this periodic variation in crystal orientation could create the optical effect of cross-striations.

Regardless of their precise structural nature, a large number of varied and diverse studies have demonstrated that cross-striations have a circadian, or 24-hour, periodicity. This is an extremely important point to address as the periodicity of the Retzius line is calculated using the cross-striations, relying entirely on the assumption that cross-striations represent a 24-hour period of enamel secretion. Many early studies tested for the 24-hour rhythm of cross-striations by counting the number of striations between marks induced through the administration of certain dyes or calciotraumatic agents (Hillson, 2014). By

injecting these markers into subjects at known intervals and creating recognisable lines, short-period markings could be counted between these markers with the assumption that cross striation counts would correspond with the actual number of days between injections. Although fewer of these studies have been conducted with enamel, dentine increments have been shown to correlate with enamel increments (Kawasaki *et al.,* 1979), therefore evidence for a circadian rhythmicity in either of these dental tissues may be considered proof of a daily periodicity in the cross-striation. While some studies utilising this type of methodology used lead acetate injections to test for circadian rhythms in dental tissue, others have argued that acetate injections may disrupt growth and so are not suitable for studies of dental or skeletal development (Yen *et al.*, 1971; Appleton, 1991). More recent studies have therefore used fluorochromes, such as tetracyclines, which do not affect development when administered in small doses, but are visible in microscopic observations under ultraviolet illumination (Kawasaki and Fearnhead, 1975). Employing sequential injections of tetracyclines, Yilmaz *et al.,* (1977) counted short period dentine increments between markers in young pigs and found that they corresponded with the number of days between injection intervals. Similar results were also reported by Bromage (1991), who found corresponding incremental lines in both the dentine and enamel of two *Macaca nemestrina*, Dean (1993) with short period dentine lines in a single *Macaca mulatta*, and Smith *et al*. (2007) with corresponding enamel cross-striations in 17 *Macaca nemestrina*.

Another approach used to provide evidence for a 24-hour periodicity in cross-striations is to make total cross-striation counts and compare them with either crown formation times, or known ages-at-death in days. Early examples comparing cross-striation counts with crown formation times can be seen by both Asper and Gysi (1916 and 1931 respectively, but read in Hillson, 2014), who counted cross-striations in human maxillary canines and found that they corresponded well with the number of days required for crown formation. More recent studies have been able to compare total cross-striation counts with known age-at-death among individuals whose tooth crowns were still forming at the time of death. In Antoine's 2000 doctoral thesis, he tested cross-striation

periodicity in the permanent dentition of eight children from the Spitalfields collection in London whose age-at-death was known in days from parish records and coffin plates. Cross-striations were counted from the neonate line (a single prominent line in enamel associated with the physiological trauma of the birthing process), to the last layers of enamel matrix formed. All eight individuals had counts that matched their independently known ages when an allowance was made for the incompletely preserved, partially mineralized, outer layers of enamel matrix. A similar study has also been conducted with five *Macaca nemestrina*, with average errors reported of only 3.5% more than the known age (Smith *et al.*, 2006). An important point to note here is even a small but consistent deviation from a 24-hour rhythm would have made a big cumulative difference to these results. Collectively then, these findings provide strong evidence that cross striations represent a daily, 24-hour incremental marking in enamel.

More recently, studies have reported the expression of circadian clock genes in enamel cells (Zheng *et al.*, 2011; Lacruz et al, 2012; Zheng et al, 2013), as well as anti-phase daily oscillations of circadian clock genes in enamel culture cells (Lacruz et al, 2012; Zheng et al, 2013), providing convincing evidence of circadian-related activity in ameloblasts. As studies have also reported the up-regulation of the essential enamel genes *Amelx* and *Klk4* in ameloblast-derived cell lines following circadian clock gene overexpression, it is possible that clock genes have a direct influence on the normal expression of enamel genes, and subsequently, aspects of enamel formation (Athanassiou-Papaefthymio et al, 2011, Lacruz et al, 2012; Zheng et al, 2013). This not only adds to the already convincing evidence that cross striations represent a 24-hour period of enamel development, but also allows researchers to formulate new hypotheses to further an understanding of what these cross-striations represent. In particular, Lacruz (2016) lays out a particularly attractive hypothesis that cross striations represent differences in mineralization along the enamel prism due to fluctuations in extra-cellular pH during enamel formation. An association between dental increments and pH levels had already been found in an early study by Okada (1943, read and cited in Lacruz, 2016), who reported how the

degree of circadian banding in dentine was influenced by a shift in the acid-base balance of blood plasma. As mentioned above, bicarbonate production and transport is an important aspect of enamel development as it functions to buffer the low pH levels that occur from the release of hydrogen ions during the nucleation of crystals (Smith, 1998). Firstly, Lacruz *et al.* (2012) reported that genes responsible for bicarbonate production and transport increase during the night in mouse molar homogenates. This fluctuation in bicarbonate activity occurs at a time when the mRNA expression of certain enamel protein genes implicated in the mediation of pH levels around neutral values decrease (Lacruz *et al.*, 2012). Among other things, this suggests that the circadian clock may schedule and regulate certain ameloblast activities to certain time periods, perhaps altering their relative intensities on a day to night basis.

Low pH levels in the enamel zone can occur from the release of hydrogen ions during the nucleation of crystals, but also from increases in CO2. As carbonic anhydrase 6 (*Car6*) is involved in CO2 production, it was thought that if a circadian fluctuation in *Car6* expression was found, that this would contribute to lower pH levels in enamel during certain periods of the 24-hour day. Testing this on mouse molar homogenates, Lacruz *et al*. (2012) reported that *Car6* mRNA expression significantly increased during the night. Combined with the other results of an increase in potentially pH-neutralizing amelogenin production during the day, and an increase in bicarbonate activity and transport during the night, a viable hypothesis was proposed that acid-base balance is disrupted in a circadian manner in the enamel zone, affecting mineralization during certain times of the 24-hour cycle and by doing so, creating the cross-striation (Lacruz, 2016). This hypothesis is supported by the findings previously described from Boyde (1979), who reported differences in carbonate composition at sites of cross-striations, and perhaps represents the most convincing and up-to-date argument for the physiological manifestation of the cross-striation.

2.3.2 Intradian Lines

Another type of short-period line that deserves mention are the intradian lines. Sometimes dismissed as artifacts of light microscopy (Boyde, 1989), intradian lines are found between cross-striations and may represent genuine sub-daily structures in enamel (Smith *et al.,* 2003a, 2004). These short-period lines are important for two reasons. Firstly, as will be described below, an early suggestion was put forward that the long-period Retzius lines could be the result of intradian line and cross-striation interactions. Secondly, Dean (1995) has discussed the difficulties of distinguishing intradian lines from cross-striations in isolated enamel, thus they become an important methodological consideration when making incremental counts. Early reports of intradian lines described "a double band effect" with cross-striations in some areas of human enamel (Gustafson and Gustafson, 1967, p.84), and a "doubling of the striations" in the outer regions of some studied *Gorilla* enamel (Shellis and Poole, 1977, p.221). More recent studies have confidently identified intradian lines in human permanent enamel (FitzGerald, 1996; Lacruz and Bromage, 2006), reporting that they may appear at any point between cross-striations and often appear in phase with other intradian lines (FitzGerald, 1996). Faint, closely spaced striations of approximately 2μm or less have also been described in the aprismatic enamel of human (Risnes, 1999), and Old World Monkey enamel (Shellis and Poole, 1977), as well as in the prismatic sub-surface and aprismatic enamel of the Miocene hominoid *A.turkanensis* (Smith *et al.,* 2003a). Smith (2004) argues that the observations of what seem to be intradian lines in these previously mentioned studies, along with numerous scanning electron microscope (SEM) and tandem scanning reflected light microscope (TSRLM) images from other studies that appear to show fine banding between cross-striations (Dean and Scandrett, 1996; Smith *et al.,* 2003a), are evidence enough to assume that "intradian lines are a real structural phenomenon" (p.50). As both SEM and TSRLM studies are not influenced by optical artifacts, this assumption seems likely. While FitzGerald (1996) suggested that intradian lines could be the result of an interference pattern of underlying cross-striations, or from the course of prism rows in a third dimension, he stated that the most likely explanation is that they "indicate a

finer eight hourly, twelve hourly, or light/dark rhythmic beat" (p.179). While not many studies have investigated the exact periodicity of intradian lines in enamel, Smith (2004) did identify a 12-hour periodicity in *Macaca nemstrina* teeth. Studies of dentine however, are seemingly more common and suggest that intradian line periodicity varies between species. In human dentine, a 12-hour periodicity has been reported (Kawasaki *et al.,* 1979), while a periodicity of 8–12 hours has been found in rabbits (Rosenberg and Simmons, 1980), and an 8-hour periodicity in rats (Ohtsuka and Shinoda, 1995).

2.3.3 Laminations

The final short-period lines to be mentioned in this section are the laminations. Found in aprismatic and/or sub-surface enamel, laminations appear parallel to Retzius lines, and have a similar spacing to cross-striations. However, unlike cross-striations, laminations do not usually cross prisms perpendicularly. Notably prominent in the enamel of ungulates (Hoppe *et al.,* 2004; Tafforeau *et al.,* 2007; Kierdorf *et al.,* 2013), laminations have also been observed in human (Kodaka *et al.,* 1989; Risnes, 1998), non-human primate (Smith, 2004), and Miocene hominoid enamel (Smith, 2003a, 2004), however a confident understanding of these aprismatic features has yet to be achieved. In particular, it is still unclear whether, or how, laminations relate to cross-striations (Smith *et al.,* 2003a). While some authors have suggested that laminations are equivalent to cross-striations (Bromage, 1991), other studies have shown that laminations may have a greater periodicity than cross-striations. Studies of both *Afropithecus turkanensis* (Smith, 2003a) and *Graecopithecus freybergi* (Smith *et al.,* 2004) have shown laminations with a greater periodicity than neighboring cross-striations when studied between pairs of Retzius lines. Regardless of their specific periodicity, Smith (2004) makes an important point in cautioning that laminations may complicate studies addressing the periodicity of Retzius lines, "as they often obscure the relationship between cross-striations and Retzius lines" (p.55). Subsequently, it is inadvisable to conduct Retzius periodicity studies in enamel regions that most commonly display laminations, such as

enamel over the dentine horn, along the EDJ in cervical enamel, and in the subsurface enamel.

2.3.4 Retzius lines

When tissue growth is periodic or intermittent, it is likely that certain growth lines will be formed within that tissue. A commonly described example of this are the annual growth rings of a tree. Risnes (1998) describes that, in order to satisfy the criteria of a growth line, such features need to represent growth planes with a three-dimensional extent and be orientated perpendicular to the direction of appositional growth. As Risnes (1998) states, "In dental enamel, we find lines which satisfy these criteria, the so-called Retzius lines" (p.343). First described in detail by the Swedish anatomist Anders Retzius in 1837, Retzius lines, or the striae of Retzius, are prominent, regularly spaced long-period structures in enamel that appear as dark bands in longitudinal sections, and concentric rings in horizontal cross-sections as they run obliquely from the EDJ to the enamel surface. Retzius lines correspond with successive outlines of the enamel forming front at a given time in development, and therefore reveal the layered growth of a tooth (Smith, 2004). The time interval between the formation of consecutive striae is called the Retzius periodicity (RP). This is determined by counting the number of daily cross striations between adjacent Retzius lines.

In lateral enamel, Retzius lines reach the enamel surface as series of shallow furrows, known as perikymata. In cuspal enamel, the striae are dome-shaped and therefore do not reach the surface (Hillson, 2014). While Retzius lines likely exist in all sections of enamel, they are not equally visible and defined throughout the entire crown. In cervical enamel, Weber and Ashrafi (1979) noted that lines were poorly defined when studied under light and electron microscopy. This led Risnes (1998) to suggest that Retzius lines are not homogenous throughout the entire enamel, and may have notable differences in their composition and/or structure, which may contribute to their differential

visibility. In cuspal enamel, Retzius lines do not reach the surface, and visibility issues are at least partially due to the presence of gnarled enamel. However, as Smith (2004) states, "there is no developmental evidence suggesting that a long-period rhythm should not be expressed throughout the formation of the entire crown" (p.59). In addition to the issues relating to decussation, Retzius line identification in cuspal enamel is also problematic due to the common presence of accentuated lines in this region of the tooth. Accentuated lines, or Wilson bands, are considered pathological Retzius lines that represent a growth-related disturbance associated with various types of physiological stress (Wilson and Schroff, 1970; Rose, 1979; Bowman, 1991). Confidently distinguishing between these periodic increments and accentuated lines seems to be an additional complication which, when combined with the fact that cross-striation counts cannot be accurately made between pairs of Retzius lines in cuspal enamel, has led Dean (1987) to suggest that only imbricational Retzius lines should be used for developmental research. Consequently, a definition of Retzius lines used by most dental histologists describes them as "long-period structures representing the position of the developing enamel front, which reach the surface of the tooth and form perikymata" (Smith, 2004, p.59).

Regarding the structural nature of Retzius lines, Boyde (1964) reviewed various early studies that have suggested that, in relation to the adjacent enamel, Retzius lines are either less or more mineralized, contain a different interprismatic substance or pigment, relate to bends or discontinuities along the enamel prism, or are the result of a robust, periodic mineralization rhythm. Early studies described 'step-like' Retzius lines formed by the lining up of transverse cross striations (Gysi, 1931, and Gwinnett, 1966, but read in Smith, 2004). The resulting 'staircase' pattern associated with this configuration is a common descriptive term used when describing the appearance of Retzius lines. For example, Weber *et al*. (1974) reported a staircase pattern and described an adjacent space above the step that appeared to display a different optical density, and an expanded or 'club-shaped' prism end below the step. It has been suggested that these features may result from a reduced concentration of crystallites in these locations, causing the sharp optical contrast observed

(Smith, 2004).

Gustafson and Gustafson (1967) have noted that under polarized light microscopy, Retzius lines commonly appear to be isotropic and positively birefringent, which may indicate that they are areas of hypo-mineralized enamel. However, they also mention that in some instances these lines may show both positive and negative birefringence, which would mean that Retzius lines would be both hypo- and hyper-mineralized in certain region of enamel. Regardless of whether they are actually differentially mineralized, Dean (1989) has suggested that both hypo- and hyper-mineralization may simply be a product of something that occurs after Retzius lines are actually formed. In fact, as early as 1958, Schmidt and Keil (cited and read in Gustafson and Gustafson, 1967) suggested that Retzius lines already exist in the enamel matrix during the secretory stage of amelogenesis, and simply become more evident after mineralization. Therefore it seems that the mineralization of enamel helps to explain the prominent appearance of Retzius lines, at least in some areas of the enamel, but does not explain the actual cause.

Regarding the theory of prism bends or discontinuities, Weber and Ashrafi (1979) described complex prism bending at the location of Retzius lines, which they suggested may give the mistaken illusion that prisms terminate at the point of the Retzius line or have a staircase configuration pattern. They also noted large pores at the points of bending that may be related to prism discontinuity. Building on this theory of prism discontinuity, Risnes (1990, 1998) conducted research that suggested that Retzius lines are characterised by both prism and interprism discontinuity, and are associated with an expansion of interprismatic enamel. Gustafson and Gustafson (1967) had already noted that interprismatic enamel sometimes showed an increase in width at the expense of prism width. Similarly, Boyde (1989) had reported an altering of the ratio of prism and interprism enamel surrounding a Retzius line. Building on this and examining tangential planes of tooth section, Risnes (1990, 1998) showed that the flat or horizontal surface of the step is enlarged at the location of a Retzius line due to a expansion of the interprismatic substance at the expense of the prismatic enamel, creating the club-shaped appearance described by Weber *et al*. (1974).

Risnes (1990, 1998) attributed this increase in interprism enamel to a constriction of the secretory Tomes' process, and an expansion of the adjacent shoulder areas of the ameloblasts. Why this would periodically occur at certain predicable points in time is unknown, however Smith (2004) has stated that this model described by Risnes (1990, 1998) represents the most comprehensive explanation of Retzius line structure proposed to date. Based on a review of the recent literature, this statement still appears to be the case.

As mentioned, the time interval between the formation of consecutive striae is termed the Retzius periodicity (RP). This is determined by counting the number of cross-striations between adjacent Retzius lines. Unlike cross-striations that appear to remain extremely consistent in their periodicity across species, RP in primates ranges from between two and 12 days (Bromage *et al.,* 2009). In humans, RP ranges from between six and 12 days in permanent teeth and therefore represents a near-weekly, or circaseptan rhythm (Reid and Dean, 2006; Reid and Ferrell, 2006). What is interesting is that while RP varies between species, and sometimes between individuals of the same species, RP does not appear to vary within an individual's permanent dentition. Counts of cross-striations between adjacent Retzius lines appear to remain highly constant throughout all permanent teeth from one individual (Reid and Dean, 2006). In a few cases, different counts have been reported between permanent teeth of the same individual or from the same tooth (Huda and Bowman, 1995; Fitzgerald, 1998), however these instances more likely reflect methodological error than actual differences in RP. As the periodicity of the Retzius line does not vary across the tooth row, and studies have also shown the occurrence of incremental lines of a similar periodicity in dentine (Dean, 1995), evidence pointed to a single underlying cause or mechanism that may be affecting multiple physiological systems (Dean and Scandrett, 1996).

While the precise structural nature of the Retzius lines still remains a relative mystery, the currently used and generalized explanation is that it represents a periodic disturbance or slowing of ameloblast activity during the secretory stage of enamel growth (Bromage *et al.,* 2012; Mahoney *et al.,* 2016a). However, no convincing hypothesis has been provided for why any aspect of ameloblast

activity would fluctuate or temporarily drop off with such a variable and extended periodicity. Furthermore, unlike the cross-striation, the periodicity of the Retzius line does not appear to align with any known environmental variable. As stated by Dean and Scandrett (1996), "it is not difficult to make the link between daily incremental markings in growing hard tissues and the 24-hour period of the Earth's rotation" (p. 240). Near-weekly rhythms however, that are variable both within and between some species, are much more difficult to explain. With presumably no data on Retzius periodicity in other mammals, or the now known variation in RP in humans, an early hypothesis suggested that the weekly rhythm in humans might have been be due to fluctuations in feeding behaviour (Gysi, 1931, but cited and read in Smith, 2004). Providing a theory that did not rely on near-weekly physiological or behavioural oscillations, Newman and Poole (1974) suggested that Retzius lines could be the result of one free-running approximately daily rhythm and one precise 24-hour rhythm that generate a Retzius line as they temporarily overlap at regular time intervals. If the periodicity of these two imperfectly synchronised circadian rhythms were 24-hours and 27-hours, for example, then when they overlapped it would create an eight-day periodicity marker. Another similar theory proposed that Retzius lines could be caused by an interaction between daily cross-striations and intradian lines (FitzGerald, 1996). However, as Smith (2004) argues "it is difficult to imagine how these rhythms could interact to produce the known ranges of Retzius line periodicity" (p. 67), when cross-striations are formed every 24-hours and intradian lines appear to be formed every 8-12 hours, depending on the species.

2.4 Interspecific long period rhythms and correlations

Dean (1995) and Dean and Scandrett (1996) appear to be the first to suggest a link between Retzius periodicity and body size across the primate order. Investigating this with data from 18 living and fossil primates, Smith *et al*. (2003b) found a significant positive relationship between average Retzius periodicity and body mass. In 2008, Smith expanded this analysis with a total of

26 living and fossil primates and similarly found a highly significant correlation between average RP and body mass. While the relationship did not hold across all primates, due to the low periodicities and large body sizes of some fossil lemur species (Schwartz *et al.,* 2002, 2005, but see Hogg *et al*. 2015 for a more recent discussion regarding the HHO in lemurs), these studies did provide the first evidence of a relationship between RP and body size across some, if not most, primate species. In 2009, Bromage *et al.* conducted a similar study looking at Retzius periodicity and body mass from 42 living and fossil primates, as well as seven living and extinct members of the *Proboscidea* genus, and also found significant positive correlations, strengthening the evidence for an association between long-period enamel growth rhythms and body mass, and extending this to some non-primate mammals. Furthermore, Bromage *et al*. (2012) reported that Retzius periodicity was also highly correlated with almost all other primate life-history variables (e.g. age at sexual maturity and lifespan) and life-history-related variables (e.g. birthweight and adult brain weight), as well as basal and mass-specific metabolic rates. In fact, of the 11 life history traits regressed against RP by Bromage *et al.* (2012), only estrous cycle length appeared to show no relationship with RP. Based on the relationship between RP and body mass, correlations between RP and other life history traits were not surprising based on the known high degree of co-variation between these variables (Harvey and Clutton- Brock, 1985; Charnov, 1991). Building on this further, Bromage and Janal (2014) used a recently compiled list of tissue and organ masses for twelve species of primate and mammal by Navarrete *et al.* (2011) to show that RP also displayed positive relationships with primate tissue and organ size. For both the correlations with the life history variables and the organ masses, statistical tests were conducted to determine whether body mass was the driving variable in these other statistical findings. Partial correlation tests were conducted, with body mass as the control variable and in all cases, no significant relationships between RP and the other variables endured (Bromage *et al.,* 2012; Bromage and Janal, 2014). This suggested that the other RP correlations observed were likely related to their dependence on body mass.

This observed relationship between RP and body mass prompted further

investigation. In particular, there was cause to investigate whether bone, the other hard tissue in vertebrates theoretically capable of retaining an incremental growth feature, expressed a similar rhythm. There was already some evidence to suggest that bone may retain evidence of incremental, periodic growth. Early studies had already noted a periodic pattern in the formation of lamellae, the basic unit of compact bone. In microscopic sections of bone, lamellae are characterised by highly orientated bands of collagen. This pattern appears from the way the bone-forming cells secrete their organic matrix (Schultz and Schmidt-Schultz, 2015). In these early studies, a 24-hour period was recorded for the formation of one lamella in three different mammals of body weight under 1kg, while a longer rhythm of unknown periodicity was observed in a larger mammal of 11.3kgs (Shinoda and Okada, 1988, and Okada and Mimura, 1940, but cited and read in Bromage *et al.,* 2011). While the significance of these findings would not have been apparent at the time, they now hinted at potential mass-related incremental growth patterns in bone. Investigating this premise on histological sections of bone and tooth from a large sample of mammalian taxa, Bromage *et al.* (2009) recorded the number of lamellae formed in primary bone between vital labels of known administration timing and compared them with the species-specific RP. Incredibly, Bromage *et al.* (2009) found that one lamella was formed in the same number of days as the species-specific RP. This demonstrated that lamellar bone is an incremental tissue with a temporal growth pattern equal to the species-specific long-period growth lines in enamel. This indicated that the periodic timing of lamellar bone growth and the formation of Retzius lines in enamel might both be responses to a similar or identical rhythmic process or fluctuation in the body. Combined with the knowledge that similar incremental lines are produced in dentine (Dean, 1995; Zheng *et al.,* 2014), these findings further suggested the existence of a rhythm not solely related to the life or activity cycle of an ameloblast, the morphology of an individual tooth, or to the collective physiology of the tooth row, but representative of an centrally regulated, systemic biological rhythm.

Importantly, this bone-related finding also suggested that RP was not just correlated with body mass, but may be related to oscillations or fluctuations in

growth that are actually driving or contributing to bone and body mass. While bone size and body mass are clearly separate variables, it is justifiable to assume that a strong relationship or similar suite of mechanisms regulating their growth would exist between them, due to the fact that bone provides the mechanical support for body mass (Schmidt-Neilsen, 1984). This conclusion also agrees with another aspect of the Bromage *et al.* (2009) study. Comparing osteocyte densities from the midshaft femur of several primate and non-primate taxa, Bromage *et al.* (2009) examined the relationship between rates of bone growth and body mass. If rates of bone growth were slower in species of larger body mass, this may help to support and explain the observation of slower long-period growth rhythms in larger mammals. Osteocyte lacuna densities were recorded as they are thought to reflect the rate of bone cell proliferation during growth. The pace at which bone is formed is significantly influenced by the number of osteoblasts that are present to lay down the bone's organic matrix. Because of this, rates of osteoblast proliferation determine the speed at which many of these cells become incorporated into lacunae within the bone matrix. Therefore, osteocyte densities are a suitable proxy for rates of cell proliferation in lamella, and ultimately the pace of bone growth (Mullender, 1996a). Bromage *et al.,* (2009) predicted that osteocyte densities should be higher in organisms with rapid growth and small body mass, whereas low osteocyte densities would be associated with slow growing, larger body sizes. Such relationships and conclusions had previously been reported for osteocyte densities in the cancellous bone tissue of five mammal species, with the authors concluding that osteocyte densities were likely related to rates of cell proliferation and metabolism (Mullender *et al.,* 1996a).

