Incremental Development of Primate Dental Enamel

A Dissertation Presented

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Tanya Michelle Smith

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Abstract of the Dissertation

Incremental Development of Primate Dental Enamel

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Tooth development is characterized by rhythmic secretions of enamel- and dentine-forming cells, permanently recorded in the mineralizing tissues, which are analogous to structural layers in marine organisms or tree trunks. Recent work has challenged the periodic nature of incremental features and the accuracy of histological analyses. In this study, experimentally labeled macaque (*Macaca nemestrina*) teeth were examined under regular and fluorescent light microscopy, and incremental features were related to injection intervals, demonstrating the existence of sub-daily, daily, and multiple-day rhythms. Using these features, the enamel crown formation time (CFT) and age at death were determined in several molars from individuals of known age at death. The accuracy of standard methodology for incremental development analysis was shown to be greater than 90%, and section obliquity is suggested to be the main source of error.

Previous analyses of non-human hominoid molar microstructure have been limited to seven individuals or fewer per genus. In this study, incremental features were examined in 267 histological sections of the mesial and distal cusps of 134 chimpanzee (*Pan troglodytes*) molars from 75 individuals. When possible, cuspal enamel secretion rate, Retzius line periodicity and Retzius line number were quantified, and CFT was determined for individual cusps. Daily secretion rate generally increases from inner to outer cuspal enamel, with an average rate of four microns per day. Retzius line periodicity generally ranges from 6 - 7 days, and appears to be consistent within teeth from the same dentition. Retzius line number varies within a cusp type, among cusps, and among molars, as does cuspal enamel thickness, resulting in CFT variation. Cusp-specific crown formation time generally ranges from two to three years, increasing from first to second molars, and often decreasing from second to third molars. These times are more similar to radiographic data than a previous histological study.

Given well-prepared material, counts and measurements of incremental features yield highly accurate estimates of the rate and duration of crown formation. Results from a large sample show that certain aspects of hominoid molar development vary considerably, which has implications for the taxonomic interpretation of small samples of living and fossil hominoids.

During the past five years, I have alternated between the profound feeling that I have been 'standing on the shoulders of Giants,' and the uncanny sense that I have been carried along by 'a great mass of Lilliputians.' This dissertation is dedicated to those Giants and those Lilliputians, in gratitude, for the village they have become and the person they have raised, knowingly or otherwise.

"I believe that much more of the intimate history of the individual is revealed to the microscopist by a study of the enamel than has been generally understood."

- Alfred Gysi, D.D.S. 1931
(As related by George Wood Clapp, D.D.S.)

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List of Abbreviations

EDJ- enamel dentine junction
HSB- Hunter-Schreger bands
DSR- daily secretion rate
SEM- scanning electron microscope
TEM- transmission electron microscope
PLM- polarized light microscope
TSRLM- tandem scanning reflected light microscope
CLSM- confocal laser scanning microscope

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Acknowledgements

This dissertation would have been impossible without the assistance of many colleagues and friends. I would first like to thank my dissertation committee: Lawrence Martin, Don Reid, Bill Jungers, and Fred Grine, for their guidance and support over the past five years. I have been truly gifted with the quality of their patient mentorship. Chris Dean, Gary Schwartz, Wendy Dirks, Anthony Olejniczak, David Beynon, Alan Boyde, and Jay Kelley have also been instrumental in shaping my understanding of incremental development and analytical methods.

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Hirsch, Mitch Irwin, Karen Samonds, Keith Metzger, Erin Vogel, Jess Lodwick, Lisa Paciulli, Eileen Larney, Michelle Pratt, Heather Smith, Felicia Brenoe and Abigail Derby. Anthony Olejniczak, a.k.a., the 5th committee member, has taught me more about friendship and loyalty in the past 4 years than I could ever repay; this thesis would not have been possible without him. Thank you to a number of Stony Brook faculty and staff who have given freely of their time, support, and encouragement, including Diane Doran, Pat Wright, John Fleagle, Dave Krause, Jack Stern, Brigitte Demes, Susan Larson, Jean Moreau, and Janet Masullo. Thank you to my friends and family in the US and abroad who have had unwavering faith in this part of my journey; including my 'support system:' Kelly Grandinette, Carl Wampole, Cara Moore, Kate Mansur, Beth Bruce, Pat Bauman, Ellen Driscoll, and Maryann Walsh. Thanks to the people in and out of the rooms for inspiration and hope, including Melissa, Ani, Bill and Bob. Finally, thanks to my mom and my family, including Allison, Callie, and Fleagle, for love, loyalty, and cat naps on my laptop.

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Smith, T.M., Martin, L.B., Reid, D.J., Bonis, L. & Koufos, G.D. (2004) An examination of dental development in *Graecopithecus freybergi* (= *Ouranopithecus macedoniensis*). *J. hum. Evol*. 46:551-577.

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Papers and Posters Presented at Professional Meetings

Smith, T.M., Reid, D.J., Dean, M.C., Olejniczak, A.J. & Martin, L.B. (in press) New perspectives on chimpanzee and human dental development. To be presented at the Max Planck Institute for Evolutionary Anthropology Dental Paleoanthropology Conference, May 26-28th.

Smith, T.M., Olejniczak, A.J., Martin, L.B., Reid, D.J.& Dean, M.C. (in press) Molar cusp formation in common chimpanzees (*Pan troglodytes*). To be presented at the 74th Annual Meeting of the American Association of Physical Anthropologists, Milwaukee, Wisconsin, April 6-9.

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Smith, T.M., Iovine, T., Anemone, R.L. & Nachman, B. (1997) Vertebrate Paleontology of the Great Divide Basin, SW Wyoming: Field and Lab Studies. Presented at the 37th Annual Meeting of the Northeastern Anthropological Association, Quebec, Canada, April 11-13.

Smith, T.M. (1997) Scanning Electron Microscope Investigations of Fossil and Living Mammalian Dentitions. Presented at the Tri-Beta Biological Honors Society Regional Meetings, Geneseo, NY, March 15.

Current Research

Incremental development in Asian hominoids. This project is an analysis of dental development as revealed by dental microstructure in large-bodied extant and extinct Asian apes. Large samples of molar teeth from *Pongo pygmaeus pygmaeus* (Bornean orang utan) and *Pongo pygmaeus abelii* (Sumatran orang utan) will be employed to provide baseline data on the degree of variation within and between sub-species. Sub-fossil orang utans will then be analyzed to provide novel information on regional and temporal variation in the dental development of *Pongo*. Finally, data will be collected on several teeth attributed to *Griphopithecus* and *Sivapithecus*, providing a broader range of geographic and temporal depth of early pongine evolution. Given that there is currently little known about dental development as it relates to geographic and temporal variation within a taxon and among subspecies, these results will be of particular value for the interpretation of data on hominid incremental development.

Thesis Research

Incremental development of primate enamel. This NSF-funded project involved an experimental approach to examine the periodicity of incremental features in developing macaque dentitions, as well as an examination of the variation of incremental features in a large sample of chimpanzee molar teeth. Variation was assessed within and between individuals, which permits greater insight into the dental development of living and fossil apes and humans.

Research in Vertebrate Paleontology

* A study of the enamel thickness and dental development of Miocene hominoids, including *Afropithecus turkanensis* (17-18 million years old), *Graecopithecus freybergi* (9-9.5 million years old), and *Griphopithecus* sp. (~14 million years old). Co-PI Dr. Lawrence Martin. 1998- present.
* Member of the SUNY at Geneseo's Great Divide Basin Project in southwestern Wyoming. Involved prospecting for new mammalian fossil localities, collecting fossils and quarrying productive localities discovered during previous field seasons. Geological work involved measuring stratigraphic sections in order to correlate localities. Principal Investigator: Dr. Robert Anemone. July 1996 & July 1997.

Research In Primatology

* A census of lemurs in three eastern rainforest sites in Madagascar, with co-PI Mitch Irwin (Stony Brook University). Faculty Advisor: Dr. Patricia C. Wright. May- August 1999.
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* Executive Committee, University Senate, 2001-2002.
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Chapter 1: A Review of Dental Development

Dissertation Objectives

This dissertation aims to provide a comprehensive review of the study of incremental features of the enamel microstructure. This begins with a review of general dental development in Chapter 1, where an emphasis is placed on critically examining unresolved or contentious issues, and numerous areas of future inquiry are identified. In the second chapter, the various methods of studying incremental development are reviewed, as well as recent applications in the anthropological literature. Additionally, Chapter 2 contains a review of several criticisms of studies of incremental development. These criticisms are addressed in Chapter 3, where the periodicities of several incremental features are demonstrated experimentally. Chapter 4 provides an experimental assessment of the accuracy of multiple methods of crown formation time and age at death estimation. These chapters are critical for the interpretation of previous studies that have provided insight into the evolutionary developmental biology of primates. Building on these methodological foundations, Chapter 5 examines the variation of incremental features and crown formation time in a large number of chimpanzee teeth. This chapter reports on dental development the largest-known histological sample of a non-human primate studied to date, and will complement recent and on-going studies of living and fossil hominoids. Chapter 6 provides a summary of each chapter as well as a review of areas for future study. In closing, it is suggested that technological advances in microscopy and medical imaging may represent breakthroughs that parallel the developments of light and scanning electron microscopy, and it is hoped that these advances permit resolution of some of the remaining mysteries of primate dental development.

Historical Overview

Boyde (1964) expertly reviewed the history of the study of enamel microstructure, which extends back several hundred years. He noted that as light microscopes became widely available during the 1800's, several important studies appeared between 1835-1840 (e.g., Retzius, 1837), followed by many reports in the next few decades. During this time, teeth were studied as 'ground sections' under a light microscope (LM), which involves sawing, thinning, and polishing sections. The application of polarized light microscopy (PLM) occurred in the 1860's, although it was not until the first third of the 1900's that a number of reports were published. This new form of microscopy provided information on the crystalline structure of enamel, which is birefringent when viewed under polarized light (i.e., it shows dramatic optical contrast due to the structural components; see Schmidt and Keil [1971] for a detailed discussion). Boyde (1964) also noted that initial studies involving X-ray diffraction in the 1920's and 1930's contributed to current views on crystallite orientation. During the 1930's and 1940's, significant increases in our understanding of the process and pattern of human dental development came from the deductive and experimental work of Schour and colleagues. Their experimental studies provided data on the rate and patterning of enamel and dentine development in a human subject and in a number of experimental animals. At the same time, Okada and his Japanese colleagues published innovative experimental work on the rate and development of incremental structures in mammalian enamel and dentine. Many of these early descriptive, theoretical, and experimental studies provided the background for breakthroughs in the 1950's and 1960's, particularly with the advent of advanced preparative techniques and the applications of novel technology such as electron microscopy.

Boyde (1964) noted that the first electron micrographs were published during the mid 1940's. Electron microscopy allows for highly controlled study of the ameloblast-prism relationship, as well as the nature and origin of crystallites. During this time, replicas of the acid-etched enamel surface were also developed and studied, which preserve details of the prismatic surface. In the 1950's, ultra-thin sections of enamel were produced, providing additional information on the crystalline organization of enamel

(although deformation of sections continues to be a problem). Studies of decalcified sections also became popular at this time, which reveal details of the organic component of enamel. During the 1960's, electron microscopes were first used to study lines known as cross-striations, which we now know to be daily features in enamel. The highly informative dissertations of Rönnholm and Boyde both came out at this time, providing insight into the nature of crystallite orientation, features of the ameloblasts and prisms, and the nature and development of incremental structures. Boyde's (1964) dissertation research involved the scanning electron microscope (SEM), which permitted high-resolution study of the intact developing enamel surface, from which he derived his well-known model of prism packing patterns. He later noted that this new technique essentially replaced transmission electron microscopy for the study of enamel (Boyde, 1989).

The decades of work following these dissertations have shown increased interest in Retzius lines and 'pathological' or accentuated lines, particularly with the application of the SEM. Additionally, after a few initial reports in the 1950's on prisms and incremental features in primates and mammals, as well as Boyde's (1964) dissertation on mammalian enamel, more attention was given to the use of these features as a potential tool to understand phylogeny (e.g., Gantt et al., 1977; Vrba and Grine, 1978a,b). Aspects of enamel microstructure became key issues in the debate over classification of fossil taxa as hominids or (non-hominid) hominoids. Martin's (1983) examination of these features in his dissertation helped to resolve some of the debate over their use, while providing a stimulus for a number of new areas of study. Additional studies by Boyde (1969b, 1979) have provided greater insight into the nature of prism decussation and the development of cross-striations. Warshawsky also published several important studies on rat enamel, providing excellent quality images using various forms of microscopy (e.g., Warshawsky, 1978, 1985). Many other LM, PLM, and SEM studies conducted since the 1960's are considered in the body of this dissertation. One of most recent breakthroughs in the study of enamel has been the development of the tandem scanning reflected light microscope (TSRLM) and confocal laser scanning microscope (CLSM), which reflect sub-surface, focal-plane-specific information. This non-invasive technique permits the study of tissue at depth of more than 100 μm below the surface (Petran et al., 1985). This form of

microscopy allows imaging that counters previous criticisms based on the possibility of preparation artifacts or interference from adjacent layers (Smith et al., 2003a).

The field of incremental enamel microstructure became 'front-page news' to most anthropologists with two 1985 *Nature* publications by Martin and by Bromage and Dean. Martin (1985) proposed a phylogeny of fossil and living hominoids based on the degree of enamel thickness and characteristics of the enamel microstructure. This report suggested that African apes share a derived condition that may group them to the exclusion of humans. Additionally, Martin (1985) showed that the condition of thick enamel does not distinguish hominids from non-human hominoids. Bromage and Dean (1985) applied knowledge of incremental development to the problem of determining the time of crown formation and the age at death of fossil hominid dental material. Their study suggested that early hominids displayed a developmental pattern more similar to extant apes than to modern humans. These studies have ushered in almost two decades of studies of enamel microstructure in fossil and living hominoids. Many of these are considered in Chapter 2 in a review of applications of enamel microstructure.

Summary of Following Sections

The first section is provided as a brief review of the embryological and cellular process of enamel development. Many standard texts on embryology, physiology and dental histology detail these concepts, therefore only a short summary of each topic is provided. Recent work involving insights from biochemical and genetic studies is also reviewed. Certain aspects of mineralization are examined in greater detail in the following section. The second section is a review of dental development at the level of the crystallite and enamel prism. Packing pattern, prism sheaths, prism width, prism path, prism decussation and aprismatic enamel are considered. Because a huge amount of research has been conducted in this area, it is not possible to produce an entirely comprehensive review. It must also be noted that certain aspects of the developmental biology of enamel formation are still not completely understood. Further, this summary was not intended to provide an exhaustive review of certain non-incremental characteristics such as prism packing patterns and Hunter-Schreger bands. Excellent

reviews on these subjects may be found in Boyde (1964, 1969b, 1989), Osborn (1973, 1981), Martin (1983), Boyde and Martin (1984a,b, 1987), Dumont (1993), and Maas and Dumont (1999).

The third section represents a review of the development of short- and long-period incremental features, specifically the structure and proposed models of formation. The periodicity of incremental structures and applications for anthropological inquiry are reviewed in the following chapters. The fourth section is a review of studies of factors that influence variation in dental development, including recent work on genetics and hormones. In addition, environmental influences on dental development are reviewed, followed by a brief review of pre- and postnatal enamel differences, as well as post-mortem changes in enamel, which has implications for the study of archaeological and paleontological material. Unresolved issues are considered at the conclusion of each section.

Enamel Development

Enamel is generally regarded as a composite material, composed of both mineral and organic components. Mature enamel is over 96% mineralized by weight, rendering it essentially fossilized when final maturation is completed. Boyde (1989) noted that the density, refractive index, and birefringence of enamel are very similar to natural hydroxyapatite, yet there are differences when the two are compared, particularly in the crystalline structure. Sakae et al. (1997) described the composition of enamel crystallites as 'biological apatite': carbonated hydroxyapatite with a variety of ion-substitutions (e.g., Mg for Ca, CO_3 for PO_4 and OH). In this review, the formation and properties of the components of this unique tissue will be discussed. As enamel grows, ‘growth tracks’ known as prisms are formed, which record the path of the secretory cells during development. Additionally, features known as Retzius lines are created that register the position of the entire enamel-forming front at successive points in time. This system of registry has often been compared to the growth lines visible in cross-sections of tree trunks. However, enamel shows a much finer resolution of temporal development, at the

scale of a day or less. As Risnes (1998) noted, an understanding of cellular and physiochemical developmental processes, as well as temporal and spatial relationships, are critical to understanding and interpreting the 'growth tracks' of enamel.

Embryology of Tooth Formation

Bud, bell and cap stages

Hominoid dental development begins prenatally and continues until an age of 18 - 25 in modern humans and 12 - 15 in great apes (Aiello and Dean, 1990). During early development, the embryonic oral cavity is lined by ectoderm, one of three types of embryonic germ layers (ectoderm, endoderm, and mesoderm) that give rise to the adult organism. One of the distinctive embryonic features of teeth is their dual composition; enamel is ectodermal in origin, while dentine, pulp, and cementum are derived from mesoderm. The early stages of tooth development are often divided into the bud, cap, and bell stages (Figure 1.1).

The *bud stage* is commonly regarded as the initial period when an epithelial thickening (dental lamina of ectoderm origin) appears, which then begins to show round or ovoid tooth buds (enamel organ, dental papilla, and dental sac); yet the cells remain poorly differentiated. Thesleff and Sharpe (1997) characterized tooth development as a series of epithelial-mesenchyme (ectoderm-mesoderm) interactions, where initial development is triggered by the epithelium, which induces the formation of a dental papilla (see also Mina and Kollar, 1987). Thesleff and Åberg (1997) and Thesleff and Sharpe (1997) reviewed evidence that has shown that the expression of growth factors during different stages of induction may serve as the specific signaling cues between tissues.[1]

[1] The genetic control of this is reviewed below, although the specifics of the signaling molecules are beyond the scope of this review.

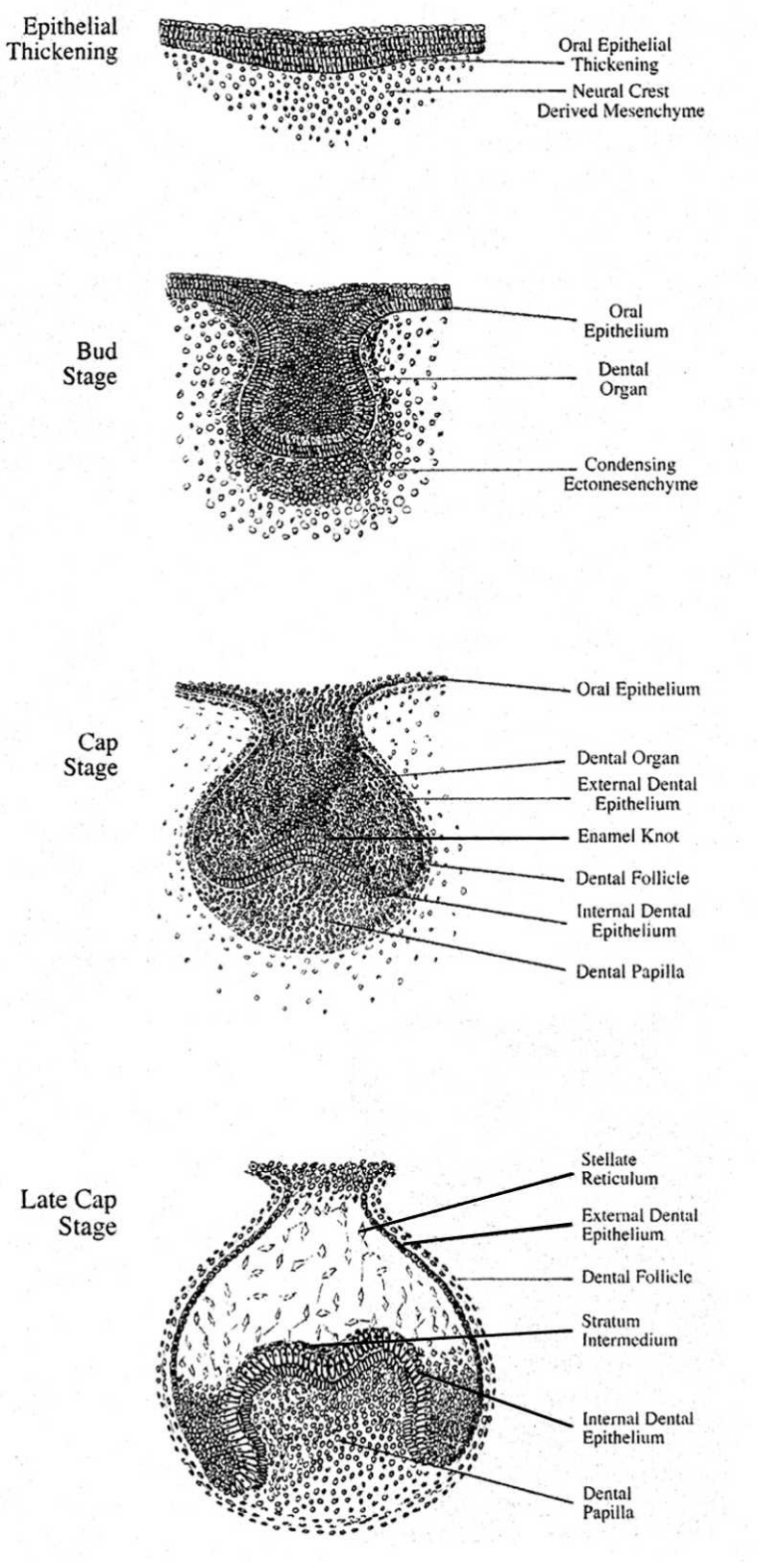


Figure 1.1 The progression of early dental development. Key structures indicated are discussed in the text. Modified from Ferguson et al. (2000).

During the late bud or early *cap stage*, the enamel knot can be identified in the epithelium, which is a transient, non-proliferating area believed to secrete a protein (or proteins) that induces mitosis in nearby cells, and may influence the future shape of the tooth (discussed further below; see also Jernvall and Thesleff, 2000). The cap stage is distinguished by the invagination of the enamel organ into a cap-shaped structure. Inner enamel epithelial cells on the internal surface of the cap become more columnar, in contrast to the outer enamel epithelial cells that remain more cuboid. The mesenchymal cells also proliferate during this stage, completing the dental papilla beneath the internal enamel epithelium, and the dental follicle (or dental sac) surrounding the tooth germ.