As anticipated, Bromage *et al.* (2009) reported that osteocyte densities were negatively correlated with body mass in their primate and non-primate mammalian sample. This relationship suggests that a comparatively larger number of cells are required to form a single micro-unit of bone in small mammals with shorter developmental timings, and that fewer cells, and therefore fewer mitoses, are required in the growth of a large mammal with a protracted developmental period. This is consistent with differences in life

history strategy, where smaller mammals have faster life histories and fast skeletal development, and therefore require faster osteoblast proliferation rates. In contrast, at the other end of the life history spectrum, large mammals that adopt a slow life history with a protracted period of skeletal development are characterised by slower cell proliferation rates. Importantly, as this slower growth rate occurs over a significantly longer developmental period of time, it is thought that the extended life history of the large mammal is great enough to eventually overcome the slower pace of growth, resulting in a larger individual (Bromage *et al.,* 2012).

In addition to these conclusions and hypotheses, Bromage *et al.* (2012) have also suggested that the direction of these interspecific cell proliferation rate and RP correlations make sense with how metabolic rates scale with body mass, and therefore help to further understand why cell proliferation and growth rates would be slower in larger mammals. Across vertebrates, whole-body basal metabolic rate (BMR) scales positively with body mass, while mass-specific metabolic rates (SMR), which represent a measure the respiration of tissue, scale negatively with body mass (Speakman, 2005). As positive correlations have been observed between SMR and osteocyte density (Bromage *et al*., 2012), and studies that have shown that reduced rates of cell division are associated with lower cellular metabolic rates (Savage *et al.,* 2007), it may be that slower cell proliferation rates and lower SMR in larger mammals represents an energy efficient strategy to mediate an otherwise larger cumulative energy expenditure of tissue over a longer period of time (Bromage *et al.,* 2012). As Speakman (2005) describes, "a gram of tissue on average expends about the same amount of energy before it dies regardless of whether that tissue is located in a shrew, a cow, an elephant or a whale" (p.1717). Consequently, horse hepatocytes consume almost 10-fold *less* oxygen per unit of time as a liver cell from a mouse (Porter and Brand, 1995). Therefore if total lifetime energy expenditure of a cell remains relatively constant across large and small body types with extended and protracted life histories, then SMR needs to be adjusted to account for this variability in lifespan. By doing so and decreasing SMR with increased body mass, cellular metabolism becomes more energy efficient (Schmidt-Nielsen,

1984). As Bromage *et al.* (2012) points out, small body size and low BMR is packaged with fast growth and high SMR "because only this combination is energetically and ecologically supportable, and then, only over a short life history" (p.138). These conclusions suggest that large mammals may have developed longer life histories and larger size by reducing mass-specific metabolic rates and cell proliferation, becoming more energy efficient. In contrast, fast growing species show high rates of cell proliferation and growth, which carry higher metabolic costs and cannot be sustained for as long. Subsequently, this may help explain why slower long-period rhythms are observed in larger mammals.

More recently, and in an attempt to characterise and actually identify this long-period rhythm, Bromage *et al*. (2016) evaluated the metabolic profiles of 33 juvenile domestic swine from daily plasma samples drawn over a two-week period. These samples were subjected to gas and liquid chromatography, coupled with mass spectrometry, to analyze the periodicities of the pig's circulating metabolites. Blood plasma was also taken at 2-hour intervals during three days of the experiment to capture the 24-hour rhythmicity of metabolites. This latter process was conducted to demonstrate the effectiveness of the methodology for capturing biological rhythms. As expected, various amino acid and fatty acid peaks were observed during these daily tests, demonstrating the effectiveness of the technique. In an attempt to identify the longer-period rhythm, metabolites were studied that oscillated with a five day periodicity, which matched the species-specific RP. Bromage *et al.* (2016) reported that 49% of the 228 metabolites studied oscillated on a 5-day period. Interestingly, the biological functions of the metabolites identified related to rates of cellular proliferation, cell apoptosis, protein synthesis, and the concentration of calcium. The overarching biological roles of these metabolites collectively relate then to the metabolic requirements of growth and the regulation and pace at which many aspects of body mass increase. Currently, it seems unlikely that these findings would be a coincidence, however similar results need to be found for other mammals with a different species-specific RP. Nevertheless, this study represents the first attempt to identify and characterise the HHO, and reports

findings that seem to corroborate with previous hypotheses regarding its nature and function.

Based on these combined findings, and described further in later sections, Bromage *et al*. (2009, 2012, 2016) have suggested that the long-period rhythm in question may be of hypothalamic origin and that, through a set of SCN-integrated nuclei, signals are transmitted to the anterior pituitary, which regulates and controls various aspects of growth, development and metabolism. Bromage *et al*. (2009) termed this hypothetical, systemic growth rhythm the Havers-Halberg Oscillation (HHO), after Clopton Havers, a 17th Century hard tissue anatomist, and Franz Halberg, a prominent researcher in chronobiology. From here onwards, this thesis will also address this hypothetical rhythm as the Havers-Halberg Oscillation (HHO).

2.5 Intraspecific long period rhythms and correlations

While positively correlated with body mass and implicated in the pace of skeletal development and metabolism across species, comparatively less is known about these RP relationships within humans. Compared to the other primate species, humans display an unusually wide range of periodicity values (Reid and Dean, 2006; Reid and Ferrell, 2006). To date, only preliminary work has been done to investigate whether RP shows a relationship with body size. Bromage *et al.* (2009) conducted preliminary research looking at RP and body height and weight among six and four human subjects respectively and reported a negative correlation between the variables. Unfortunately, the sample size in this study was too small to be able to draw meaningful conclusions. Furthermore, when Bromage *et al*. (2009) attempted to investigate whether the timing of lamella formation matched RP as it did for other species, the administration timings of an assumed 7-10 day treatment course of tetracycline antibiotic used to investigate this were not precisely known. Therefore while the authors reported that the formation of one lamella was consistent with the range of human Retzius periodicity, it is not currently known whether the individual periodicity of

lamella formation accurately mirrors the individual and highly variable periodicity of Retzius lines in humans. Just as RP and body size appears to have become decoupled among domestic dogs, which have significantly different body masses yet display relatively similar RPs across species (Hogg *et al.,* 2016), so too could RP be decoupled from body size in humans. Consequently, RP could have no relationship with bone growth or body size in humans. Currently then, research on the relationship between Retzius periodicity and body mass is lacking. This research therefore aims to investigate the relationship between RP and body size. As mentioned above, while body size is an abstract concept not a concrete parameter (Hemmer, 2015), the term is used here to refer to both height and body mass variables.

2.6 Biological rhythms

As Retzius periodicity represents an unknown biological rhythm, the first part of this second section of the literature review briefly describes different biological rhythms in nature and whether rhythms of similar periodicity, function or entrainment are applicable to humans. Importantly, as the precise nature and etiology of Retzius lines are unknown, this section remains notably broad and inclusive of many different biological rhythms. For example, while biorhythms with periodicities of less than 24 hours may appear irrelevant to the discussion of long-period Retzius lines, these rhythms become important when considering the potential interplay, overlap and synchronisation between multiple rhythms. The latter part of this section then describes the basic features, mechanisms and cues that maintain and entrain biological rhythms. In most cases, these descriptions are exclusively related to circadian systems and the mammalian control center for circadian rhythms; the suprachiasmatic nucleus (SCN). While the SCN and the mechanisms responsible for maintaining circadian rhythms are unlikely to be the sole contributors and sustainers of the HHO in RP>1 species, these mechanisms are discussed here for numerous reasons. Firstly, circadian rhythms are the most extensively studied and understood of the biological rhythms. Significantly less is known about the precise mechanisms or processes

involved in non-circadian systems. Therefore, at this time, circadian rhythms provide the only solid frame of reference for attempting to understand non-circadian patterns. Secondly, as will be described later, the SCN appears to influence rhythmic expression in non-SCN hypothalamic nuclei. Thirdly, while the HHO does not represent a circadian rhythm in RP>1 species, the expression of RP in units of whole days suggests the circadian system has an important role in the process. Finally, circadian rhythms play an important role in certain earlier theories of Retzius formation, and therefore deserve adequate discussion. This review ends with a focus on the complexity of biological rhythms in nature and how unusual rhythmic expression may occur through the interplay between rhythms.

2.6.1 General description

Biological rhythms, broadly defined as the cyclic change or fluctuation in the level of a bodily chemical or function, are common features of almost all living things, having been identified in plants, animals, fungi and even single celled organisms (Hastings, 1997). Biorhythms exert significant regulatory control over various aspects of an organism's physiology, development and behaviour. As biorhythms can be observed in so many different processes or functions, the periodicity of a rhythm can vary from fractions of seconds to decades (Schibler and Naef, 2005). Arbitrarily, these biological rhythms can be divided into ultradian, circadian and infradian time categories. While circadian rhythms represent cycles that occur approximately once every 24 hours, ultradian and infradian rhythms generally represent cycle times under 20 and over 30 hours respectively. Due to the broadness of these categories, ultradian rhythms are associated with a wide variety of different physiological and behavioural processes including the electrical firing of neurons, heartbeats, sleep episodes, yeast respiration, and certain mammalian foraging behaviours (Refinetti, 2005). At the other end of the spectrum, infradian rhythms are associated with processes such as the menstrual/estrus cycle, many mammalian mating patterns and behaviours, and the migratory and hibernation behaviours of various

species (Schibler and Naef, 2005). Retzius periodicity falls into this latter category. While the periodicity of some rhythms are directly related to the size and morphology of the organism (for example, heart rate and breathing rate are inversely proportioned to body size) (Refinetti, 2005), others rhythms, especially in the circadian and infradian categories, are actually anticipated reactions or direct responses to the geophysical cycles on this planet. These daily (24 hours), tidal (12.4 hours), lunar (29.5 days), and annual (365.24 days) cycles, and the environmental cues they produce, function as important temporal signals for the organization and maintenance of many biological processes for many species (Tessmar-Raible *et al.,* 2011).

Interestingly, while some of the biological rhythms associated with the geophysical cycles are purely direct responses to external cues, in that when the stimulus is removed, the corresponding response in the physiology or behaviour of the organism no longer occurs, others actually persist when the stimulus is removed (Johnsson, 2008). The persistence of a biorhythm after the external influence is removed is an important criterion in the identification of a *biological clock,* and indicates an endogenous component to the observed rhythmic process. Critical evidence for a biological clock is that the rhythm must *free-run,* meaning that after being *entrained,* or synchronised, by an environmental cue (known as a *zeitgeber*), the rhythm must persist with a relatively similar periodicity to its original cycle (Johnsson, 2008). Commonly referenced examples of Zeitgebers include environmental temperature cycles, changes in photoperiod, shifts in the tide, and lunar phases. It is assumed that biological clocks evolved to increase the fitness of the organism by enabling it to anticipate environmental change, rather than just react to it, and optimise their physiology and behaviour (Johnsson, 2008). As will be described, in some cases various external stimuli may work in combination with one another to produce, suppress, or entrain an organism's response. In other cases, the interaction between endogenous rhythms and external stimuli, or between two endogenous rhythms in the same organism, may produce or suppress a particular reaction or response. The fact that biorhythms often free-run with slightly deviated periodicities in constant conditions suggests that the accurate maintenance of a

naturally observed rhythm often involves the interplay between both internal and external factors (Smith, 2004). As almost all organs, tissues and cells seem to display some type of rhythm, one of the difficulties in the study of biological rhythms is determining the specific nature and etiology of an observed rhythmic physiological or behavioural process. The complexity of biological rhythms, especially in multicellular organism that display an enormous number of quasi-autonomous oscillatory systems, renders their study difficult and explains why current knowledge is somewhat limited to circadian rhythms and clocks.

2.6.2 Circadian rhythms

The 24-hour rotation of the Earth causes predictable changes in environmental light and ambient temperature. Consequently, many physiological, developmental and behavioural processes also occur with predictably similar periodicities (Johnsson, 2008). While certain processes or functions simply reflect a direct passive response to a solar-related environmental change, many organisms have been shown to display circadian clocks that actually anticipate these environmental signals and allow the organism to predict and synchronise aspects of their physiology and behaviour. While much less is known about what organisms display ultradian and infradian rhythms, circadian rhythms are comparatively well understood and have been found in almost all light-sensitive organisms (Bass, 2012). In humans, as well as many other mammals, 24-hour rhythms are seen in body temperature, sleep-wake cycles, blood pressure, metabolism, retinal electroretinogram responses, endocrine secretion, as well as numerous other physiological and behavioural processes (Hastings *et al.,* 2003). Furthermore, many of these are tightly regulated by circadian clocks. For example, humans experimentally subjected to constant light conditions continue to exhibit sleep/wake cycles with a free-running periodicity of approximately 25-hours (Czeisler *et al.,* 1999). As described in section 2.3.1, there is strong evidence to suggest that the activity of ameloblasts are under circadian clock regulation. In fact, even before the discovery of clock genes in the ameloblast, observations of cross-striations in enamel already showed many of the properties Roenneberg *et al.* (2008) suggest are required for evidence of a

circadian clock. Cross-striations are rhythmic in nature, have an approximately 24-hour periodicity, have a rhythm with amplitude strong enough to drive outputs, and have a rhythm that does not seem to dampen under constant conditions. While substantial evidence suggests that circadian rhythms are present in ameloblast activity, osteoblasts have also been shown to express clock genes (Fu *et al.,* 2005). Interestingly, studies have shown that mice with mutations in circadian clock genes show increased numbers of osteoblasts and subsequently develop high bone mass (Fu *et al.,* 2006).

2.6.3 Ultradian rhythms

The designation *ultradian* is generally reserved for biological rhythms with periodicities of less than 20 hours (although this varies slightly between authors). As mentioned above, while these rhythms may seem irrelevant to a discussion of the nature and etiology of Retzius lines and the HHO, ultradian rhythms become important when one considers the potential interplay and overlap between multiple rhythms. Of the seemingly unsynchronised, purely physiological rhythms, heart rate and breathing rate are the most commonly cited. As mentioned, across species these are generally inversely proportioned to body size. The periodicity of heart rate is driven by a pacemaker in the sinoatrial node of the heart and is regulated by the sympathetic and parasympathetic nervous systems (Refinetti, 2005). For breathing rate, while the concerted contraction of various muscles in the chest and abdomen cause the actual flow of air through the lungs, the rhythm seems to be generated by various respiratory bulbospinal premotor neurons in the lower brainstem (Refinetti, 2005). While circadian clock rhythms appear to exist in endocrine function (Hastings *et al.,* 2007), the system also exhibits ultradian rhythms. For example. luteinizing hormone and follicle-stimulating hormone, as well as cortisol and insulin, are secreted rhythmically approximately every hour in humans. In the case of lutenizung hormone, periodic secretion is initiated by the hypothalamus, which stimulates the anterior pituitary gland to secrete lutenizing hormone, thus stimulating the gonads to secrete estogens, progestins and androgens (Refinetti,

2005). Ultradian rhythms have also been noted in contractions of the smooth muscles of the gastrointestinal tract, with 3-10 cycles per minute (Moore, 1992), and bursts in human locomotor activity in experimental studies reporting 0.5-2 hour periodicities that appear to remain relatively stable within an individual (Grau *et al.,* 1995). Unfortunately, due to the significant variability of many of these rhythms between individuals, interpretations and meaningful conclusions are often difficult (Aschoff, 1981). Additionally, unlike Retzius periodicity, which appears to remain consistent throughout the entire period of permanent tooth crown formation, many of these rhythms are extremely sensitive to both external and internal factors, sometimes varying in periodicity from month-to-month or moment-to-moment (Glass, 2001).

Also within the ultradian category are tidal rhythms. Unlike the ultradian rhythms previously mentioned, these often represent anticipated reactions to predictable environmental changes produced by the tides. The periodicity of rhythmic processes associated with the tides therefore remains relatively stable both within and across species. Furthermore, while some of these physiological, behavioural or developmental responses are simply direct reactions to environmental change, some marine species, especially in intertidal zones, have been shown to display endogenous circatidal clocks that free-run in constant conditions (Tessmar-Raible *et al.,* 2011). However, while circatidal clocks have been identified in various marine crustaceans, there is no evidence and no reason to expect that the tides have any effect on human physiology.

2.6.4 Infradian rhythms

In contrast to ultradian rhythms, infradian rhythms have periods longer than that of a circadian cycle. One of the most prominent is the estrous/menstrual cycle. While the menstrual cycle is approximately 28 days in female humans, the duration varies significantly between species and bears no clear relationship to environmental cycles or body sizes (Refinetti, 2005). Furthermore, it also varies in periodicity within species and within individuals on a month-by-month basis.

Nevertheless, the estrous/menstrual cycle is associated with numerous hormonal and behavioural shifts and fluctuations, and sometimes free-runs with longer and shorter durations in constant conditions, in some cases eventually leading to a condition of persistent estrus in some species (Refinetti, 2005).

An infradian environmental cue that certain species appear to synchronise aspects of their physiology or behaviour is the 29.5-day lunar cycle (this can be further broken down into 14.8-day circasemilunar cycles). The lunar cycle affects numerous organisms through variation in nighttime luminosity and changes in the tide (Tessmar-Raible *et al*., 2011). For example, variation in nighttime luminosity creates unusual behavioural activity patterns in the Argentinean owl monkey (Fernandez-Duque and Erkert, 2006), and the spawning of mummichog fish (Taylor *et al.,* 1979). In some cases, experimental studies have implicated potential endogenous circalunar clocks in these behavioural and reproductive processes (Tessmar-Raible *et al.,* 2011). However, while the phases of the moon appear to have a slight effect on the activity patterns of certain nocturnal mammals (Bachleitner *et al.,* 2007), and lunar phases clearly have some influence on human culture (Foster and Roenneberg, 2008), there is little convincing evidence that the moon has any effect on human physiology or that humans display lunar-influenced endogenous rhythms. Despite some mental health professions showing a belief that the full moon can alter behaviour (Vance, 1995), and a belief among midwives that more babies are born during full moon than new moon, statistics prove that these are simply subjective associations (Arliss *et al.*, 2005). Additionally, while studies have reported a circalunar periodicity of epileptic seizures in women (Quigg *et al.,* 2008), this finding is perhaps better explained by the potentially disturbing effect of nighttime light on sleep or through changes in the menstrual cycle. Building on an earlier study reporting that humans slept an average of 19mins less on full moon nights (Röösli *et al.,* 2006), one study reported evidence that moon phases had significant influences on both objective and subjective sleep measures when conducted under strict controlled laboratory conditions to exclude any biases in lunar perception or light influence (Cajochen *et al*., 2013). Since the effects of light were controlled, and the moon does not have any explicit gravitational

effect on the human body, the authors concluded that the observed result likely represents an endogenous circalunar rhythm. However, while they describe that circalunar clocks "tick inside many animals" (p.1486), presumably to strengthen an argument that they are such a common feature of life that they could exist in humans, all examples they provide are marine species and no mention is made as to how or why lunar rhythms might exist in humans. Additionally, while it is unlikely that lunar cycles have any notable impact on diurnal, terrestrial species, it is even more difficult to reconcile how the lunar cycle would have any influence on RP or any other aspect of hard tissue growth. While Bromage *et al*. (2009) report a long-period rhythm in *Paranthropus robustus* lamellar bone "resembling the lunar cycle" (p.400), it is assumed that the authors acknowledge nothing more than a coincidental relationship in this finding. Seasonal and annual rhythms are also examples of infradian rhythms, however their timescales are far beyond anything that could correspond with RP, and are therefore not discussed here.

2.6.5 Circaseptan rhythms

Also within the infradian category are the circaseptan (or 7-day) rhythms. As RP roughly corresponds with a circaseptan rhythm in humans, this infradian sub-category is discussed separately. Early research reported various weekly rhythms from laboratory experiments, such as fluctuations in the locomotor activity of a centipede (Mead, 1970, but cited and read in Aschoff, 1981), the larviposition of a mosquito (Nash and Trewern, 1972) and in the enzyme activity of the rat pineal (Vollrath *et al*., 1975). Unfortunately, many of these are now considered to be due to periodic disturbances in laboratory conditions and not actually endogenous rhythms (Aschoff, 1981). In humans, Pöllmann (1984) has suggested that wounds heal with a circaseptan periodicity, and that kidney transplants often display circaseptan peaks of rejection. Research has also observed elevated levels of testosterone secretion in males on Sundays, and reports of higher blood pressure and higher frequencies of heart attacks on Mondays (Refinetti, 2005). However, these latter examples are unlikely to be

endogenous rhythms, and instead appear to be the result of behavioural habits, such as variation in food intake throughout the week (Debry *et al.,* 1975), increased mobility, sexual activity, or stress on certain days of the week (Refinetti, 2005), or based on treatment schedules of patients (Undt, 1976, but cited and read in Aschoff, 1981). For example, a study investigating weekly rhythms in horses found no rhythms in lactic acid, blood pressure, or rectal temperature in the sedentary group, but a weak 7-day rhythm in lactic acid concentration for those imposed on a weekly schedule of fitness training (Piccione *et al.,* 2004). Subsequently, although numerous studies have reported circaseptan periodicities in human physiology, some of these findings may need to be cautiously interpreted. For example, although Rawson *et al.* (2000) claimed to identify a weak circaseptan rhythm in heart rate and blood pressure, these findings were based on a single female and the study failed to address or control for any potential social or behavioural influences. Furthermore, while Lee *et al.* (2003) reported a 7-day rhythm in human heart rate and blood pressure from a sample of five individuals, one author has commented that the actual data presented is "unconvincing at best" (Refinetti, 2005, p.132). Importantly, both Bromage *et al*. (2009) and Appenzeller *et al*. (2005) have suggested that reported weekly heart rate and blood pressure oscillations may directly relate to Retzius periodicity, in that they are both physiological manifestations of the same centrally regulated biological rhythm. However to date, substantial and convincing evidence for a circaseptan periodicity in heart rate and blood pressure appears to be limited. Providing perhaps more compelling data, Cornélissen *et al*. (2000) identified circaseptan rhythms in the blood pressure of an 11 pair sample of newborn twins. Equally interesting, the researchers reported smaller rhythmic variability between each twin set than there was across unrelated infants, indicating a potential genetic influence on the exact periodicity. Ultimately, while approximately 7-day rhythms appear to exist in heart rate and blood pressure, and the dental enamel of some individuals, the periodicity is completely arbitrarily linked with the socially constructed week, which has no environmental counterpart. Nevertheless, the current data available is suggestive of an approximately 7-day rhythm in some human physiological processes. Further investigation into physiological rhythms of this

periodicity may provide interesting results. Equally important however, for the study of RP and the HHO, will be the identification of notable variability in these rhythms within humans.

Unlike many of the purely physiological rhythms that vary in periodicity within an individual on a month-by-month or even moment-to-moment basis, Retzius periodicity appears to remain stable during the entire period of permanent crown formation. Seemingly resilient to physiological or environmental perpetration, RP appears in some ways to resemble some of the robust characteristics of an endogenous biological clock. However, unlike a biological clock, the periodicity of this long-period rhythm does not seem to align with any known external signal or cue and also varies significantly between individuals. Currently then, RP appears to represent a comparatively novel, if not slightly bewildering, physiological biorhythm.