During the early *bell stage*, the enamel organ continues to grow into a bell-shaped structure with four distinct layers: outer enamel epithelium, stellate reticulum, stratum intermedium, and inner enamel epithelium. Berkovitz and Moxham (1981) suggested that the outer enamel epithelium, a one-cell thick layer, may help to maintain the shape of the enamel organ and regulate substance exchange between the enamel organ and the dental follicle. The stellate reticulum, which lies between the outer enamel epithelium and the stratum intermedium, is likely to have a protective and a nutritive role during development. The stratum intermedium is a flattened layer two or three cells thick which overlays the inner enamel epithelium. It is proposed to function in protein synthesis or transport to and from ameloblasts.[2] The inner enamel epithelium is composed of a single layer of cells that become increasingly columnar, eventually becoming ameloblasts (enamel-forming cells). This layer is separated from the dental papilla by a basement membrane and a cell-free zone that is 1 - 2 μm thick (Berkovitz and Moxham, 1981).

It is during the late bell stage that hard tissue formation begins. The inner enamel epithelial cells begin to divide and expand along the interface with the mesenchymal dental papilla, giving form to the characteristic cusps and basins of the future enamel-dentine junction (EDJ). While this permanent outline is forming, an inductive force from the inner enamel epithelium causes an adjacent layer of cells in the mesenchyme to mature into dentine-forming cells, known as odontoblasts. Kollar and Baird (1969) demonstrated that the mesenchyme (dental papilla) is responsible for the gross shape of a

[2] Reid (pers. com.) noted that it has several roles throughout the process of development and eruption, which are essential for enamel development.

tooth, as transplanted molar mesenchyme has been shown to develop molar teeth under incisor epithelium and vice versa. Jernvall and Thesleff (2000) noted that although this is commonly regarded as the inductive method of enamel formation, the exact mechanism of induction is unclear. Sharpe (2000) suggested that it may be the expression of homeobox genes in the mesenchyme that directs the initiation of enamel knot cell differentiation in the epithelial tooth bud. An interesting related issue is the discovery that in mutant mouse embryos, mandibular mesenchyme cells produce cartilage in the absence of epithelial signals (reviewed in Ferguson et al., 2000). The authors suggested that this is consistent with the evolutionary history of the development of cartilage preceding the evolution of true teeth. It is also consistent with the proposed role of the enamel knot (discussed below).

Sharawy and Yeager (1991) suggested that the differentiation of the ameloblasts is progressive, with those in the incisal edge or cusp tip being more advanced than those in the cervical region. They note that the free border of the enamel organ contains a cervical loop, which is a zone of transition between outer and inner epithelial cells. Some hold that the shape of a tooth is determined by the cessation of mitotic division of inner enamel epithelial cells, which occurs when cells begin to differentiate and eventually become ameloblasts. These cells are constrained at the cervical loop, which, in addition to the proliferating cells on either side of the zone of maturation, may cause the epithelium to buckle and form the cuspal outline (Ten Cate, 1998). However, several recent studies have suggested that primary and secondary enamel knots may be responsible for the position and shape of future cusps (Jernvall et al., 1994; Thesleff and Sharpe, 1997; Pispa et al., 1999; Jernvall and Thesleff, 2000). Jernvall et al. (1994) documented the presence of enamel knots over the tips of future cusps just prior to hard tissue development, which may influence the shape of the cusp by initiating development at a specific location and time through specific gene expression. In mutant mice with a reduced number of molar cusps, it was shown that, prior to cusp development, the primary enamel knot was reduced and the secondary knots were fused, representing strong evidence for their role in cuspal development (Pispa et al., 1999)

Once mature, the odontoblasts begin secreting dentine under the cusp tips of the future EDJ, which is then closely followed by the maturation of inner enamel epithelial

cells (pre-ameloblasts) into ameloblasts by reciprocal induction (reviewed in Thesleff and Åberg, 1997). The production of dentine briefly precedes the production of enamel, which forms on top of the dentine. The maturation of ameloblasts is indicated anatomically by a reorientation of the nucleus to the proximal end of the cell in response to a changing nutrient supply, as vascular channels have penetrated the layers of the enamel organ and begin to nourish the cells. This revascularization is necessary as deposition of the initial layers of dentine cuts these cells off from the dental papilla, which had been the source of support during the previous stages. The ameloblasts secrete enamel matrix proteins that rapidly initiate mineralization and form enamel on top of the dentine, moving away from this surface and towards the future crown surface.

After the initial layer of enamel is formed, the ameloblasts develop secretory processes. These Tomes' processes are extensions of the distal end of the cell, which secrete enamel precursor from one side that is roughly perpendicular to the resulting enamel prism (discussed below). Once reaching a certain distance from the EDJ, the ameloblasts withdraw their Tomes' processes, stop secreting enamel matrix, reduce in size, and undergo a maturation stage followed by a protection stage until eruption when they die and/or fall off, leaving the completed surface free of cells. The odontoblasts continue secreting dentine while moving towards the inner pulp of the tooth, and later create secondary dentine slowly throughout the life of a tooth (reviewed in Aiello and Dean, 1990).

Amelogenesis and mineralization

Moss-Salentijn et al. (1997) described three stages in the life cycle of an ameloblast: cytodifferentiation, matrix secretion, and enamel maturation. *Cytodifferentiation* occurs after the mitotic proliferation of inner epithelial cells ceases, and it is defined as the two-step process of epithelial cell transformation into pre-ameloblasts, which eventually become ameloblasts. During this transition, cells become tall and columnar, show nuclear polarity, increase the number of organelles, increase protein synthesis, and eventually develop a Tomes' process at the secretory end of the cell (after initial enamel matrix is secreted). Boyde (1964) described inner terminal bars and a terminal web that separate the body of the cell from the Tomes' process. Junctional

complexes, made up of tight junctions and gap junctions, also form between neighboring cells, which may allow cells to coordinate secretory activity (Warshawsky, 1978), and regulate what may enter or leave the enamel (Ten Cate, 1998). During *matrix secretion*, ameloblasts begin depositing enamel matrix as soon as initial calcification of the adjacent dentine begins. This matrix is made up of 80 - 90% matrix protein and 10 - 20% mineral. Numerous studies have been conducted on the various matrix proteins, which may be divided into several different groups (e.g., amelogenin, enamelin, ameloblastin, tuftelin, etc.; see Robinson et al., 1998). It appears that these proteins regulate mineralization, although the process is not completely understood (Moss-Salentijn et al. 1997; Robinson et al., 1998). Further consideration of specific matrix proteins is beyond the scope of this review, and the process of crystallite growth is reviewed in the following section.

Enamel maturation begins after the ameloblasts have completed their principal matrix secretion, lost their Tomes' processes, and the enamel has reached its final (local) thickness. During this stage, matrix is degraded rapidly and replaced by tissue fluid, and the mineral content increases (Robinson et al., 1995; Moss-Salentijn et al., 1997). Shellis (1981) and several others suggested that when the ameloblasts have secreted matrix to its full thickness, the cells then secrete only mineral ions (but see Boyde, 1989). Maturation ameloblasts also become less columnar, degrade many internal organelles, and participate in re-absorption of the matrix proteins. Other layers of the enamel organ such as the external enamel epithelium and the stellate reticulum also become reduced or lost.

There are two forms of maturation ameloblasts: ruffled-ended and smooth-ended, which cohorts of ameloblasts may switch between (reviewed in Boyde, 1989; Moss-Salentijn et al., 1997; Ten Cate, 1998). The more common ruffle-ended ameloblasts may be important for the incorporation of calcium into maturing enamel, although there is no currently accepted explanation for how calcium and phosphate ions enter enamel (Moss-Salentijn et al., 1997). Smooth-ended ameloblasts appear to be associated with the removal of protein and water (Ten Cate, 1998). There is also a final *protective stage* where ameloblasts develop hemidesmosomes that anchor the cells to the enamel surface until tooth eruption, when they are shed from the surface (Ten Cate, 1998).[3] During this

[3] The lifecycle of the ameloblast from cytodifferentiation to maturation is clearly illustrated in Nanci et al., 1992: Figure 1, p. 336.

stage enamel composition may still be modified, as it has been shown that fluoride may be incorporated into an unerupted tooth.

Based on X-ray emission microanalysis, Boyde (1964) suggested that mineral content increases from the developing front inwards to the EDJ. Beynon et al. (1998a) illustrated this in a microradiograph of developing enamel (Beynon et al., 1998a: Figure 2, p. 354). Ten Cate (1998) described mineralization more specifically as a four-stage process (likely based on the work of Suga [1989]). During the first stage, partially mineralized enamel matrix is formed (30% mineralized except near the EDJ, where a heavily mineralized 8 μm thick layer is initially deposited). In the second stage, a wave of mineralization begins at the surface of the enamel and quickly sweeps to the deeper layers. The third stage is marked by an additional wave of mineralization moving from the innermost layer out to the enamel surface. At the surface a 15 μm layer exists that mineralizes more slowly. Finally, during the last stage, this outer layer mineralizes very quickly and becomes the most heavily mineralized region of the enamel. Ten Cate (1998) reported that the final mineralization pattern is a decreasing gradient running from this outer layer inwards until the more heavily mineralized inner layer is reached at the EDJ (see also Wilson and Beynon, 1987). Thus, it appears that the pattern of mineralization reverses from the developing condition to the completed condition.

Robinson and Kirkham (1985) and several others have shown that in a developing tooth there is are simultaneously a forming edge (enamel matrix secretion zone), transition zone (where enamel matrix begins to be lost), maturation zone (where final mineralization begins), and finally a fully mineralized mature crown. Robinson et al. (1995) also noted that mineralization is not homogeneous in enamel of differing thicknesses, as the inner tissue in thicker enamel is often less well mineralized than outer tissue. Enamel in cervical areas and in fissures also contains more protein than in other areas (Robinson and Kirkham, 1985). Although the authors pointed out that the reason is not clear, they speculate that the local capacity of the matrix (which facilitates crystal growth) may influence this. It is possible that differences in mineralization/protein content may affect the appearance of incremental features, which is considered further below and in Chapter 3.

Genetics of developmental biology

Significant progress has been made in understanding the genetic control of dental development, largely due to the work of Thesleff, Sharpe, Jernvall and colleagues (reviewed in Thesleff and Sharpe, 1997; Ferguson et al., 2000; Sharpe, 2000). Insight into the molecular biology of dental developmental is particularly valuable for the study of mammalian and primate evolution (e.g., Jernvall et al., 1994; Zhao et al., 2000; McCollum and Sharpe, 2001). Many of the biomolecular pathways that lead to dental development are found in invertebrates, which is surprising given that they do not develop homologous structures (Thesleff and Sharpe, 1997). The system appears even more conserved when considering that the same genes and gene interactions are sometimes 'recycled' within a system independently, resulting in both redundancy and novel functions (Weiss et al., 1998). Although a review of the evolutionary development of the mammalian dentition is beyond the scope of this chapter, it is worthwhile to briefly review the control of dental development and the genetic basis of dental patterning.

It has been proposed that the patterning of tooth shape and position appears to be controlled by different homeobox genes expressed in mesenchyme (Sharpe, 1995). The homeobox is a small conserved region of DNA that was originally identified in *Drosophila* (reviewed in Sharpe, 2000). Studies of homeotic mutants have shown that some homeobox genes are responsible for the morphogenesis of embryonic tissue into different structures. The products of these homeobox regions are DNA-binding proteins that regulate transcription, and in effect may hierarchically control gene expression in morphogenesis. In mammals, homeobox genes that are similar to homeotic genes are known as Hox genes, which are important for somatic patterning and development. However, initial dental development appears to be driven by the expression of a number of non-Hox homeobox genes (reviewed in Thomas and Sharpe, 1998; Sharpe, 2000).

Sharpe's (1995) model, termed the 'odontogenic homeobox gene code', suggested that homeobox genes in ectomesenchyme (derived from neural crest cells) are responsible for the patterning of the dentition. He predicted that this tissue is pre-programmed to form a specific type of tooth in a specific region given the appropriate signaling molecule or homeobox gene product. Thesleff and Sharpe (1997) reviewed studies of 'knockout mice' that showed that the expression of these genes differs

spatially, implying that the developmental control of different teeth (incisors and molars) in different jaw positions (maxillary and mandibular) is spatially independent (see also Thomas et al., 1998; Weiss et al., 1998; Keranen et al., 1999; and images of embryonic gene expression in Thomas and Sharpe, 1998). Transplantation studies have shown that maxillary and mandibular cells continue to express specific genes unique to their jaw position, which may be due to their different origin very early in development (McCollum and Sharpe, 2001) These authors have also suggested that canines and premolars may arise from the influence of overlapping domains of gene expression, which is similar to Butler's (1939) field theory of development (but see Butler, 1982).

Concluding remarks

A review of several recent oral biology texts illustrates the need for a universally accepted developmental model of tooth formation.[4] It appears that certain developmental issues remain unresolved, such as the specifics of how induction between tissues occurs, what determines the final form of the tooth, what the roles of matrix proteins are in mineralization, and how ions become added to the maturing enamel. Additional insight into the process of mineralization may be gained from more studies like those of Robinson et al. (1995, 1998) on the chemistry of enamel development. Developmental issues such as the ontogeny of tooth form will continue to benefit from additional work in the field of developmental genetics and cell signaling, particularly as greater emphasis is placed on understanding the molecular biology of dental development as a means of understanding the process of evolution (Jernvall et al., 1994; Zhao et al., 2000; Kangas et al., 2004).

Cellular Level Development

Crystallites

The study of the basic units of enamel, often referred to as the ultrastructure, requires higher resolution techniques than are possible through conventional light

[4] Additionally, different authors insist on using alternative names for structures (e.g., internal enamel epithelium vs. inner enamel epithelium; enamel rod vs. enamel prism), a convention that introduces additional confusion to students of this field.

microscopy. Transmission electron microscopy (TEM) was first applied to replicas of fractured enamel surfaces (Wolf, 1940 [in German]: as cited by Boyde, 1989), and has since been used in numerous studies of ameloblast morphology, crystallite orientation, crystallite size and shape, prism composition, and enamel matrix proteins. As Boyde (1964) noted, an understanding of the patterning and orientation of crystallites, particularly the planes of abrupt change in orientation, is critical to understanding the overall prism structure. Various TEM studies have been conducted in the last 40 years by a number of researchers, including Fearnhead, Rönnholm, Boyde, Frank, Helmcke, Warshawsky, and Nanci. This technique has proven to be invaluable in the study of enamel development, particularly for the visualization of the Tomes' process and the orientation of developing crystallites (illustrated below). Boyde (1989) discussed several of the pros and cons associated with this method, and also provided some highly informative TEM images of mammalian developing enamel surfaces. One of the drawbacks of this method is that it involves generating ultra-thin sections of a highly mineralized structure, which means that enamel crystallites may be broken or deformed (Boyde, 1989). As noted above, other forms of microscopy have been used in the last few decades to evaluate and supplement evidence gathered from TEM studies.

During the 1960's several important discoveries were made regarding the development of enamel. In 1960, two separate workers (Fearnhead and Watson) proved that enamel develops in an extracellular location, as opposed to a model of formation within ameloblasts (reviewed in Gustafson, 1959). Rönnholm (1962a,b) described the boundary between the ameloblasts and the developing enamel surface as a jagged, serrated edge made up of Tomes' processes from the distal ends of ameloblasts, which are each continuous with the cellular plasma membrane (Figure 1.2). The cell membrane, specialized for exocytosis (cell secretion), has many deep infoldings. He described the cross-sectional triangular shape of the Tomes' process as an extension of the lateral wall of the cell on one side (main secretory side) and an ‘end surface’ on the other projecting side (see also images in Gustafson, 1959; Boyde, 1964; Warshawsky, 1978). Crystallites develop roughly perpendicular to this ‘lateral secretory surface’ and are roughly parallel to the 'end surface’ of the next process. Rönnholm (1962a) noted that the boundary between enamel prisms developing from the secretory face of the Tomes' processes is

related to the point of the process, referred to as the 'interprismatic gap', which has also been regarded as the prism sheath or interprismatic region (discussed below). Risnes (1999) recently illustrated differences between rodent and human enamel, showing that the crystallites in the former do not develop perpendicular to the Tomes' process. He suggested that additional factors must also influence crystallite orientation. Boyde (1964) also cited some evidence from comparative mammalian studies to suggest that the length of the Tomes' process may be related to the rate of enamel formation. However, Skobe et al. (1981) reported that rhesus macaque Tomes' process length is greater than that of humans. Given reported similarities in rate between the two, it does not appear that Tomes' process length may predict secretion rates among primates.

An important aspect of enamel formation is that the composition does not differ between prism bodies and interprismatic regions (Rönnholm, 1962a,b; Boyde, 1964). They are both made up of closely packed apatite crystals and small amounts of organic material. The boundaries are due to abrupt changes in crystallite orientation. Risnes (1998) recently noted that it was commonly accepted that the transition in crystallite orientation is intimately related to the distal surface of the ameloblastema (surface of the Tomes' processes and the membrane topography between). He reviewed work that suggested that secretion occurs from two areas: the rim around the base of the Tomes' process, and a flat aspect of the Tomes' process (which was not clear during the earlier work of the 1960's). Rönnholm (1962b) reported that the orientation of crystallites within a prism shows a gradual transition from a perpendicular to a more parallel position relative to the prism axis. Boyde (1964) reported that the majority of crystallites tend to be oriented perpendicular to the mineralizing front, and the crystallites in the lateral and cuspal walls of the Tomes' process pits may be parallel to those surfaces. These crystallites undergo 'stroking' by the 'end surface' (*sensu* Rönnholm, 1962b), which affects their orientation and implies further that this surface is not secretory. Meckel et al. (1965) stated that crystallites may range from an orientation of 0° - 70° to the long axis of the prism, where the smallest deviation corresponds to crystallites in the prism 'head' and the largest corresponds to the 'tail' (distinctions discussed below; see also Risnes, 1999). Helmcke (1967) described the orientation of crystallites within a prism as fan-shaped, with an axis that is not directly in the center of the prism.

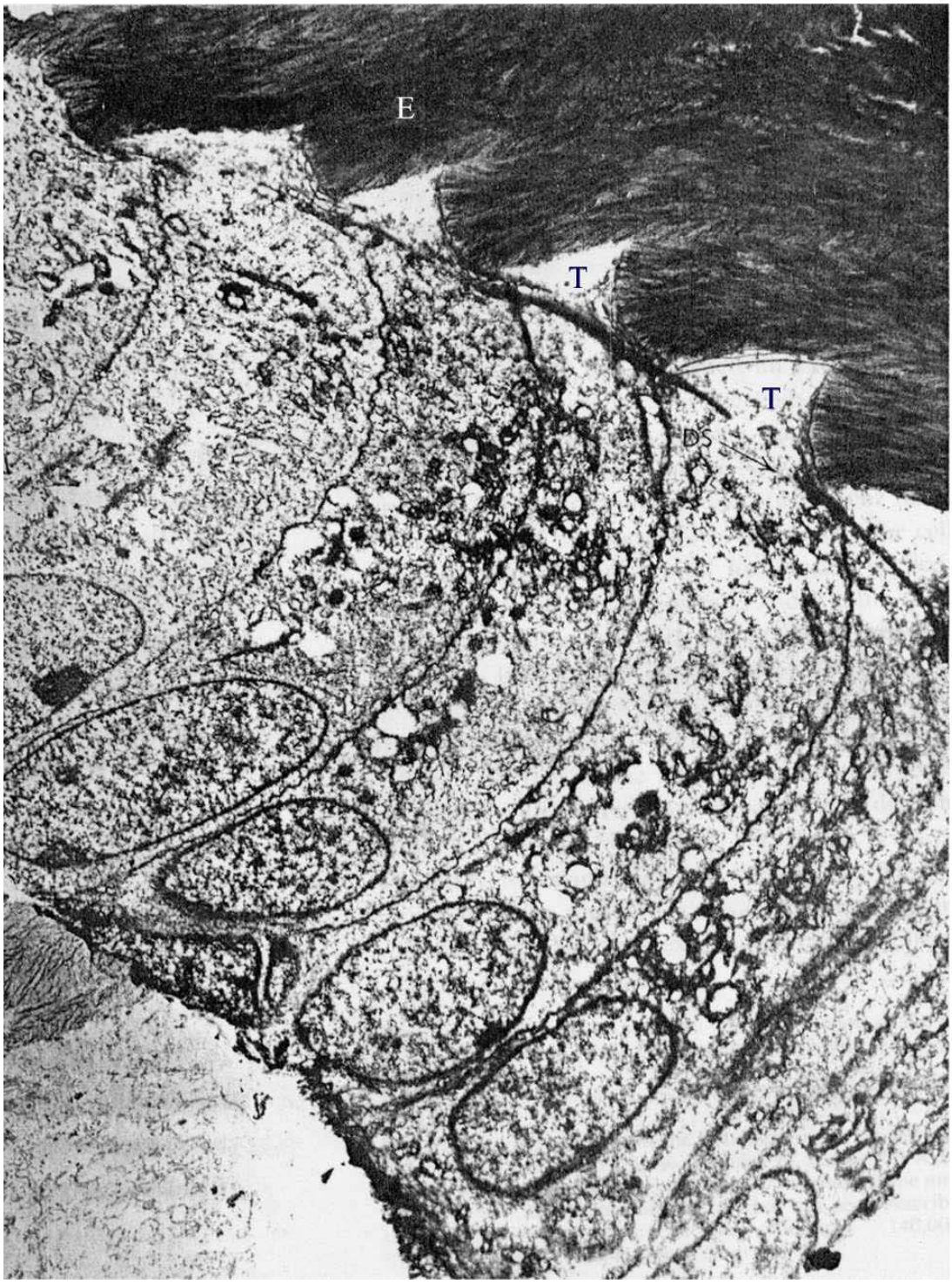


Figure 1.2 Transmission electron microscope (TEM) image of the developing enamel surface of human enamel. The Tomes' processes coming from the ameloblasts (below) are indicated (T) and the enamel is indicated (E) near the top of the image, which is made up of thin crystallites. Modified from Rönnholm (1962a).

Robinson et al. (1995) suggested that the first enamel crystallites are likely nucleated by apatite crystallites in dentine, although a specific enamel protein has also been implicated in the nucleating role. Once started, the crystals within the organic matrix grow rapidly in length (and in width, although not as quickly), adding minerals and eventually displacing the organic matrix. Robinson et al. (1995) described the role of the matrix as 'an ephemeral informational support' that guides and directs crystallite and prism morphology, modulating their growth. The ribbon or lath-like crystallites increase in length and width until they are tightly packed, resulting in an increase in overall mineralization. They may also fuse with neighboring crystallites during maturation (Sakae et al., 1997). It is important to consider that some researchers believe once the enamel is initially 'seeded' at or near the dentine, no new crystals are added (Robinson et al., 1995). However, Palamara et al. (1980) suggested that new crystals must start within the enamel, based on the overall pattern of increasing and decreasing crystallite width (from the EDJ to the surface of the tooth), which is in contrast to the increase in overall surface area (relative to the EDJ).

There are several different theories regarding the size and shape of crystallites (reviewed in Rönnholm, 1962b; Boyde, 1964, 1989; Sakae et al., 1997). Boyde (1989) reported that crystallite diameter is relatively uniform throughout the enamel, and that approximately 10,000 crystallites constitute a prism (although he did not specify prism width or position in the enamel). However, several studies have shown that crystallite width is larger in the outer layers of enamel, which Sakae (1988) attributed to less ionic substitutions than in the inner enamel. Palamara et al. (1980) provided data that showed that crystallite width increases up to the sub-surface enamel, where it then decreases to the surface.

Prism features

a) Packing pattern

The development and application of the SEM in the 1960's provided a novel form of high resolution investigation. Boyde (1964) was the first to apply SEM to study dental tissues, particularly the developing enamel surface. One of Boyde's most frequently cited models is of enamel prism packing pattern. His 1964 dissertation detailed previous work

in this area, which he integrated with observations of the developing enamel surface in his description of patterns 1, 2, and 3 (along with variants of 2 and 3) (Figure 1.3). He credited the influential work of Shobusawa (1952) and a few others who attempted to describe the various mammalian prism cross-sectional shapes. Boyde (1964) also predicted the specific orientation of crystallites in each pattern with a model of different planes of section (Figure 1.4). Each pattern of prism cross-sectional shape was described as the "outline of the plane of sudden changes in crystallite orientation, which is determined by the way in which the depressions in the developing front fill in" (p. 159).

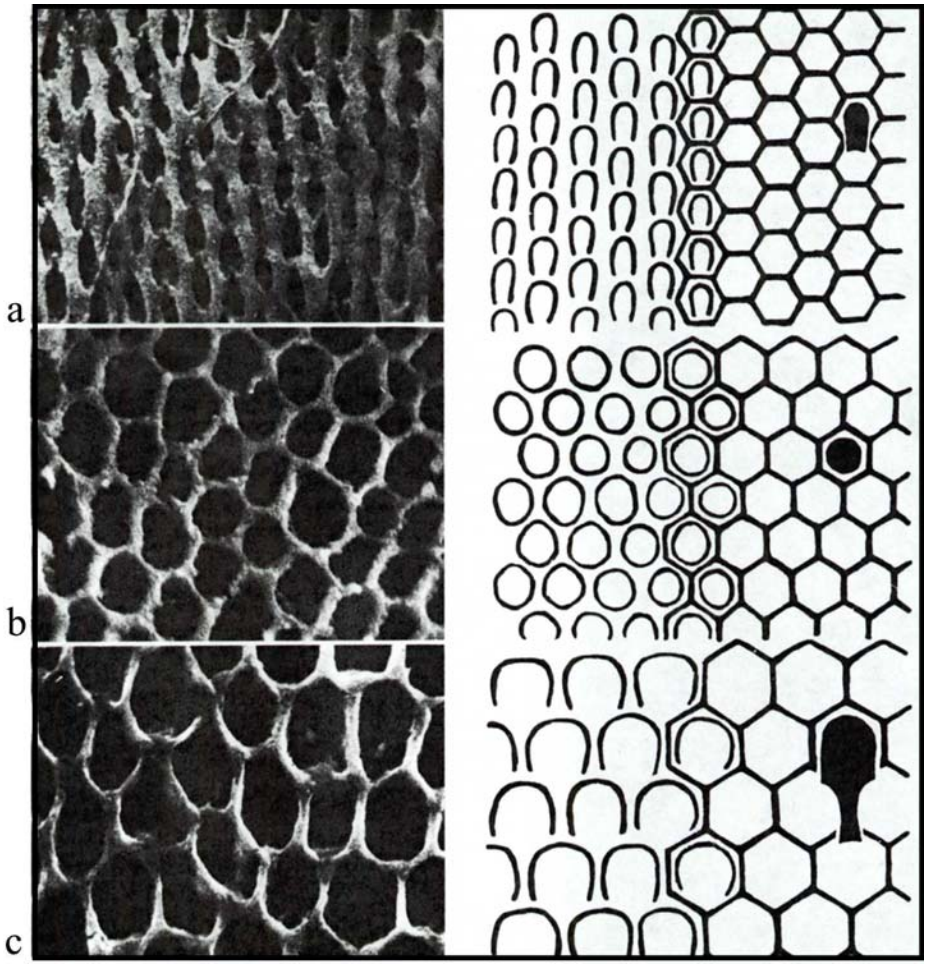


Figure 1.3 (a-c) Enamel prism packing patterns arranged by size, with scanning electron microscope images on the left, and the relationship between the prism (dark outline) and developing enamel surface diagrammed on the right (cuspal direction is to the top). a) Pattern 2 enamel, b) Pattern 1 enamel, c) Pattern 3 enamel. Modified from Boyde (1969a).

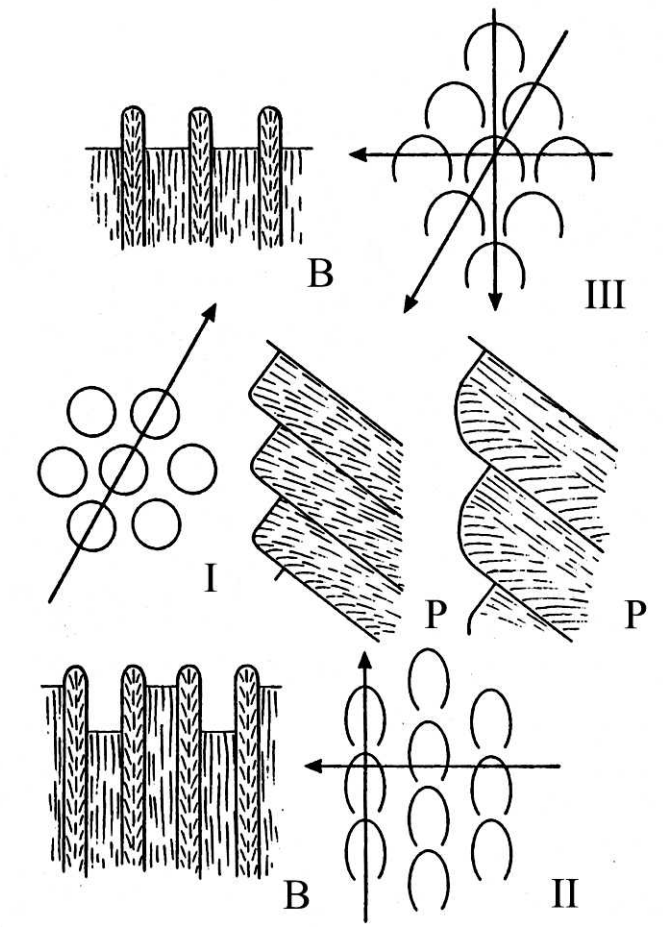


Figure 1.4 Model of the relationship between prism packing pattern and crystallite orientation within prisms and interprismatic spaces. Packing pattern is indicated to the right of the circular outlines: I, II, and III for patterns 1, 2, and 3 respectively. Profiles are labeled as ‘B’ for battlements and ‘P’ for picket-fence profile. Direction of section is indicated with the arrow, which points to the resulting profile. Modified from Boyde (1989).

He described two common arrangements of crystallite bundles when developing enamel is sectioned in a longitudinal plane: the 'picket-fence' (or 'saw tooth') arrangement and the 'battlements' arrangement. Boyde (1964, 1989) described the picket-fence profile as having a single discontinuity (i.e., ‘prism sheath’ or boundary) per Tomes' process pit with a change in crystallite orientation on either side of the Tomes' process (related to the tip of the Tomes' process). Only pattern 2 and pattern 3 could be sectioned to create this profile, and differences between the spacing of the 'pickets' and the transition in crystallite orientation may distinguish the patterns, but not necessarily. The battlements

arrangement may result from a section of pattern 1, 2 or 3 prisms. In this arrangement, a prism discontinuity will exist at either side of the Tomes' process pit (walls) that relates to the non-secretory sides of the Tomes' process. Crystallites in the battlements profile of the prisms will be nearly parallel with the long axis of the prism, and any deviation will not be as marked as in the picket-fence plane of section. This profile will be generated from any direction of pattern 1, but only a transverse section of pattern 2 or 3. Pattern 2 may be distinguished as the battlement depressions appear at alternate depths due to the staggered rows of prisms, but the pattern 3 battlements may be indistinguishable from pattern 1. Boyde's (1964) model suggested that additional variant battlements patterns may also permit distinction between the three packing patterns, which will not be discussed here. From an examination of published TEM photographs in various planes of section, it is clear that the picket-fence and battlements profiles are found at the developing enamel surface, but quite often these patterns and variants of both appear in the same area.

Boyde (1964) also diagramed the 'honeycomb' plane of section, which is observed when the ameloblasts and/or prisms are cut transversely. He illustrated several scenarios of differing oblique section planes, which passed through the inner ends of the ameloblasts, terminal bars, Tomes' processes, and the honeycomb of enamel, as well as the filled-in honeycomb areas (e.g., Figure 1.5). He noted that the honeycomb may be regarded as interprismatic enamel in the case of pattern 1 and 2, but not in pattern 3, as the tails make up the honeycomb. In patterns 2 and 3, the crystallites in the depressions show a different orientation than those in the honeycomb. In pattern 1, Boyde (1964) noted that all crystallites run in roughly the same direction.[5] It is clear in this plane that one ameloblast/Tomes' process sits in each ‘hole’ of the hexagonal honeycomb (Boyde, 1964), and depending on the angle of section, the shape of the Tomes' process may be reconstructed. An examination of several of these sections suggests that the Tomes' process has a slightly concave or flat secretory face that relates to the area that is filling in, as well as a concave outer border which corresponds to the boundary or walls of the pit.

[5] This is not apparent from his model shown in Figure 1.4.

Prior to this work, Rönnholm (1962b) speculated that a minimum of 3 cells contributed to a prism, but no one had attempted to relate this to the various patterns of cross-sectional prism arrangement. Boyde (1964) related the formation of each pattern to a characteristic number of ameloblasts: pattern 1 was formed by 1 cell, pattern 2 by 2 cells, and the majority of pattern 3 by 3 cells with a small contribution by a fourth ameloblast (cells covered by dark outlines on right in Figure 1.3). Note that in patterns 1 and 2, interprismatic material lies between the true prisms, whereas in pattern 3, prism 'heads' (circular regions) are separated by prism 'tails', and there is no true interprismatic enamel. Recent work by Risnes (1999) and Radlanski et al. (2001) has demonstrated the two- and three-dimensional spatial oscillations of prism tails, relative to the position of the heads of neighboring prisms.

Boyde (1964) also described the various patterns found in different mammals, and noted that patterns 1, 2 and 3 are found in primate enamel (reviewed in Boyde, 1989). Pattern 1 frequently appears over the tip of cusps and in the incisal edges, whereas pattern 3 is more common than pattern 2 in other regions, specifically in hominoids. Boyde (1969a) also showed that the type of packing pattern correlates with the ameloblast size, ranging from large pattern 3 ameloblasts to the smallest pattern 2 ameloblasts. Several studies have been conducted on the type and distribution of packing patterns; these have been reviewed in numerous sources (e.g., Shellis and Poole, 1977; Martin, 1983; Boyde and Martin, 1984a,b, 1987; Martin et al. 1988; Boyde, 1989; Maas and Dumont, 1999).

b) Prism sheaths and prism width

Boyde (1964) and Helmcke (1967) reviewed the nature of the ‘prism sheath,’ which Rönnholm (1962b) showed was the result of planes of discontinuity in crystallite orientation between prisms. Boyde (1964, 1989) noted that the actual discontinuity is quite small (~0.2 μm), and should not be visible under light microscopy. The reason for the appearance of relatively large spaces between prisms in light microscopy is due to large differences in the refractive index of the two regions, causing an optically exaggerated boundary. It is commonly held that the organic content is higher in these regions, which causes a lower refractive index than that of the surrounding enamel. This effect led early workers to believe that there were actual sheaths surrounding prisms.

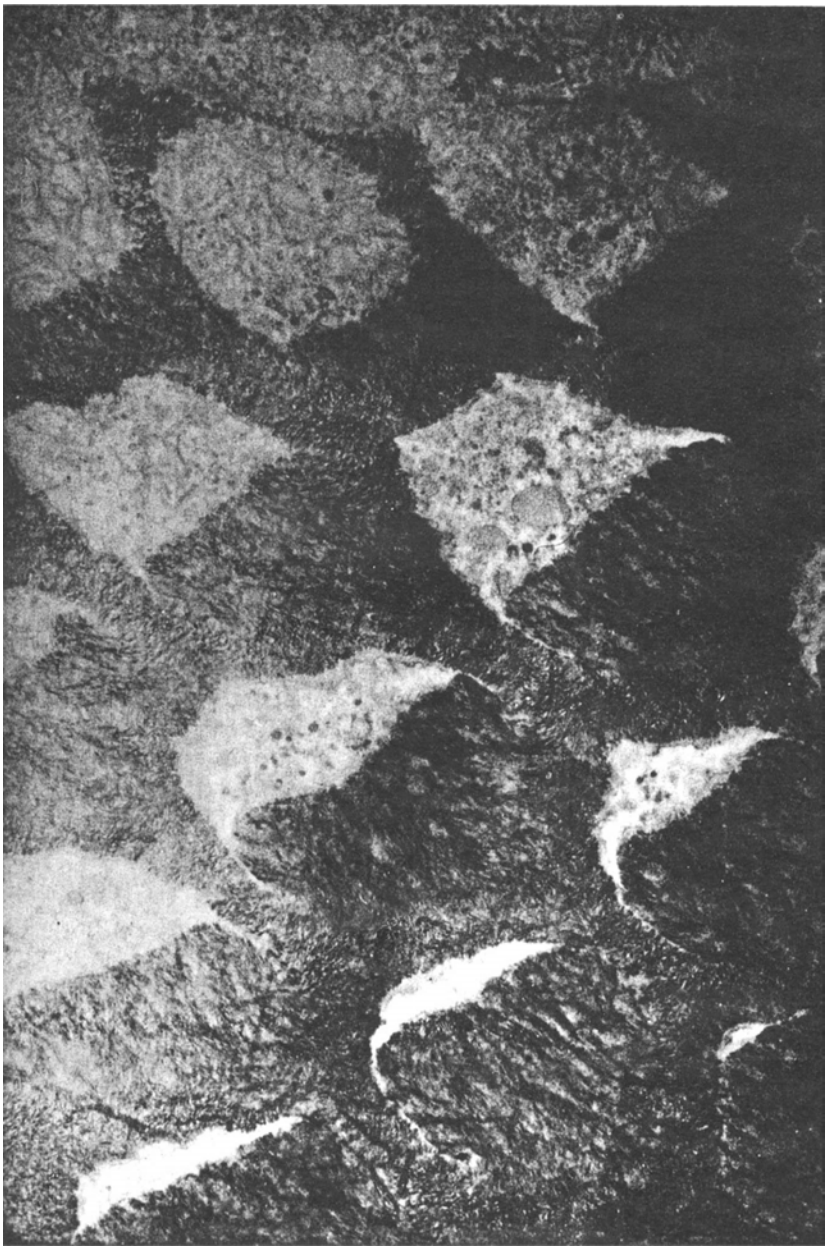


Figure 1.5 Transmission electron micrograph of a transverse plane of section illustrating the 'honeycomb pattern,' or plane of section across Tomes' processes (light structures) and Pattern 3 enamel prisms (dark circles), with interprismatic material making up the tails of the prisms. Field width 5 is μm. Reproduced from Boyde (1989).

Published evidence is equivocal regarding changes in prism width from the EDJ to the tooth surface. Pickerill (1913) reported that human prism width near the EDJ ranges from 2.5 - 3.1 μm, and at the surface it ranges from 5.7 - 6.5 μm (see also similar values in Skobe and Stern, 1980; Osborn, 1981). Prior to this work, it had been assumed

that ‘supplemental prisms’ existed in the middle and outer enamel, which accounted for the increased area of the surface relative to the EDJ (proposed by Retzius, 1837). Rönnholm (1962b) suggested that the increase in prism width towards the surface of the enamel is due to increases in the diameter of the crystallites. Risnes (1998) stated that the distance between the ameloblasts and/or prisms increases towards the enamel surface (but did not provide evidence that the actual prisms become wider). He suggested that the ameloblastema may stretch as it approaches the surface, which is reflected in the extension rate and the morphology of the Retzius lines (discussed below). He also cited work by Radlanski et al. (1986, 1988) that suggested that, rather than increasing width to generate an increased surface area (relative to the EDJ), the prisms approach the enamel surface at an angle (increasing cross-sectional area) while width remains constant. Skobe and Stern (1980) also supported this theory of 'oblique approach' as a factor contributing to the increased surface area, but also suggested that prism width increases. However, Risnes (1999) recently compared prism widths at the surface of a hypoplastic event and in the surrounding enamel, where the latter were much greater than those at the surface of the hypoplasias, and did not appear to show oblique angulation.

Shellis and Poole (1977) noted an average prism width of 5 μm for several families of non-human primates. Boyde and Martin (1987) stated that primate prism widths are approximately 6 - 7 μm. Dean (1989) illustrated a prism width of 6 μm in a micrograph of *Parathropus boisei* enamel. However, none of these sources mentioned changes in width. Ockerse (1963) reported that the prism width in vervet monkeys ranges from 1.3 - 5 μm, increasing from the EDJ to the surface. Dean and Shellis (1998) provided data suggesting that in *Hylobates*, *Pongo*, and *Proconsul*, prism width either remains the same or actually decreases from the EDJ to the tooth surface (from 4.5 - 5.6 μm near the EDJ to 4.1 - 3.8 μm at the surface), which results in characteristic ‘S-shaped’ Retzius lines in the lateral enamel (discussed below). Radlanski et al. (2001) reported that prism width in human cervical enamel appeared to remain constant from the EDJ to the surface, which was measured in optical and physical serial sections. More recently, Dean (2004) characterized prism width in *Pan*, *Pongo*, and *Homo* as a fairly uniform 4.5 - 5.5 μm spacing in the inner and outer lateral enamel, and sometimes increasing to 6.0 - 6.8 μm in the outer cuspal enamel.

c) Prism paths and decussation

Pickerill (1913) quoted Tomes (no date) as stating that the path of the individual prism is 'more or less a spiral' running from the EDJ to the surface of the tooth. Pickerill (1913) discussed the importance and difficulty of cutting sections to study the nature of the prism path, which he described as a corkscrew path near the EDJ, becoming a wavy longitudinal path as the surface is approached. Following the course of an individual prism was difficult at the time, as the three-dimensional path was nearly impossible to image. Helmcke (1967) illustrated the course of five prisms for a short distance, which was determined from 90 serial sections (imaged via electron microscopy) that were used to create a solid reconstruction (but see criticisms and models of Osborn, 1973). This method required an immense amount of labor, and is necessarily discontinuous.

Boyde (1989) described the course of a prism as sinusoidal or helicoidal, with several changes in direction along its path. Osborn (1973, 1981, 1990), Ten Cate (1998), and Macho and colleagues (Jiang et al., 2003; Macho et al., 2003) also proposed theoretical models of prism course and arrangement. Jiang et al. (2003) and Macho et al. (2003) used algorithms to develop a graphical model from images of naturally fractured teeth. Their work suggested that a simple sinusoidal or helicoidal explanation did not account for the fact that the wavelength of prism path in a transverse plane differed from the wavelength in a longitudinal plane (e.g., Jiang et al., 2003: Figure 6, p. 456; Macho et al., 2003: Figure 1, p. 83). However, these studies were only able to sample a small area of lateral enamel, and it is not clear how applicable these models are to other regions such as the cuspal enamel. Radlanski et al. (2001) recently demonstrated that it is possible to follow a prism from the EDJ to the surface of the tooth using SEM serial sections or CLSM imaging. They found that prisms change positions relative to other adjacent prisms, but do not appear to show dramatic oscillations in human cervical enamel.

Massler and Schour (1941, 1946) were the first to state that the length of a prism relates directly to the rate and duration of secretion. They showed that the time of formation could be determined by dividing the 'normal' prism length by the 'characteristic rate of apposition.' However, it is unlikely that they corrected for the three-dimensional prism deviation from a straight path. Risnes (1986) attempted to correct for two- and three-dimensional prism path to determine the rate and time of enamel formation in

human enamel. He assumed that prisms mainly deviate in a transverse plane in lateral enamel, and used a trigonometric formula based on a two-dimensional model of the prism path from the EDJ (modeled in Risnes, 1986: Figure 3, p. 396). For cuspal enamel, he devised an equation based on a three-dimensional model of a spiral prism path (modeled in Risnes, 1986: Figure 4, p. 397). Based on the results of this study, many researchers have used 1.15 as a correction factor for cuspal prism decussation in fossil and extant primates, although this value was based on the prism path in the lateral enamel of human premolars. Macho et al. (2003) recently noted that this factor is an overestimation of the true prism deviation (discussed further in Chapter 2).

Another characteristic of prism path is the phenomenon of decussation, which is prism crossing in the form of 'X'. It has been formally described in rodent enamel, where single alternate layers of prisms cross each other at right angles, but may also be loosely applied in cases where groups of prisms are oriented in different directions to one another. Boyde (1964, 1969b) provided an excellent review of early descriptions of this phenomenon, and described the origin of the term 'Hunter-Schreger band,' which refers to successive layers of prisms in differing orientations running from the EDJ towards the tooth surface (Figure 1.6a). Pickerill (1913) built upon the work of Tomes, describing longitudinally and transversely cut groups of prisms in the same section, known as parazones and diazones respectively (Figure 1.6b). These zones may involve a more subtle transition in prism orientation in primates than in rodent enamel (Kawai, 1955; Boyde, 1969b).

Several explanations and models for the development of decussation have been put forth, but the developmental basis of this phenomenon is not completely understood (Boyde, 1989). Boyde (1964) suggested that in rodent enamel, unequal growth in the Tomes' process pit causes decussation, and that the pressure of filling in the pits actually drives the ameloblasts and causes their movement. Osborn published several models of prism paths and decussation in human enamel (e.g., Osborn, 1967, 1973, 1981, 1990), including three-dimensional models of prism path, prism decussation, and Hunter-Schreger bands (Osborn, 1981: Figure 6.21, p. 179; Osborn, 1990: Figure 11, p. 877).