2.7 Clock mechanisms

At the molecular level, one of the key mechanisms that keeps the circadian cellular clock ticking after entrainment is a transcriptional positive-negative feedback loop that drives rhythmic expression of core clock components (Allada, 2003). These clock components are genes responsible for the generation and regulation of circadian rhythms. The primary feedback system is controlled by the transcriptional activators BMAL1 and CLOCK, which heterodimerize and initiate transcriptions of their target genes *Per* (period) and *Cry* (cryptochrome). Negative feedback is achieved through the resulting activation of the repressors PER(1-3) and CRY(1-2). PER and CRY are made in the cytoplasm and upon reaching certain levels, they translocate back to the nucleus to repress their own transcription by binding to CLOCK and BMAL1 (Dibner *et al*. 2010). Degradation of the PER and CRY proteins terminates the repression phase and results in a new cycle of transcription. Post-translational factors such as phosphorylation and ubiquitination add complexity to the system but the cycle takes approximately 24 hours to complete and thus constitutes a circadian molecular

clock (Dibner *et al*. 2010).

It should be noted that while this transcription and translation mechanism is commonly attributed to the majority of circadian systems, it appears not to be the only mechanism used to maintain circadian rhythms. For example, human red blood cells lack the nuclei required for such processes and yet peroxiredoxins are oxidized with a circadian rhythm and are entrainable to temperature cycles (O'Neill and Reddy, 2011). In fact, researchers have concluded that rhythmic oscillations in cellular redox state seem to represent another mechanism for the regulation and maintenance of a circadian rhythm (Tomita *et al*. 2005). From this, authors have conceded that there may be even more non-transcriptional circadian mechanisms that remain to be discovered and, importantly, that potential communication between them may "reveal a whole new level of regulation of circadian functions within a single cell" (Buhr and Takahashi, 2013, p.5).

Currently, it is unknown whether the HHO would function through an oscillatory system, however it certainly seems to be the more likely scenario when compared with hourglass mechanisms that explain single life events such as menopause (Schibler, 2005). Certainly, all forms of circadian clock appear to be based on oscillator mechanisms, and evidence is growing that similar oscillatory mechanisms are also suspected to lie at the basis of certain ultradian and infradian timing systems. For example, Toda *et al.* (2014) has implicated circadian clock genes in the circalunar clock functioning of the Goldlined spinefoot fish (*Siganus guttatus*), and from this suggested that similar mechanisms may exist between rhythms of different periodicity. Similarly, Causton *et al.* (2015) identified rhythmic oscillations in cellular redox state that appear to be responsible for the 1-5 hour respiratory rhythms in budding yeast (*Saccharomyces cerevisae*). Causton *et al*. (2015) suggests that their data also points to common mechanisms underlying ultradian and circadian rhythms. Finally, O'Neill *et al*. (2015) have shown that oscillations in cellular redox state may also follow a circatidal pattern in the speckled sea louse (*Eurydice pulchra)* . Thus, certain key oscillator mechanisms appear to be common not only for circadian rhythms, but perhaps also for ultradian and infradian rhythms. These

mechanisms, and their presence and role in non-circadian systems, point to a hypothetical ways in which the HHO may maintain such a robust, predicable rhythm.

2.8 The SCN and peripheral clocks

To adapt or synchronise an organism's physiology or behaviour to changing environmental parameters, information from the outside world has to reach physiological structures that can interpret and integrate this information, and be able to send the appropriate signal responses to the various tissues and organs. The suprachiasmatic nucleus (SCN) has the ability to act as a physiological relay between these external and internal worlds (Dibner *et al*., 2010). This is particularly true for biological rhythms and responses of a circadian nature (Yamazaki *et al.,* 2000). Groundbreaking studies in the field of chronobiology showed that transplantation of fetal SCN tissue into SCN-lesioned hamsters re-established circadian locomotor activity (Drucker-Colín *et al*., 1984), while mouse SCN tissue transplantation was similarly shown to restore circadian rhythmicity in genetically arrhythmic individuals (Sujino *et al*., 2003).

While the SCN appears to be influenced via numerous major input pathways, including signals sent through the geniculohypothalamic tract (GHT), and from serotonergic (5HT) input, the most significant appears to be signals transmitted through the retinohypothalamic tract (RHT) (Dibner *et al.,* 2010). Mammals primarily perceive light through the retina of the eye, where photosensitive retinal ganglion cells respond by sending photonic information through the RNT to the SCN. The release of neurotransmitter molecules implicated in this photonic signaling causes the activation of several signaling pathways and the subsequent phosphorylation of BMAL1 and CLOCK that bind to their respective clock genes, activating their transcription and consequently inducing a phase-shift (Hirota and Fukada, 2004).

To function as a master pacemaker and synchroniser for other regions of the

body and brain, this timekeeping signal from the SCN needs to be transmitted. To synchronise and communicate with other regions of the hypothalamus, these timekeeping signals are transmitted through SCN efferents to various other regions of the hypothalamus (Hermes *et al*., 1996; Castel and Morris, 2000). These SCN efferents terminate most densely in the subparaventricular zone, the preoptic area, the lateral septum, the dorsomedial hypothalamus, and the arcuate nucleus (Morin *et al.,* 1994; Morin, 2007), suggesting that communication between these regions may be particularly important for several physiological, rhythmical processes. Interestingly however, these SCN efferents are not always required for the establishment of some rhythms. As previously mentioned, SCN tissue transplanted into certain brain regions can reestablish rhythmic activity. This suggests that currently unknown paracrine factors released from SCN tissue can synchronise and maintain the expression of certain rhythms (Dibner *et al.,* 2010). Transplanted SCN tissue does not however reestablish rhythms in the neuroendocrine axis, suggesting that SCN efferents are important in maintaining hormonal rhythms (LeSauter and Silver, 1998). Subsequently, if the SCN and other hypothalamic nuclei are involved in the regulation of the HHO, it is likely that SCN efferents are important in these specific communications.

Beyond the brain, the discovery of the clock genes mentioned in section 2.7 have facilitated the identification of circadian clocks in most, if not all, peripheral organs and tissues, including the heart, the pancreas, the liver, adipose tissue, and bone and cartilage tissue (Balsalobre *et al.,* 1998; Yamazaki *et al.,* 2000; Green *et al*., 2008; Bass & Takahashi, 2010). In individual cells, the most studied evidence of peripheral clocks comes from the self-sustaining circadian clocks in individual fibroblasts. In cultured fibroblasts, circadian gene expression has been observed, and actually continues during and after cell division (Nagoshi *et al.,* 2004). In cartilage, clock genes have been implicated in circadian rhythmic activity associated with chondrogenic differentiation and longitudinal bone growth (Takarada *et al.,* 2012; Okubo *et al*., 2013). Similarly, circadian clock activity appears to be present in bone. For example, studies have shown that circadian gene KO mice display significant increases in osteoblasts numbers in

bone, which result in increased bone formation (Fu *et al.,* 2005; Maronde *et al*., 2010). Importantly however, while circadian rhythms are implicated in bone growth, these processes appear to be common to all mammals. Thus it seems difficult to attribute these daily oscillations to the enormous variation in body mass observed between species or individuals.

While it was initially thought that only oscillators in the SCN were truly self-sustained, and that peripheral clocks would fade significantly after a few cycles (Dibner *et al.,* 2010), research has shown that circadian rhythms in cultured lung and liver cells can produce robust cycles of *Per2* luciferase expression for more than 20 days (Yoo *et al.,* 2004). However, while peripheral clocks may continue to display rhythmic cycles, the phases of these oscillations often lose their synchronicity without the SCN. For example, while peripheral clocks continue to oscillate in SCN-lesioned mice, their phases are no longer synchronised with each other (Yoo *et al.,* 2004). In fact, one study has shown that a functional SCN is required to maintain phase synchronicity between hepatocytes of the same liver (Guo *et al.,* 2006). Losing this phase synchronicity between peripheral organs and tissue can sometimes lead to the appearance of multiple rhythms in the physiology or behavior of the organism (Schwartz *et al.,* 2009). As stated by Dibner *et al*. (2010) the SCN is probably best then viewed as "a conductor of an orchestra of clocks" (p.520), rather than a structure imposing a rhythm on otherwise arrhythmic cells. The SCN transmits signals to help synchronise the peripheral clocks to a uniform internal time.

To synchronise peripheral clocks, the SCN uses various direct and indirect routes to establish phase coherence. For example, feeding-fasting cycles, influenced by rest-activity cycles controlled by the SCN, are strong Zeitgebers for several peripheral organs, including the liver, kidney and the heart (Damiola *et al.,* 2000; Yamazaki *et al.,* 2000). Body temperature cycles also appear to be important entrainment cues for various organisms, either directly through the SCN or through SCN-controlled activity cycles (Brown *et al.,* 2002). In addition to these indirect pathways, the SCN also uses more direct timing cues, such as humoral and neuronal outputs, to synchronise the phases of peripheral clocks (Vujovic *et al.,* 2008; Cailotto *et al.,* 2009). For example, glucocorticoid levels show circadian

oscillations in both mice and humans that are driven by the SCN via the hypothalamic–pituitary–adrenal axis (Oster *et al.,* 2006). As the glucocorticoid nuclear hormone receptor is expressed in almost all cell types (Balsalobre *et al.,* 2000), glucocorticoid hormones likely represent an important route for SCN phase synchronisation. Studies have also suggested that the autonomic nervous system may be another synchronisation route used by the SCN. Blocking humoral and neuronal pathways to the liver, studies have shown that light still affected circadian liver gene expression, with the researchers attributed this to autonomic input (Cailotto *et al*., 2009). Ultimately, difficulties in identifying specific synchronisation routes are at least partially a consequence of the numerous signaling pathways that can reset or synchronise peripheral phases in the same organ or cell. Schibler and Naef (2005) concede that for circadian rhythms in cultured fibroblasts, there are "a bewildering number of signaling pathways can synchronise circadian oscillators" (p.224). While many of the synchronisation pathways used by the SCN still need to be identified and separated to properly understand their individual significance, these examples show a few of the numerous signalling routes used in the phase entrainment of peripheral clocks.

2.9 Interplay between rhythms

Difficulties in studying biological rhythms, their mechanisms, and their end products, are likely exacerbated by the knowledge that, in certain circumstances, different entrainment cues, signaling pathways, or oscillators may interact or conflict with one other. The interplay between different cues can cause either the suppression of one of the usual responses, or produce an observed rhythmical reaction of seemingly unusual nature, periodicity, or intensity. Furthermore, a suggestion that individual cells may display multiple circadian oscillators controlling different functions (Roenneberg and Merrow, 2001), that rhythms may drift out of synchronicity and 'phase jump' back into line in a seemingly periodic fashion (Roenneberg and Morse, 1993), and that certain organs and tissues require longer periods of time to readjust their cycles after SCN phase

shifts than others (Yamazaki *et al.,* 2000), again emphasizes the complexity of biorhythms and highlights some of the numerous reasons why certain tissues and organisms display rhythmic processes of unusual periodicity or intensity.

To provide a few examples of this, while the feeding-fasting cycle is normally in phase with the SCN-driven rest-activity cycle, food availability during an animal's inactive period can actually override SCN signals. This inverts the phase of gene expression in related peripheral organs like the liver, and in doing so temporarily decouples these clocks from the master SCN (Le Minh *et al.,* 2001; Kornmann *et al.,* 2007). Conversely, in the submaxillary salivary gland, temporal alteration in food availability has no impact on circadian gene expression. Interestingly however, if the salivary gland is subjected to sympathetic denervation, the oscillators swiftly adjust their phase to the new feeding schedule (Vujovic *et al*., 2008). This not only shows that certain peripheral organs respond differently to altered entrainment cues, but also provides another example of how multiple synchronisation pathways can contribute to peripheral oscillator synchronisation in the same tissue.

When looking for examples of how two biological clocks may interact, the most common examples come from the study of marine species. In particular, certain species of crab (e.g. *Carcinus maenas*) show unusual but predictable intensities and durations of locomotor activity under constant laboratory conditions that cannot be attributed to one clock alone (Tessmar-Raible *et al.,* 2011). Currently, there are two dominant hypotheses to explain their unusually timed activity bursts. The first is that a circadian oscillator controls basic activity levels but is then combined with a circatidal clock that suppresses this activity during certain stages of the cycle (Naylor, 1958). The second hypothesis is that the phenomenon represents two circalunar clocks, normally running in 180 anti-phase to each other, but drift in period length in the absence of external cues, decoupling the two clocks and creating predicable but irregularly timed behavioural responses (Palmer, 1995).

Finally, and of particular relevance to the study of RP, Roenneberg and Morse (1993) documented the presence of two circadian oscillators in one unicellular

organism. Identifying two separate oscillators, one regulating circadian rhythm in bioluminescence and the other regulating circadian locomotor activity, these rhythms persisted in constant light but displayed separate phase peaks. Perhaps more interestingly however, while both the free-running circadian rhythms shortened their periodicity slightly, as is common in constant conditions, the activity-related rhythm did so with an even shorter periodicity but subsequently 'phase jumped' roughly every 7 days, returning closer to the phase timing of the other rhythm. Importantly, this discovery provided at least circumstantial evidence to support the hypothesis postulated by Newman and Poole (1974, 1993) regarding the formation of Retzius lines. To briefly recap, they hypothesized that Retzius lines were formed when a precise circadian rhythm and a free-running circadian rhythm were either most offset from each other, or when the free-running rhythm 'phase jumped' to correct itself. Unfortunately however, to account for the current known range of RP in humans, the timing of the free-running rhythm would have to vary significantly between individuals so as to create a different numbers of days between phase jumps. Interestingly, studies do suggest that variations in the periodicity of some free-running rhythms in humans are substantially greater than for many other species. For example, while the percentage coefficient of variation (PCV) in free-running activity rhythms is only 0.08% in rats, 0.3% in hamsters, and 0.7% in mice (Czeisler *et al.,* 1999), humans display free-running circadian activity rhythms with periodicities varying from 13 to 65 hours, reflected in a PCV of 30.3% (Wever, 2013). However, whether the periodicity of a free-running rhythm could remain consistent within an individual over many years, as RP appears to, is currently unknown.

2.10 Literature review conclusion

This literature review briefly describes the stages of tooth development and the current knowledge regarding the formation and nature of the incremental structures observable in histological sections of enamel. After this, the interspecific correlations with Retzius periodicity were described and the

hypothetical HHO was introduced. Collectively, this first section provides the groundwork for understanding the aim and justification of this research. In the second section, biological rhythms were introduced. As previously mentioned, this second section of the literature review is not essential for understanding the basic aims of this research but functions to emphasize the comparatively novel and unusual nature of Retzius periodicity, while also laying a broad foundation for interpreting and discussing the possible origin of the rhythmic biological processes and mechanisms involved. Importantly, the nature of these discussions will vary significantly based on the results of this research. To briefly recap, Retzius periodicity positively correlates with body mass and the pace of skeletal development and metabolism across species and so appears to represent a centralized, systemic rhythm that is driving or regulating growth and adult body mass (Bromage *et al.,* 2012). However, it is unknown if the same relationship exists within humans, who display an unusually wide range of RP values. This research therefore aims to investigate the relationship between RP and body size in a modern human population. If Retzius periodicity correlates with adult body size, this will provide an exciting new direction with which to understand and interpret RP in humans. If RP does not correlate with adult body size, other explanations will be required for understanding the unusual variability in RP seen within humans. In either case however, this research represents an important step in developing an understanding of the rhythmic process(es) involved in this unusual biological rhythm.

3. Study sample

The sample used in this study represents an archeological collection of modern humans from Late Medieval Canterbury, UK. Between the years of 1988 and 1991, Canterbury Archeological Trust excavated a total of 1,339 Late Medieval skeletons from St. Gregory's Priory and Cemetery within the Northgate area of Canterbury. While the extensive use of this graveyard made it difficult to establish a date for the earliest burials, Canterbury Archeological Trust concluded that the graveyard appeared to be in continuous use from the 11th century when the priory was established, until shortly after its dissolution in 1537 (Brent, 1879; Hicks and Hicks, 2001). Historical literature and archaeological evidence also suggests that there was a clear divide in the socio-economic status of individuals buried within the cemetery and priory (Tatton-Brown, 1995). High status individuals were buried within the Priory, as it was a popular way of displaying status (Ottaway, 1992; Daniell, 1997), while the low status individuals were buried in the cemetery. A collection of these skeletal remains from both burial sites is currently curated at the Skeletal Biology Research Centre, School of Anthropology and Conservation, University of Kent (UK). Importantly however, only individuals of cemetery burial, and therefore of low status, were sampled in this study. As environmental variables or lifestyles may significantly but disparately influence aspects of stature and body mass, by only selecting individuals from one socio-economic group it was hoped that differences in environmental exposure or insult on body size would be minimized. While it is likely that there are certain disadvantages to choosing the low status group, which will be addressed further in the discussion, both the University of Kent sample and the entire St. Gregory's collection contain significantly fewer high status individuals. Therefore, to increase initial sample sizes, but minimize potential differences in environmental stress, only individuals of cemetery burial were included in the sample. Additionally, as it is unknown whether a potential relationship between RP and body size would differ between males and females, only individuals of one sex were chosen for final histological sampling. The decision on which sex to choose was initially based on the group that showed a greater range of body sizes.

4. Methodology

4.1 Osteological methodology

Estimates of stature and body mass were essential requirements in the later analysis with Retzius periodicity. However, stature and body mass estimates were also a part of the specimen sampling process. As the aim of this study was to investigate the relationship between Retzius periodicity and body size, the largest and smallest individuals from the skeletal collection were specifically identified and chosen. Had this not been done and skeletal individuals had been randomly chosen for histological preparation, body sizes might not have varied significantly enough between the sample to test the assumptions of this research. Additionally, as previously stated, while only individuals of one sex were to be chosen for the final histological preparations, estimates of stature and body mass, along with visual assessments of dental preservation, were initially made for both male and female specimens. These results would then determine whether males or females were chosen for the later stages of the methodology. Therefore, initial osteological methods described below were conducted on a random selection of 188 male and female individuals from the St. Gregory's skeletal collection. All specimens included in the study were adults, as determined by confirmation of fusion in all long bone epiphyses. The specimens also had no evidence of obvious skeletal pathology.

4.1.1 Sex determination

Sex determination was carried out using multiple methods and morphological characteristics of the pelvis and cranium. The methods were chosen based on their documented reliability in osteological research (White *et al.,* 2011), and their familiarity to the observer. The pelvic methods included the three Phenice characteristics (1969), and the greater sciatic notch described in Buikstra and Ubelaker (1994). Cranial features used included the mastoid process, supraorbital margin, mental eminence, and nuchal crest, as described by

Buikstra & Ubelaker (1994). When determinations from cranial and pelvic features conflicted, priority was given to the pelvic criteria (White *et al.,* 2011). For statistical purposes, probable males were identified as male, and probable females as female.

4.1.2 Stature estimation

Generally, there are two approaches that are employed in the estimation of stature from skeletal remains. The first is the anatomic method, which involves summing the superoinferior measurements of all skeletal elements that contribute to stature (femur, tibia, vertebral column, height of the talus and calcaneus, and cranial height), followed by the application of a correction factor to account for soft tissue (Raxter *et al*., 2006). The alternative is the mathematical approach, which involves estimating stature from individual skeletal measurements through the utilization of regression equations or ratios that take advantage of the high linear correlations between certain bone measurements and stature (Trotter and Gleser, 1952). While it has been shown that anatomically derived stature estimates strongly correlate with living stature, have an average predictive error of less than 1cm, and are not sensitive to population differences in body proportions or unusual body types (Lundy, 1985), the method does require that all previously mentioned bones are both present and display minimal damage. Although the anatomical approach may have been possible for some individuals in the St. Gregory's collection, using such a method in this study would have drastically reduced histological sampling options and perhaps the final sample size. Stature estimation was therefore conducted with the mathematical approach. Unlike the anatomical approach, this method can be conducted with only a single bone measurement, and so is much less limited by bone preservation. While stature regression formula have been created for cranial height (Ryan and Bidmos, 2007), scapula, clavicle and os coxa (Shulin and Fangwu, 1983), metatarsals (Byers *et al.,* 1989), tarsals (Holland, 1995), vertebrae (Nagesh and Kumar, 2006) and even fragmented remains (Wright and Vasquez, 2003), the best estimations are made from limb bone

measurements. However, such methods do require the careful choosing of an appropriate regression formula. Due to differences in body size and proportions between different populations, specific regression formulas need to be chosen that have been created from reference populations that best represent the study population in question. Similarly, males and females also require separate formulae due to differences in body proportions between the sexes (Ruff *et al.,* 2012; Sládek *et al.,* 2015). Unfortunately, as Ruff *et al.* (2012) notes, when dealing with archeological material, it is often not possible to closely match reference and study populations. In addition to these requirements, a regression formula should also be chosen that has been formulated from a suitably large sample size, has obtained reliable stature data from living individuals or cadavers, and has the smallest prediction interval (Ruff *et al.,* 2012; Sládek *et al.,* 2015).

While numerous different formulae have been created to estimate stature in European archaeological material (e.g., Formicola, 1993; Giannecchini and Moggi-Cecchi, 2008; Vercellotti *et al.,* 2009; Meiklejohn and Babb, 2011), some of the most widely used are those of Trotter and Gleser (1952, 1958). The regression equations by Trotter and Gleser (1952, 1958) were actually formulated from a large collection of 5027 individuals, mostly comprised of male soldiers from World War II and the Korean War. The rest of their sample consisted of 855 men and women from the Terry Collection, which was composed of unclaimed cadavers from the low socioeconomic classes of Missouri (Hunt and Albanese, 2005). While differing in both temporal and spatial similarity to the study population, these equations appear to have performed well when tested on other European archaeological material (Giannecchini and Moggi-Cecchi, 2008), have previously been used in other contexts to estimate the stature of the St. Gregory's collection (Hicks and Hicks, 2001), and adequately fulfil the other requirements previously mentioned. This study therefore used the White male and female regression equations formulated by Trotter and Gleser (1952, 1958) for stature estimation.

Maximum lengths of the femur, humerus, ulna, and radius were measured to the nearest millimeter with a ScienceFirst® 60cm scale osteometric board. Tibia

lengths were measured from the most proximal part of the lateral half of the lateral condyle to the most distal projection of the bone, not including the malleolus, as suggested by Jantz *et al.* (1994). No measurements were made on the fibula due to the rarely intact nature of the bone in the present sample. For all measurements, left and right sides were taken and an average was calculated to reduce lateral biases. When only one side was present or adequately preserved, a single side value was used. Each measurement was then entered into the appropriate regression equation based on the determined sex of the individual, and a stature estimate was calculated.

4.1.3 Body mass estimation

As with stature estimation, there are two commonly used approaches to estimate body mass from skeletal remains. The first is the mechanical method, which relies on the functional relationship between a weight-bearing skeletal element and body mass. Mechanical methods are often subdivided into those that utilise cross-sections dimensions or diaphyseal breadths, and those that use articular surface dimensions. The second approach is the morphometric method, which attempts to directly reconstruct overall body shape from measures of stature and bi-iliac breadth. While many authors have suggested that mechanical methods utilising femoral head size are more accurate because the femur bears the majority of an individual's body weight (Jungers, 1988; Aiello and Wood, 1994; Churchhill *et al.,* 2012), others have suggested a greater reliability with the currently formulated and used morphometric equations as they were created from larger and more diverse sample sizes (Auerbach and Ruff, 2004). One of the limitations of the morphometric approach however, is that bi-iliac breadth is not easy to obtain in archaeological samples due issues of skeletal preservation and so can notable reduce sample sizes (Pomeroy and Zakrzewski, 2009). Additionally, it has been suggested that morphometric methods fail to account for body mass extremes (Moore, 2008), which are the actual targets of this study's sampling process. This study therefore used a mechanical method based on measurements of the femoral head. This specific method was chosen because

it is one of the mostly widely used body mass estimation methods, has been shown to perform as well as the commonly used morphometric method when tested on individuals of known body mass, and also has the advantage of being influenced less by activity levels or muscular loadings during life (Trinkaus *et al.,* 1994; Lieberman *et al.,* 2001; Aurbach and Ruff, 2004).

As with the majority of body size estimation approaches, femoral head size methods are based on regression equations. Consequently, some authors have argued that population-specific equations need to be used, especially for particularly small or large bodied groups (McHenry 1992; Kurki *et al.,* 2010). Additionally, while some have suggested that combined-sex equations are more accurate than sex-specific equations because they generally have larger reference samples with broader ranges of variation (Henneberg *et al*., 2005), or raised concern that the level of sexual dimorphism in the reference sample may not be the same as that of the target specimen (Niskanen and Junno, 2009), sex-specific equations are generally considered to be more accurate due to the differences in body size and shape between the sexes (Ruff *et al.,* 1991). Therefore the sex-specific versions of the equation provided by Ruff *et al*. (2012) were used in this study. As the sex-specific equations are formulated from European populations presumed to be broadly comparable to the individuals in this study, it is hoped that the level of sexual dimorphism is also roughly comparable and that the populations are relatively similar in body size.

Body mass was estimated by measuring femoral head anteroposterior breadth in millimeters using a Draper® 150mm plastic caliper gauge. Both femoral heads were measured where possible, and an average of these was calculated. When only one femoral head was available, the one available side was used in the equation as directional asymmetry in the lower limbs is usually small and deemed inconsequential for the purposes of estimating body mass (Auerbach and Ruff 2004; Ruff *et al*., 2012). Measurements were then entered into the sex-specific calculations to obtain a body mass estimate for each individual. As stature was considered the more important variable in this study, body mass was only estimated for individuals who had intact long bones for stature

estimation. Of the 188 individuals with stature estimation, 169 had adequately preserved femoral heads for a body mass estimate.

Table 1. Male body size descriptive statistics

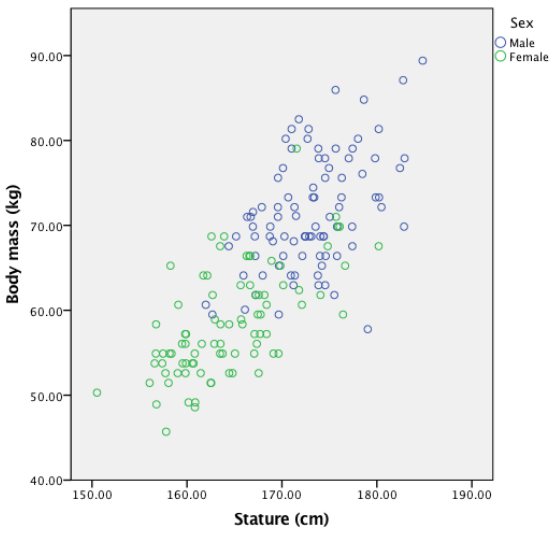
	N	Range	Mean	Std. Deviation
Stature (cm)	95	22.84	172.98	4.80
Body mass (kg)	82	31.62	71.43	6.71

Table 2. Female body size descriptive statistics

	N	Range	Mean	Std. Deviation
Stature (cm)	93	29.66	164.42	5.58
Body mass (kg)	87	33.35	58.48	6.26

Tables 1 and 2 show the descriptive statistics for male and female body size variables respectively. Overall, while body mass was relatively similar in both range and standard deviation between the sexes, females displayed a greater range and standard deviation in stature. Therefore initially, females were chosen to represent the histological sampling. Unfortunately, when collecting the individual teeth from each individual, it was found that many of the females occupying these extreme ends of body size spectrum either had no preserved teeth or the enamel was significantly worn. Conversely, the particularly large and small individuals of the male sample had comparatively better dental preservation. Why this trend was found is unknown and beyond the scope of this thesis to discuss. Consequently, the male

Figure 1. Stature and body mass for male and female samples



individuals of St. Gregory's were chosen for histological processing. While an attempt was made to gather individual teeth for the 25 largest and 25 smallest male individuals, dental preservation meant the size difference between some of the individuals from each category were negligible. This methodology did however ensure that large and small body types were represented in the final methodological stage. Additionally, five males who did not have intact femoral heads for body mass estimation were included in the sample because they were of particularly desirable stature and had good dental preservation.

4.2 Enamel histology methodology

To calculate RP, histological methods were used to prepare the thin ground sections of single teeth from each of the 50 male individuals chosen. While molars were primarily chosen for histological sectioning, anterior teeth were sampled when molars were not present. In the following section, this histological process is described in detail (See similar published examples in Mahoney, 2013, 2015). Importantly, this section also describes many of the reasons why such techniques were employed and some of the alternatives mentioned by other authors. Additionally, for some stages of the process it was possible to experiment with slightly different techniques or products in an attempt to improve the visibility of the incremental structures, and these are also described here. Collectively, this section describes the histological methodology used in this study but also aims to contribute to a dialogue on conducting enamel histology. Even with careful histological practices, errors can occur at any one of the stages of preparation and result in damaged or useless tooth sections. Furthermore, in some cases, even with the best methodology, incremental structures cannot be clearly observed. As enamel histology is a destructive and irreversible process, it is important to develop and test techniques to ensure the best possible outcomes. Through collaborative trial and error, it may be possible to subtly but gradually improve enamel histology methods for future research.

4.2.1 Embedding and Sectioning

After the individual teeth were selected, cusp tips and planes of section for each tooth were marked with a permanent fine-tipped marker on both sides of the enamel. This helps to orientate the tooth and cut an accurate section during the later stages of the process (Mahoney, 2015). The section should be made through the point of maximum thickness of enamel above the dentine horn and travel non-obliquely down the long-axis of the cusp and tooth, capturing the most cervical extension of enamel (Mahoney, 2013). For incisors, the sections were made where the central mamelon would have been positioned, with the section plane running from lingual to labial. For canines, the section plane was the same, but was positioned where the highest point of the tooth would have been. For molars, there were two possible section planes cutting along either the mesiobuccal and mesiolingual or the distobuccal and distolingual cusps, running from buccal to lingual and parallel with the vertical axis of the tooth. In some cases, both section planes were cut from one molar in this study. It should be noted that while accurate section planes ensure a complete record of the enamel incremental structures (Antoine *et al.,* 2009), they are also extremely important for studies of enamel thickness, as bad section planes with significant obliquity can result in considerably inaccurate values (Smith *et al.,* 2005; Suwa and Kono, 2005).

Each tooth was then embedded in a solid medium before cutting. While freshly extracted teeth are often durable enough to be sectioned without such treatment and can sometimes simply be coated in cyanoacrylate to reduce the risk of splintering (Schwartz *et al.*, 2002; Hillson, 2014), Li and Risnes (2004) have highlighted several benefits to embedding a tooth prior to sectioning and grinding. These include ease of handling, protection of the enamel surface during etching, and a significant reduction in the risk of splintering or fracturing. The latter point is of particular concern in this study as splintering and fracturing risk is much higher with archeological specimens, compared with freshly extracted or fossilized teeth (Paine, 2007; Hillson, 2014). Frequently in histological preparations, paraffin wax is used as an embedding agent as it has a similar density to most soft tissue (Lim *et al.,* 2010). However, while paraffin wax

may be a suitable embedding medium for embryonic or decalcified teeth in some instances, calcium deposits in non-embryonic and untreated enamel have a different density to the paraffin and therefore cannot be sectioned with this medium (Ohazama, 2012). Alternative embedding mediums include epoxy, acrylic, polyester and araldite resins. However, while resins are more suitable for enamel sectioning, some are not without their own weaknesses. Noting various issues associated with some embedding resins (including resin infiltration and subsequent interference of dental morphology, resin smearing during grinding or polishing, and poor adaptation to the tooth surface resulting in a gap between the enamel and resin), Li and Risnes (2004) conducted a comparison and evaluation of eight commercially available embedding resins for dental histology preparation. Based on their performance in relation to these issues mentioned, along with consideration of other favorable properties including strength and flow, Li and Risnes (2004) reported the best results with the epoxy resins, and the worst with the acrylic resins. Conversely, Hillson (2014) has suggested that it is important to use the hardest resin possible so that sections polish evenly, suggesting that this is currently methyl-methacrylate (acrylic resin). However, embedding in methyl-methacrylate can take several days and requires a fume cupboard (Hillson, 2014). This study therefore used an epoxy resin system, made from four parts Buehler EpoxiCure 2 Resin, and one part EpoxiCure 2 Hardener. Accurate ratios of resin to hardener were achieved by weighing the quantities of each substance on a digital scale in grams as they were poured into a vial. These liquids were then mixed with a spatula using a lift and stir motion until there were no visible streaks or traces of unmixed substance in the vial. The benefits of using this epoxy system is that it is a transparent resin, produces sufficiently hard mounts, can be air cured, is solvent resistant, and has a low enough viscosity that it can fill all the microscopic pores and voids of the tooth without the need for vacuum impregnation equipment.

Both the marked tooth and resin were added to a Buehler SamplKup mounting cup. Prior to this, the inside of the mounting cup was lightly brushed with Buehler Release agent to ensure the section could be easily removed from the cup after polymerization. The specifications of the epoxy resin state a cure time

of six to eight hours at room temperature, however approximately 24 hours was observed before advancing to the next stage of the process. This ensured that the resin had fully cured, and accounted for possible room temperatures below 20°C during various periods in the laboratory. While it may be possible to decrease the cure times of these epoxy resins by gently heating them in an oven (Caropreso *et al.,* 2000), this was not conducted in this study.

After polymerization of the embedding agent, tooth sections were cut using a gravity-fed, diamond-wafering blade (Buehler IsoMet 1000 precision saw). The embedded tooth was carefully orientated in a Buehler single saddle chuck so that the saw cut directly through the tooth at the points and angles indicated from the permanent marker lines previously made. Sections with the Buehler IsoMet 1000 were made at between 100-300 revolutions per minute. Silva *et al.* (2011) have stated that saw speeds above 300rpm tend to damage the specimen surface and should be avoided. Water and Buehler Isocut Plus Cutting fluid was added to the saw's coolant tank as a lubricant and coolant. After the sections were cut, and before mounting them on microscope slides, embedded sections were washed with water and given a single swipe along a coarse abrasive grinding pad to improve adhesion of the section to the slide during the next stage of the process.

An alternative method to cutting the sample with a precision saw is to manually grind the embedded tooth down to the center line with a coarse abrasive pad (Hillson, 2014). While significantly more time consuming, the benefits of this method may be the accuracy of the section plane and a lack of saw marks on the sample. This manual grinding technique was attempted with an isolated archeological tooth of unknown ownership to test the applicability of such a method for this study. As expected, this method achieved a near perfect section plane across the tooth. This is because it was possible to consistently check the progress being made as the ground surface slowly reached the desired section plane. Unfortunately however, while this method did achieve great results regarding section plane accuracy, it was very difficult to achieve an even thickness and a completely flat ground surface. This is an issue that has previously been experienced and documented by De Boer *et al.* (2013) and Haas

and Stora (2015) when attempting such methods for bone histology preparation. Additionally, the nature of this method means that half of the tooth is ground away and lost. Subsequently, this manual grinding method was not implemented further in this study.

4.2.2 Mounting, grinding, and polishing

After sectioning with the saw, the samples were mounted on 1-1.2mm microscope slides with various epoxy resins (Gorilla Epoxy, Bostik Evo-Stik Adhesive Express, and UniBond Repair Power Epoxy All Purpose). After carefully mixing the epoxy resin and coating the cut side of the section with the glue, the sample was pressed down onto the slide. It is important at this point to make gentle depressed circular motions with the section to release any bubbles that have formed under the section (Maggiano, 2012). Essential specifications regarding the resins are that they set translucently, adhere well to both the glass slide and the embedded tooth, are strong and durable, and are water and solvent resistant. While all of these features are important, the strength of the resin is particularly important due to the forces applied to the section during later grinding and polishing. The chosen epoxy also needs to make a parallel layer between the glass slide and section, otherwise the tooth may be ground and polished more on one side than the other. Hillson (2014) suggests that the best results for an even parallel finish are achieved not just with the right choice of resin, but also "by using a spring-loaded bonding jig while the adhesive sets" (p.264). In this study however, either notably uneven layers did not occur, or they were small enough that they did not seem to effect later observations. It is also important at this stage however to achieve an adhesive layer that is as thin as possible, known as a zero bond (Hillson, 1996), and it is likely that spring-loaded bonding jig would also help in achieving this.

Of the various epoxy resins that were tested, the best results were observed with the Gorilla Epoxy. In particular, teeth mounted with the Gorilla Epoxy appeared to have significantly less bubbles in the section during later microscopy. In

addition to these epoxies tested, and the numerous others on the market, some researchers choose to use the original embedding mixture (in this case the EpoxiCure Resin and Hardener mixture) as a glue for mounting the sections to the microscope slides (See Kierdorf *et al*., 2013, and Haas and Stora, 2015). However this study found that when the EpoxiCure mixture was used in this way, more air bubbles were observed in the section during later microscopy. Although some of the setting times for the chosen epoxy resins in this study were stated by the manufacturer to be as low as two hours with a room temperature of 20^{o}C, approximately 24 hours was given before progressing to the next stage of preparation. Waiting 24 hours both ensured that the resin had fully set, and accounted for possible room temperatures below 20^{o}C during various periods in the laboratory. While it may also be possible to decrease the cure times of these resins by gently heating them in an oven, this was not attempted in this study. Haas and Stora (2015) attempted this by increasing the temperature of their samples to 60^{o}C and reported fractures in four of their five histological slides.

After roughly 24 hours, mounted sections were attached to a glass slide chuck and more of the tooth and resin was cut with the Buehler IsoMet 1000 so that the attached tooth section was reduced to approximately 1-2mm in thickness. From there, sections were further reduced in thickness using a graded series of Buehler silicon carbide (SiC) abrasive grinding pads (P400, P600 and P1200), and a heavy jig. The heavy jig holds the glass slide by vacuum, keeping the tooth section sturdy and in a plane parallel to the grinding pads as it is reduced in thickness. This ensures a more uniform thickness is achieved along the section, and provides the histologist with a sturdy structure to grip. Grinding was conducted by hand, using the heavy jig and grinding pads, and also with the use of a Buehler Eco-Met 300 Grinder-Polisher machine. This grinding and lapping machine has a rotating base with which grinding pads or polishing cloths can be attached. While this machine can speed up the process of grinding, it can quickly reduce the thickness of the section and should be used carefully and sparingly, especially when dealing with particularly thin sections, deciduous teeth, or fossil specimens. Additionally, care and attention needs to be paid when replacing worn abrasive pads. Unlike diamond abrasive pads, SiC pads have a relatively

short usable life and need to be replaced frequently. When transferring from a worn to a fresh abrasive pad, this study found that, perhaps obviously, the new paper is significantly more effective and therefore can drastically reduce the thickness of a section, potentially destroying a specimen if care is not taken. It should also be noted here that there is significant importance in progressing through the graded system of abrasive pads. The different grades are not just for how fast or intensely one wants to reduce the thickness of the section, but functions to remove the scratches produced by the previous abrasive pad. Without progressing through the graded system, later polishing will not be able to remove the large scratches made by the coarser pads. Sections were then lapped with a fine grade 0.3 micron aluminum oxide slurry (mixture of Buehler Micropolish II powder and water) on a Buehler premium polishing cloth. This was also conducted using the Buehler Eco-Met 300 and the heavy jig. Lapping the tooth section functions to remove additional minor fractions of enamel and dentine, reduce the number of saw marks on the surface, and increase the visibility of incremental features (Hillson, 2014). While FitzGerald and Rose (2000) advocate using a 9-micron and 3-micron aluminum oxide slurry before then using a finer grade 0.5 micron powder, Hillson (2014) suggests that it is best to use a fine abrasive of 0.3 microns throughout the entire polishing procedure, and that this successfully removes the majority of the remaining scratches on the section.

This stage of the process marks the point when the final thickness of the section is achieved. FitzGerald and Rose (2000) suggest that the desired final thickness of a tooth section after grinding and lapping should be 100μm, while others have suggested thicknesses of between 30μm (Silva *et al.,* 2011) and 150μm (Hillson, 1996). Determining and achieving such a precise measurement, however, can be challenging. One method is to use the micrometer adjustment on the jig, which stops grinding and/or polishing at the indicated thickness. Another option would be to measure the thickness routinely with a hand-held thickness gauge (Hillson, 2014). While it would be interesting to test the reliability of such methods in the future, this study found that different samples seemed to display incremental features at variable thicknesses, perhaps because a zero bond was not

consistently achieved between the section and slide. Instead then, a desirable final thickness for each section was achieved through short intermittent periods of grinding and polishing until incremental features were visible and enamel displayed a semi-transparent, whitish appearance at magnification on a GX L1000A Microscope. While perhaps slightly time consuming, cyclic repetitions of brief grinding followed by visual checks with the microscope ensured that optimal thicknesses were achieved for each individual specimen.

4.2.3 Final histological preparation

After polishing, sections were placed, but not fully submerged, in a shallow ultrasonic bath and intermittently sprayed with water for approximately two minutes to remove additional surface debris. Ultrasonic cleaning uses cavitation bubbles induced by high frequency sounds waves to agitate the water. This agitation produces high forces on any contaminants and debris clinging to the tooth section, and is an effective way of cleaning the specimen. At this stage, some dental histologists choose to acid-etch their samples. Acid-etching is usually carried out by submerging a section into a phosphoric acid solution for a short period of time (FitzGerald and Rose, 2000). Boyde (1989) demonstrated that acid-etching can exaggerate the appearance and visibility of incremental markings. Additionally, Grine (2005) has pointed out that etching may also remove any smeared enamel. This technique was attempted on a small sample of teeth, however subjective analysis did not find any substantial or even particularly noticeable differences in incremental visibility before and after enamel etching. However, there are several potential reasons for this. Firstly, in an effort to ensure a specimen was not damaged, this study immersed the tooth sections for one minute using 2% (v/v) phosphoric acid, as suggested by Hillson (1996). However, other studies have etched samples in much stronger concentrations. For example, Kierdorf *et al*. (2013) etched soay sheep enamel for five seconds with 34% phosphoric acid to enhance the visibility of the enamel structure. Similarly, Sabel *et al.* (2008) studied deciduous enamel increments and sections were etched with 30% phosphoric acid for 30 seconds. While the

submersion times were shorter in these other studies, it is not known how this may have influenced the effectiveness of the technique. Secondly, while every effort was made to ensure the concentration of the solution was as close to 2% (v/v) phosphoric acid as possible, slight errors may have occurred in the exact measurements of distilled water and acid. Subsequently, the final solution may have been lower in concentration that was assumed. Unfortunately then, no definitive comment can be made on the value of this technique for studying incremental markings, however the reported success in other studies suggests it is an effective technique.

Sections were then dehydrated through an ascending series of alcohol baths. This involves submerging the sections in 95% and 100% ethanol for approximately two minutes each. This dehydration exercise functions to extract any lipids, water, and lipid-soluble or water-soluble constituents from the sections (Hillman, 2000). After this, sections were cleared. Clearing the sections increases the refractive index of the tissue, removes any dehydrating agents, and makes the tooth more transparent (Hillman, 2000). A suitable clearing agent, as specified by Anderson and Gordon (1996), has minimum toxicity, causes minimal tissue damage, and effectively and quickly removes any dehydrating agents. In the past, tissue histologists commonly used xylene as a clearing agent (Matthews, 1981; Pollard *et al.,* 1987; Buesa and Peshkov, 2009). However a large number of animal studies have shown that excessive exposure to xylene can have significant adverse effects on the nervous system (Gamberale *et al.,* 1978), the liver (Kum *et al.,* 2007), the lungs (Sandikci *et al.,* 2009) and the skin (Chatterjee *et al.,* 2005). Additionally, xylene is also highly flammable and volatile (Kunhua *et al.,* 2012). Subsequently, many studies now use xylene alternatives such as Histoclear (Schwartz *et al.,* 2002), methyl salicylate (Skinner, 1986), and certain vegetable and mineral oils (Buesa and Peshkov, 2009). Histoclear, in particular, is an orange oil-based clearing agent that is significantly less hazardous than xylene, but still retains the other suitable characteristics described by Anderson and Gordon (1996). This study used Fisherband Histoclear as a clearing agent, as seen in Schwartz *et al.* (2002, 2003) and Mahoney (2015).

Prepared sections were then mounted with a 0.13-0.17mm cover slip using a mounting medium composed of distyrene, a plasticizer, and xylene (DPX). As with many of the consumables used in this study, there are many options and variables to consider when choosing a suitable mounting medium for dental histology. Early histological research commonly used aqueous medias such as gelatin, glycerine, and polyethylene glycol, however these mounting mediums often leaked after the solvent evaporated and sometimes became contaminated with bacteria and fungi (Hillman, 2000). More recently, sections are mounted in non-aqueous resins, such as DPX, Canada Balsam, or Euparal. Silva *et al.* (2011) recommend using Entellan, which is a rapid cure resin that polymerizes in approximately 24 hours. Alternatively, they recommend the cheaper Canada balsam, but point out that this medium requires about 4–5 days for polymerization. Besides polymerization time, another important feature of mounting mediums that needs to be considered are the refractive indexes (RI) of the substance. Cross striation visibility, for example, is thought to be due to reflection phenomena at the boundaries between materials with different refractive indexes. Therefore much depends on the refractive index of the medium with which the cover slip is mounted (Hillson, 1996). As apatite has a refractive index of 1.65 (McDonnell, 2012), an arguably better mounting medium will have a refractive index that significantly differs from that value. Comparing some commonly used mounting mediums (Canada Balsam RI–1.54, DPX RI–1.52, xylene RI–1.49), it is clear however that there is a need to balance some of the various desired characteristics with the refractive index, toxicity, and cost of each medium. This study used Thermo Scientific DPX, as it has a better refractive index than Canada balsam, does not colour with age, and is less carcinogenic and teratogenic than some of the purer xylene alternatives. Interestingly, Hillson (2014) suggests that cover slips can be mounted temporarily using a drop of water, arguing that "water is optically a much better mounting medium than… [an] equivalent mounting medium" (p.264). With a refractive index of 1.33, this not only seems to be a good option, but also means that cover slips can be removed at a later time if the section needs to be manipulated or further reduced in thickness in the future. However, to repeat Hillman's (2000) comment, as

aqueous mediums can leak and become contaminated with bacteria and fungi, water mounting was only ever used as a temporary measure in this study.

Regardless of what mounting medium is used, Maggiano (2012) emphasizes that cover-slipping is a very important part of the preparation procedure. After losing over 45 non-covered histological bone samples in a partial flood, Maggiano (2012) explains that coverslips make samples much more resilient to humidity and fractures. At the point of applying the cover slip, it was common in this study for bubbles to sometimes appear under the glass. When this occurred it was usually possible to safely remove the cover slip if only a short amount of time had gone by, and reapply another slip. Another technique for minimizing the number of bubbles under the cover slip, proposed by Maggiano (2012) and successfully implemented in this study, is to exert pressure on the slide, in this case using a cotton bud, and introduce DPX to the edge of the slide nearest the bubble. As the pressure is then lifted off the cover slip, the DPX often replaces the air bubble, pushing it out to the side. Once cover slips were successfully placed, the mounted sections were then left for approximately 24 hours to set. It is important at this final stage to ensure that the surface in which the section sits is completely flat. If the samples are not on a flat surface, there is a tendency for the cover slip to slide off as the DPX is setting.

Throughout this entire histological procedure, glass slides, tooth sections, and cover slips were checked for cleanliness, and cleaned with ethanol and a cotton bud if found to be dirty. This ensured that no dirt existed that may have directly interfered with the visibility of the enamel features, that particles were not present that could scratch any of the surfaces, and that surfaces were kept clean to help the various adhesives bond suitably. Drying these materials at each point of the process was also important to ensure that water particles or bubbles were not formed or trapped, and that the integrity of the liquids was not compromised by introducing a wet slide to the bottle. While some researcher recommend drying samples in a vacuum desiccator (FitzGerald and Rose, 2000; Hillson, 2014), sections and slides were dried adequately using a heat lamp in this study.

It is also important to label the tooth sections during the histological preparation, especially when preparing multiple sections at the same time.