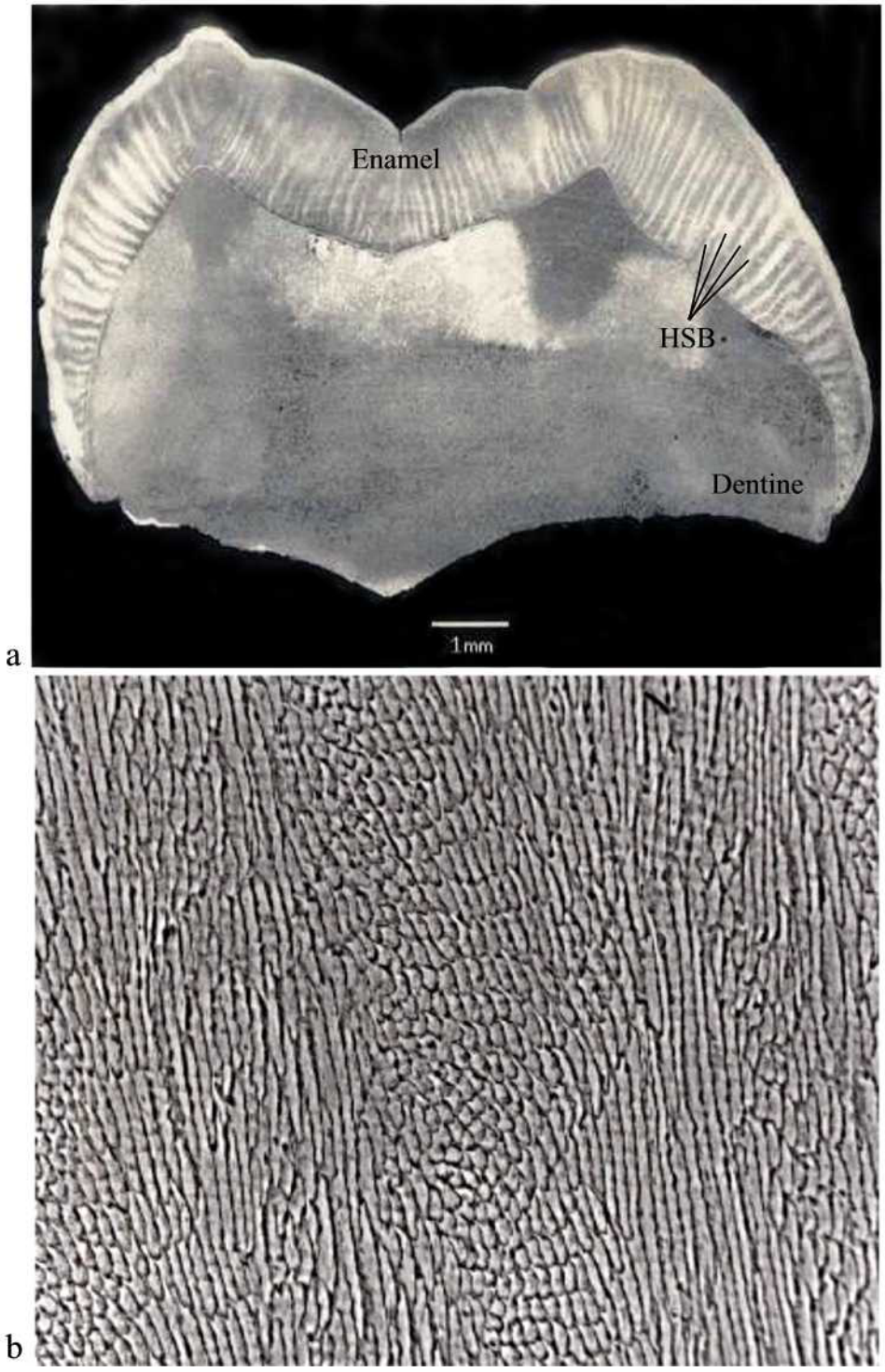


Figure 1.6 (a,b) Scanning election micrographs of *Afropithecus turkanensis*. a) Sectioned molar overview showing Hunter-Schreger bands (HSB) running from the enamel dentine junction towards the tooth surface. b) Lateral enamel showing parazones (horizontally oriented longitudinal prisms) and diazones (cross-cut prism viewed end-on). The tooth surface is toward the top of the image. Smith and Martin (unpublished images).

Osborn examined the effects of changing certain parameters of his model, such as the shape of the EDJ and the growth front, enamel thickness, amount of prism width increase, amount the transverse prism path oscillations are dampened, and the prism path in a longitudinal plane. Certain alterations of these factors produced Hunter-Schreger bands that are very similar to those seen in human enamel. Osborn (1990) also noted that his model suggested that both parazones and diazones may be seen within the same Hunter-Schreger band, and that the borders of the zones do not necessarily match the bands (although this is difficult to assess from his illustrations, see Osborn, 1990: Figure 7b, p. 875 and Figure 11, p. 877). This model seems to contradict the convention that Hunter-Schreger bands are defined by differences in prism orientation. Additional models for the formation of Hunter-Schreger bands will not be considered here (see also Skobe and Stern, 1980; Moss-Salentijn et al., 1997; Jiang et al., 2003; Macho et al., 2003).

An additional aspect of prism path and decussation is the 'gnarled enamel' found typically in the cuspal region over the dentine horns (Figure 1.7a,b). It appeared to be recognized as far back as Retzius (1837), where it is depicted in one of his figures (Retzius, 1837: Figure 7, plate XXI). Boyde (1964) noted that the curved, convex surface of the EDJ at the dentine horn may contribute to an exaggerated angular relationship between ameloblasts and prisms, resulting in this phenomenon. Enamel within the cingulum over an incipient dentine horn also supports this to a lesser degree (Figure 1.7c,d). Osborn (1967) proposed that the appearance of gnarled enamel is due to the tight spiral arrangement of the prisms that are arranged in adjacent groups of opposite orientation. Unfortunately, prism decussation and gnarled enamel frequently obscure or distort incremental features in the cuspal and lateral enamel.

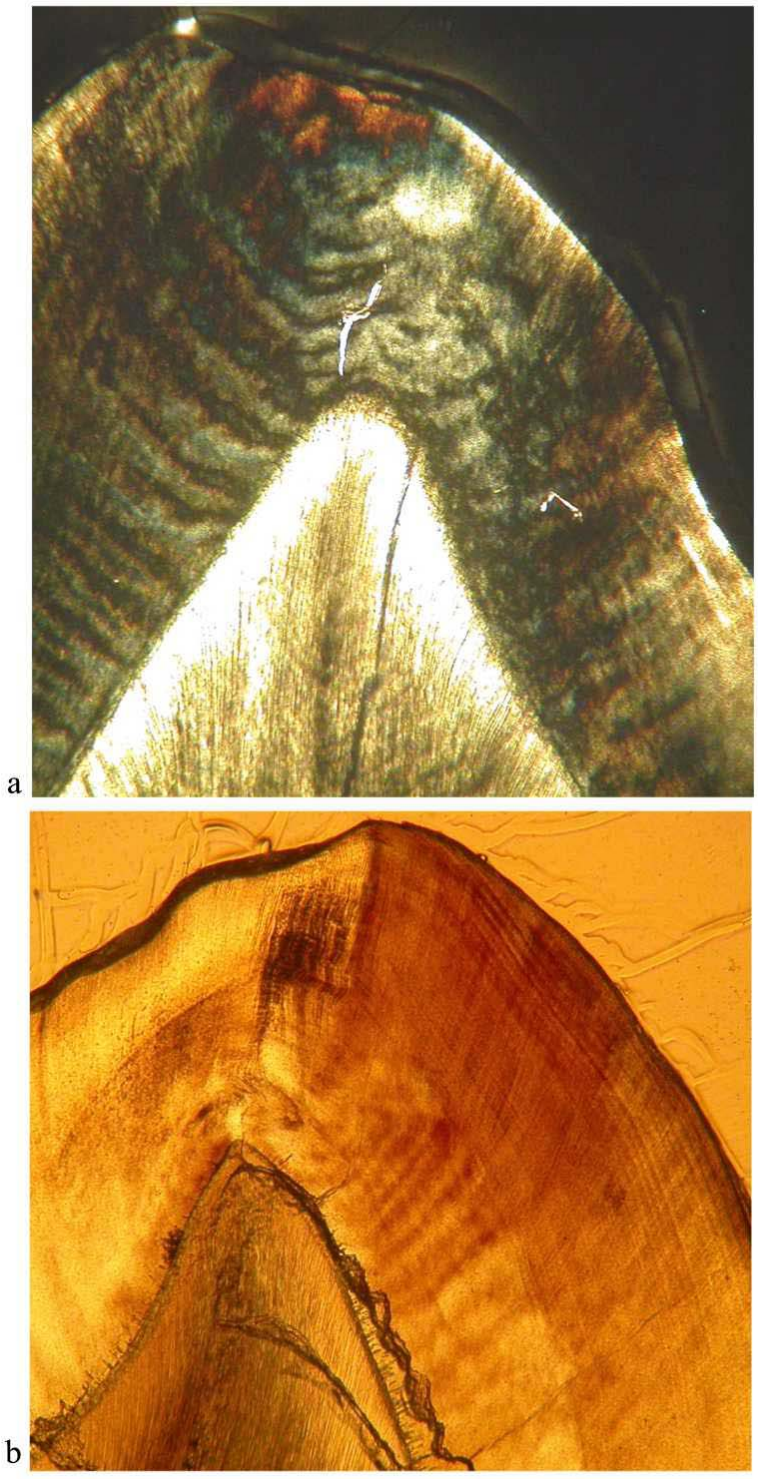


Figure 1.7 (a-d) Polarized light micrographs of gnarled enamel over the dentine horn in chimpanzee molar cuspal enamel. a) Full polarization, b) half polarization. Typical Hunter-Schreger bands may be seen in the enamel on the sides of the horns running to the tooth surface. (Figures [c-d] are on the following page.)

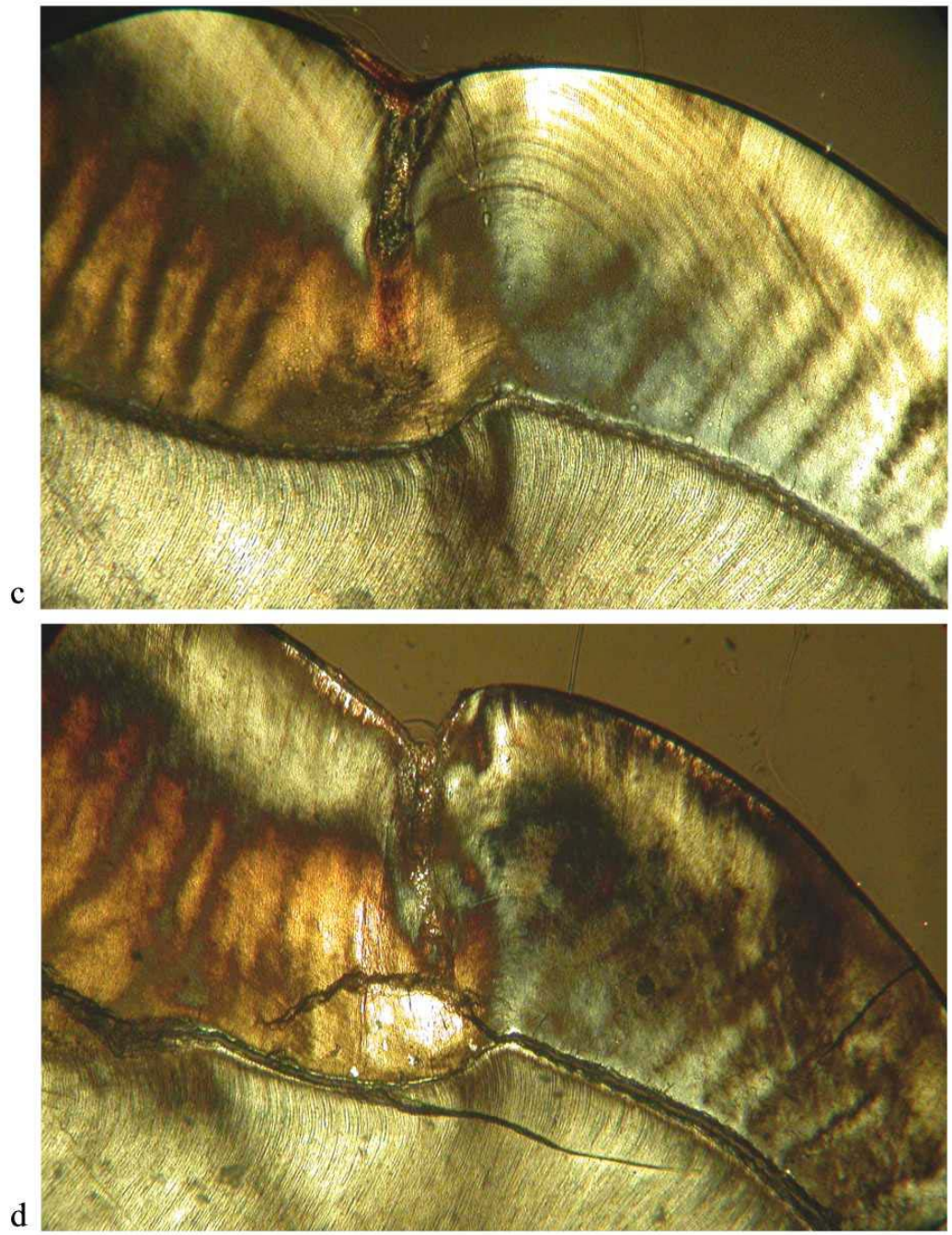


Figure 1.7 (c,d) Polarized light micrographs of gnarled enamel in the cingulum, located over an incipient dentine horn in chimpanzee molar enamel. In each image, the tip of the cusp is to the left, and the cervix is to the right. Typical Hunter-Schreger bands may be seen in the enamel on both sides of the incipient horns.

d) Aprismatic enamel

The first- and last-formed layers of enamel at the EDJ and tooth surface are often characterized by irregular prisms or a structureless, aprismatic (or prism-free) layer. Pickerill (1913) referred to this in 'soft' enamel, where he described a layer with a 'faint striation' perpendicular to where the prisms cease just below the surface. Gustafson (1959) also noted and illustrated aprismatic enamel, explaining it as a broad layer of ‘compressed sections’. Boyde (1964) suggested that aprismatic enamel may be related to relatively slow enamel production, as the ameloblasts at the EDJ have yet to form a Tomes' process, and have lost it in the sub-surface enamel. Ripa et al. (1966), Gwinnett (1967), and Whittaker (1982) specifically addressed this feature in deciduous and permanent surface enamel. Ripa et al. (1966) suggested that it is more common in deciduous teeth, occurring in all of the deciduous teeth they examined (n=28), and that it is not more prevalent in any specific tooth type. They described two types of aprismatic enamel: the ‘continuous band’ type (parallel to the surface), and the ‘onion layer’ type (related to Retzius lines at the surface) (Figure 1.8), which is less common in deciduous teeth. They noted that this layer is not entirely devoid of features, as fine striations or ‘surface parallel laminations’ are often visible, which may be similar to either cross-striations or Retzius lines (discussed below).

Whittaker's (1982) study on 550 deciduous and permanent human teeth may represent the most thorough examination of sub-surface enamel, supporting many of the conclusions above. He documented the variation and depth of aprismatic sub-surface enamel in different regions of different tooth types, and also found that deciduous teeth were more likely to show aprismatic enamel than permanent teeth. Additionally, he reported that the thickness of aprismatic zones appeared to increase from anterior to posterior teeth (in deciduous and permanent teeth). Kodaka et al. (1989) also examined this feature in human deciduous teeth with multiple forms of microscopy, which they used to document variation and categorize four different classes of aprismatic enamel based the distinction of prism boundaries, crystallite orientation, and the presence of a laminated appearance. However, they did not mention aprismatic enamel found in association with Retzius lines (as noted by Gwinnett [1967]).

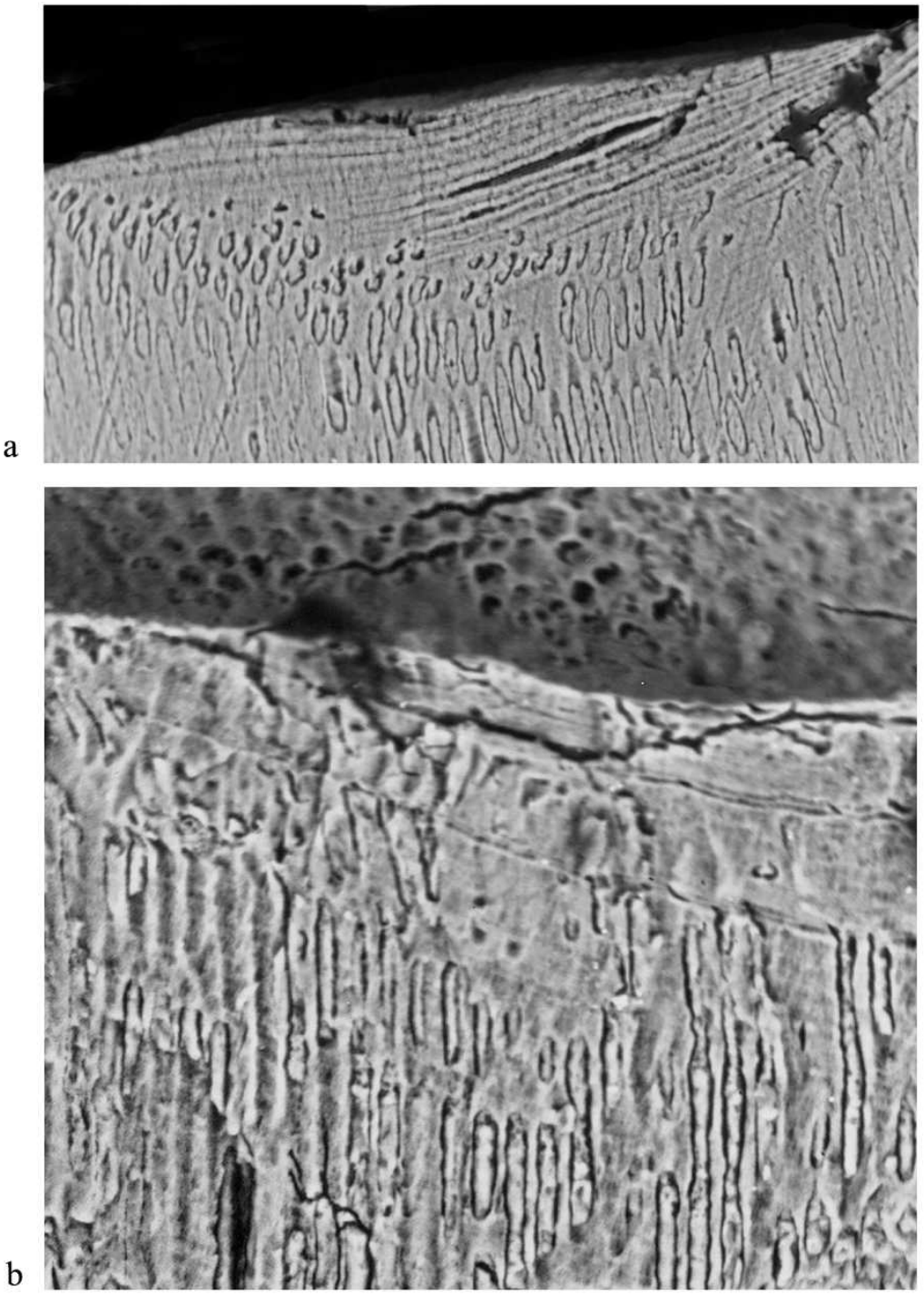


Figure 1.8 (a,b) Scanning electron micrographs of aprismatic enamel in *Afropithecus turkanensis* molar enamel. a) Parallel to the surface in a polished and etched block, and b) related to Retzius lines in a naturally fractured preparation. Prisms run vertically and the surface is at the top. See text for reference to Ripa et al. (1966) and a description of the continuous band and onion layer aprismatic enamel types. Smith and Martin (unpublished images).

Martin (1983) did not address this layer specifically, but it may be seen in his SEM images of *Pongo* and *Hylobates* enamel (Martin, 1983: Fig. 5.7d, p. 342; Fig. 5.8a,d, p. 345). Boyde and Martin (1987) used TSRLM to examine prism packing pattern and aprismatic layers in a large number of extant and fossil primates. They reported that this layer is common in primates, extends a variable distance below the surface, and often grades into pattern 1 enamel beneath (see also Shellis and Poole, 1977; Dirks, 1998; and Smith et al., 2003a, 2004 for additional descriptions in all major groups of extant non-human primates as well as in Miocene hominoids).

Boyde (1964) noted that crystallites in aprismatic enamel show a more uniform, parallel orientation, and that mineralization is greater than in the adjacent prismatic enamel. Gwinnett (1967) also noted that crystallites in aprismatic enamel commonly show a uniform orientation, which he suggested is approximately equivalent to the mean deviation of the underlying prismatic crystallites (illustrated in Gwinnett, 1967: Figure 12, p. 385). He elaborated on the onion layer (scale-like) appearance of areas associated with Retzius lines, noting that crystallites almost showed a perpendicular orientation (to Retzius lines) as they reached the surface. However, Weber et al. (1974) noted that curvilinear Retzius lines in this layer exhibited a decreased concentration of crystallites, but no significant deviation in orientation. Additionally, Palamara et al. (1980) noted that crystallite size in the aprismatic sub-surface enamel is very similar to the aprismatic enamel at the EDJ, suggesting similar formation.

A few studies provided estimates of the width of aprismatic layers, which may be variable in appearance and location, and may show some type(s) of incremental features (e.g., Shellis and Poole, 1977; Whittaker, 1982; Smith et al. 2003a). Whittaker reported that the maximum thickness of aprismatic enamel in deciduous and permanent teeth was 100 and 90 μm, respectively, but was generally much less than these values. Smith et al. (2003a) reported that, when present, this layer ranges from 21 - 63 μm in thickness in two molars of *Afropithecus turkanensis*. Shellis and Poole (1977) reported a very thin 5 - 10 μm superficial layer in *Gorilla* and *Pan*, taxa they noted are not generally characterized by the aprismatic 'scaly or layered' surface enamel of other non-hominoid primates. Other studies have reported on the nature and spacing of associated incremental features. Whittaker (1982) reported that the average spacing of increments in human aprismatic

enamel was 5 μm. Shellis and Poole (1977) reported 'finer-striations' with a repeat interval of 2 μm in the aprismatic outer cervical enamel of Old World monkeys. In some regions of *A. turkanensis*, closely packed striations were seen with the SEM that were more closely spaced than nearby cross-striations, which may represent the final few days or hours of enamel secretion (Figure 1.8a) (Smith et al., 2003a). Risnes (1998) and Li and Risnes (2004) published several excellent images of aprismatic human enamel and also suggested that the incremental layers in these regions show a relationship with cross-striations (Risnes, 1998: Figures 2,3, p. 336; Li and Risnes, 2004: Figure 1, p. 47). Li and Risnes (2004) suggested that cross-striations continued into aprismatic enamel, and that these increments are equivalent to daily lines (discussed below in the section on laminations). They also illustrated an area of aprismatic enamel that shows light and dark bands, which became more pronounced with longer periods of acid etching. They suggested that the dark bands represent areas with less densely packed crystals than the light bands, which may have implications for the formation of incremental lines (but the effects of acid etching must be considered as well).