4.2.4 Microscopy

Sections were examined at magnification under a high-powered microscope (Olympus BX51) using transmitted and polarized light. Under transmitted light, the tooth section is illuminated by the condenser lens, which is positioned under the stage holding the slide. To observe the incremental structures, the condenser lens was centered and focused, and the condenser iris diaphragm was closed down to a small aperture. Areas of interest were initially found at magnifications of x4 and x10, with counts and measurements then being made at magnifications of either x40 or x60. The x10 objective on the BX51 has a resolving power of about 1μm and a field depth of about 10μm, while the x40 objective has a 0.4μm resolving power and a 2μm depth of field (Hillson, 2014). An understanding of these field depths, and the knowledge that Retzius line visibility depends on a cumulative effect while cross-striations do not, may explain why Retzius lines were often visible at x10 and then not at x40 in this study, and why Retzius lines and cross-striations were often not seen together.

For transmitted light microscopy, another way to increase the contrast and help incremental observations is to use dark field illumination (Boyde, 1989; Suvarna *et al.,* 2013). This technique is often accomplished by positioning a perfectly sized opaque circular disc on the glass filter carrier below the condenser lens (Hillson, 2014). By doing so, only oblique rays of light enter the slide, making the background of the image dark, but the light scattered or reflected from the discontinuities within the enamel show up brightly. This method was briefly attempted on a GX L1000A microscope, however efforts to create a perfectly sized disc were unsuccessful and further attempts were abandoned.

As well as using transmitted light, this study also utilised polarizing light microscopy. Polarizing light helps to define incremental features and provides better lighting for image capturing by taking advantage of enamel's ability to

refract light at multiple indices (Paine, 2007). In polarized light microscopy, the tooth section is illuminated from a special polarizing filter built into the condenser assembly, and observed through another polarizing filter (the analyser) fitted above the objective with a vibration plane perpendicular to that of the polarizer (Hillson, 2014). With this setup, a beam of light is constrained into two waves, each polarized perpendicular to each other, with different refractive indexes (RI). When the light waves emerge from the apatite crystal, they recombine slightly out of phase, and are resolved by the analyzer filter to produce a range of colours related to the birefringence and thickness of the section. While apatite crystals are obviously too small to see individually, it is their grouped arrangement in similar orientation that results in a visible image (De Medeiros, 2012). Once areas of interest were found within a section of enamel, and focused at magnifications of either x40 or x60, images were captured through a trinocular head. These images were then manipulated and analyzed with Cell^D Olympus imaging software. This software contains a range of image acquisition tools, standard image processing, and measurement functions that ensure accurate incremental enamel data could be collected.

4.2.5 Incremental data collection

Retzius periodicity can be determined using two methods. The first involves directly counting the number of cross-striations between Retzius lines. When this is not possible, RP can be calculated by measuring the spacing between Retzius lines and dividing this figure by the average local daily secretion rates (DSR), as described by Dean *et al.* (1993) and Swindler and Beynon (1993). While this latter method has been questioned by Smith (2004), who stated that "DSR was not constant within Retzius line intervals, and [therefore]… may not consistently yield reliable periodicity estimations" (p.251), Smith (2004) also admits that counting cross striations between Retzius lines "is often difficult to do with certainty" (p.95). While some studies conceivable jump between RP methodologies depending on the visibility of the incremental structures in an individual tooth, this study only used the direct count method. While the

measurement method could have been employed to potentially increase sample sizes, this study considered accurate, clear periodicity counts using one method to be more desirable than a less accurate and methodologically inconsistent data set of larger size. RP was only calculated between Retzius lines that visibly met the surface in lateral enamel, as suggested by Dean (1987). Also, measurements were only made between lines that displayed the characteristic curve of a genuine Retzius line. This study found that, in a small number of cases, small scratch marks from cutting or grinding that were not completely removed by polishing sometimes left relatively evenly spaced marks that looked very much like Retzius lines at high magnification. However, what distinguished the two was the slight curve to the Retzius line through the enamel, compared with the extremely straight lines of a cut or scratch. For all specimens, RP was calculated throughout multiple sections of lateral enamel. If RP could only be calculated from one count, the individual was not included in the final results. Additionally, three separate, independent observations were made for each tooth that displayed the necessary incremental marking for making RP counts. Any individual with RP counts that differed by more than one day during these three observations was excluded. When one of the observations differed by one day, the individual's RP was assumed from the two other agreeing observations. In no cases did all three observations differ in recorded RP. While RP had initially been calculated for 27 individuals, four individuals failed the intra-observer error test, leading to their exclusion and reducing the final sample size to 23.

For each of these 23 individuals, daily secretion rates (DSR) were calculated near the location of the RP count. This was primarily done to ensure that individuals were normal in respect to their enamel growth and also to test for correlations between DSR and RP. When calculating DSR, a minimum length of three cross striations were measured, and a minimum of three prisms were measured from each location. Additional measurements were made when the quality of the sections permitted, and in some cases up to 10 successive cross striations were accurately measured along a prism. Cross-striations were counted or measured from the center of the first dark band to the center of the last and care was taken to not count intradian lines as cross-striations.

4.3 Statistical analysis

From the initial 50 individuals chosen for this study, confident RP data could accurately be made from 23 male individuals. The strength of the relationship between RP and stature, and RP and body mass, was measured with correlation and regression statistics for 23 and 18 individuals respectively (linear, log, quadratic). The strength of relationships between stature and body mass, RP and daily secretion rates, and RP and the distance between Retzius lines, was also measured with correlation and regression statistics (linear, log, quadratic). Following this, allometric scaling relationships between log-transformed Retzius periodicity, and log-transformed stature and body mass, was examined through reduced major axis regression (RMA). Scaling relationships between log-transformed stature and body mass, log-transformed daily enamel secretion rates and RP, and log-transformed RP and the distance between Retzius lines was also examined through RMA. RMA, also known as the standardized major axis (Warton *et al.,* 2006), is commonly used for examining scaling relationships, particularly when the X-axis and Y-axis variables are measured in different units, and there is no information about error variances (McArdle, 1988). Unlike ordinary least squares regression, RMA is specifically formulated to handle errors in both the x and y variables, as this study will have. An RMA slope equal to one reflects a perfect isometric relationship. Positive allometry, represented by a slope greater than one, and negative allometry, represented by a slope less than one, are identified using the 95% confidence intervals of the slope (CI's). When the confidence intervals cut through a value of one, the relationship is regarded as isometric. Statistical analyses were conduced using SPSS and PAST (Hammer *et al*., 2001).

5. Results

This chapter presents the results of the analysis. First, descriptive statistics are presented for daily enamel secretion rates, Retzius periodicity, stature, and body mass, along with comparative data sets from other studies. Inferential statistics are then presented that explore the relationships between Retzius periodicity and the other variables in this study.

5.1 Daily enamel secretion rates in μm

Table 3 and 4 show the descriptive statistics for daily enamel secretion rates (DSR). The DSR measurements in this study were calculated close to the location of the Retzius periodicity counts, and therefore come from mid- and outer lateral enamel. Where possible, comparative data from other studies are taken from equivalent enamel regions. Daily secretion rate values for anterior teeth in general, and for lateral enamel along the tooth row, are currently not well represented in the literature. Incisor and canine DSR's fall within maximum and minimum values described in other studies, and the mean is within one standard deviation of a mean reported for outer lateral enamel described by Schwartz *et al.* (2001a). The standard deviation in this study however, is much higher than for other published results. This is likely due to the variable location of these measurements throughout the mid and outermost lateral enamel in this study. For example, while many of these other studies calculated DSR throughout the tooth at very specific locations and depths, thereby creating relatively homologous results, DSR measurements in this study were taken at more variable locations that corresponded with the clear identification of Retzius periodicity counts. Daily secretion rates for molars do not exceed maximum or minimum values from other published findings, and with a mean of 4.42μm/day, DSR falls within one standard deviation of these other reported results.

Table 3. DSR (µm) in incisors and canines					
	N	Min	Max	Mean	SD
This study	6	3.02	5.45	4.36	1.05
Reid *et al,* 1998[1]	21	3.00	4.00	-	-
Schwartz *et al,* 2001a[2]	19	3.98	4.97	4.38	0.32
Schwartz *et al,* 2001a[3]	19	4.18	5.77	4.92	0.42

[1]DSR measurements from incisor and canine cuspal enamel. [2]Average DSR in canine outer lateral enamel. [3]Average DSR in canine outer cuspal enamel.

Table 4. DSR (µm) in premolars and molars					
	N	Min	Max	Mean	SD
This study	17	2.98	5.80	4.42	0.77
Reid *et al,* 1998[1]	33	3.00	5.80	-	-
Beynon *et al,* 1991[2]	39	2.60	5.00	-	-
Lacruz and Bromage, 2006[3]	10	-	-	4.80	0.67
Lacruz and Bromage, 2006[4]	10	-	-	4.30	0.5
Smith *et al,* 2007[1]	21	-	-	4.11	-
Mahoney, 2008[5]	15	3.64	5.45	4.55	0.61

[1]DSR from cuspal enamel. [2]DSR from lateral enamel. [3]DSR from outer lateral enamel. [4]DSR from mid-lateral enamel. [5]DSR from outer cuspal enamel.

5.2 Retzius periodicity in days

Table 5 shows the Retzius periodicity (RP) distribution from this sample by tooth type. RP is higher for the incisors and canines, however sample sizes are small. Figure 1 shows the RP distribution as a percentage, and includes data from two other large studies that have been transformed into percentage distributions for comparison. The histogram indicates that there is a slight positive skew to the RP data generated for this study. A slight skew to RP data has been reported previously for human samples (Smith *et al.,* 2007). However the RP values generated from this study were normally distributed ($p>.05$). The most notable difference between the findings shown in figure 2 is the much greater contribution of 6-day periodicities in this study.

Table 5. Retzius periodicity distribution by tooth type								
		Tooth type					Total frequency	Frequency percentage
		I	C	P	M			
Retzius periodicity in days	6.00	-	-	-	4		4	17.4
	7.00	-	-	1	3		4	17.4
	8.00	1	-	2	4		7	30.4
	9.00	2	1	-	1		4	17.4
	10.00	-	1	-	1		2	8.7
	11.00	-	1	-	-		1	4.3
	12.00	-	-	-	1		1	4.3
	Total	3	3	3	14		23	100
	Mean	8.67	10	7.67	7.71			
	Mode	9	-	8	-			

For tooth type, I – incisors, C – canines, P – premolars, M – molars.

Tables 6 and 7 show the descriptive statistics for Retzius periodicity, along with comparative data sets. Table 4 shows RP sub-divided into anterior and posterior teeth. As previously mentioned, RP is slightly higher in the anterior teeth but the sample size is small. The data in table 5 represent RP values for all the teeth in this study, and separates archeological and modern comparative data sets. Both the mean (RP of 8.2) and mode (RP of 8) from all teeth in this study are similar to other published results (Reid and Dean, 2006; Smith *et al.*, 2007). Retzius periodicity in this study covers the full range of currently known periodicities for permanent teeth (E.g. Smith *et al.*, 2007). Interestingly, it does this despite having a relatively small sample size. In fact, this sample of 23 individuals shows a greater range than the 298 Northern European and 191 North American individuals studied by Reid and Dean (2006).

Figure 2. Retzius Periodicity (in days)

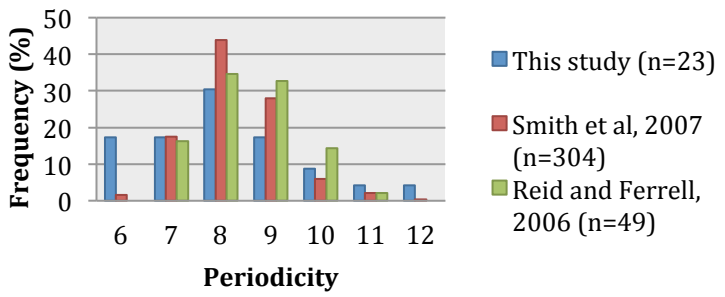


Fig.1 The periodicity distribution data for Smith *et al* (2007) is estimated from the histogram on p.183.

Table 6. Retzius periodicity, subdivided into anterior and posterior teeth						
	Population	N[1]	Range	Mean	Mode	SD
Anterior teeth						
This study	Medieval Canterbury, UK	6	8-11	9.33	9	1.03
Fitzgerald, 1998	Various populations	96	-	9.7	-	1.2
Reid and Ferrell, 2006	Medieval, Denmark	49	7-11	8.5	8	1.0
Reid and Dean, 2006	Various populations	115	6-12	8.99	-	1.08
Posterior teeth						
This study	Medieval Canterbury, UK	17	6-12	7.71	8	1.57
Reid and Ferrell, 2006	Medieval, Denmark	35	6-12	8.5	8	1.1
Mahoney, 2008	Bronze age, UK	15	6-9	7.67	7	1.04
Reid and Dean, 2006	Various populations	643	6-12	8.22	-	0.97

[1]In some cases for the other studies included here, the *n* value may not indicate the number of individuals in the sample, but the number of teeth. [2]While Reid and Dean (2006) separated their data for anterior and posterior teeth, combined figures have been calculated for this table. [3]The mode values for Reid and Dean (2006) represent figures provided on a smaller sub-set (n=304) of these same individuals by Smith *et al,* (2007).

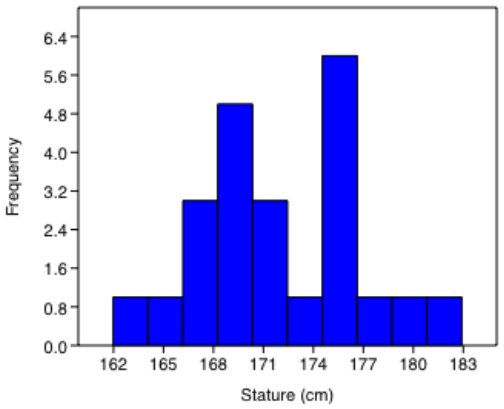
Table 7. Retzius periodicity, separating archeological and modern populations						
	Population	N[1]	Range	Mean	Mode	SD
Archeological population						
This study	Medieval Canterbury, UK	23	6-12	8.2	8	1.59
Fitzgerald, 1998	Medieval UK	31	7.8-11.2	9.87	9.20	0.86
Reid and Ferrell, 2006	Medieval, Denmark	84	6-12	8.5	8	1.1
Mahoney, 2008	Bronze age, UK	15	6-9	7.67	7	1.04
Modern day population						
Fitzgerald, 1998	South African	30	7.95-12.12	9.87	10	1.13
Reid and Dean, 2006[2]	South African	424	6-12	8.77	9[3]	1.1
Reid and Dean, 2006[2]	Northern European	298	6-11	8.37	8[3]	1.05
Reid and Dean, 2006[2]	North American	191	7-9	7.8	8[3]	0.63

[1]In some cases for the other studies included here, the *n* value may not indicate the number of individuals in the sample, but the number of teeth. [2]While Reid and Dean (2006) separated their data for anterior and posterior teeth, combined figures have been calculated for this table.

5.3 Stature and body mass

Despite specifically choosing the tallest and shortest individuals from the skeletal collection for this study, maximum and minimum values for both stature and body mass are within published ranges for adult medieval European males. As seen in Table

Figure 3. Stature distribution



8, a mean height of 172.16cm is similar to the mean height in cm of other medieval European samples and modern populations. The histogram in Figure 3 shows the distribution of height for this sample. This double peaked distribution is as expected, based on the nature of the sampling process. However, stature is still normally distributed ($p > .05$).

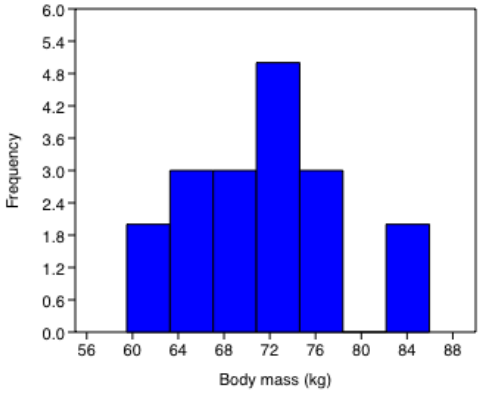
Table 8. Stature for male individuals (cm)*

	N	Population	Min	Max	Mean	SD
Medieval and archeological populations						
This study	23	Medieval Canterbury, UK	161.98	182.91	172.16	5.07
Hicks and Hicks (2001)[1]	<38	Medieval Canterbury, UK	163.6	184.1	173.6	-
Dawes and Magilton (1980)	240	10th – 16th century York, UK	153.0	184.0	169.3	-
Mays (1991)	9	13th-16th century Ipswich, UK	166.3	182.8	171.9	5.11
Lilley *et al,* (1994)	205	Medieval York, UK	155.0	190.0	171.2	
Gilde (2013)	37	Medieval Netherlands	160.18	181.84	171.69	-
Modern day Populations						
Moody (2013)	3154	British males	-	-	175.3	-
DoH, South Africa (2007)	121	South African males	-	-	168.0	-
Fryar *et al,* (2012)	5647	US males	-	-	175.9	-

*In all archeological data presented, stature estimates were made using the method of Trotter and Gleser (1958). For the modern data samples, standing height of living individuals was measured with a stadiometer. [1]Stature figures from Hicks and Hicks (2001) represent individuals from the same archeological collection as this study. For consistency and comparison, data is specifically chosen for male individuals of cemetery burial. Only one individual (SK 66) is represented in both Hicks and Hicks (2001) and this study.

For body mass, the sample size is slightly smaller than for the other variables, due to skeletal preservation. The comparative archeological data in Table 9 was selected because these previously published studies employed the same methodology as this study. At 71.26kg the mean body mass is slightly greater than other medieval UK collections mentioned here, but is notably

Figure 4. Body mass distribution



less than recent figures for modern day UK and US males. While lower than recent figures from the UK and US, average body mass from this medieval population is still greater than the body mass of contemporary South African males. Both in stature and body mass, the South African male is on average smaller than individuals from this study, and from modern UK and US populations. The standard deviation for body mass is larger than other medieval samples mentioned here, however once again, that is expected based on the sampling process. Body mass is normally distributed ($p > .05$), and is illustrated by the histogram in Figure 4.

Table 9. Body mass for male individuals (kg)

	N	Population	Min	Max	Mean	SD
Medieval and archeological populations						
This study	18	Medieval Canterbury, UK	59.50	85.95	71.26	7.19
Vercellotti *et al,* (2011)	31	Medieval Trino Vercellese, Italy	-	-	68.36	4.55
Lilley *et al,* (1994)[1]	125	Medieval York, UK	50.53	81.35	66.4	-
Pomeroy and Stock (2012)[2]	108	Archeological samples from central Andean coast and highlands of South America	-	-	59.6	5.28
Siegmund and Papageorgopoulou (2008)	291	Medieval Switzerland (various locations)	43.3	95.9	71.2	6.8
Weiss (2006)	58	6th-16th century California, USA	-	-	66.71	4.58
Modern day populations						
Moody (2013)	3172	British males			84	0.33
McDowell *et al* (2008)	4489	US males	-	-	88.3	0.46
DoH, South Africa (2007)	3275	South African males	-	-	65.8	0.42

[1]Body mass estimates have been calculated from femoral head measurements published by Lilley *et al,* (1994) using the equation by Ruff *et al,* (2001). [2]Figures included from the Ruff *et al,* (2001) methodology only.

5.4. Relationship between stature and body mass

Stature was tested as a linear, log and quadratic function of body mass (Fig.5). Although there was a significant and positive correlation between stature and body mass in each model (n=17, $p<0.01$), the quadratic function was the best fit, with the highest r^2 value of 0.446. A reduced major axis regression line was fitted

to the data to assess the relationship between stature and body mass in this sample (Fig.6). There was a positive correlation between log-stature and log-body mass that scaled with positive allometry (n= 18, r=0.67, r^2=0.45, *p*=0.002, slope = 3.37, 95% CI = 1.41 – 4.51, intercept = -5.69).

Figure 5. Curve fit for stature and body mass

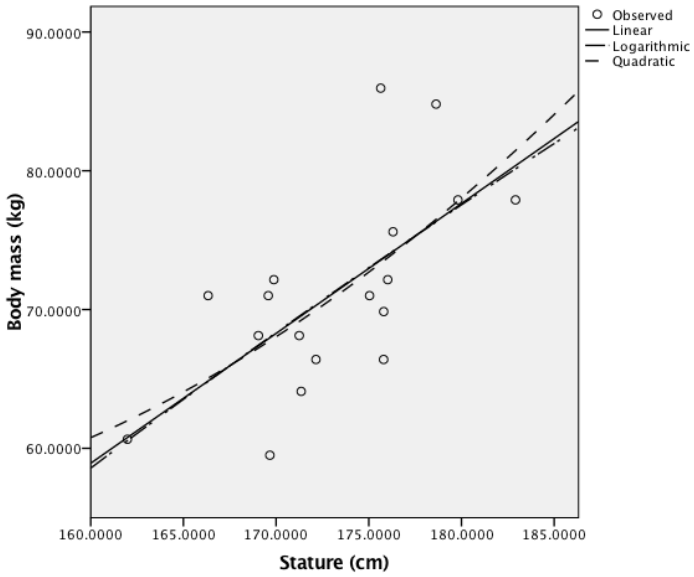
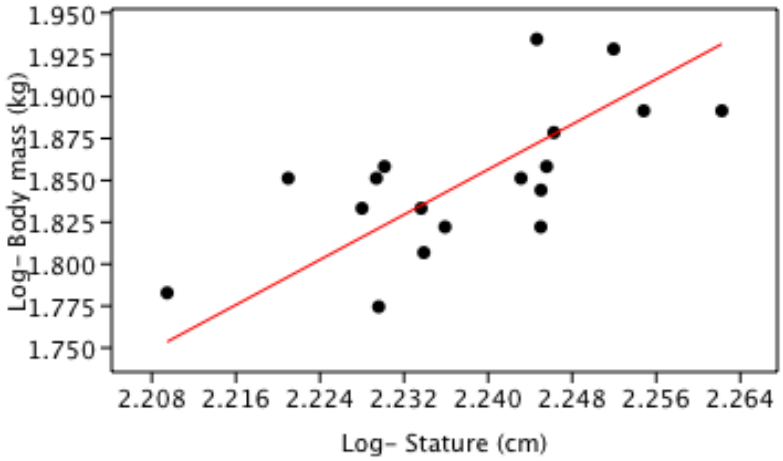


Figure 6. Stature and body mass

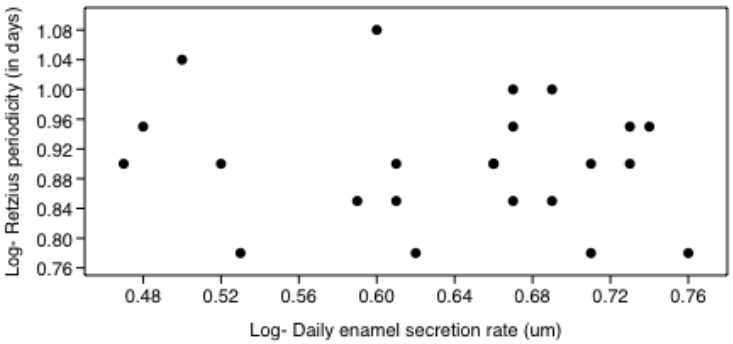


5.5 Exploring Associations with Retzius Periodicity

5.5.1 Retzius periodicity in days and daily enamel secretion rates

A reduced major axis regression line was fitted to the data to assess the relationship between Retzius periodicity and daily enamel secretion rates (Fig.7). The analysis found no correlation between Retzius periodicity and daily enamel secretion rates (n= 23, r= -0.15, r^2=0.02, p=0.48).

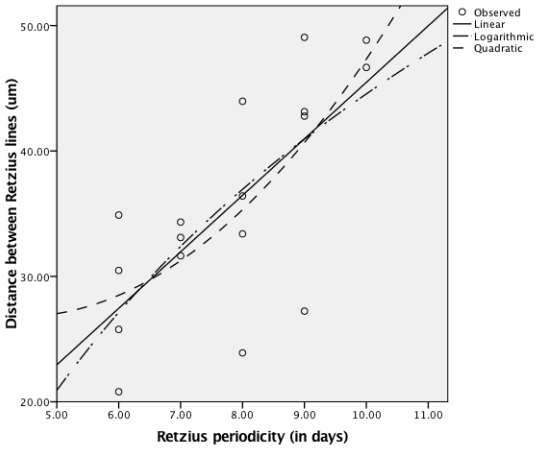
Figure 7. Retzius periodicity and DSR



5.5.2 Retzius periodicity in days and distance between adjacent Retzius lines in μm

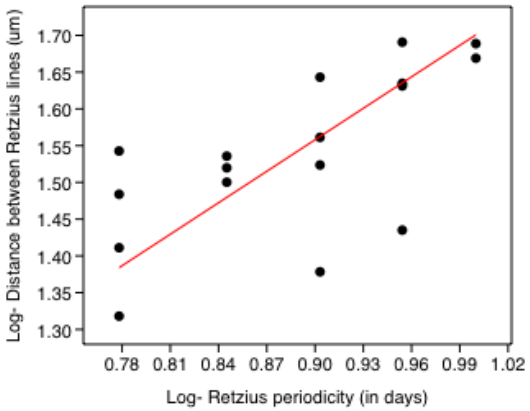
Retzius periodicity was tested as a linear, log, and quadratic function of the distance between adjacent lines, measured along an enamel prism (Fig.8). Although there was a significant and positive correlation between RP and the distance between adjacent Retzius lines in each model (n=17, p<0.05), the quadratic function was the best fit, with the highest r^2 value of 0.558. A reduced major axis regression line was fitted to the data to assess the scaling relationship between Retzius periodicity and the distance between adjacent Retzius lines (Fig.9). The RMA revealed a positive correlation between Retzius periodicity and the distance between

Figure 8. Curve fit for Retzius periodicity and Retzius line distances



Retzius lines that scales isometrically (n= 17, r=0.71, r^2=0.50, *p*=0.002, slope = 1.43, 95% CI = 0.96 – 1.85, intercept = 0.27; Fig.8). The coefficient of determination (or r-squared value) is likely lower here than expected due to the variation in enamel regions and teeth for which Retzius interval distances were measured.

Figure 9. Retzius periodicity and Retzius line distance



5.5.3 Retzius periodicity in days and stature in cm

Retzius periodicity was tested as a linear, log, and quadratic function of stature (Fig.10). Only the linear and log functions showed a significant negative correlation (n=23, *p*<0.05), with the log model displaying the highest r^2 value of 0.231. A reduced major axis regression line was fitted to the data to assess the scaling relationship between log-Retzius periodicity and log-stature. The analysis revealed that RP and stature are negatively correlated, and scales with negative allometry (n= 23, r= -0.49, r^2=0.24, *p*=0.02, slope = -6.58, 95% CI = -8.67 - -3.88, intercept = 15.61; Fig.11). When the analysis was repeated using just one tooth type, the molars, there was a slightly stronger negative correlation between periodicity and stature (n=14, r= -0.56, r^2=0.32, *p*=0.03, slope = -6.35, 95% CI = -8.99 - -2.39, intercept = 15.09; Fig.12).

Figure 10. Curve fit for Retzius periodicity and stature

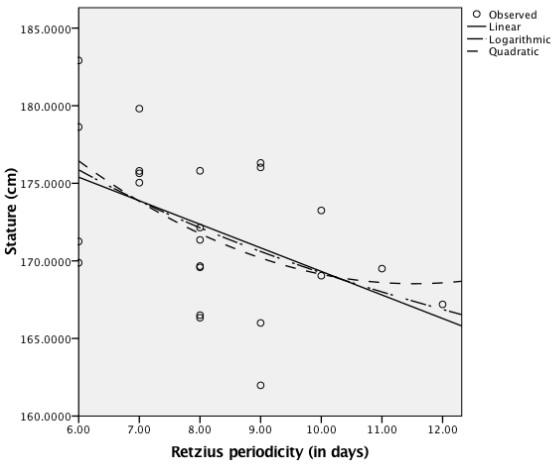


Figure 11. Retzius periodicity and stature (all teeth)

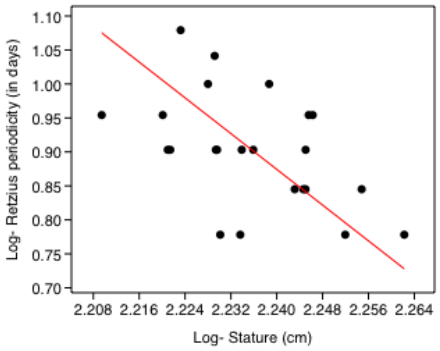
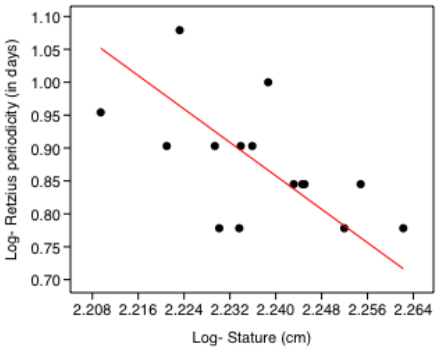


Figure 12. Retzius periodicity and stature (molars)



5.5.4 Retzius periodicity in days and body mass in kg

Retzius periodicity was tested as a linear, log and quadratic function of body mass (Fig.13). Although all models showed that the two variables were negatively correlated, none of the functions were statistically significant. A reduced major axis regression line was fitted to assess the scaling relationship between log-Retzius periodicity and log-body mass. The analysis revealed that RP and body mass are negatively correlated and scale with negative allometry (n= 18, r= -0.44, r^2=0.19, *p*=0.06, slope = -1.59, 95% CI = -2.16 - -0.73, intercept = 3.81; Fig.14). When the analysis was repeated with just the molar teeth from this study, a stronger negative correlation was found (n=12, r= -0.68, r^2=0.45, *p*=0.02, slope = -1.55, 95% CI = -1.95 – -0.69, intercept = 3.72; Fig.15).

Figure 13. Curve fit for Retzius periodicity and body mass

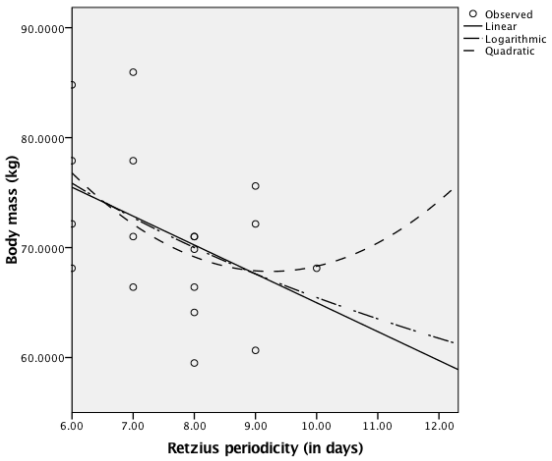


Figure 14. Retzius periodicity and body mass (all teeth)

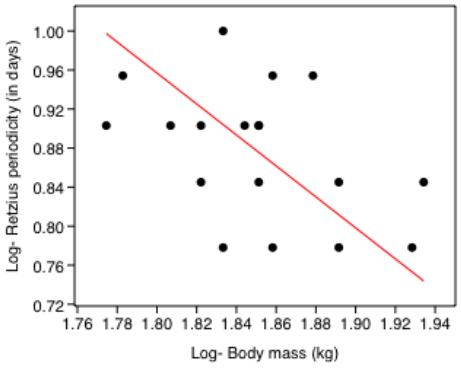
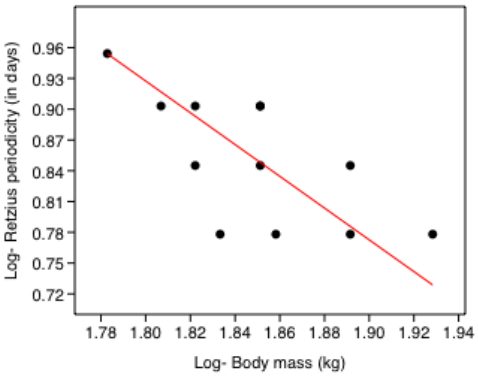


Figure 15. Retzius periodicity and body mass (molars)



6. Discussion

6.1.1 Retzius periodicity and body mass and stature

Results from the analysis of Retzius periodicity with body mass and stature support the first hypothesis (section 1.1), that Retzius periodicity and body size are negatively correlated in humans. Subsequently, these results reject both the null hypothesis and hypothesis two. While the relationship between Retzius periodicity and stature had initially been described for just six humans of known life history (Bromage *et al.,* 2015), this study provides evidence for a negative correlation between RP and stature with data from 23 male individuals of medieval UK origin (section 5.5.3). Similarly, while a negative correlation between Retzius periodicity and body mass has been reported for four individuals (Bromage *et al.,* 2015), this study provides further support for such a relationship with 18 male individuals (section 5.5.4). Overall, these results provide support for the existence of an intraspecific HHO, in which increases in body size are achieved with an accelerated biorhythm, and subsequent lower Retzius periodicity. Individuals with higher RPs were of smaller stature and body mass than those with a lower RP.

Interestingly, these results agree with another component of the Bromage *et al*. (2009, 2015) studies not yet discussed. As was described in section 2.4, bone cell proliferation rates negatively correlate with body mass across the primate order, demonstrating that smaller mammals have faster life histories and increased osteoblast proliferation rates, while in contrast, larger mammals with slow life histories are characterised by slower cell proliferation rates. Importantly, Bromage *et al*. also collected similar data on a small sample of modern humans as part of two studies. From both studies, the first with five females from the Melbourne Femur Collection in Australia (2009), and the second with a mixed-sex group of twelve individuals of sub-Saharan African (Malawi) Bantu origin (2015), the relationship between osteocyte density and body height showed a positive correlation between the variables. As with the results of this study, this

finding is also the opposite of what is seen between species. In the more recent and larger human study, the relationship was only statistically significant after an outlier with unusually thin cortical bone and a history of malnutrition was removed. However, that Bromage *et al.* (2015) found a significant correlation despite not controlling for age, which is known to affect osteocyte density (Mullender *et al.,* 1996b), suggests that a relatively stable positive relationship may exist between osteocyte densities and body size. Based on this positive correlation, with taller individuals showing greater osteocyte densities, and smaller individuals showing less osteocyte lacuna per millimeter, these results suggest that bone and body size increases within humans are accomplished with increased rates of cell proliferation.

Subsequently, it appears that while interspecific cell proliferation rates and the HHO are slower with larger body size, the opposite is true intra-specifically, with larger humans displaying increased rates of cell proliferation and accelerated HHOs. The important factor in understanding these intraspecific results in the context of what is known inter-specifically may relate to the timing of life history events, or specifically, the duration of growth. Across species, larger mammals display reduced cell proliferation rates and a slower HHO, but grow over a longer period of time. As mentioned in 2.4, it is thought that this extended growth period is long enough to eventually overcome the slower pace of growth (Bromage *et al.,* 2012). Differences in developmental time then are important for understanding the general relationship seen across species. However, within humans, the duration of growth is relatively constrained, especially within a single human population (Eveleth and Tanner, 1990). Thus, to achieve a larger body size in humans, rates of cellular proliferation and growth must increase, which correspond with an accelerated HHO and lower RP.

With evidence of a negative correlation between Retzius periodicity and body mass in a suitably sized sample of modern humans (section 5.5), and a positive correlation between cell proliferation rates and body size (Bromage *et al.,* 2015), evidence seems to suggest that the HHO may also influence growth in humans. While the direction of the relationships are the opposite of what is seen across

species, this can be explained by differences in life history timing across species, and constraints in such timings within humans. Ultimately, it is likely then that similar physiological mechanisms exist both inter-specifically and intra-specifically. As will be described in further detail below, these findings provide important early insights into what RP variability in humans represents. Consequently, variation in RP in humans turns from a seemingly random and unusual feature of enamel, into a potentially exciting new variable in the study of human life history and growth. Furthermore, these results may help to understand previously unexplained trends observed in RP expression across human populations, while also extending it's reach to the study of fossil hominins and, perhaps in the future, will provide researchers with a powerful new analytical tool for studying growth, life history and evolution in humans.

6.1.2 Retzius periodicity between different tooth categories

The relationship between periodicity and body size showed a moderate negative correlation. When the same statistical tests were conducted with just the molar teeth in this study, the strength of the relationship increased (section 5.5.3 and 5.5.4). However, based on the widely accepted notion of RP homogeneity along the tooth row (Fitzgerald, 1998), it is assumed that the increased statistical strength observed is simply random and that if anterior teeth were sampled from the same molar individuals, an identical statistical result would have been found. Similarly, while this study showed a slightly higher average periodicity in anterior than posterior teeth, this finding is likely due to the small sample size of anterior teeth and not indicative of an actual difference between tooth categories. When comparing anterior and posterior teeth from the comparative data sets provided, Reid and Ferrell (2006) reported very similar results between the two tooth categories, and Reid and Dean (2006) a slightly higher mean periodicity for anterior teeth. While Fitzgerald (1998) reported a comparatively high mean periodicity of 9.7 days for a sample of 96 anterior teeth, molars were not sampled for comparison. It is assumed that had molars been sampled from the same individuals, a similar result would have been found.

The high periodicity reported by Fitzgerald (1998) may be due to an unknown sampling bias of individuals with particularly small body size, a methodological overestimation, or another currently unknown factor.

6.1.3 Daily secretion rates

Daily secretion rates from all teeth fell within maximum and minimum values described in other studies, and mean values for both anterior and posterior teeth were also comparable to published data sets (section 5.1). The standard deviation in this study however, was much higher than for other published results. As previously mentioned, this was likely due to the variable location of these measurements throughout the enamel in this study. For example, while many of the other data sets calculated and reported DSR at very specific locations and depths (Reid *et al.,* 1998; Schwartz *et al.,* 2001), thereby creating relatively homologous results, DSR measurements in this study were taken at more variable locations that corresponded with the clear identification of Retzius periodicity counts. Subsequently, while the majority of DSR calculations were made from mid to outer lateral enamel, in some cases DSR was measured in inner lateral enamel, at the border between cuspal and lateral enamel, and towards the outermost lateral enamel where convergent Retzius lines were occasionally observed. As DSR is known to differ throughout the tooth, generally increasing from inner to outer regions and greater in cuspal than lateral enamel (Smith, 2004), this contributes to an explanation of why the standard deviation is higher than reported for other studies. Additionally, as sample sizes for certain tooth types were small, DSR data was grouped into anterior and posterior tooth categories, meaning that descriptive statistics for DSR represent not just different enamel regions but also different tooth types. Had a different approach been taken and DSR been calculated at consistent regions of the enamel crown for each tooth irrespective of RP count locations, it is assumed that the standard deviation would have been much lower and comparable to the other data sets provided. While it is not expected that unusual DSR values would have affected RP counts, especially as all RP was visually determined, these results provide some indication of normal enamel growth in this sample, especially surrounding

the region of RP count locations, and demonstrate that the individuals in this study are likely to be normal representations of modern humans.

Data was also used to assess the relationship between DSR and RP in the sample. The analysis found no correlation between DSR and RP (section 5.5.1). However important considerations need to be made regarding this result. As mentioned, DSR was taken from variable locations throughout the enamel. Therefore it is very possible that a relationship might not have been found even if it did exist. Mahoney *et al.* (2016a) found that RP and enamel thickness were positively correlated in deciduous teeth, however they also reported that RP was positively correlated with enamel formation times. Therefore it is likely that higher RP and longer formation times are linked in the production of thicker enamel, and not through variation in DSR. Currently the relationship between RP, DSR and enamel thickness has not been confidently and directly addressed yet for permanent teeth. Reid and Ferrell (2006) reported that enamel formation times in permanent canines were longer with lower RPs, while shorter formation times were associated with higher RPs. If DSR does not vary significantly between high and low RPs, then this relationship, along with the relationship between RP and body size confirmed by this research, should result in thicker enamel for individuals of larger body size, and thinner enamel for those of smaller body size, as should be perhaps expected.

6.1.4 Retzius periodicity and distance between Retzius lines

Results show that Retzius periodicity scaled positively and isometrically with the distance measured between adjacent Retzius lines (section 5.5.2). As previously mentioned, RP does not then seem to correlates with or influence the rate at which enamel is deposited. However, as expected, it does correlate with the amount of enamel deposited between Retzius lines, i.e. higher RPs result in thicker layers of enamel between adjacent Retzius lines. Importantly, if DSR was correlated or associated with RP in some way, this isometric positive relationship may not have been observed. While the strength of the relationship is perhaps lower than may be expected, this is likely due to the variable locations

in the enamel for which Retzius interval distances were measured. Ultimately, this once again suggests that if RP and enamel thickness are correlated in any way, it is likely going to be through a relationship with enamel formation time and not DSR. If a relationship is found between the RP and enamel thickness, as it is for deciduous teeth, it will be interesting to consider why and what linking variables, if any, are involved.

6.1.5 Retzius periodicity

In some respects, the data presented here for Retzius periodicity is comparable to other published data sets. Specifically, RP for each individual fell within the known periodicity values for permanent teeth and a slightly positive skew was observed in the distribution (section 5.2). In other ways however, the data differs from previously published results. For example, there was a notable difference in the standard deviation of RP in this study. Without consideration or knowledge of the primary findings of this study, such a comparatively large standard deviation for Retzius periodicity may be considered unusual. Now, with an understanding of the relationship between RP and body size, the large standard deviation in periodicity simply becomes a consequence of the body size sampling process. This may then also contribute to an explanation of why RP in this study covers the full range of currently known periodicities values for permanent teeth, despite having a relatively small sample size. However, as was mentioned in the results section, this small sample of 23 male individuals show a greater range in periodicity than the 298 Northern European and 191 North American individuals studied by Reid and Dean (2006). If the scaling factor and strength of the observed relationship between Retzius periodicity and body size holds relatively stable for all human populations, it seems unlikely that these other samples did not include individuals of body sizes that would correspond with periodicities of both six and 12 days. Whether other factors contribute or influence the distribution and expression of Retzius periodicities in certain populations is worthy of consideration. Alternatively, the range and distribution of periodicity values here may relate to the accuracy of the methodology. For

example, as many early studies would have relied on an eyepiece reticle and employed a non-digital version of the measurement method to calculate Retzius periodicity, this could have introduced a certain degree of subjectivity or error in data collection and prompted more conservative rounding of decimal values. In comparison, this study was able to report clear and confident, visually apparent Retzius periodicities using a high-powered microscope and imaging software. Whether a growing number of studies will also begin to report higher frequencies of extreme RP values remains to be seen.

6.1.6 Stature and body mass

Despite specifically choosing the largest and smallest individuals from the skeletal collection, maximum and minimum values for both stature and body mass are within published ranges for adult medieval European males (section 5.3). Additionally, despite their medieval origin, mean stature was comparable to the modern day values provided and actually exceeded the mean stature of modern day South African males. As will be described in section 6.6.1, environmental influences and insults on adult height and growth in medieval Europe may not have been as pronounced and severe as classic historical texts suggest. Subsequently, this sample is likely to be fair representation of a European medieval population, and a modern human sample in general.

While comparable to other medieval populations, average body mass in this sample is significantly lower than recent figures from the UK and US (section 5.3). Importantly though, notable differences exist in how these measurements were made. For the medieval collection, body mass is calculated from skeletal dimensions that do not significantly change with behavioural or environmental influence throughout life, and so more accurately reflect a genetically-determined young adult body mass (Trinkaus *et al.*, 1994; Lieberman *et al.*, 2001). Conversely, for the modern populations, body mass was calculated from living body weights and therefore accurately reflects current environmental and behavioural body transformations, such as obesity caused by excessive food intake. Consequently, it is not unusual that body mass in this sample would be

lower than most modern values. What is perhaps unusual is that the average body mass from this medieval population is greater than the body mass of contemporary South African males. Both in stature and body mass, the South African male is on average smaller than individuals from this study, and from modern UK and US populations. As the average South African Retzius periodicity is also higher that these other populations, this may be an interesting point of consideration in relation to the main assumptions and findings of this study.

6.2 The origin of the HHO

Although the interspecific and intraspecific RP and body size correlations are in opposite directions, this is likely due to differences in life history timing and growth duration in the former, and constraints of these in the latter. Ultimately, it seems highly likely that similar physiological structures and mechanisms are responsible in both cases. As the periodicity of this rhythm in enamel has been shown, for some mammal species so far, to match the periodicity of growth increments in bone (Bromage *et al*., 2009), and also correlate with almost all life history traits across species (Bromage *et al.,* 2012), a strong case exists for the presence of a centrally regulated, systemic biological rhythm. Centrally regulated processes associated with growth ensure that physiological components grow at the right ratios to each other, at the right times, and at the right speeds (Raff, 1996). To coordinate and synchronise the combined growth, metabolism, and reproductive development and function associated with the mammalian life history package, a centrally regulated systemic rhythm would appear to be necessary. Additionally, some evidence has been presented that suggests tissue-specific metabolic rates, and perhaps therefore cell proliferation rates, also require a centrally regulated mechanism to function in a species-specific manner. For example, numerous studies have shown that cultured mammalian cells do not scale their mass-dependent metabolic behaviors as they do *in vivo* (West *et al*., 2002; Brown *et al*., 2007; Robb *et al*., 2012). This would indicate that mammalian cells do not have intrinsic, species-specific, metabolic rate set points controlled by peripheral clocks, but that differences in SMR, and therefore cell

proliferation rates, are the result of centralized, systemic control mechanisms. Subsequently then, if one attempts to search for the potential sources and pathways of a systemic rhythm related to growth and reproductive development and function, the anterior pituitary looks to be a strong candidate in the process.

The anterior pituitary is one of two lobes in humans that make up the pituitary gland, and has a central role in mammalian physiology and development. Through the release and inhibition of various hormones that target the thyroid gland, gonads, liver, and adrenal gland, the anterior pituitary plays an important role in growth, metabolism, lactation and reproduction (Le Tissier *et al.,* 2012). Subsequently, anterior pituitary outputs appear to be directly related to many of the processes shaping RP-correlated life history variation. As anterior pituitary activity is regulated by hormones secreted by the hypothalamus, this also suggests that the HHO may have a hypothalamic origin. There are several hypothalamic nuclei that are known to influence anterior pituitary outputs. These include the arcuate nuclues, supraoptic nucleus, preoptic area, paraventricular nucleus, and among them, the rhythmic SCN (Dibner *et al.,* 2010). Importantly, based on the HHOs seemingly wide influence throughout the body, it would seem unlikely that a single hypothalamic nucleus would be solely implicated in HHO expression. As described in section 2.8, the dense hypothalamic efferents that exist between many of these hypothalamic nuclei suggest that important endocrine-related communications and shared functions exist between these hypothalamic structures. Also, as was outlined in section 2.9, the interplay between nuclei and their signaling pathways are often essential for generating or suppressing a specific response. Providing one final example of this, the median preoptic nucleus (MnPO) is the main integration center for thermoregulatory processes (Guzman-Ruiz *et al.,* 2015). As the time of day plays an important role in determining the temperature set point, anticipatory regulation by the MnPO is partially influenced by the SCN (Buijs and Kalsbeek, 2001; Scheer *et al*., 2005). However, core body temperature is also influenced by feeding times (Dibner *et al.,* 2010). As the arcuate nucleus (ARC) is known to regulate feeding behaviours, rhythms in core body temperature are therefore thought to depend on the interplay between temporal signals from the SCN and

metabolic signals from the ARC (Dibner *et al.,* 2010). Importantly, as the ARC is implicated in energy homeostasis, metabolism, and the regulation of body weight (Biebermann *et al*., 2006; Lee *et al*., 2006), Bromage *et al*. (2012) has suggested that this nuclei, located in the mediobasal hypothalamus, may be a particularly important candidate in the regulation and expression of the HHO. Based on its specific functions, this certainly seems possible. However, studies have shown that SCN-lesioned rats show a complete disappearance of rhythmic activity in the ARC (Angeles-Castellanos *et al.,* 2010; Li *et al.,* 2012). Therefore, even if the ARC is responsible for the HHO, the SCN is likely to occupy a role in its regulation or expression. Currently then, the most promising hypothetical scenario points to the HHO being of hypothalamic origin and that, through a set of SCN-integrated nuclei, signals are transmitted to the anterior pituitary, which in turn regulates and controls various aspects of growth, development, metabolism, and reproductive function, and consequently, variation in body size and life history. While the intraspecific influences of the HHO are currently limited to adult body size, it is likely that similar mechanisms exist in both cases and that some of the same physiological structures described here are responsible.