Concluding remarks

Additional studies on crystallite growth and dimensions may resolve some of the debate over their size throughout the enamel, as well as the theory that all crystallites begin near the EDJ. Considering that crystallite discontinuities characterize prism boundaries, it is hard to imagine that new crystallites do not begin within prisms (a view also advocated in Boyde [1989] and Risnes [1999]). It does not appear that the number and size of crystallites in individual prisms has been thoroughly examined in the various patterns of enamel prisms, nor has the width or daily volume of prisms been related to packing pattern.[6] It is hoped that improvements in electron microscopy will lead to greater insight, as additional work is needed to determine the nature of crystallite properties throughout the enamel. Due to the nature of TEM studies, it is critical that artifacts are carefully controlled for, as it is easy to imagine how crystallites may be inadvertently modified during preparation.

[6] However, see Boyde (1969a) regarding ameloblast secretory territory

Boyde (1964) cautioned that his model of prism packing pattern should be considered a diagrammatic representation, intended for assistance in visualizing the development of enamel (rather than as a comprehensive and precise model). One criticisms of Boyde's model is that it does not account for decussation (Martin, pers. com.). Ameloblasts must change or alternate spatial positions relative to neighboring cells to produce this structural feature (illustrated in Radlanski et al., 2001). As noted above, Boyde (1964) did consider the characteristic form of decussation in *Murinae*, which he explained and illustrated as a 'filling in from alternative sides of alternative zones'. He noted that decussation is common in areas with arcade-shaped prisms (patterns 2 and 3), but not in pattern 1, as the ameloblasts and prisms are in the same straight line. Similarly, his model does not explain the formation of parazones and diazones. Nor is it clear how regions of prisms may change from one pattern to another, nor how a single prism may change patterns.[7]

A major difficulty in testing Boyde's model of crystallite orientation and packing pattern is that it is very difficult to section prisms in the appropriate plane, especially as ameloblasts and prisms form at an angle to one another. Quite often only a portion of the Tomes' process is visible. It is empirically difficult to determine when the different types of packing patterns have been sectioned in a manner that would confirm these predictions. It appears that Boyde's model does partially explain the relationship between changes in crystallite orientation and the differing sections of the Tomes' processes, yet there is also a wide range of observed variation. Additional work on this topic, particularly on modeling the shape of the Tomes' process and documenting morphological variation in mammals, may facilitate a deeper understanding of the variation of crystallite orientation and the resultant prism patterns (e.g., Risnes, 1999).

Although a large body of published work suggests that prisms increase in diameter from the EDJ to the enamel surface, additional work may help to clarify the relationship between prism width, prism path, packing pattern, and daily secretion rate (DSR). As noted above, Skobe and Stern (1980) suggested that the increase in surface area between the EDJ and the tooth surface is due to a number of factors, including an increase in prism width and an oblique cross-sectional approach towards the surface.

[7] Illustrated with serial SEM sections in Radlanski et al. (2001: Figure 10, p. 411).

They suggested that regional differences exist in ratios of surface area to EDJ area: ~1:1 in cervical enamel, ~2:1 in lateral enamel, and ~ 4:1 in the cuspal enamel. Cuspal enamel may increase more substantially in volume due to the circumferential winding of prisms in parazones (similar to a ball of yarn), an increase in prism width, and the migration of prisms from an upper-lateral origin at the EDJ to the cuspal surface. Lateral surface area was suggested to increase through an increase in prism diameter and some winding of the prisms, but less so than in the cuspal enamel. Cervical enamel prism width may not change at all, which is consistent with the findings of Radlanski et al. (2001).

These suggestions have not been investigated thoroughly, and illustrate the need for an integrated model of prism path that considers changes in prism width, as well as changes in the spatial position of prisms relative to neighboring prisms in cuspal, lateral, and cervical enamel. Such models may be generated from TSRLM or CLSM (e.g., Radlanski et al., 2001; Dean, 2004). One of the reasons that various authors have reported differences in prism width and prism path may be due to difference in the regions of enamel sampled. Additionally, preparations that involve sectioned, ground, and etched enamel may be subject to the influences of prism obliquity, which may affect prisms viewed from end on, or in a longitudinal plane (Figure 1.9). It is empirically difficult to know when the true width of a prism has been captured. It is possible that in some instances, only a portion of the prism head or tail has been sectioned (e.g., see narrow prisms in Figure 1.9b), or that the apparent width represents the prism head and tail, leading to under- and overestimates, respectively (e.g., see very wide prisms in Figure 1.9b).

A final remark on the issue of prism width is based on a consideration of the relationship between prism width and DSR (Smith, unpublished 2001 IPDAS Essay; Dean, 2004). Several studies have noted that average prism at the EDJ is roughly 3 μm wide, and is formed at a rate of approximately 3 μm/day in the cuspal enamel (discussed in Chapter 2). Several accounts of outer enamel suggested the width of the prism is 5 - 6 μm, and the secretion rate is approximately 5 - 6 μm/day. The implications of this relationship are that when the dimensions of the daily secretion product (prism) double, the ameloblasts must increase the volume of enamel production by a factor of eight.

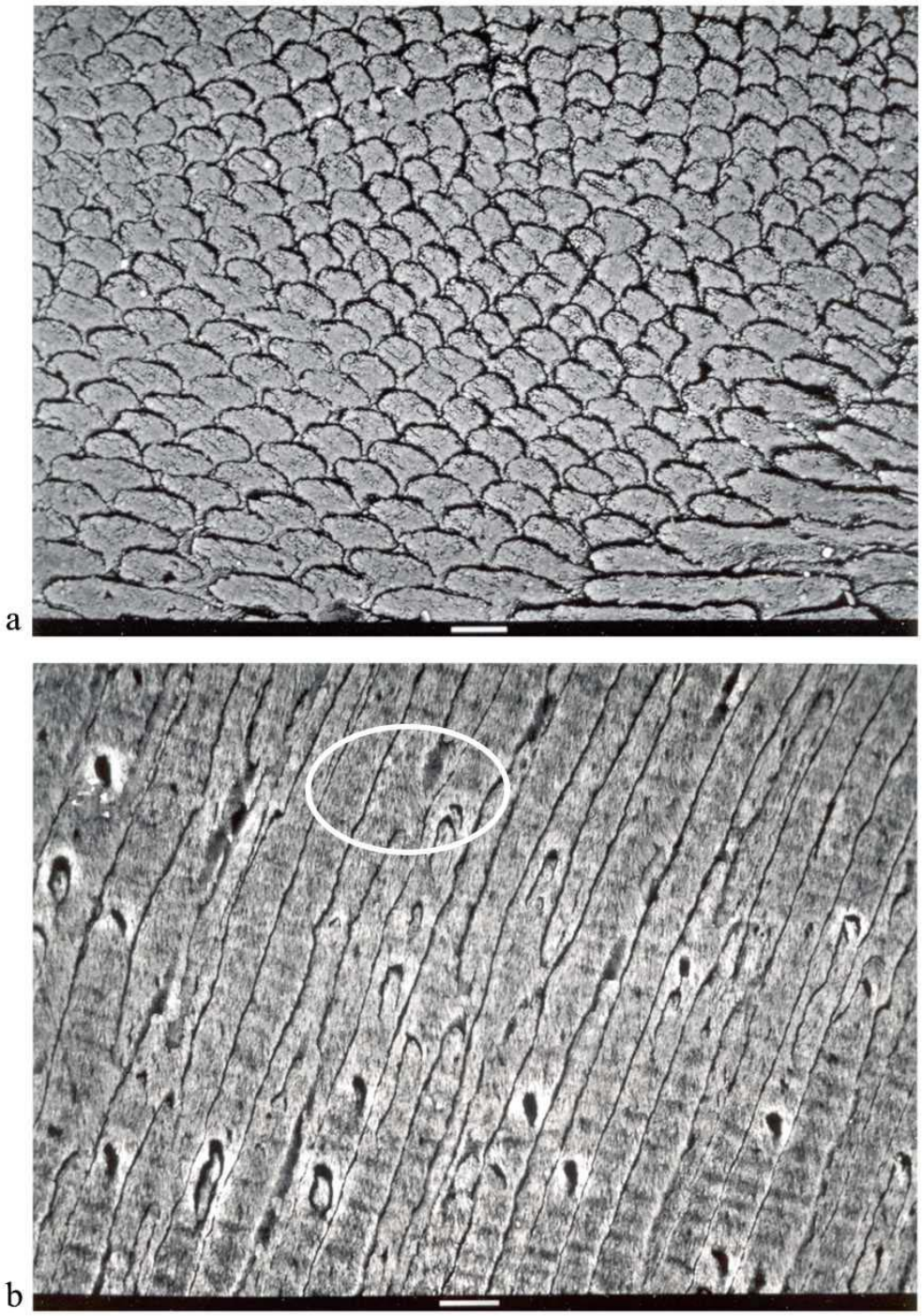


Figure 1.9 (a,b) Scanning electron micrographs illustrating the difficulty of determining prism width in polished and etched sections. a) Enamel prisms of *Afropithecus turkanensis* viewed end-on, showing a change in prism diameter due to a progressively more oblique orientation (at the bottom of the image). b) Enamel prisms of *Graecopithecus freybergi* running almost vertically, showing variation in width. Prism heads and tails are sometimes continuous (white circle), leading to exaggerated width. Scale bar at bottom is equivalent to 10 μm. Smith and Martin (unpublished images).

The volume of a cylinder is represented by the formula:

$$V = \pi * r^2 * h$$

where r = radius and h = height

The volume of the product of one day of secretion at the EDJ (~3 μm by 3 μm) where r=1.5 and h=3 would be 6.75* π cubic micrometers. This may be compared to the daily secretion of a prism in the outer enamel (~6 μm by 6 μm), where r=3 and h= 6, and the volume is equivalent to 54* π cubic micrometers. If this model is correct, ameloblasts must undergo a dramatic increase in enamel matrix production as the surface is approached (also noted by Dean, 2004). It would be interesting to explore the cellular and vascular changes that may occur in this instance, as well as these relationships in teeth that show less dramatic differences between the surface area of the EDJ and the tooth surface (e.g., deciduous teeth, thin-enameled teeth).

Regarding aprismatic enamel, there are no developmental models to date that account for the variation seen in aprismatic sub-surface enamel or the incremental features associated with this layer. Development models (in primates) should consider the prevalence of pattern 1 enamel adjacent to this layer in sub-surface enamel and near the EDJ (e.g., Figure 1.8). Boyde and Martin (1987) suggested that it is common to observe a transition in mid-thickness enamel from pattern 3 to pattern 1 to aprismatic enamel as the surface is approached, which is the opposite pattern commonly seen at the EDJ. Theoretically, this could be accomplished by a change in the Tomes' process morphology and size of secretory territory, followed by a loss (or acquisition at the EDJ) of the Tomes' process. The additional significance of pattern 1 enamel adjacent to aprismatic enamel is that crystallite orientation may remain the same from one type of enamel to the next. Further study on the morphology of the Tomes' process at the beginning and end of formation, as well as the nature of changes in prism patterns may increase our understanding of this feature.

Additional work is necessary to document the periodicity and etiology of incremental features in aprismatic enamel, as current models (discussed below) do not provide adequate explanation. Risnes (1990) proposed a theory about the relationship between Retzius line formation and aprismatic enamel, which is considered in the next

section. Additionally, a possible relationship between the 'fine-striations' seen in this layer and cross-striations is considered below. A confounding factor in the study of aprismatic enamel relates to methodological differences, as light and electron microscopy may yield different impressions due to the nature of imaging techniques (Whittaker, 1982). Ideally, this feature should be studied using SEM, TSRLM, or CLSM (e.g., Boyde and Martin, 1987; Li and Risnes, 2004). One final area of future inquiry concerns the frequency and distribution of aprismatic enamel within the primate order. Images of prosimian and monkey enamel may show qualitative differences and/or a higher frequency of aprismatic enamel when compared to some hominoids (Shellis and Poole, 1977; Martin and Smith, unpublished data). However, a truly systematic study of this feature in non-human primates has yet to be undertaken.

Incremental Structures

In the following section, several types of incremental feature will be described, including each respective structural manifestation, model of formation, and proposed etiology. Incremental features in enamel will be the main focus of this review, although relevant information on analogous aspects of dentine formation will be noted, as well as the results of several experimental studies on dentine. The temporal nature, or periodicity, of each feature will be considered at length in the following chapters, and will be only briefly mentioned here. The applications of these features for anthropological inquiry will also be reviewed in the following chapters.

Short-period features

a) Cross-striations: building blocks of enamel

One of the earliest descriptions of cross-striations was by Retzius (1837), who illustrated the classic pattern of a group of relatively straight 'enamel threads' (prisms) viewed under transmitted light and bisected with a series of 'cross-lines' (cross-striations) (Retzius, 1837: Fig. 8, Tab. XXI). Leeuwenhoeck (1674) may have been the first to illustrate these structures, describing fine transparent 'pipes' that are likely the enamel prisms, which appear to be illustrated with cross-striations along their length

(Leeuwenhoeck, 1674: Fig. 1 and 2, p. 1002).[8] Many authors have subsequently described them using various forms of microscopy, including SEM, TEM, PLM, TSRLM, and CLSM.

Boyde (1989) provided what may be considered a working definition of cross-striations: light and dark bands or stripes that cross prisms perpendicularly with an interval of about 2 - 5 μm and a periodicity of 24 hours (although other studies have suggested a slightly different range of repeat intervals). Experimental work by Schour and Poncher (1937), Mimura (1939), Schour and Hoffman (1939b), Okada (1943), Kawasaki et al. (1977, 1980), Bromage (1991), Ohtsuka and Shinoda (1995), Rinaldi (1999), and Ohtsuka-Isoya et al. (2001) has confirmed the presence of daily lines in both enamel (cross-striations) and dentine (von Ebner's lines) of various mammals, including humans and macaques, suggesting analogous short-period structures in both tissues. The quantification of secretion rate and the circadian nature of cross-striations will be considered further in Chapters 2 and 3 respectively.

b) Prism varicosities vs. light/dark bands

The majority of work on incremental features has suggested that 'gentle constrictions or varicosities' along the prisms are equivalent to light and dark bands crossing the prisms perpendicularly (cross-striations *sensu stricto*) (e.g., Helmcke et al., 1961; Boyde, 1964; Helmcke, 1967; Boyde, 1979; Martin 1983; Dean, 1987a, 1989; Swindler and Beynon, 1993; Dean and Scandrett, 1996). Dean (1989) noted that while light and dark bands may be confused with artifacts of light microscopy, prism varicosities (equivalent to cross-striations) are visible under SEM and confocal microscopy (illustrated in Boyde, 1979: Figure 2, p. 982; Boyde, 1989: Figure 37, p. 379). This is in keeping with the developmental model of Boyde (1964, 1979, 1989), which stated that prism width changes with the rate of formation (illustrated below).

However, Hinrichsen and Engel (1966), Simmelink and Nygaard (1982), Risnes (1986, 1998), and Li and Risnes (2004) have suggested that varicosities do not always conform to cross-striations. Warshawsky and Bai (1983) reviewed the literature

[8] He did not discuss them specifically in his text, where he stated that enamel is composed of 'globules', which appear to be prisms viewed end-on, although other authors since have used this term to refer to cross-striations.

supporting the proposed relationship between cross-striations and varicosities, and noted the discrepancy between the commonly reported varicosity interval (4 - 8 μm) and the typical cross-striation repeat interval (2 - 4 μm). Risnes (1986) also discussed this difference and suggested that the relationship is unclear and requires additional investigation. In light of the 2:1 ratio of reported values, he speculated that cross-striations may relate to places along the prism where the curvature changes, implying that a cross-striation may represent one half of the undulation wavelength (of a varicosity).

Osborn (1967, 1973, 1981) generated three-dimensional reconstructions of prisms that showed undulations with a periodicity of 4 - 8 μm that he considered likely to be related to cross-striations. In 1981, he suggested that prisms do not show true varicosities and constrictions, instead they simply undulate (with a constant width) (illustrated in Osborn, 1981: Figure 6.20, p. 178). Osborn suggested that sectioned prisms and fractured surface prisms that appear to show increases and decreases in width are simply optical artifacts. He argued that the impression of constrictions is created when undulating prisms of equal width are cut or viewed from one side. He also made the observation that if cross-striations (as lines or dark bands) correspond with prism varicosities/constrictions, the cross-striations should appear staggered. Yet very often, as Boyde (1964) and many others have noted or illustrated, groups of cross-striations appear to be in phase across a number of adjacent prisms. Martin (pers. com.) suggested that they appear to be in phase because prisms extend in whole day units, which means that each line 'in phase' (horizontally across a group of prisms) is separated by one day of formation. He interpreted the apparent contradiction (between what Osborn proposed about a staggered arrangement and what has been noted as an 'in phase' arrangement) as the result of prism heads and tails lining up in one plane of section, which would cause cross-striations to appear in phase (provided they form in a similar manner in both head and tail regions). However, Risnes (1990, 1998, 1999) has shown that cross-striations in prism tails tend to bend cervically, which may prohibit the appearance of horizontal rows.

Examination of scanning electron micrographs of *A. turkanensis* suggests that varicosities and cross-striations do not always appear to be in phase, as varicosities may show a greater spacing than cross-striations, or do not always appear where incremental lines may be seen along prisms (Figure 1.10).

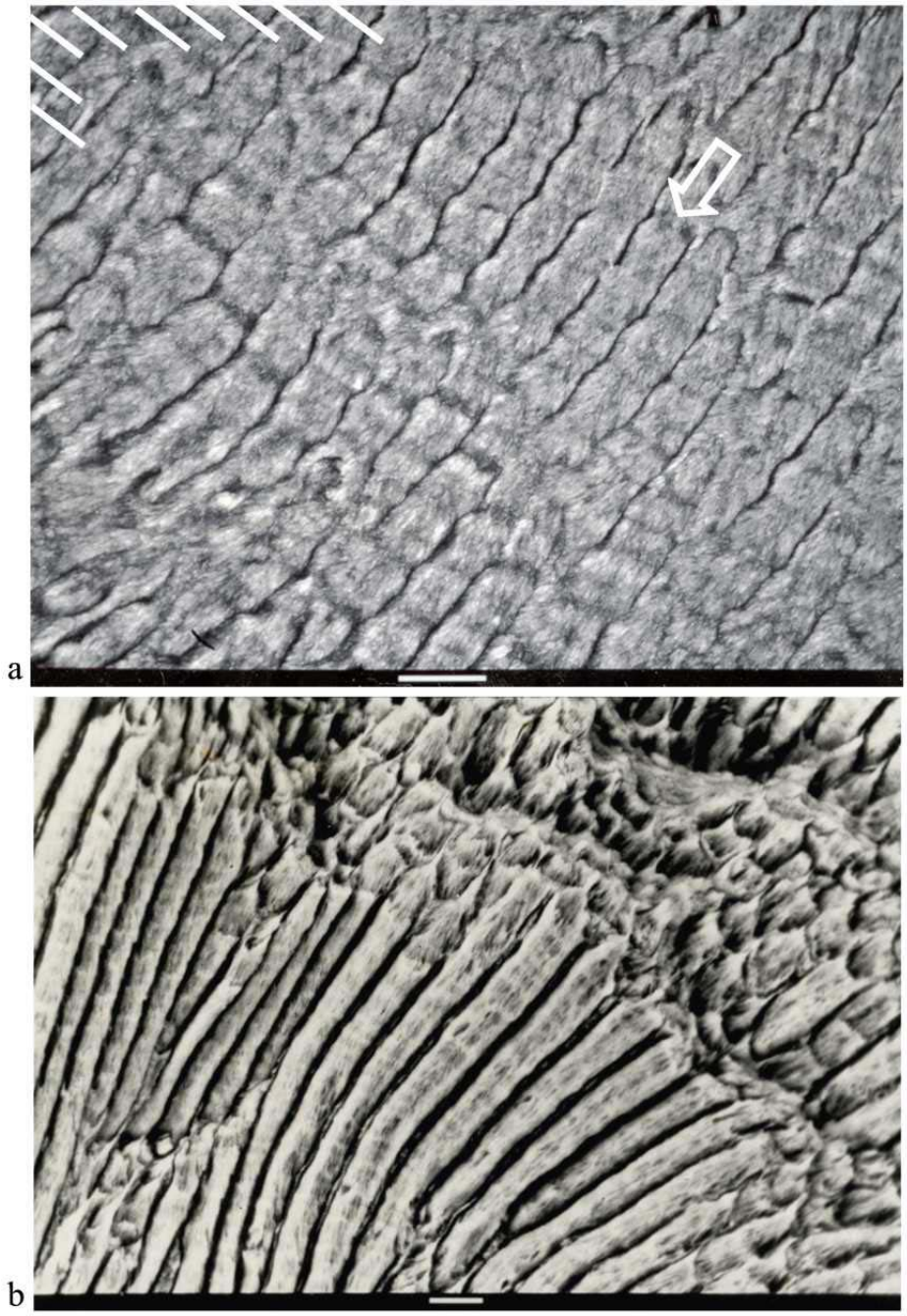


Figure 1.10 (a,b) Scanning electron micrographs of *Afropithecus turkanensis*. a) Polished and etched section showing prisms running from upper right to lower left (white arrow), with alternating varicosities/constrictions approximately 8 μm apart, and cross-striations approximately 4 μm apart. A field of cross-striations in register can been seen by holding the page at eye level and looking from the bottom right corner to the upper left corner, where white lines denote the approximate spacing of cross-striations. b) Naturally fractured (not polished or etched) tooth showing prisms in the same orientation as a), with faint incremental lines (on central prisms), but no obvious varicosities. The scale bar at the bottom of each image is 10 μm. Smith and Martin (unpublished images).

An earlier figure (Figure 1.6b) showed cross-striations perpendicular to the vertically running prism, which appear in some areas to be in register with varicosities (upper right corner in particular), yet other areas in this tooth show more than one cross-striation per varicosity. An additional earlier figure (Figure 1.9b) showed relatively straight enamel prisms with perpendicular cross-striations that do not appear to relate to varicosities or constrictions. Risnes (1998) and Li and Risnes (2004) recently examined this issue, showing a number of SEM images where light and/or dark bands did not conform to respective varicosities and/or constrictions (Risnes, 1998: Figure 11, p. 341; Li and Risnes, 2004: Figure 3, p. 49). Li and Risnes (2004) demonstrated that the degree of etching influences the appearance of this relationship, and stated that, "although there was tendency for the dark bands to coincide with prism constrictions and for the light bands to coincide with prism expansions, this configuration was not consistent, several exceptions were observed" (p. 50). They noted that when differences were found, undulations were more widely spaced than cross-striations. For the remainder of this dissertation, cross-striations will be strictly defined as noted above (light and dark bands crossing enamel prisms, illustrated in Chapter 2, Figure 2.1) without reference to the appearance of prism boundaries.

c) Models of formation and physiological causes of cross-striations

Boyde (1964) thoroughly reviewed the early development of theories on the nature of cross-striations. At the time of his dissertation, most workers believed that they represented a pattern of decreased calcification/increased organic material relative to other regions in the enamel. Boyde (1964) credited Helmcke et al. (1961) with the proposal that cross-striations are associated with changes in the width of the prisms and the orientation of the long axes of the crystallites, which was highly influential on his theory of formation. Boyde's (1964) model suggested that, because the shape of the mineralizing front and the rate of ameloblast secretion are related to the orientation of the crystallites, a periodic change in ameloblast activity would cause a periodic change in the mineralizing front (Figure 1.11).