Importantly, based on the results of this research, and the current hypothetical explanations regarding the origin of the HHO, researchers can now begin developing new hypotheses for what RP may represent, how hypothalamic and anterior pituitary functions may regulate both bone and body mass, and how growth-related variables may manifest in dental enamel. For example, based on the premise that the HHO is of hypothalamic origin and influences both bone and body mass, Bromage *et al*. (2009) has suggested that the system might be intricately related to the neuroendocrine functions of leptin. Leptin is a circulating hormone that primarily regulates energy balance, satiety and body mass, but also contributes to many other physiological processes (Friedman and Halaas, 1998). In particular, studies have reported that leptin-deficient and leptin receptor–deficient mice display increased bone formation leading to high bone mass, and that hypothalamic administration of leptin decreased bone mass in these mice by inhibiting bone formation through a pathway involving increased sympathetic signaling (Ducy *et al.,* 2000; Karsenty, 2006).

Subsequently, while it was assumed that bone formation was regulated by autocrine and paracrine mechanisms, these studies provided evidence that endocrine regulators are involved in the process. The authors also suggested that leptin used similar receptors to control both body weight and bone mass, therein providing a mechanism linking the control of bone mass with the regulation of body weight. From this, Takeda *et al.* (2003) described numerous hypothalamic structures that could be implicated in this leptin-mediated regulation of bone and body mass. Consequently, these findings prompted Bromage *et al.* (2009) to suggest that leptin may be an important factor in the regulation of the HHO. Unfortunately however, more recent studies have reported conflicting evidence. For example, Turner *et al.* (2013) reported that bone formation rates were *lower* in leptin-deficient mice, and that leptin appeared to increase bone formation by increasing osteoblast numbers. Turner *et al*. (2013) also provided evidence that while increasing leptin levels in the hypothalamus of leptin-deficent mice did normalized bone mass, leptin primarily acts through peripheral pathways, and not through a hypothalamic relay as was previously suggested. Importantly, these studies report contradictory evidence regarding the precise role leptin plays in bone formation, and also how these processes are regulated. They also suggest that peripheral clocks, and not centrally regulated endocrine factors, play the more important role in bone growth. Leptin appears to have numerous functions as a growth factor in a range of different cell types. Acting as an endocrine, paracrine, and perhaps an autocrine factor (Margetic *et al.,* 2002), the general complexity of the leptin axis renders current hypotheses regarding its relative importance or relevance in a centrally regulated growth rhythm difficult. Nevertheless, it still remains plausible that leptin plays a contributory role in HHO expression or function.

To provide one theoretical possibility on how hypothalamic growth-related variables may influence dental enamel, attention is drawn to transforming growth factor beta-1 (TGFβ1). TGFβ1, synthesized by the thyroid gland, is a protein that circulates in blood plasma and regulates cellular proliferation, cell growth and cell differentiation in a wide variety of different tissues (Pisarev *et al.,* 2009). In skeletal tissue specifically, TGFβ1 plays an important role in the

development, maintenance and metabolism of bone and cartilage (Janssens *et al.,* 2005). In addition to its role in cell proliferation and growth, TGFβ1 is also expressed in ameloblasts and promotes MMP20 expression (Gao *et al*., 2009). As mentioned in section 2.2, MMP20 is the predominant secretory stage protease that functions to degrade the enamel proteins between the crystal ribbons, allowing the crystals to grow in width and thickness. *Mmp20* null mice do not degrade amelogenin properly, resulting in altered enamel rod patterns and hypoplastic enamel (Guan and Bartlett, 2013). Interestingly, Shin *et al.* (2014) have shown that the overexpression of MMP20 also results in poorly mineralized enamel. While the precise structural or chemical nature of Retzius lines are unknown, it is not completely implausible that they may resemble the poorly mineralized enamel associated with MMP20 overexpression. Hypothetically then, rhythmic fluctuations in TGFβ1 could influence both cell proliferation rates, and secretory activity in ameloblasts, causing the formation of a Retzius line. While there appears to be little to no evidence for robust long-period rhythmic activity in TGFβ1 or TGFβ1 activators, TGFβ1 does play an important role in wound healing (Beck *et al.,* 1993; O'Kane and Ferguson, 1997), which, as mentioned in section 2.6.5, appears to display a circaseptan periodicity in humans (Pöllmann, 1984).

Obviously, these early and tentative hypotheses may be limited or flawed in various respects. However, both the influences of leptin discussed by Bromage *et al.* (2009), and the rough TGFβ1-based theory provided here, are examples of how future research may build on the primary findings of this research by developing theoretical and empirical frameworks linking hypothalamic outputs and growth factors, with bone and body masses, and fluctuations in ameloblast activity. Importantly, these hypotheses and considerations regarding the contributions the HHO makes to adult body size and life history should, for now, remain widely inclusive of all growth factors and not limited to hypothalamic and anterior pituitary outputs. While the hypothalamic HHO remains the most promising candidate for the regulation of body size and life history across and perhaps within species, the precise contribution of the HHO to bone and body growth remains to be determined. As described, while some studies have

provided evidence to suggest that leptin-mediated hypothalamic processes are important in bone and body growth (Ducy *et al.,* 2000; Karsenty, 2006), other have reported the opposite and suggested that bone growth is primarily guided through peripheral clocks and pathways (Turner *et al.,* 2013). Furthermore, while evidence was described earlier that cultured mammalian cells do not scale their mass-dependent metabolic behaviors and that differences in SMR are the result of a centralized, systemic control mechanisms (West *et al.,* 2002; Brown *et al*., 2007; Robb *et al*., 2012), all of the these studies have been criticized for the types of cells used (Glazier, 2014). The additional difficulty in unraveling and determining the central and peripheral contributions to growth is that systemic control of growth appears to vary between organs. For example, transplanting multiple fetal thymus glands into a developing mouse causes each gland to continue to grow to normal adult size (Metcalf, 1963). This suggests that growth in this case is mainly controlled by peripheral mechanisms. However, if a similar experiment is carried out with multiple fetal spleens, the opposite result is observed and the combined mass of the two spleens only equals the mass of one normal adult spleen (Metcalf, 1964). This would suggest that spleen growth is mainly controlled by centrally regulated mechanisms.

There is clearly no way of overestimating the importance of centrally regulated growth within an organism. As stated, centrally regulated mechanisms ensure that physiological components grow with the right ratios to each other and at the same times (Raff, 1996). Growth hormone (GH), thyroid-stimulating hormone (TSH), and follicle-stimulating hormone (FSH) are some examples of anterior pituitary outputs that regulate and coordinate numerous aspects of growth and reproductive development for numerous different organs and tissues (Norman, 1997). Consequently, at this time, it appears right to attribute the findings of this research to a hypothetical, hypothalamic HHO. However, directly determining this, and unraveling the specific contributions that the HHO makes to growth and development, remains a significant but exciting challenge for the future.

6.3 The HHO in deciduous teeth

An additional point of interest regarding the current intraspecific HHO hypothesis relates to the recent findings by Mahoney *et al*. (2016a). Specifically, Mahoney *et al.* (2016a) have shown that HHO patterns and correlations may not be as simple as are shown and described here. Testing the intraspecific predictions of the HHO hypothesis on a skeletal sample of human juveniles from the same archeological collection using in this study, Mahoney *et al*. (2016a) calculated RP in the deciduous enamel of 25 age-matched individuals and compared these values with the average area of primary osteon vascular canals in their humeri. While this bone growth variable differs from those used by Bromage *et al*. (2009), canal size is also a good indication of bone growth, with smaller canal sizes reflecting increased primary bone deposition. While there appeared to be a relationship between RP and bone growth in their juvenile sample, as the HHO would predict, Mahoney *et al.* (2016a) reported that higher RP values corresponded with *increased* primary bone deposition. Children with 7-8-day periodicities had smaller osteon canal areas than those with 6-day periodicities. Unexpectedly then, the direction of this relationship is the opposite of what the HHO hypothesis would predict. While lower RP correlates with increased bone formation and body size in adults, a slower oscillation appears to correlate with increased bone formation in children. Additionally, Mahoney *et al.* (2016a) compared deciduous RP in this sample with average enamel thickness, and reported a positive correlation between these variables. Higher RPs correlated with thicker enamel, while low RP corresponded with thinner enamel. While this supports a prediction that RP and enamel thickness may be related, perhaps through their associations with body size, the direction of the relationship was again the opposite of what the intraspecific HHO would predict. While these findings collectively support a hypothesis that RP and bone growth variables are related, they show that the direction of the relationship differs between deciduous and permanent teeth.

Currently then, it is difficult to reconcile how or why the opposite relationship would be found with deciduous RP. Importantly, for these findings and also for a

general understanding of the HHO, Mahoney *et al.* (2016a) report a lower range of RP values in their deciduous sample than is currently known for permanent enamel. With a range between four and 11 days, and with 26% of their values occupying periodicities outside the known range reported for permanent teeth, their data suggests that RP may not be the same between deciduous and permanent teeth. While Mahoney (2012) reported that RP did not vary between a deciduous and permanent molar from one individual, it may be that in some cases, the HHO changes with age. Mahoney *et al*. (2016a) have tentatively suggested that this shift in periodicity may indicate that humans follow an inter-specific, ancestral HHO pattern early in development, but adjust to an intraspecific, derived version later in childhood. Overall, these findings by Mahoney *et al.* (2016a) do not necessarily refute the intraspecific HHO hypothesis as described throughout this discussion. They do however indicate that the HHO may be more complex in its nature or function than originally suggested. Furthermore, while the HHO appears remarkably robust throughout the period of permanent lateral enamel formation, Mahoney *et al.* (2016a) provides the first evidence that the HHO may change with age. Subsequently, studies may need to consider age as an important variable when conducting future research on this topic.

6.4 Retzius periodicity and body mass variation

Another important aspect of human Retzius periodicity that requires brief mention is large range of observed RP compared with other primates and mammals. The known human permanent Retzius periodicity ranges from 6-12 days (or from 4-12 days with the inclusion of deciduous teeth). In comparison, the commonly reported range of periodicity values is only 6-7 days in *Pan troglogytes*, 7-10 days in *Gorilla gorilla*, and 8-11 days in *Pongo pygmaeus* (Schwartz *et al.,* 2001b). Importantly, this does not appear to be due to small sample sizes. For example, studies reporting RPs of 6-7 days in *Pan troglogytes* are based on combined sample sizes of 135 individuals, while RPs of only eight days have been found among 98 *Macaca nemestrina* individuals (Hu *et al.,* 2012

and references therein). Briefly addressing this, Bromage *et al.* (2009) state that "repeat intervals in Homo sapiens are highly variable, but modern humans also have an unusually high body mass variability for a primate"(p.398). However, a brief read through the literature on primate body mass variability suggests this statement may not be as accurate as it implies. For example, Smith and Jungers (1997) conducted a meta-analysis on primate body mass variability for 19 species, including in their analysis both wild and laboratory animals, and reported coefficients of variation (CV) that ranged from 4.3 to 19.5, with a mean of 12.5. While humans occupied the higher end of the spectrum with a CV of 15.8, body mass variability was greater in numerous other primate species. In *Pan troglogytes,* body mass CV was 11.2 and 14.1 for males and females respectively. Therefore, while *P. troglogytes* body mass variability does appear to be slightly lower than in humans, the difference is not as great as their periodicity values would suggest. Smith and Jungers (1997) do mention that laboratory animals are included in some of their figures. As laboratory and zoo animals can be up to 80% heavier than their wild counterparts (Fooden and Izor, 1983; Leigh 1992), this will have significantly influenced some of their CV figures. However, the data for the *P. troglogytes* body mass variability come from wild chimpanzees populations in Mahale, Tanzani, and so is unlikely to include the extreme or unusual values associated with captive individuals. Whether larger sample sizes would reveal different results is unknown. However currently this data does indicate that some non-human primate body masses are at least as variable, if not more variable relative in size, as humans.

It is perhaps justifiable to concede that human body size variability may be greater than other primate species when pathological body types, pygmy populations, and clinically obese and anorexic individuals are included in this statement. However these extreme examples are not represented in this study, yet the entire range of human periodicity values were observed. While the largest and smallest individuals from the initial sample were chosen, the results and comparative data sets provided in section 5.3 show that the sample in this study does not represent individuals of extreme or abnormal body type. Subsequently, it may be that an additional explanation is needed for why humans

display such a large range of periodicity values. Whether such an explanation can be found when accounting for the high brain mass variability that may accompany high body mass variability in humans (Herculano-Houzel, 2009), remains to be determined and is one of many hypotheses that are beyond the scope of this thesis to discuss further. Interestingly, whatever the cause may be, whether purely mass related or combined with other factors, its discovery will likely provide fascinating insights into hominin evolution. While non-human primates display a significantly smaller range of RP compared with humans, the australopithecines appear to more closely resemble humans. For example, reported periodicities for *Paranthropus robustus* range from 6-12 days, while *Australopithecus africanus* range from 6-11 days (Smith *et al.,* 2015). Whether this suggests that profound changes in growth rate variability and body size had evolved in the hominin lineage as early as the Late Pliocene, remains to be investigated.

6.5 Population differences and sexual dimorphism

While the recent mass-related RP findings have arguably created more questions than they have answered, the results of this study do now provide an opportunity to look back on old questions or trends with fresh perspectives. For example, very little was known about whether Retzius periodicity differed between the sexes. In the few cases where differences were reported, it was unclear as to why. For example, from two large human samples of known-sex, Smith *et al.* (2007) reported that South African females displayed a higher periodicity than their male counterparts, but found no differences between the sexes in their North American sample. Now, with an understanding of the relationship between Retzius periodicity and body size, these findings can be reconsidered.

Sexual size dimorphism is a common feature of the animal kingdom, and for most mammals and birds, the male is the larger sex. In humans, average sexual size dimorphism (SSD) is approximately 1.07. Importantly however, variation

exists in the degree of SSD between populations (Gaulin and Boster, 1985; Gustafsson and Lindenfors, 2004). For example, the Mountain Ok population of Papua New Guinea show a SSD approximately 8% higher than some other populations of low SSD (Eveleth and Tanner, 1990). While some authors have argued that population differences in SSD do not actually exist and that statistical findings are mainly a function of within-population sample sizes (Gaulin and Boster, 1985), or that differences in SSD do exist but reflect cultural and/or nutritional differences and not genetic factors (Gaulin and Boster, 1992; Holden and Mace, 1999), other studies have reported that sexual size dimorphism shows a highly significant association with phylogeny and genetics (Reeve and Fairbairn, 1996; Badyaev, 2002; Mank, 2009). It is conceivable then that individuals in the South African sample studied by Smith *et al.* (2007) showed a large enough degree of sexual size dimorphism to result in statistically significant higher periodicities among the females, whereas the other populations did not. Unfortunately, the degree of sexual size dimorphism in this population is unknown and therefore this assumption remains speculative.

It should also be considered that differences in RP might exist between the sexes that are independent of body size. While sexual size dimorphism is a simple explanation for why some studies have reported differences in RP between the sexes, the relationship may be more complex than this. In particular, differences may be somewhat complicated by the differing growth durations between males and females. As males have approximately three additional years to grow to adult size (Eveleth and Tanner, 1990), they may not need a faster HHO to achieve a comparatively larger body size. Unfortunately, as the primary objective of this study was to test the relationship between Retzius periodicity and body size, only individuals of one sex were chosen for histological sampling. This decision was made so that any confounding factors or unknown differences between the sexes, in any of the studied variables, would not create unwanted statistical noise. However by doing so, this study could not test for sex differences in periodicity that were independent of body size.

The results of this study may also now shed light on why population differences in RP have been observed. Initially, it was not understood why slight population

differences were observed in RP. For example, mean RP was 9.87 in a South African population, but 7.8 in a larger North American sample. With the results of this study, these differences may now be attributed to differences in body size. While the body size figures are not from the same individuals that RP was calculated, modern day South African male mean stature is 7.8cm shorter than US and UK populations, and body masses are approximately 20kg lighter on average. While these body size differences may partially be attributed to cultural or nutritional disparities between the populations, the general trend nevertheless aligns with their periodicity values. Consequently, population differences in RP may partially reflect differences in body size. Obviously, as with the sex differences, this assertion is likely to be more complex than is stated here. For example, growth durations and age at skeletal and sexual maturity differ across populations based on genetic and non-genetic factors (Cavalli-Sforza and Bodmer, 1999). Also, just as the elephant and male gorilla share similar RP values but have notably distinct body sizes due to phylogenetic differences, so too could different populations but to a lesser extent. While the precise relationship between RP and body size is likely to differ between individuals of different population or sex, the results of this study provide previously absent hypotheses for why studies have observed differences in average RP across different human groups.

6.6 Limitations

6.6.1 Environmental influences on body size

While observations of incremental development or growth rate variables may be limited somewhat in their accuracy due to methodological limitations, adult body size as a variable used for assessing growth is additionally and significantly limited by environmental conditions experienced during development. As the skeletal sample in this study represents a low status medieval population, there are many reasons to assume that environmental insult would have been high. The following discussion briefly addresses the genetic contribution to body size,

and discusses some of the more influential and severe environmental influences on growth and how prevalent these may have been for this medieval population. This section primarily focuses on discussing the genetic and environmental influences on stature, with only brief mention of body mass. This is because body mass is extremely variable throughout the entire lifespan of an individual, and adequate discussion of all factors that influence mass is far beyond the scope of this section.

As stated, final adult body height is significantly influenced by environmental conditions experienced during growth. However there is also undoubtedly an underlying genetic basis to body size. Large-scale twin studies and genomic analyses have shown that genetics have a significant influence on growth trajectory (Ruff, 2002; Dubois *et al*., 2007), and long bone length and stature (Silventoinen *et al.,* 2003; Morris *et al.,* 2012). Current research suggests that in modern, affluent populations, approximately 80% of the observed variation in body height is genetic (Silventoinen *et al.,* 2003). However, many other studies have reported estimates of between 40% and 80% (Garn *et al.,* 1979; Stunkard *et al.,* 1986). Importantly, it is unlikely that these reported differences are all due to methodological flaws and inaccuracies, but instead that the heritability of body height is not a constant factor. In particular, lower heritability is generally consistent with poorer environments. Mueller (1976) reviewed parent–child correlations for body height across 24 studies, and found that correlations were higher in European countries than non-European countries where environmental conditions were worse. Similarly, Silventoinen *et al.* (2000) reported that actual body heights, as well as the heritability of body height, increased in Finland during the first half of the 20th century. This marked a period of time in which there was a significant improvement in the standard of living. As these increases in average body height occurred in such a short period of time, these changes could not be attributed to changes in the gene pool. Instead they are attributed to difference in environmental quality during growth and development.

Studies have identified numerous environmental factors that influence adult height. These include diet and nutrition (Allen, 1994), parasitic infestation and infectious disease (Stephensen, 1999), pollution and contamination (Lombard, 2014; Gong *et al.,* 2004), behavioral toxicants (Ulijaszek *et al*., 1998), and psychosocial stress (Sandberg and Voss, 2002), among many others. Additionally, some of these factors may interact with each other to create increased or extended periods of insult (Scrimshaw *et al.,* 1968). In all cases however, growth is disrupted or even arrested (Kuzawa and Bragg, 2012). While each of these factors can influence growth, it is often the duration and intensity of these insults that determines their precise and lasting impact. For example, evidence from developing countries has shown that chronic insults have a greater effect on adult body size than intense, episodic insults (Golden, 1994). This can partially be explained by catch-up growth, where less severe or prolonged disruptions can be recovered either through increased growth rates or delayed maturation (Martorell *et al.,* 1994). Importantly however, this is only possible if the quality of the environment improves. Insults also vary in their respective impact according to the individual's stage of life. In particular, the prenatal period to the age of three years are generally identified as the most sensitive to environmental insult. This is because the pace of growth is much faster and nutritional requirements are greater at these early stages of development (Kuzawa and Bragg, 2012). Additionally, weaning during this time often introduces extra nutritional stress as the previously balanced nutritional milk resource is replaced with less balanced and less sterile foods. Subsequently, growth during infancy is particularly sensitive to infections, such as diarrheal diseases, which in some environments can be frequent and severe (Kuzawa and Bragg, 2012). Later stages of development are also sensitive to environmental insult, however nutritional deficiencies primarily slow the pace of maturity, and have less of an impact on final adult body size. In particular, infection appears to have much less of an impact on growth during these late developmental stages. This is because the immune system of an adolescent individual has matured and adaptive immunity is largely in place (Karlberg. 1989).

In most human populations, much of the variation in stature can attributed to

differences in both dietary quality and quantity. While protein intake is the most essential single nutrient (Allen, 1994), deficiencies in zinc (Chen *et al.,* 2003), and vitamins A and D (Torun *et al.,* 1996), have been implicated in growth faltering. A study of differences in stature across various European populations identified the consumption of high-quality proteins from pork, fish, and wheat, as important foods associated with growth and adult height (Grasgruber *et al.,* 2014). Similarly, increased consumption of cow's milk has been associated with increases in linear growth (Hoppe *et al.,* 2006). Compared with protein deficiencies, micronutrient deficiencies appear to only have a modest effect on linear growth, and only cause growth faltering when they are severe (Ulijaszek, 2006). While historical records and archeological evidence suggests that high status individuals in medieval Europe consumed a variety of meats and fish (Harvey, 1993; Dyer, 2000; Hicks and Hicks, 2001), low status individuals had limited access to these sources of protein (Dyer, 1983, 2002; Van der Veen, 2003). For children in Medieval Europe, historical and isotopic evidence suggests that the gradual introduction of non-milk foods into an infants diet, and the eventual point of final weaning, were comparatively early. Mixed feeding often occurred between the ages of seven to nine months, and infants were weaned between 12 and 18 months (Mays *et al*., 2002; Richards *et al*., 2002; Burt, 2013, 2015; Mahoney *et al*., 2016b). This early age of weaning could have had a significant impact on growth trajectories in this sample. Furthermore, the foods introduced during mixed-feeding were unlikely to deliver the adequate nutritional requirements needed for proper growth. Common examples of mixed feeding meals were pap, which was a mixture of flour, milk, and egg yolk, or panada, which was bread in broth with either butter or oil (Fildes, 1986; Orme, 2003). Isotopic studies also suggest that children consumed a diet that was lower in protein than that of older members of society (Richards *et al.,* 2002). After mixed feeding, historical textual evidence suggests the diet of the low status cemetery individuals sampled in this study likely consisted primarily of cereal based foods, along with some eggs and cheese (Dyer, 1983; Dunn, 2004). Collectively, these studies indicate an early age of weaning, and a replacement diet with a limited amount of the nutrients required for growth. As the low status diet appears to remain relatively poor throughout the later stages of

development, with limited protein intake, it is unlikely that any significant catch-up growth would occur.