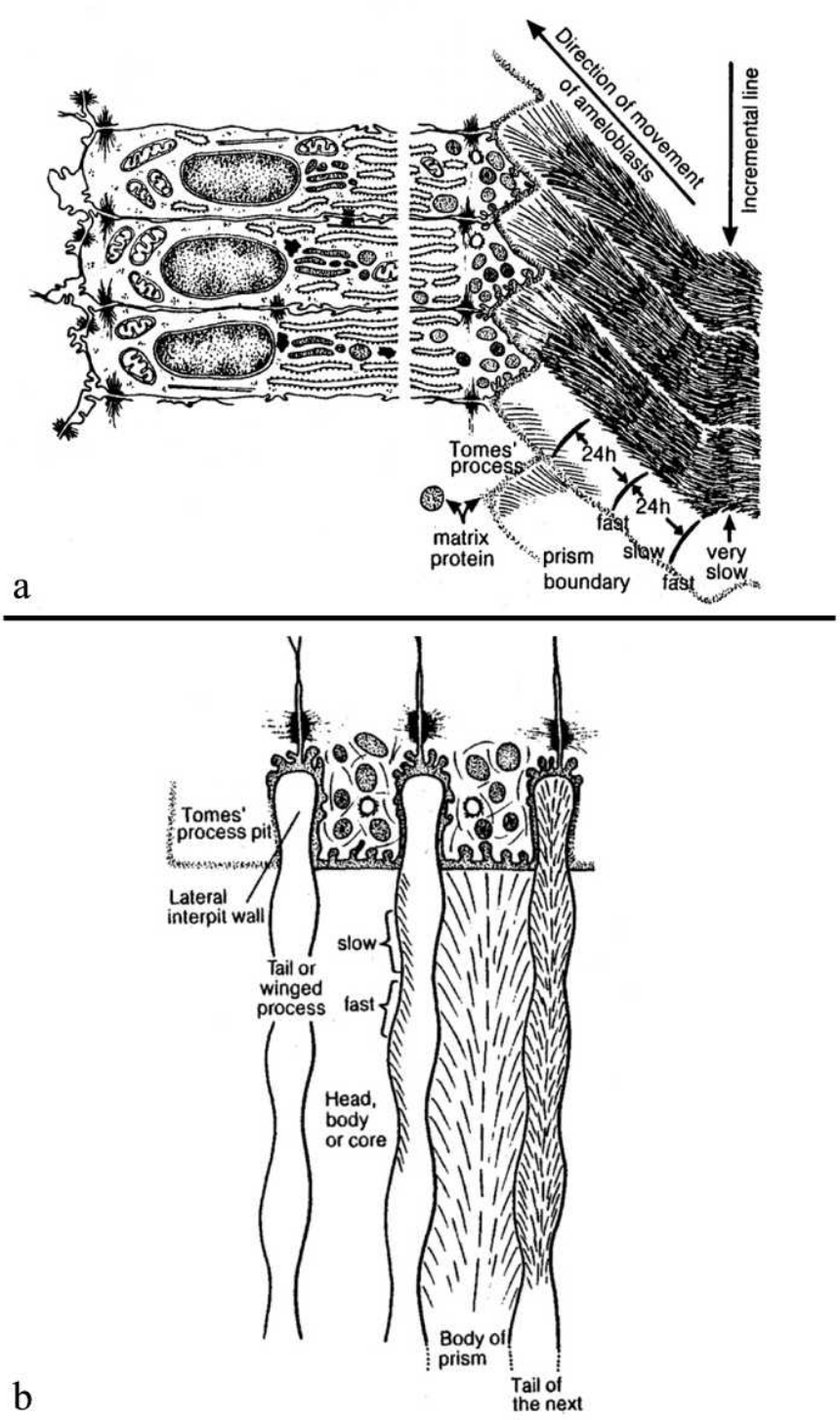


Figure 1.11(a,b) Boyde's model of the development of cross-striations, viewed from a) the picket-fence orientation (patterns 2 and 3) and b) the battlements orientation (all patterns). Ameloblasts are oriented at an angle to the forming prism, and the direction of growth is parallel to the prism axis. See text for more detail. Modified and reproduced from Boyde (1989).

This may result in: 1) a change in the ratio of intra-depression crystallites (within the floor of the Tomes' process pit) to the circum-depression crystallites (i.e., pattern 1 interprismatic enamel, pattern 2 interrow sheets, pattern 3 tails), and 2) changes in the orientation of the crystallites. Further, a change in the rate of secretion would influence the amount of 'stroking' of the crystallites by the non-secretory face, causing an additional change in crystallite orientation. From this, he predicted that during the slow phase of secretion, crystallites in the circum-depression areas will diverge more from the long axis of the prism, as there is less stroking or sliding of the mineralizing front past the Tomes' process walls. Also, he predicted that these regions become more favorable sites for crystallite growth, resulting in a widening of the interprismatic/interrow/prism tail enamel (depending on the pattern).[9] In 1989, he clarified that the distinction between favorable and unfavorable sites for matrix release is critical, with the former being the interpit and pit floor locations, and the latter being the pit walls.

In his later work, Boyde (1979, 1989) noted that available evidence suggests that enamel crystallite centers contain high levels of carbonate relative to the rest of the surrounding tissue. He demonstrated that acid-etching (or caries development) tends to exaggerate cross-striations, due to the relationship between cross-striations and variation in the level of carbonate.[10] He reviewed work that demonstrated that carbonate was more likely to dissolve than phosphate under these conditions. Further, variations in the carbonate composition may translate into differences in the refractive index of prisms, creating alternating bands along their long axis when viewed under polarized light microscopy (i.e., the typical light/dark patterning). Boyde theorized that if ameloblasts experience circadian variation in metabolic activity, pCO_2 should also vary, which may translate into carbonate differences within the enamel. He predicted that during fast or active growth, carbonate content should be higher in the wide or widening portions of the prisms (varicosities). In 1989, Boyde reviewed work that suggested that a high level of magnesium is found in association with carbonate-rich areas, and suggested that cross-striations in micromilled (topography-free) preparations may be explained by changes in

[9] This aspect of his model is similar to Risnes' (1990, 1998) model of Retzius line formation, which is discussed below.

[10] See Boyde et al. (1978) for a discussion of the effects of acid etching on prism structure.

the ratio of calcium and phosphate to magnesium and carbonate. During a fast growth period, higher levels of magnesium and carbonate would be incorporated into the prism, and the mean atomic number or density would decrease (creating a dark band under backscattered electron microscopy). He noted that the commonly held alternative explanation is that changes in crystallite orientation may cause this optical phenomenon, rather than (or possibly in addition to) chemical changes.

The innovative experimental work of Okada and Mimura, largely published in Japanese until a 1943 review paper in English, was reviewed in a video presented in 1963 at the International Physiological Association meetings in Leiden, Holland (Shinoda, 1984). Okada's (1943) review detailed his demonstration of daily lines in both enamel and dentine using a 'vital labeling' procedure (lead acetate injections, discussed further in Chapter 3). Based on hematoxylin staining of decalcified sections, daily lines in dentine were shown to consist of pairs of light and dark bands (under light microscopy), calcium-poor and calcium-rich areas respectively. Okada (1943) then examined the effects of various factors on the production of daily lines in rabbit dentine: constant dark, starvation, sleep, sympathetic nervous excitation, parasympathetic nervous excitation, increased alkalinity, and increased acidity of the blood plasma. Daily lines continued to be produced in constant dark or during starvation, although the lines became less pronounced with time. He reported that dark bands were formed during induced periods of sleep and/or during parasympathetic nervous excitation, while white bands were formed during sympathetic nervous excitation.

Okada (1943) then examined how daily lines in dentine related to changing acid/base levels. The blood plasma levels of rabbits were examined over time, which showed increases in the alkalinity of blood plasma at night, and decreases during the day (and also during starvation); higher levels of alkalinity related to dark band production at night, and higher levels of acidity related to diurnal light band formation. Following this, he measured the CO_2 capacity of the blood plasma in pregnant rabbits, and found that this level decreased towards the end of pregnancy, was minimal the day of parturition, and increased dramatically after birth; corresponding dentine was dominated by white lines (or a lack of dark lines) just prior to the end of pregnancy, a strong white line on the day or parturition, and followed immediately with marked dark and light lines. Finally, he

demonstrated that during the day, calcium levels in the blood plasma remained fairly constant, but at night there was a steady decrease. He suggested that this evidence provided a physiological explanation for the production of light bands during the day and dark bands at night; when blood plasma became more acidic (during the day), calcium levels in the blood increased, which decreased the precipitation of calcium in hard tissues, and produced a white, calcium-poor line. The opposite scenario at night produced a dark, calcium-rich line. In addition to a chemical basis for daily lines in dentine, Okada (1943) also demonstrated a structural basis. He found that collagen fibers showed a 'double refraction' under polarizing light microscopy, corresponding to diurnal changes in orientation.

Okada (1943, 1963) also reported that maximum growth occurred during dark conditions and minimum growth occurred during light conditions, which has been confirmed in several subsequent studies.[11] Miani and Miani (1971) used a 12-hour tetracycline injection protocol in dogs to examine the relationship between light and dark cycles, feeding times, and circadian dentine production. They showed that the maximum advancement of dentine occurred at or shortly after the beginning of a dark period, and the minimum advancement occurred at or shortly after the beginning of the light period, which also represented the time of maximal adrenal cortex activity.[12] Work by Shinoda (1984: translated by Yoshiko Abe) showed that the phase of circadian dentinogenesis is related to the activity patterns of the animal; darkly staining-bands were formed during the period of activity in both diurnal and nocturnal rodents. Shinoda (1984) also showed that the timing of feeding may influence the production of light and dark staining bands, which suggests that results from studies with different feeding protocols (or animals with different activity patterns) may not be directly comparable. Another study that explicitly measured incremental lines and activity cycles was concerned with suprachiasmatic nucleus functioning (Ohtsuka-Isoya et al., 2001), but they did not relate the production of specific dentine lines to the phase of activity.

[11] This was based primarily on studies of rabbits, which are generally considered to be nocturnal. Thus, dentine production was greatest when the animal was active.

[12] This was found to be true in semi-starved dogs, while the timing of feeding was shown to shift the phase of the advancement, demonstrating the influence of an addition exogenous signal on a circadian rhythm, discussed further in Chapter 3 and in Shinoda (1984).

d) Intradian lines

An additional class of short-period lines is found between cross-striations, often termed intradian, ultradian, or infradian lines, which have received passing mention in recent literature, but relatively little explanation (Dean, 1995a; Dean and Scandrett, 1996; Dean, 1998b; Antoine et al., 1999; Antoine, 2000; Dean, 2000; but see FitzGerald, 1996).[13] For the purpose of this work, the term intradian lines will be used to refer to structures that are believed to be formed in less than one day (Figure 1.12). Although some workers have dismissed these lines as artifacts of light microscopy (e.g., Boyde, 1989; Shellis, 1998; Antoine et al., 1999), evidence from multiple forms of microscopy has confirmed their existence (Smith et al., 2003a, 2004).

A review of the literature shows that intradian lines have been noted and described a number of times prior to the last decade. Gustafson (1959) and Gustafson and Gustafson (1967) described a 'double-band effect' in relation to typical cross-striations. They suggested that, although this effect may appear to be an optical artifact, careful examination shows that they are real structures (see numerous images in Gustafson, 1959; Gustafson and Gustafson, 1967: Figure 6, p. 85). Shellis and Poole (1977) noted that *Gorilla* enamel showed a 'doubling of the striations', particularly in the outer enamel. They also reported subdivision of the cross-striations into two or more faint lines, with a repeat interval of 2 μm, in the aprismatic outer cervical enamel of two old world monkeys. Risnes (1999) illustrated a sub-surface region of human cuspal enamel with increments spaced 2 μm apart (Risnes, 1999: Figure 4c, p. 316). As noted above, closely spaced striations (~ 2 μm or less) were also seen in the aprismatic sub-surface enamel of *A. turkanensis* (shown above in Figure 1.8a), as well as in the prismatic sub-surface enamel (Figure 1.12a).

A number of published light and scanning electron microscope images have shown fine banding between cross-striations, which was not specifically addressed in the respective texts (e.g., Gustafson, 1955: Figure 9; Martin, 1983: Figure 5.4e, p. 333; Martin, 1985: Figure 2b, p. 262; Dean and Beynon, 1991: Figures 6, p. 223; Sharawy and Yeager, 1991: Figure 3-11, p. 57; Moss-Salentijn et al., 1997: Figure 3, p. 17; FitzGerald,

[13] Technically, the appropriate terms for something that occurs with a periodicity of less than one day is intradian or infradian; 'ultradian' implies an exaggerated day.

1998: Figure 1, p. 378) Dean and Scandrett (1996) presented a CLSM image of a human premolar that shows either highly variable cross-striations, or intradian lines (Dean and Scandrett, 1996: Figure 3, p. 237) (also see TSRLM images from Smith et al., 2003a, 2004). Evidence of this nature demonstrates that intradian lines are a real structural phenomenon, as CLSM or TSRLM images of enamel below the surface have a very limited focal plane, making interference from layers of prisms negligible (which is true of SEM as well). As Dean (1995a) noted, one of the complications in determining the presence of this feature is the difficulty of distinguishing intradian lines from cross-striations in isolated enamel (particularly without associated Retzius lines or a known developmental chronology). Due in part to this difficulty, there has not been a formal model proposed to explain either their structural or etiological basis.

FitzGerald (1996) addressed intradian lines in his dissertation, reporting that they may appear at any point between successive cross-striations (in a number of anterior human teeth examined under light microscopy). Also, he noted that they appeared in phase with other intradian lines, may be paired or single between cross-striations, and were found most frequently away from the occlusal surface. He considered three explanations for the lines; based on Boyde (1989) he suggested that they could result from an interference pattern of underlying cross-striations and/or the course of prism rows in a third dimension. The third explanation he also credited to Boyde and others (via Dean, pers. com.); these lines "indicate a finer eight hourly, twelve hourly, or light/dark rhythmic beat" (p. 179). Of the three hypotheses, FitzGerald (1996) noted that the latter one is the most likely based on available evidence, including experimental work. Studies of dentine have suggested two to three short-period lines between daily lines, which is theoretically equivalent to the relationship between intradian lines and cross-striations in enamel (Kawasaki et al., 1977, 1980; Rosenberg and Simmons, 1980a; Shinoda, 1984; Ohtsuka and Shinoda, 1995; Ohtsuka-Isoya et al., 2001). The periodicity of these structures will be considered further in Chapter 3.

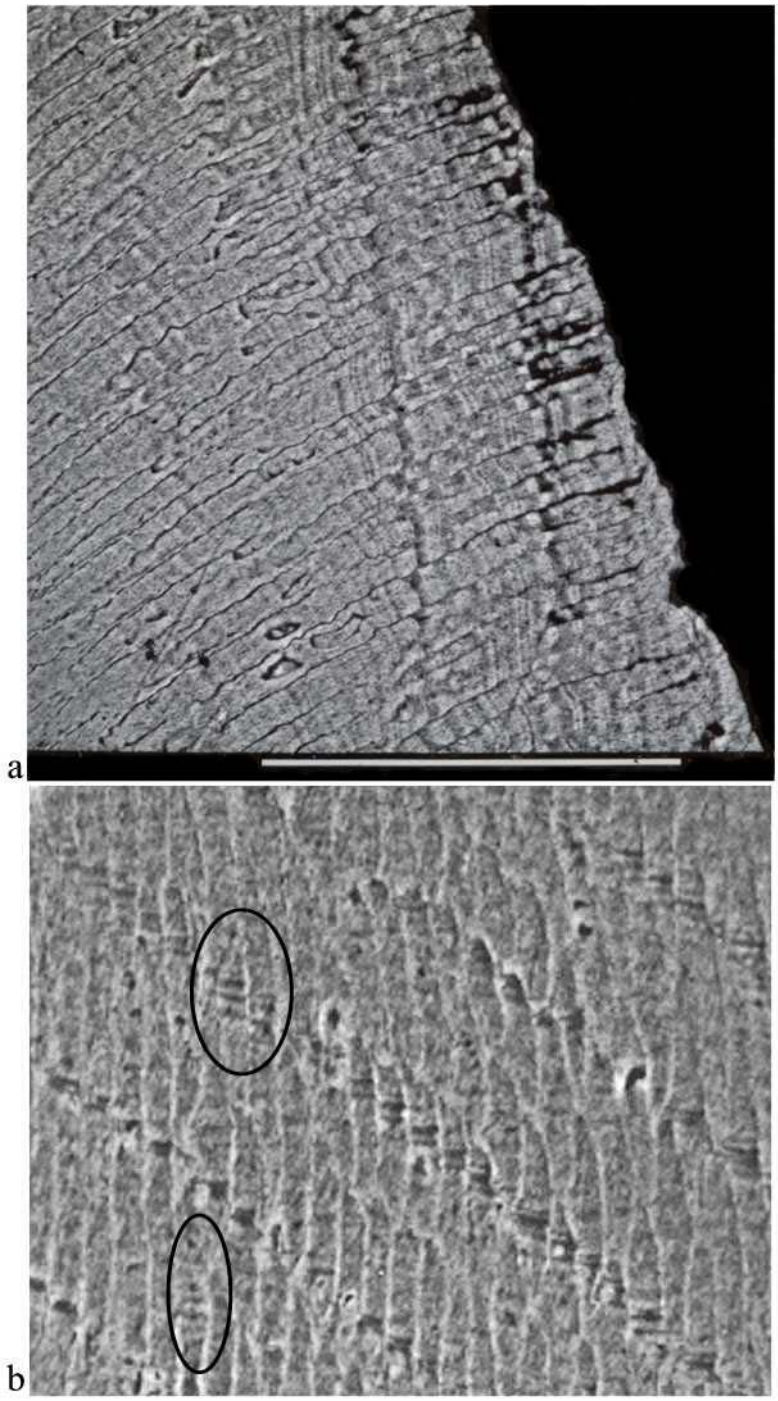


Figure 1.12 (a,b) Scanning electron micrographs of intradian lines in polished and etched enamel. a) Prisms in *Afropithecus turkanensis* run from lower left to upper right, Retzius lines run vertically, and intradian lines cross prisms. Scale bar equals 100 μm. Smith and Martin (unpublished image). b) Prisms in *Graecopithecus freybergi* run vertically, Retzius lines run from lower right to upper left, and intradian lines (circled in black) are evident near the plane of the Retzius lines. The field width of this image is approximately 145 μm. Both teeth have an eight day periodicity. Published in Smith et al. (2004).

e) Laminations

Smith et al. (2003a, 2004) have recently re-described laminations, an additional class of short-period feature that appears to have been noted originally in reports of aprismatic enamel (Figure 1.13). They defined laminations as closely spaced features that run parallel to Retzius lines (or the developing enamel surface) (Smith et al., 2003a: Figure 5, p. 300; Smith et al., 2004: Figures 2, 3, p. 557-558). Ripa et al. (1966) originally referred to 'surface parallel laminations' in the outer aprismatic enamel, which they described in association with 'continuous band' aprismatic enamel (discussed above and illustrated in Figure 1.8a). Whittaker (1982) also described regions of sub-surface aprismatic enamel with laminations at an interval of approximately 5 μm, reported to be in register with cross-striations in the adjacent prismatic enamel. An examination of Whittaker's original figures shows a region similar to the 'onion layer' aprismatic enamel described by Ripa et al. (1966) (e.g., Ripa et al., 1966: Figure 1, Plate I; Whittaker, 1982, Figure 3, Plate I, Figure 7, Plate II), where distinctive, closely spaced bands running parallel to Retzius lines appear to be in register with cross-striations (perpendicular bands crossing the prisms). It appears that these two authors referred to laminations as either two separate features of the aprismatic enamel, or the same feature with a slightly different orientation. The distinction may have been related to differences in the orientation of the Retzius lines and the nature of aprismatic enamel in deciduous and permanent enamel. Although they did not refer to these structures as laminations, Li and Risnes (2004) also provided several images of them in aprismatic human enamel (e.g., Li and Risnes, 2004: Figure 3, p. 47), and stated that these features were equivalent to cross-striations.

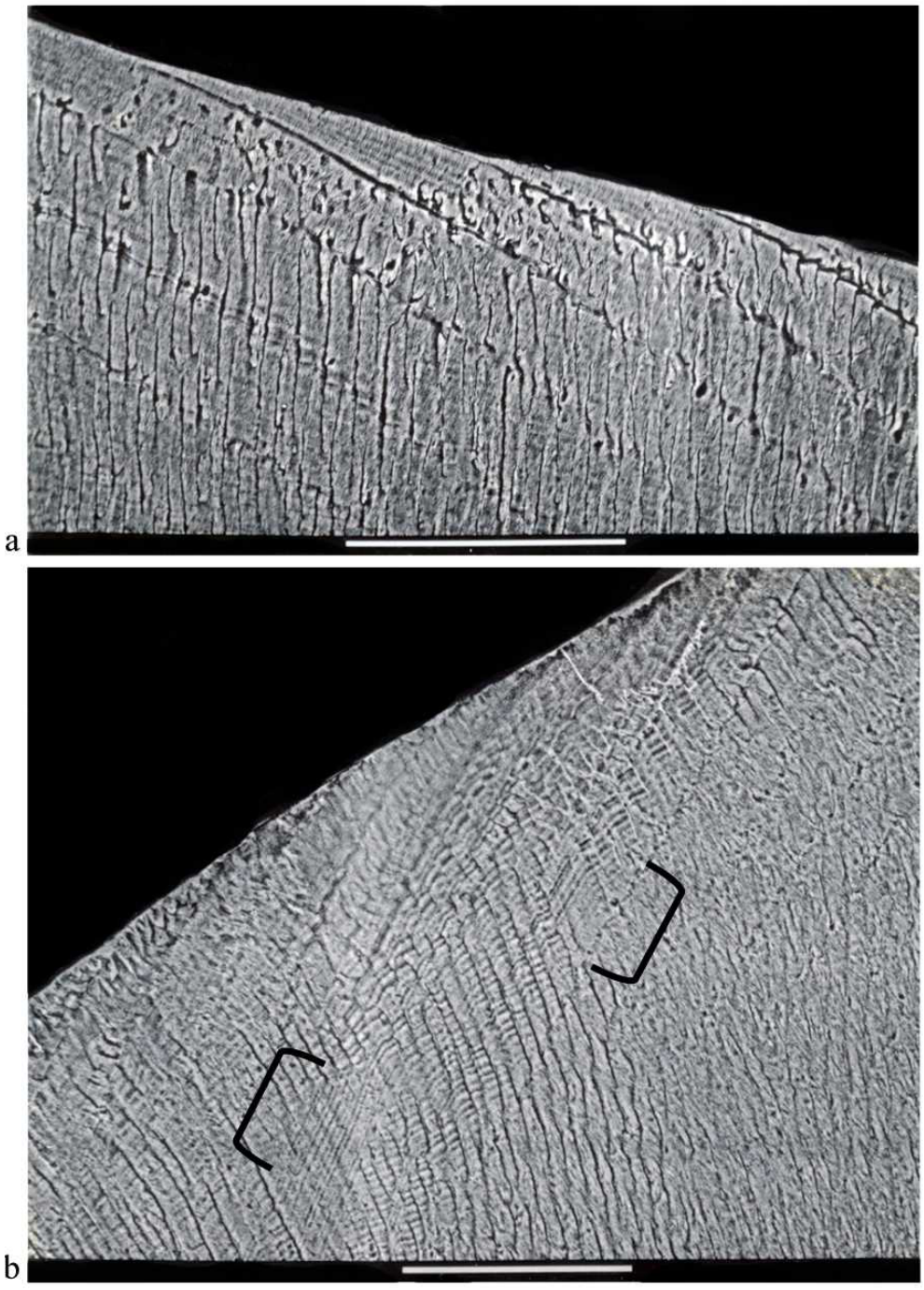


Figure 1.13 (a-d) Scanning electron micrographs of laminations in the sub-surface enamel. Prisms run vertically and Retzius lines run diagonally. Scale bar equals 100 μm. a) Aprismatic (onion layer) enamel with laminations between Retzius lines near the surface in *Graecopithecus freybergi*. Smith and Martin (unpublished image).b) Prismatic enamel showing what appear to be cross-striations (perpendicular to prisms) in register with laminations (black brackets) in *Pongo pygmaeus*. Martin and Smith (unpublished image). (Figures [c-d] are on the following page.)

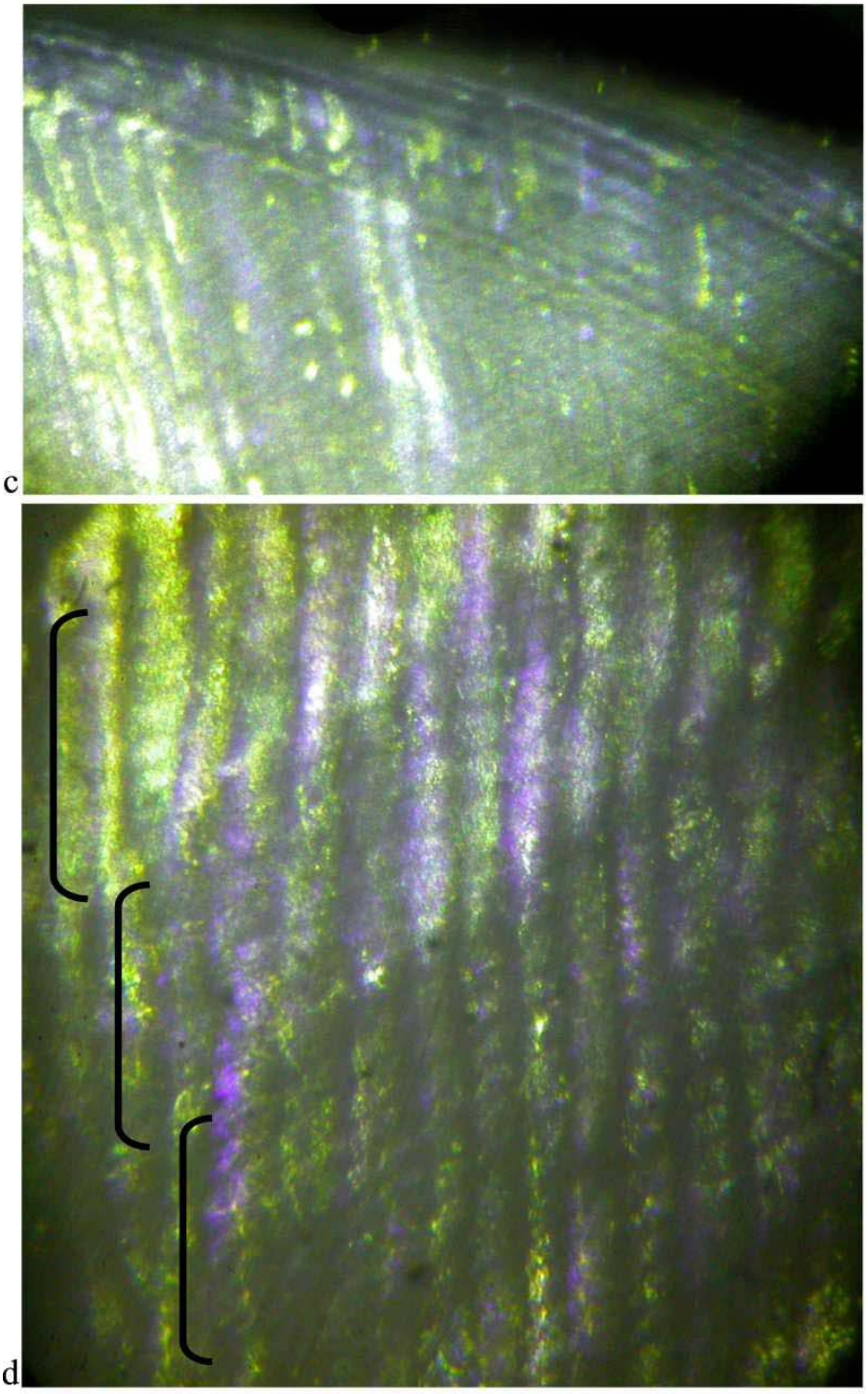


Figure 1.13 (c,d) Tandem scanning reflected light micrographs of laminations in *Graecopithecus freybergi*. a) Sub-surface enamel showing a potential transition from prismatic enamel below to aprismatic enamel above (with laminations). b) Diagonal Retzius line intervals (black brackets) with cross-striations (perpendicular to vertical prisms) in the upper two intervals, and laminations (parallel to Retzius lines) in the lower intervals. Scale information is not available. Smith and Martin (unpublished images).

Smith et al. (2003a) argued that laminations complicate the determination of Retzius line periodicity, as they often obscure the relationship between cross-striations and Retzius lines. They suggested that laminations may not show a daily repeat interval, as they generally appeared to be more widely spaced than nearby cross-striations (e.g., 6.5 - 6.9 μm apart in sub-surface cuspal enamel, which was greater than nearby cross-striations), and did not appear to be equal in number to the cross-striations between Retzius lines (Smith et al., 2003a: Figure 5, p. 300). Smith et al. (2003a, 2004) hypothesized that these features may be related to aprismatic or pattern 1 enamel production, particularly in light of their frequency near the enamel surface and the EDJ. Additionally, there is evidence that laminations may also show a sub-division, similar to the relationship between intradian lines and cross-striations. For example, close examination of Whittaker's (1982) images reveals additional finely-spaced lines parallel to the laminations (and Retzius lines) (e.g., Whittaker, 1982: Figure 7, Plate II). Smith et al. (2004) also found what appeared to be sub-divisions of laminations in *Graecopithecus freybergi*. Lamination and intradian line periodicities, as well as their relationships to other incremental features, are discussed further in Chapter 3.

Long-period features

Some of the more prominent features in human and primate enamel are the regularly spaced incremental lines running from the EDJ to the surface of the lateral and cervical enamel. These lines were described and diagrammed by Retzius (1837), and have since been referred to as the striae of Retzius, or Retzius lines.[14] Since then, several studies have attempted to explain their structural basis and physiological cause. Risnes (1985a, 1987) illustrated a novel method for studying Retzius lines with SEM. He showed that by sectioning and grinding a tooth in a minimum of two planes, the three-dimensional nature may be visualized through the continuity of these lines in different planes, representing the position of the ameloblast sheet during enamel formation. It is also well-established that these lines manifest on the tooth surface as perikymata, row-like depressions that extend around the circumference of the tooth (illustrated below).

[14] See Risnes (1990) for a detailed history of the original description of these lines concurrent with and prior to Retzius.

Many studies have shown that Retzius lines in hominoids have an approximately circaseptan nature, demonstrated by what is believed to be a uniform number of cross-striations between regularly spaced Retzius lines within an individual's dentition (see Smith et al., 2003a: Table 3, p. 293 for a summary of published values). The regularity of these increments and ease of observation, through both invasive and non-destructive techniques, have allowed researchers to establish crown formation times with less effort than by counting individual cross-striations (discussed further in Chapter 2). Developmental patterns may also be inferred from their relative lengths and angulation with the EDJ. These applications and the temporal nature of Retzius lines will be considered in the following chapters.

a) Retzius line structure

Boyde (1964) provided a thorough review of descriptions and explanations of Retzius lines in the early literature. Some of these sources suggested that, in relation to the adjacent enamel, Retzius lines are either less mineralized, more mineralized, contain a different interprismatic substance (such as pigment), relate to prism bends or discontinuities, or are subjected to a mineralization rhythm (see also Risnes [1990] for a thorough and current review of the various theories). Several explanations for the structural basis of Retzius lines will be reviewed here, including the lining-up of cross-striations into 'step-like', or 'stair-step' lines, prism bending along the plane of the Retzius lines, and mineralization or compositional differences along the line.

Gysi (1931) described 'step-like' Retzius lines formed by the lining up of 'transverse striations' (cross-striations) of enamel prisms (Figure 1.14a; also shown above in Figure 1.12b). The 'staircase configuration' is a common descriptive term for this appearance of Retzius lines. Gwinnett (1966) reported that only 15% of Retzius lines showed this profile (under phase contrast light microscopy). However, Weber et al. (1974) reported that this was found for all Retzius lines in the outer third of the cervical enamel (in a light and electron microscopy study). They illustrated the staircase pattern and described an adjacent 'triangular space' above the 'stairs' that appeared to show a different optical density. This may result from a reduced concentration of crystallites relative to the adjacent step below it (causing a sharp optical contrast), although there is

not a significant deviation in crystallite orientation in this area (but see Risnes, 1998, 1999). Weber et al. (1974) also described an expanded or 'club-shaped' prism end below the step, which showed a dense crystallite band. These features were subsequently considered in Risnes' model of Retzius line formation, which is discussed below.

Retzius (1837 [in German]: as cited by Boyde, 1964) proposed that these incremental lines are derived from a pigment and from the bending of prisms. Gustafson (1959) and Gustafson and Gustafson (1967) also noted and illustrated an apparent change in prism direction, which they suggested is associated with Retzius lines in some instances. This has been supported by a number of studies, some of which suggest a transverse deviation (e.g., Osborn, 1971), a longitudinal deviation (e.g., Gustafson, 1959; Hinrichsen and Engel, 1966), a cervical deviation (e.g., Boyde and Martin, 1984a; Boyde, 1989), or both (e.g., Weber and Ashrafi, 1979). Weber and Ashrafi (1979) described complex translocation (prism bending) at Retzius lines, which they suggested may give an erroneous impression that prisms end at the line or have a 'staircase configuration'. They also noted that large pores are frequently found at the point of bending. These pores, or micropores, may be related to prism discontinuity, and Risnes (1998) suggested that they may contain organic material (which remains to be verified).

Since Boyde's (1964) review, other research has supported the idea that Retzius lines are areas of hypomineralized enamel (e.g., Gustafson and Gustafson, 1967; Frank, 1978). Gustafson and Gustafson (1967) noted that under the PLM, these lines frequently appear isotropic and positively birefringent (appearing as dark, contrasting bands). These authors provided a number of images of variable Retzius lines, and noted that in some instances they may show both negative and positive birefringence and may be either hypo- or hypermineralized. Dean (1987a, 1989) reviewed earlier work and suggested that although enamel appears to be hypomineralized or less uniform at Retzius lines, the maturation of enamel may obscure the clarity of the initial formative event. He suggested that hypo- or hypermineralization may be simply an artifact and is something that occurs after Retzius line formation. Boyde (1989) also supported the idea that Retzius lines do not represent a change in the degree of mineralization. Risnes (1990, 1998, 1999) attributed the 'hypomineralized effect' to prism discontinuities at the Retzius line and a local region of poorly formed prisms directly above.

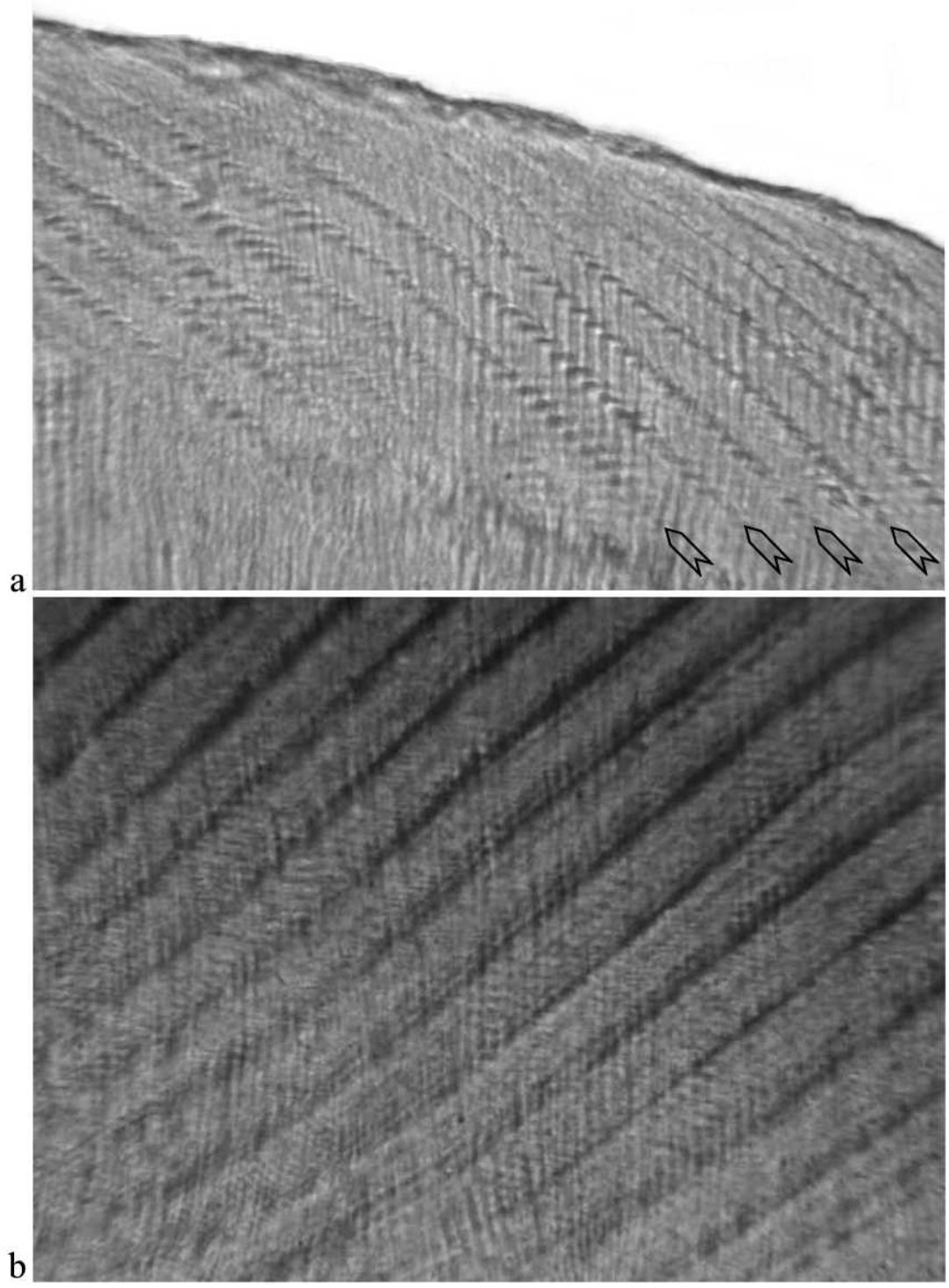


Figure 1.14 (a,b) Transmitted light micrographs of Retzius lines in chimpanzee enamel. a) Stair-step profile of diagonal Retzius lines (indicated with open black arrows). Note how the profiles transition to narrow clefts in the sub-surface enamel, where cross-striations are not evident. b) Image of potentially 'hypomineralized' (dark) appearance of diagonally running Retzius lines. Note the apparent change in definition from the inner enamel (below) to the middle/outer enamel (above).

Several studies have noted compositional differences along individual Retzius lines (Gustafson and Gustafson, 1967; Weber et al., 1974; Weber and Ashrafi, 1979; Risnes, 1990, 1998). Weber and Ashrafi (1979) noted that in inner cervical enamel, Retzius lines are poorly defined under light and electron microscopy (Figure 1.14b, also shown in Figure 1.15 below). Risnes (1990, 1998) addressed this issue, and suggested that Retzius lines are not homogeneous throughout the enamel. He stated that differences in the structure and/or composition may influence their visibility, noting that Retzius lines are characterized by a prism and interprism discontinuity, and an interprism expansion and prism constriction. Risnes (1998) demonstrated that in the inner enamel, the discontinuity is lost, while the interprism expansions are still visible. He suggested that these are unrelated structures, and that the interprism expansion is more fundamental to the structure of a Retzius line (discussed further below). Another reason Retzius lines may not appear as clearly in inner enamel may relate to the greater incidence of prism decussation and the prevalence of Hunter-Schreger bands.

The discussion above pertains to Retzius lines implicitly defined as long-period structures representing the position of the developing enamel front, which reach the surface of the tooth and form perikymata (the traditional definition used in the work of Beynon, Dean, Reid, and Martin). However, Bullion (1987), Ramirez Rozzi (see Smith et al., 2003a for review of his works), and Dirks (1998) have referred to Retzius lines found in cuspal enamel, which do not reach the surface. Ramirez Rozzi has utilized the technique of immersing naturally fractured fossilized teeth in ethanol and viewing them under polarized incident light, which he reported may show regularly spaced Retzius lines throughout cuspal enamel, termed 'appositional' or 'hidden striae' (in contrast to imbricational Retzius lines that form perikymata). Unfortunately, it was not possible to validate these estimates with those derived from counts or measurements of cross-striations. However, Dirks (pers. com.) noted that estimates of cuspal formation time derived from counts of Retzius lines were very similar to estimates derived from other methods (discussed further in Chapter 2).

Although there is no developmental evidence suggesting that a long-period rhythm should not be expressed throughout the formation of the entire crown, particularly

given the appearance of long-period lines in all but the earliest-formed coronal dentine, micrographs of cuspal enamel with a succession of Retzius lines have not been published to date. If these features are formed in the cuspal enamel, it is likely that they are frequently obscured by the course of prisms in taxa with decussation or gnarled enamel, as they appear to be rare in histological sections or SEM preparations of hominoid enamel. An additional complication of Retzius line identification in cuspal enamel is the lack of criteria distinguishing them from accentuated lines, which appear to be common in the teeth of hominoids, and do not represent intrinsic periodic phenomena (discussed further below) (Figure 1.15, see also Smith et al., 2004: Figure 11, p. 569). Dean (1987a) noted that only Retzius lines that meet the enamel surface should be assumed to be evenly spaced incremental lines. Alternatively, it would be necessary to demonstrate that 'cuspal Retzius lines' were formed with a consistent periodicity by counting the number of cross-striations between pairs of lines, which must be equivalent to the periodicity between imbricational Retzius lines. Because this has not been possible, the definition of Retzius lines given above will be used for the rest of this dissertation.

b) Perikymata

As Retzius lines terminate at the surface of the tooth in the imbricational (lateral and cervical) enamel, they form slightly depressed ridges or troughs that run around the circumference of the tooth, known as perikymata (Figure 1.16). Boyde (1964) credited Retzius (1837) as the first to note this association between internal long-period lines and their manifestation on the surface of the tooth (see also Asper, 1916; Frank, 1978). Risnes (1985b) described them as "parallel, alternating ridges and grooves circumscribing the enamel surface in a plane roughly transverse to the long axis to the tooth" (p. 185), which he demonstrated are closed circles around the tooth. Because of the equivalence between these features and Retzius lines, counts of perikymata have often been used to reconstruct the time of crown formation (discussed further in Chapter 2).

Figure 1.15 Transmitted light microscope montage of approximately 130 Retzius lines (dark bands) running to the surface of cervical and lateral chimpanzee enamel. In cuspal enamel (top of image over the dentine horn), accentuated features give the impression of regularly spaced lines, but close examination shows that these lines do not display a consistent periodicity. For scale, the length of the enamel dentine junction between the dentine horn and the cervix is approximately 5.5 mm.

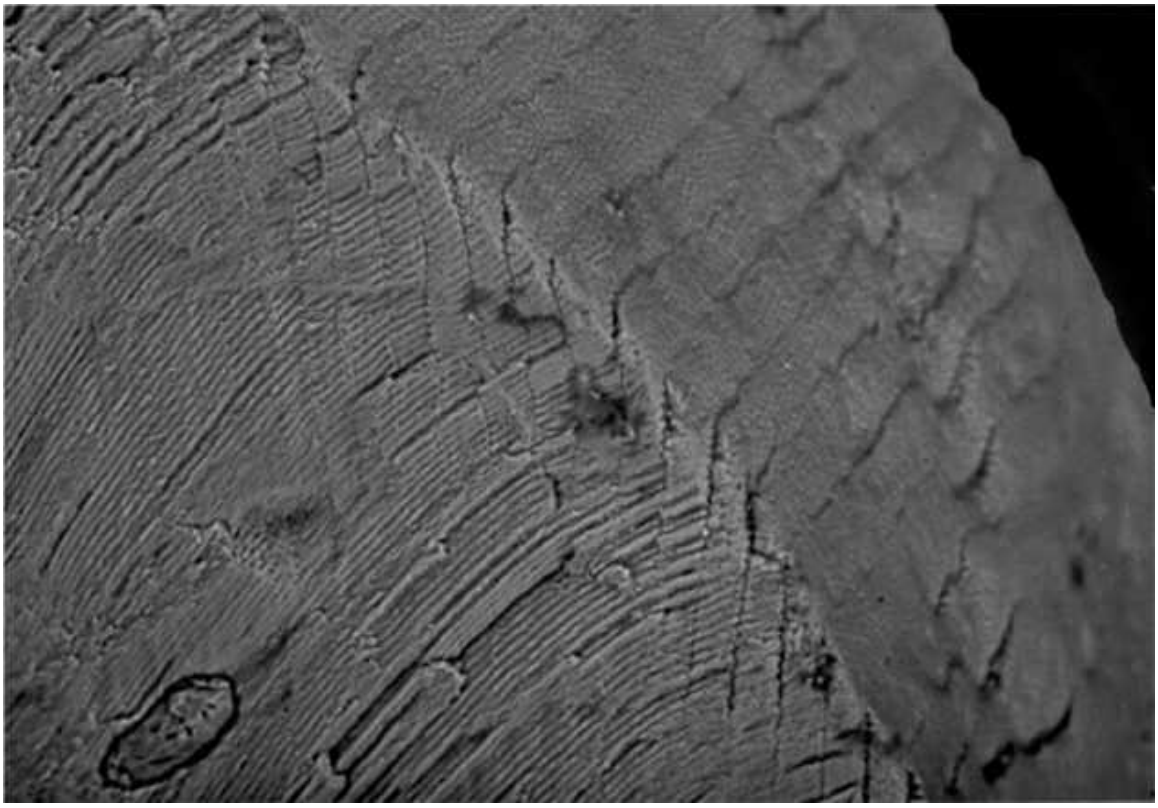


Figure 1.16 Scanning electron micrograph of the lateral enamel of *Afropithecus turkanensis*, illustrating the correspondence of Retzius lines (originating below the image) and perikymata on the tooth surface (running into the background in the upper right). Published in Kelley and Smith (2003).