While diet remains the most influential environmental factor in growth and adult stature, disease is also an important cause of growth retardation. Commonly described disease categories associated with growth faltering are diarrheal diseases (Moore *et al.,* 2001), respiratory tract infections (Liu *et al.,* 1999), and intestinal parasitic infections (Wilson *et al.,* 1999). Importantly, many of these are associated with poor nutrition by decreasing food intake and appetite, impairing nutrient absorption, and/or increasing metabolic requirements (Ulijaszek, 2006). Correspondingly, decreased nutrient intake can then make disease more likely (Victora *et al.*, 1990; Walter *et al.*, 1997). In developed countries, associations between disease and growth are not as strong due to a significantly lower prevalence of serious disease and access to effective treatment methods. However in medieval European cities, serious diseases were significantly more common, and effective treatment methods were lacking. Furthermore, disease prevalence was likely to be higher among the low status individuals sampled here. Several authors have described how low status individuals in medieval society were often affected by infectious diseases such as tuberculosis and leprosy (Roberts and Manchester, 2007). While the entire sample in this study was visually inspected for skeletal pathology, some of the sampled individuals may have suffered from a chronic disease that did not manifest on their skeleton before they died. For example, tuberculosis only affects the skeleton in 1-5% of cases (Rankin and Tuli, 2010), and treponemal disease in only 5–15% of cases (Steinbock, 1976). Furthermore, a large number of diseases that historical records suggest were prevalent in medieval Europe have no known skeletal manifestations at all (Ortner, 2003).

Nutrition and disease are the most dominant and commonly cited environmental influences on stature, however pollution, food contamination and behaviour toxicants are a few examples of other environmental factors that can also significantly influence growth and may have relevance to this study population. To expand on one example, high exposure to lead has been associated with

growth and development faltering, perhaps due to its effect on endocrine function (Selevan *et al.,* 2003; Wu *et al*., 2003; Ulijaszek, 2006). Lead was widespread in medieval Europe, and was found in various coins and kitchenware, in stained glass windows, and lead tiled roofs (Hicks and Hicks, 2001). Importantly, many of these sources were in close contact with food and drinking water. Recent chemical analysis of bone tissue from a large medieval European sample has shown that lead exposure was so prevalent that even rural communities, where there was presumably less contact with lead-based commodities, showed high levels of lead exposure (Rasmussen *et al*., 2015). A final environmental influence on stature that receives much less attention is mechanical loading. Both clinical observations and animal studies have shown that mechanical loading and compression can inhibit the rate of longitudinal growth (Stokes, 2002). As medieval children, especially of low social status, were often required to work alongside parents or independently from a young age (Fleming, 2001; Bailey *et al*., 2008), it is possible that occupational mechanical strain could have contributed to a reduced final adult stature.

As with body height, there are various genetic, environmental and behavioural influences on adult body mass. However unlike stature, these components continue to change after maturation and therefore longer-term cumulative environmental responses, reversed trajectories, and extreme deviations from genetic prediction are common (Shephard and Shephard, 1991). This is why the preliminary study by Bromage *et al*. (2009) focused on human RP correlations with height and not weight. However, while living body weight measures are perhaps too variable to gain meaningful conclusions or findings, the results of this study attest to the benefit of using skeletally derived measurements to estimate body mass, possibly even when living body mass measures are available. Importantly, the femoral head measurement used in this study is thought to be relatively unresponsive to external influences and load changes throughout adult life (Trinkaus *et al*., 1994; Lieberman *et al*., 2001). Therefore this measure may better represent a genetically predicted estimate of body mass for an individual than actual living body weight measures in some cases. That is not to say however that femoral head growth is not responsive to environmental

perturbation when the bone is growing. In fact, authors have suggested that the head of the femur likely follows a similar trajectory of growth as the length of the bone (Auerbach, 2011). Therefore if environmental insults disrupt femoral length growth, as many are known to do, similar insults presumably also affect the development of the femoral head with a similar intensity.

This section highlights a few of the many environmental factors that may have influenced the growth and final adult body size of this sample. As individuals in this study represent a low status medieval population, it is likely that many would have experienced some degree of environmental growth disruption. It is important to note however that while historical evidence described here suggests that these individuals are likely to have suffered some degree of growth disruption, these factors may not have been as pronounced as this discussion has implied so far. Importantly, average stature in this study sample, and in the comparative medieval data sets provided, are only slightly shorter than modern day UK and US populations (section 5.3). If environmental insults had been particularly severe for all individuals in this sample, it would be unusual to observe relatively comparable average body heights across these populations. In fact, stature estimates from various other European populations of different time period suggest that average medieval European body heights were relatively comparable to modern day heights, and that significant decreases in stature only occurred during the seventeenth and eighteenth century, coinciding with the onset of the Little Ice Age and increased urbanization and the spread of disease at that time (Steckel, 2004; Cardoso and Garcia, 2008). This is no way implies that the environmental factors described throughout this section were not a significant influence on final adult body size in this sample. Importantly, even modern western individuals are influenced by some degree of growth disruption, especially among low status populations (Kuzawa and Bragg, 2012). Stature figures reported here do however suggest that they may have been less pronounced, as least for some individuals, than this discussion may have initially implied.

An additional issue regarding these environmental factors is that it is unlikely

that each individual would have suffered a relatively comparable level of insult. For example, the risk of malnutrition and infection may have differed slightly or significantly between low and middle class individuals in medieval Canterbury. While the St. Gregory's burial locations sort individuals into high and low status groups, class systems are rarely so binary and it seems likely that middle class individuals would also have been buried in the cemetery and are represented in this sample. Perhaps suggestive of different levels of insult, figures 11 and 14 in section 5.5.3 and 5.5.4 show significant differences in stature and body mass between some individuals of the same RP. Unfortunately, as environmental insults cannot be quantified, controlled for, or even predicted with a reasonable degree of certainty, it is unknown whether this variability in body size actually reflects differences in environmental impact, or whether other unknown, intrinsic factors also contribute to adult body size in humans. The variable and unquantifiable nature of environmental stress therefore also limits further considerations regarding the contribution the HHO actually makes to body size in humans. Currently it is too early to hypothesize on whether the HHO would buffer or accentuate any of these factors to differentially exaggerate or minimize their effects based on an individual's periodicity. Similarly, while it is conceivable that particularly harsh environmental conditions experienced in early life could act as an epigenetic signal to influence or alter growth rates and the HHO, this is also far beyond the scope of this discussion. Ultimately, environmental factors represent a significant and unavoidable limitation in attempting to accurately determine the strength of the relationship between RP and growth when final adult body size is used as a variable to infer the latter. Determining the relationship between RP and adult body size is, however, an important first step in developing an understanding of the HHO in humans, and now justifies future research on how other, more robust growth related variables may scale with RP.

6.6.2 Methodological limitations

One of the most significant methodological limitations relates to the accurate determination of Retzius periodicity, as it has an impact on the accuracy of the

data, and also on final sample sizes. For the visual counting approach specifically, the method requires Retzius lines and cross-striations to be clearly visible in the same enamel location and depth of focus. When these requirements were not met, a periodicity count could not be made and the individual was excluded from further analysis. While the measurement method could have been employed to potentially increase sample sizes, this study considered accurate, clear periodicity counts using one method to be more desirable than a less accurate and methodologically inconsistent data set of larger size. A criticism of the visual method could be that the determination of a particular periodicity is vulnerable to subjectivity (Smith, 2004). However, where a measurement should begin and end using the alternative method also appears to have the potential for subjective error. Nevertheless, as several authors have stated in relation to their own data (Reid and Ferrell, 2006; Mahoney *et al*., 2016a), if an error was made, it is unlikely that the periodicity would have been miscalculated by more than one day. This would seem especially unlikely over three independent observations on multiple regions of the same section. While one early study reported inter-observer errors of up to two days in RP (Huda and Bowman, 1994), they also reported that RP appeared to vary within the same tooth by up to three days. Therefore it may be safe to assume that the methodology in this case was flawed in some way. Subsequently, while a significant histological methodological limitation existed with regard to the sample size, it is hoped that the data presented in this research is relatively accurate.

Significant sources of potential error in this study also relate to the stature and body mass estimations. Errors in stature and body mass estimation can arise from using equations derived from populations that do not accuracy represent the body types of the sample population, if the equation itself was flawed in it's creation, and from direct measurement error (Ruff *et al.,* 2012; Elliott *et al.,* 2015). For stature estimation, this study chose an equation that has been shown to perform well when tested against known body heights, and is commonly used for archeological European populations (Giannecchini and Moggi-Cecchi, 2008). However it is impossible to know how accurate these estimates are for archeological populations. Currently, the only way to assess the accuracy of these

equations for archeological populations is to see how closely they match the results from others equations (Auerbach and Ruff 2004; Kurki *et al*., 2010; Pomeroy and Stock 2012). The obvious issue with this is that the correspondence between results only demonstrates that both equations produce similar results, it does not demonstrate whether they are accurate. Similarly, the stature figures in this study do correspond with other estimates of medieval European stature, but as these other studies used the same method and equation as was employed in this research, these similarities once again only demonstrate that the equation yielded similar results, not that the result are actually accurate. Duyar *et al.* (2006) and Ruff *et al*. (2012) have suggested that because stature regression equations are based on the average stature of a target population, the heights of particularly large individuals will tend to be underestimated, while the heights of short individuals will tend to be overestimated. Consequently, the large individuals in this study may have been even larger than was estimated, and the small individuals smaller than estimated. This may contribute to an explanation of why the full range of permanent tooth periodicity values were observed, despite an apparent lack of extreme values in body height among the sample.

Regarding direct measurement error with stature estimation, it is hoped that by collecting multiple long bone measures for each individual, and averaging both left and right sides of the same bone when available, the impact of any isolated random measurement errors would have been minimized. As long bone measurements are less susceptible to subjective error, it is unlikely that consistent errors were made. A final point to make regarding stature estimation is that while individuals were only selected if they had multiple intact long bones, in two cases these long bones were only of the upper limb. As the upper limbs appear to have a lower correlation with stature than the lower limbs (Trotter and Gleser, 1958), it is possible that stature estimation for these individuals was not as accurate as for the rest of the sample. Ultimately, a significant limitation exists in attempting to accurately investigate the relationship between RP and body height when the latter measurement is estimated from skeletal remains. In this case, more accurate and confident

relationships could be observed using direct measures of living body heights. As mentioned above however, there may be some benefit to using skeletally derived body mass estimates.

As joint dimensions commonly used to estimate body mass are thought to be relatively unresponsive to external influences throughout adult life, this measure has an advantage over highly variable, environmentally influenced, actual body weight measures. Nevertheless, body mass estimates from skeletal remains are not without their own significant limitations. As with stature estimates, potential inaccuracies in skeletal body mass estimation are contingent on how accurately the reference population represents the sample in question, and how accurately the measurements are made (Lorkiewicz-Muszyńska *et al.,* 2013; Elliott *et al.,* 2015). While it is hoped that the reference population used to generate the body mass equation used in this study adequately represents the Medieval St. Gregory's population, it is unknown how accurately it does so. Generally, intraspecific studies have suggested that an appropriately matched equation should estimate the majority of a sample to within 10 to 15 % of their actual body mass (Ruff *et al.,* 2005; Lorkiewicz-Muszyńska *et al.,* 2013). However, it is conceivable that these percentages could in some cases be higher due to the possibility of slight errors made in the measurement of the femoral head. Unlike long bone lengths, there is a slight subjective element in determining the precise dimension to use to measure the breadth of the femoral head. Additionally, slight cortical wear or unidentified lipping could have influenced the accuracy of the measurement. Finally, measurements were made with a plastic caliper than could only measure to a precision of 1mm. If a digital caliper with increased precision had been used, the accuracy of the measurement may have been better, and may have provided a more precise body mass estimate for each individual. The strong positive correlation between stature and body mass in this study does point to a broadly accurate estimate in each variable for each individual, however whether this is partially a reflection of similar degrees of over and underestimation in each body size variable is unknown. Ultimately, even though slight errors could have been made in all three of the variables of this study, and environmental factors may have notably influenced the final outcome of two of

them, significant relationships were still observed between RP and the body size variables. This perhaps indicates that a much stronger relationship exists between RP and body size than this research currently reports.

As one final methodological limitation, it has to be considered that while sex estimations were made using multiple methods, some of these may have been inaccurately assigned. In particular, for statistical purposes, probable males were seen as male, and probable females as female. If only those with a confident sex determination had been included, sample sizes would have been notably reduced. It is therefore not implausible that this study actually comprises a mixed-sex sample. As is it possible that the relationship between RP and body size differs between the sexes (Smith *et al.,* 2007), this may have influenced the strength of the observed relationship. This is another limitation of using skeletal material instead of living subjects.

Broader limitations are that the specific strength and scaling factors from these results are likely to be population specific and not broadly applicable to all humans. For example, as mentioned earlier, the 191 North American individuals studied by Reid and Dean (2006) only had periodicities varying from 7 to 9 days, yet it seems unlikely that some particularly large and small body types would not be present in this sizeable sample. Similarly, the graphical figures provided by Bromage *et al*. (2015) appear to show differences of approximately 20kg in weight and 50cm in height between individuals with periodicity counts that only differed by one day. While this study reports negative allometry between RP and body size, it may be that RP scales isometrically or even positively in other populations. Whether these differences will be shown to reflect different degrees of environmentally influenced growth, or implicate other currently unknown factors in the scaling of these relationships, it seems likely that the scaling factor and/or strength of the correlation may differ between human populations.

Finally and importantly, while growing evidence suggests that the HHO, manifest in dental enamel as RP, regulates growth and adult body size in humans, this study was only able to show that RP correlates with body size, not that it directly influences it. Providing direct evidence of causation, instead of just correlation as

this study has provided, will be a challenging but pivotal step in the future of intraspecific HHO research.

6.7 Future studies

As this research is still in its infancy, there is so much still to be investigated that full discussion of all potential avenues is far beyond the range of this thesis. Subsequently, this section limits its future study considerations to humans and intraspecific correlations. This in no way suggests that interspecific investigations are no longer of interest or use. After all, the discovery of interspecific RP relationships preceded and initiated intraspecific investigations. In the same way, future interspecific findings may guide future RP and HHO studies in humans. Furthermore, there are many important methods, procedures and experiments that cannot be carried out on human subjects. For example, much of what is known about clock genes in enamel and bone comes from transgenic and gene KO mice. In the following section however, discussion is limited to intraspecific future research that either has not already been addressed in previous sections of this discussion, or deserves additional comment.

As described briefly in section 2.4, Bromage *et al.* (2016) identified metabolomic signature fluctuations in the domestic pig with periodicities that matched the RP of that species. While these results need to be repeated in other species with different RP to test whether these findings are simply coincidental, it may also be possible to conduct the same tests within humans. For example, using gas- and liquid-chromatography in combination with mass spectrometry, studies have analysed the human plasma metabolome in clinical and physiological experiments (Lawton *et al.,* 2008), including one in particular that investigated the effects of the circadian clock on human metabolic pathways (Dallmann *et al.,* 2012). While such experiments would also require the histological sectioning of a tooth from each study individual, it is not completely unreasonable to assume that this could be achieved if the sample was comprised of individuals who had,

or were to, undergo clinical tooth extraction. The issue would be that if the HHO changes with age into later life, the periodicity between the current metabolomics fluctuations and the structural evidence of the rhythm in enamel may be different. Ultimately, this emphasizes another important aspect of the HHO that needs investigating. While Bromage *et al*. (2009) believed it to be unlikely that a centrally regulated rhythm would change with age, Mahoney *et al*. (2016a) has provided the first evidence to suggest that it might. Whether this represents a one-off, abrupt event is currently unknown. This too is an important area of future investigation. Additionally, it will be interesting to discover whether periodicities always increase with age, or whether some individuals retain their periodicities throughout their entire period of deciduous and permanent enamel development. Mahoney (2012) did report that RP did not vary between a deciduous and permanent molar from one individual, however larger sample sizes are required to verify these observations.

Regarding periodicities changing, it may also be interesting to investigate whether periodicities shift temporarily after particularly stressful physiological events or insults. As disease can cause disruptions in growth, it is possible that a particularly severe episode of physiological stress could temporarily alter RP. While all current evidence suggests that RP does not change within permanent teeth, areas of enamel with accentuated striae associated with physiological or psychosocial stress events are perhaps avoided in RP counts as they are considered a methodological concern. However in doing so, research misses the opportunity to investigate this question. Unfortunately, the sample in this study had no particularly prominent accentuated striae to examine this. Future histological studies with teeth displaying prominent accentuated striae and linear enamel hypoplasia, along with clear incremental markings before and after the accentuated line, may provide evidence of such an event.

As mentioned in section 6.6.1, body height is significantly influenced by genetic factors. Genome-wide SNP-association analyses (GWAS) have identified numerous genomic variants that are associated with adult height (Lettre *et al*., 2008; Weedon *et al*., 2008). It would appear likely then that RP may have a genetic component to its expression. Interestingly however, these genomic

variants explain only a fraction of the observed genetic contribution to human height, suggesting that significant influences are missed through SNP analysis alone (Simeone and Alberti, 2014). One particular candidate for such non-SNP-linked information is through epigenetic modification. Epigenetics is the study of cellular and physiological variation that results from environmental factors that modify gene expression. In some cases, these gene modifications appear to be heritable (Goldberg *et al.,* 2007). Importantly, numerous height-associated genes have been found to contain the necessary structure required for epigenetic manipulation (Simeone and Alberti, 2014). Epigenetic heredity thus also appears to be a determinant of adult height. Whether RP has a genetic or epigenetic component remains to be determined, however it would seem likely that some heritability exists. Additionally, whether shifts in RP reported by Mahoney *et al.* (2016a) represent epigenetically mediated responses to environmental conditions in early life, also remains plausible but highly speculative at this time. While the relationship between RP and body size indicates a potential genetic component to the expression of the former, other evidence is also suggestive of such a link. As described in section 2.6.5, Cornélissen *et al.* (2000) identified circaseptan rhythms in the blood pressure of newborn twins, and also reported smaller rhythmic variability between each twin set than there was across unrelated infants. While the study sample was small, this may indicate a genetic influence on the exact periodicity of oscillations in blood pressure. If RP and circaseptan fluctuations in blood pressure both represent physiological manifestations of the same biological rhythm as Bromage *et al*. (2009) suggest, this may also then indicate that a genetic or epigenetic component influences the HHO.

Another area of future work will be investigating whether RP correlates with any other life history traits in humans, as it does across species. Unfortunately, this may be challenging. Interspecific life history variation is studied based on species averages, and reflects historical constraint and organismal design (Dunham and Miles, 1985; Harvey and Clutton-Brock 1985). However, intraspecific variation in many cases reflects phenotypically plastic responses to environmental conditions (James, 1983; Brown, 1985; Myers *et al.,* 1985). In this

way, many other life history traits likely suffer from the same mass-based limitations described in this study. Additionally, certain life history traits in modern humans, such as average number of offspring, weaning age, and age at first reproduction, are likely to be significantly influenced by various cultural practices and behaviours (Aunger, 1994; Barrett and Stulp, 2013). As birth weight roughly predicts adult body size (Sørensen *et al*., 1997), it is possible that a correlation with RP in the former may be observable. Additionally, as cell division rates appear to regulate longevity *in vivo* (Magalhães and Faragher, 2008), and some theories have suggested that ageing is a process regulated by tissue specific metabolic rates (Tolmasoff *et al*., 1980; Adelman *et al.,* 1988), it is not unreasonable to consider that long period rhythms could correlate with local aging mechanisms and lifespan. However, as lifespan is also highly influenced by environmental factors, it alone is perhaps an unreliable study variable. Nevertheless, reports have shown that shorter individuals may have greater longevity potential than taller individuals when nutrition, lifestyle, and other environmental factors are similar (Samaras and Storms, 1992; Samaras *et al.,* 2003), suggesting that mass-related correlations may exist.

As considerable body size variability exists between species of the same RP, the HHO is clearly not the only factor regulating growth and adult body size. This may also be true for humans. As notable differences in height were observed between individuals with the same RP in this study (Figure 11, section 5.5.3), it is possible that the HHO is not the only factor regulating growth in humans. Unfortunately, was described in section 6.6.1, there are significant and unavoidable environmental limitations in studying final adult body size over growth rhythms or incremental markings that make intraspecific considerations of this kind impossible. Interestingly, Bromage *et al*. (2015) also mentioned that the observed variability in lacuna area between both large and small body types in their human study indicated that the HHO appears to be "only one moderating factor responsible for the rate of cell proliferation and achievement of body size" (p.8). Determining the contribution the HHO makes to adult body size will be a difficult but necessary future step in understanding the impact the HHO has on human growth and development.

While these examples above provide a few indications of where future research might head, further work needs to be conducted to bolster and reinforce the relationships and correlations currently found. In particular, measuring osteocyte densities, lamella bone increments, body sizes, and RP from the same human subjects will be an important and necessary step in solidifying the intraspecific findings and hypotheses presented to date. Additionally, studies conducting the same research as presented here for larger sample sizes of different population and sex may provide better understandings of the strength and nature of the correlations observed, and the potential variability among them. These future directions, combined with any further interspecific discoveries, are essential for confidently understanding the HHO, and how it may be used in the future. Importantly, while the elucidation of the HHO is in itself likely to be fascinating, the rhythm may also become useful as an analytical tool for investigating aspects of human and mammal life history variation and evolution. In addition to this, it may even function as an analytical tool for disciplines one would not initially consider. For example, if a large population specific regression plot for RP and body size were constructed from an archeological population, and a few certain individuals fell far from the regression line and their population-specific, RP-predicted body size, it may raise interesting questions regarding their individual environmental health during development relative to the rest of the population. In this way, RP and body size correlations may have use in bioarcheology.

The study of RP and the HHO holds much promise for the science of organismal life history, biological anthropology, chronobiology and even endocrinology. Similarly, a full and concise understanding of the HHO might require a collaborative effort between these disciplines. In doing so however, future studies are likely to unearth many more fascinating aspects of this seemingly novel and unusual biological rhythm.

7. Conclusion

The aim of this study was to explore the relationship between Retzius periodicity and body size in humans. Based on previous research, three hypotheses were established. The first was that Retzius periodicity would negatively correlate with body size. This was based on the preliminary work conducted on a small sample of humans by Bromage *et al.* (2009, 2015). The second hypothesis was that Retzius periodicity would positively correlate with body size, as it does with body mass across most primate species (Bromage *et al.,* 2009). The null hypothesis was that no relationship exists between Retzius periodicity and body size in humans. Addressing this, stature and body mass were estimated in an archeological sample of modern humans. Retzius periodicity was then reconstructed from permanent teeth from a selection of individuals of different body size. Reduced major axis regression revealed that RP was significantly and negatively correlated with stature and body mass in adult humans, supporting the first hypothesis. Individuals with higher RPs were of smaller stature and body mass than those with lower RPs. Thus, while RP correlates positively with body mass across species, and appears to reflect a strategy in which mass increases are achieved by slowing developmental rates over longer life histories, larger body sizes within humans appear to be achieved by increasing the developmental rate within the same constrained growth duration. That growth-related life history timings differ between species, but remain relatively consistent within humans, is an important point to consider when attempting to understand these intraspecific results in the context of what is known inter-specifically. Notably, the findings of this research also agree with data from two studies showing how human osteocyte densities scale with body size.

Retzius lines have been observed in human dental enamel for over a century, yet they have remained an etiological mystery. The findings of this research provide new perspectives on RP variability in humans, while also offering a new context with which to understand the previously unexplained trends in RP expression across populations or between the sexes. Additionally, they also generate a plethora of new questions regarding the specific nature of the HHO and its

relationship with other physiological systems in humans. Initially just an unusual and variable periodic feature in enamel, in the future RP may become a powerful analytical tool for investigating aspects of human and mammal life history variation and evolution. In particular, these intraspecific results may have interesting applications and implications to the study of human fossil ancestors. Before that however, future research is needed to bolster these conclusions and unravel the precise relationships and factors involved. For example, a recent study of deciduous RP and bone growth has suggested the relationship may be more complex than the results here suggest. Also, the direct cause or mechanisms involved in regulating and maintaining the HHO remain completely unknown at this time. This research advances the knowledge on what this rhythm may represent in humans. Building on this and even opening up the investigation beyond hard tissue, more research can be directed towards examining aspects of human physiology, metabolism and growth that may display rhythmic behaviour with a similar periodicity and variability to that observed in human RP. While the justification or reasoning for such research may have been lacking before and subsequently only received limited attention, such investigation may now be justifiably fruitful. In doing so, and in collaboration with dental histologists, future research will hopefully broaden and develop an understanding of how the HHO operates in humans and what this rhythm, manifest in dental enamel as Retzius periodicity, actually represents.

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