It is possible that, in certain tooth types or taxa, Retzius lines tend to decrease in width and clarity as they meet the enamel surface, possibly due to convergence and/or the production of aprismatic enamel. Beynon and Dean (1991) illustrated this in human enamel, as they showed contrasting micrographs of sub-surface enamel with perikymata and with smooth enamel (similar to Figure 1.13b above) (Beynon and Dean, 1991; Figures a,b, p. 196). Beynon (1992) explained this variation as the result of rapid ameloblast slowing near the surface that produces visible perikymata, versus progressive slowing, which produces very curved lines that become almost parallel with the surface (and do not leave a notable depression). Boyde (1989) suggested that perikymata result from ameloblasts that stop just prematurely relative to where they are 'programmed' to finish. This results in a slight shift in the prism boundary, which is in keeping with Risnes' (1990, 1998) explanation of Retzius line formation (discussed below).

c) Models of formation and physiological basis of Retzius lines

Boyde (1964) stated that, at the time of his dissertation, the most common belief was that Retzius lines represent a reduction or disturbance in the rate of enamel formation, which he argued leads to an alteration of the developing front and the orientation of crystallites. He argued that this may influence the proportions of interprismatic material, and may cause the formation of fewer but larger crystallites. If this holds, the resulting pattern of large crystallites and large spaces between them would explain the common brown color (under transmitted light microscopy). Schmidt and Keil (1958 [in German]: as cited by Gustafson and Gustafson, 1967) noted that Retzius lines are preformed in the enamel matrix, and become more evident after mineralization, as the crystallite precipitation/orientation depends on the organic matrix. In keeping with Boyde (1964), Risnes (1990) suggested that ameloblasts pause during Retzius line formation (corresponding to the discontinuity), but that the cause of this is unknown.

Gustafson and Gustafson (1967) noted that, in rare instances, the interprismatic substance shows an increase in width at the expense of the prism width. Boyde (1989) also noted that enamel prism boundaries shift toward the center of the prism in the plane of a Retzius line, altering the ratio of pit to interpit enamel. He related this to his model of cross-striation production, suggesting that Retzius lines represent an extra-slow phase, and show more extreme 'inward necking of the prism boundary'. He suggested that this accounts for the cervical kink in the prism boundary, and the production of crystallites that are parallel to the plane of the Retzius line (see right side of Figure 1.11a above). Subsequently, Risnes (1990) proposed a model of Retzius line formation that synthesizes a number of these observations (Figures 1.17 and 1.18).

In his 1990 description of the staircase configuration, Risnes noted that the horizontal part of the stairs results from a 'cleft-like defect' across the prisms at a right angle, which he refers to as the transverse cleft (Risnes, 1990: Figures 3b, 4b, p. 137). Risnes illustrated that the ‘post-cleft’ transverse surface has a higher crystallite density than the ‘pre-cleft’ surface immediately below (Risnes, 1990: Figures 5, 6, p. 139). He noted that the penetration of these defects is variable, and that some appear to represent a complete prism interruption. The vertical part of the stairs is the adjacent prism boundary/sheath.

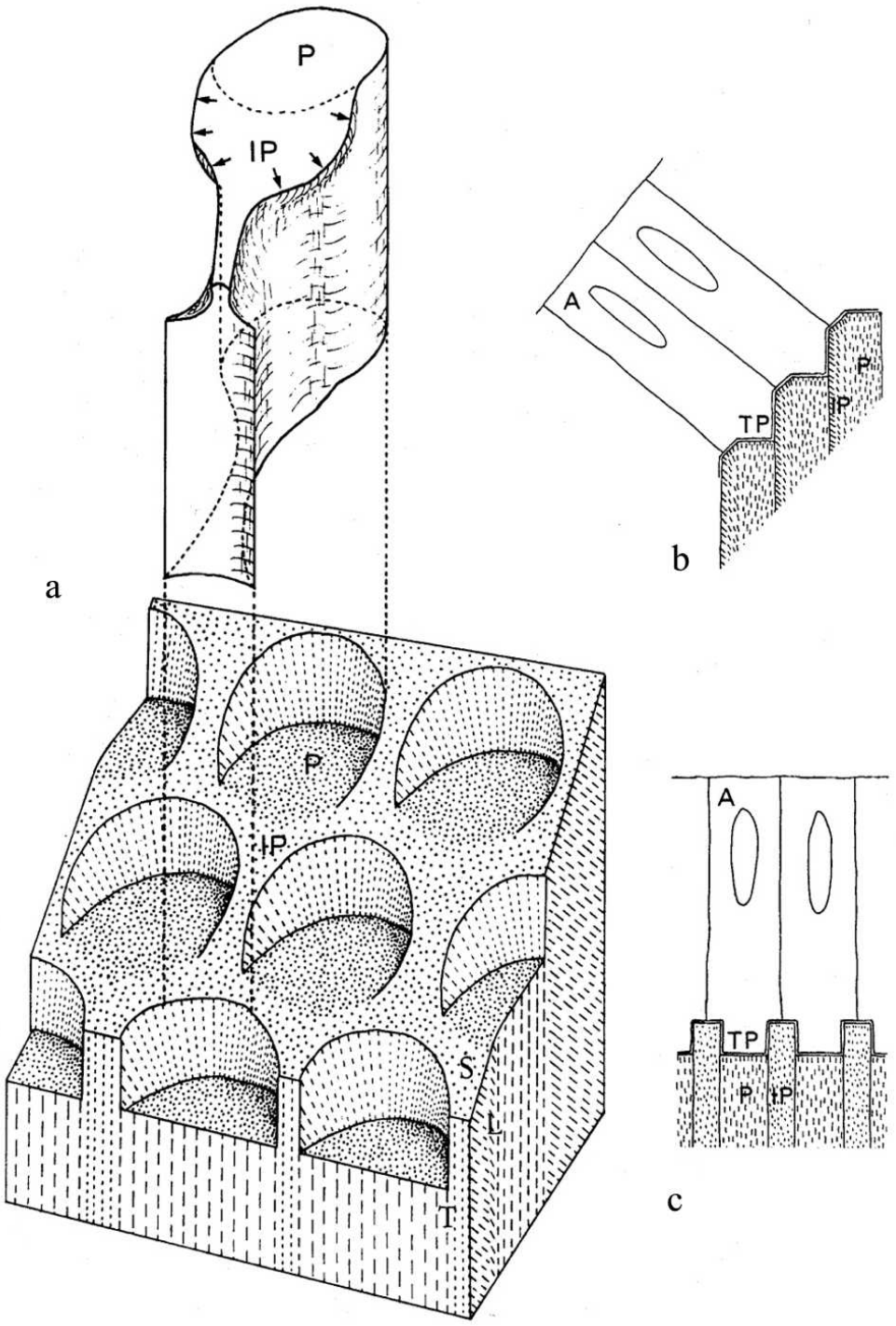


Figure 1.17 (a-c) Risnes' model of enamel development prior to the formation of a Retzius line. P- prism, IP- interprism, TP- Tomes' process, A- ameloblast, S- surface, T- transverse plane, L- longitudinal plane. a) Relationship between the developing prism and the profile of the developing enamel surface, showing the interprism expansion (arrows) that characterizes the pre-cleft surface. b) Picket-fence profile of ameloblasts and forming prisms. c) Battlements profile of ameloblasts and forming prisms. Modified and reproduced from Risnes (1990).

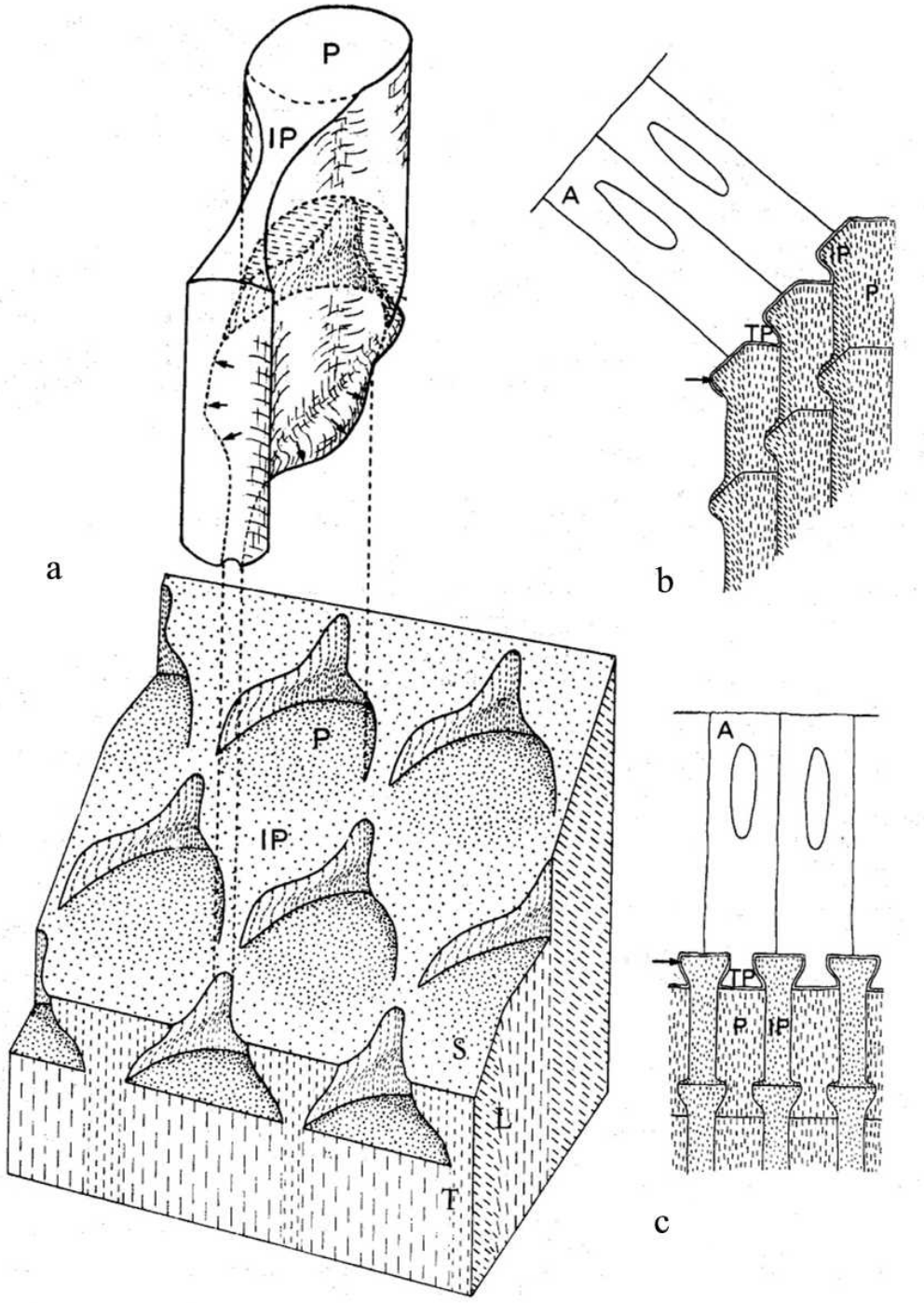


Figure 1.18 (a-c) Risnes' model of enamel development during the formation of a Retzius line. Codes and orientations are as in Figure 1.17. a) Interprism expansions (arrows) that characterize the post-cleft surface have forced a narrowing of the pits on the developing enamel surface, forcing the cervical neighboring prisms to narrow. b) Picket-fence profile shows the interprismatic expansions formed by the shoulders of the ameloblasts as oblique ridges, displacing the adjacent cervical prisms. c) Battlements profile shows the narrow Tomes' processes and expanded shoulders forming interprismatic expansions. Modified and reproduced from Risnes (1990).

By examining tangential planes of section (prisms end-on at the plane of the Retzius line), Risnes (1990, 1998) showed that the horizontal part of the stairs, or the 'end surface' of the prism, appears flat and enlarged, while the adjacent cervical prism appears small and irregular (Risnes, 1990: Figures 10, 11, p. 140). He illustrated that the end surface is enlarged due to a cervical expansion of the interprismatic substance, creating a club-shaped appearance at the edge of the transverse cleft (Figure 1.18b).[15] This interprism expansion forces adjacent prisms to reduce in width. He attributed this to constriction of the Tomes' processes that are forming the end surface, and an expansion of the adjacent shoulder areas of the ameloblasts (Figures 1.18b, 1.18c).

Risnes (1990) also noted that oblique clefts or ridges are parallel to the oblique trend of the staircase Retzius line, or are superimposed on it, and that they are due to 'defects' of the interprism material (framing the triangular space defined by Weber et al., 1974). Risnes (1990) explained that as the prism continues to form above the transverse cleft, a more typical diameter is achieved, which may be accompanied by the oblique ridge. As shown in Figure 1.18a, this is formed at the transition from the narrowed prism to the regular prism width (stippled area at the back of the base of the prism), and corresponds to secretions from shoulders of the ameloblasts. Between the transverse and oblique clefts, Risnes (1990) noted that prisms may show irregular clefts or cavities, resulting from the Tomes' process sliding by the overhanging edge of the expanded interprism, which presumably affects the quality of the local enamel (see also Whittaker and Richards, 1978; Weber and Ashrafi, 1979). This model represents the most comprehensive model of Retzius line expansion proposed to date (see numerous supporting micrographs in Risnes, 1990, 1998, 1999).

Dean and Scandrett (1996) noted that, in contrast to circadian incremental features, it is more difficult to explain the physiological cause of a long-period line. Gysi (1931) attributed the presence of circaseptan Retzius lines in humans to dietary factors, such as feast days (Sundays). However, this cultural explanation does not account for the presence of these regularly spaced lines in other mammals, nor does it account for the range of human periodicity values (discussed below and in Chapter 2). Dean and Scandrett (1996) suggested that the best causal explanation is that of Newman and Poole

[15] Previously described by Weber et al. (1974).

(1974, 1993), who suggested that the eight-day rhythmicity of Retzius lines may be the result of two imperfectly synchronized circadian rhythms, such as a 24-hour and 27-hour cycle. As these two rhythms run, they generate a 'beats frequency' as they interfere with each other, which may manifest as a Retzius line every eight days. Alternatively, they suggested that if the temporal separation of two cycles (one free-running approximately circadian and one 24-hour body rhythm) reaches a threshold, a physiological effect may manifest as a depositional delay and subsequent restoration of secretion, resulting in a Retzius line. However, it is difficult to image how this may explain the range of Retzius line periodicities seen within humans (6 - 12 days), or among primates (2 - 12 days). FitzGerald (1996) stated that Dean (via pers. com.) had suggested that Retzius lines may result from interference between cross-striations and intradian lines. Yet, considering current evidence that suggesting that cross-striations are formed every 24 hours and intradian lines are formed every 8 - 12 hours, it is difficult to imagine how these rhythms could interact to produce the known range of Retzius line periodicities. **B**iological rhythms and incremental feature periodicities are discussed further in Chapter 3.

d) Retzius line morphology and enamel extension

Numerous studies of primate enamel in the last decade have examined Retzius line spacing, relative lengths, and angles of intersection with the EDJ (reviewed in Chapter 2). Recent work has also considered the pattern or course of these growth lines in enamel. Dean and Shellis (1998) suggested that 'S-shaped' Retzius lines are occasionally found in the enamel of *Proconsul*, *Pongo*, and *Hylobates.* These lines show a sinuous course from the EDJ to the tooth surface (Dean and Shellis, 1998: Figure 1, p. 403; see also Chapter 2, Figure 2.8). Dean and Shellis (1998) provided a model that demonstrates that this morphology may result when prism width remains constant or decreases from the EDJ to the enamel surface. They stated that these distinctive lines are also characterized by increases in DSR, a cervical inclination of the prisms, and an increased angle between the Retzius line and the EDJ. It is unknown why these 'S-shaped' Retzius lines are variably found in certain regions of the enamel, or if they are more apparent in specific tooth types or taxa (discussed further in Chapter 2).

Boyde (1964) discussed the relationship between the angle of Retzius lines at the EDJ and the process of enamel extension, suggesting that smaller angles of intersection indicate more active cells and rapid development (extension), as there is less difference in angle between the ameloblasts and developing prisms. This angle has been taken to be indicative of the rate of ameloblast differentiation and/or the extension of enamel forming cells along the future EDJ. Shellis (1984a,b) suggested that the extension rate of enamel formation may be calculated from the angle of the Retzius line at the EDJ, the angle of the prism at the EDJ, and the daily secretion rate at the EDJ. From these parameters, he provided a model and trigonometric formula that theoretically yields the daily length of extension along the EDJ (discussed further in Chapters 2 - 4).

e) Neonatal lines and accentuated lines

FitzGerald (1996) provided a thorough review of the neonatal line, defined as an accentuated line parallel to the developing enamel front that is formed at birth. This line is of particular importance for determining the age of individuals that died while still developing their teeth, as well as for establishing the chronology of dental development (discussed in Chapter 2). The neonatal line has been documented in both deciduous and permanent human teeth (first molars) (e.g., Rushton, 1933; Schour, 1936; Whittaker and Richards, 1978), in both dentine and enamel (Schour, 1936). Schour (1936) first demonstrated deductively that these accentuated lines are formed at birth. He suggested that the physiological trauma experienced during 'regular' birth causes this line, which was present in over 90% of 350 deciduous teeth examined. Additional studies have shown that the width or appearance of this line may relate to the difficulty of the birth process (Eli et al., 1989), as well as to deficiencies in the neonatal environment (Noren, 1984). Noren (1983, 1984) suggested that the neonatal line may be caused by a decrease of plasma calcium during or just after birth. Noren (1984) showed that neonatal lines in infants born to diabetic mothers were wider than infants of normal mothers, which he related to the prevalence of hypocalcemia. Using microradiography, he demonstrated that this line was hypomineralized, lending support to his etiological explanation.

Based on a study of ten children between the ages of one hour to six months old, Schour and Massler (1937) reported an arrest in growth that averaged 14 days after the

deposition of the neonatal line. Massler and Schour (1946) corrected estimates of dentine formation time by 15 days to compensate for this delay. Whittaker and Richards (1978) suggested that the effects of birth may be seen in the next three to four days of development, but that the average distance and period of effected formation had been overestimated in light microscopy studies (see also Whittaker and MacDonald, 1989). Boyde (1989) also suggested that this line may involve a week or more of enamel formation, but was not specific about whether he meant an arrest in growth or a pronounced week-long band. Recent reports of crown formation time determined from incremental features do not include a period of developmental delay relating to this line (reviewed in Chapter 2). The current consensus may be that this line does not involve a true cessation in formation. This could be assessed in material that was of known age at death, or in material that had been experimentally labeled before and after birth. Antoine (2000) did not specifically consider this possibility in his discussion of the accuracy of crown formation time estimation in a large sample of known-aged human material, which may be a good sample to test this in.

Whittaker and Richards (1978) reviewed the history of structural descriptions of the neonatal line, including hypo- and hypermineralization, changes in the organic composition, changes in the crystallite concentration and prism sheaths, and/or prism bending. Using SEM, they documented a structural change in the width and orientation of prisms, which appeared to show a 0.2 μm 'interruption' (but not discontinuity) as they crossed the line. They noted that prism structure immediately after the line seemed to show a lower crystallite concentration (similar to Risnes' [1990] observations on the structure of the 'post-cleft' prism in the plane of a Retzius line). Whittaker and Richards (1978) noted that it was unclear if the neonatal line was equivalent to a Retzius line, which a number of previous researchers had suggested. Weber and Eisenmann (1971) illustrated that the neonatal line had a staircase configuration that appeared to be identical to Retzius lines. However, given the definition of Retzius lines stated above (regularly spaced features that meet the surface and form perikymata), the neonatal line will not be considered equivalent to a Retzius line in this dissertation. Other sources have suggested that this line is similar or equivalent to 'pathological' or 'accentuated Retzius lines' (e.g., Gustafson and Gustafson, 1967; Risnes, 2001).

Numerous studies have noted the presence of accentuated lines parallel to the developing enamel surface, often considering them structurally equivalent to the neonatal line or Retzius lines in general. Gustafson (1959) distinguished between 'functional' Retzius lines that result from prism bending, and 'pathological' Retzius lines that result from causes related to mineralization or compression of sections. Gustafson and Gustafson (1967) later stated that there are many variations in the appearance of pathological Retzius lines, shown in the many forms of these (often hypomineralized) lines (see also Frank, 1978; Goodman and Rose, 1990). Wilson and Schroff (1970) reviewed the manifestation of 'incremental lines' in enamel (e.g., Retzius lines and 'pathological' lines), and proposed the existence of three categories: line striae, band striae, and pathological striae, respectively equivalent to imbricational Retzius lines, cuspal Retzius lines, and what will be termed here accentuated lines. They regarded pathological striae as 'relatively broad bands', formed by changes in prism direction or composition. It is unclear how these lines were distinguished from band striae, as they were both noted to show bends in prism direction, and although Wilson and Schroff (1970) speculated that there may be differences in formation between Retzius lines and pathological lines, they did not provide any evidence for this. Rose (1977) subsequently noted the lack of diagnostic criteria to distinguish normal from pathological features, and proposed three different classes of pathological bands, distinguished by differences in prism structure. Rose et al. (1978) coined the term 'Wilson bands' (after Wilson and Schroff, 1970) for accentuated lines (for the purpose of not implying a pathological etiological basis), and suggested that analyses of the position and frequency of these lines may be used to infer the relative health of a population (discussed further in Chapter 2; see also Goodman and Rose, 1990; Thomas, 2003). Rose (1979) re-categorized these lines into five categories (four actual and one artifactual) based on morphological characteristics of the enamel prisms. He noted that in one instance, a band changed from one category to another.

Neither the specific physiological causes nor the developmental processes responsible for the formation of accentuated lines have been conclusively demonstrated (similar to the case of Retzius lines *sensu stricto*). Gysi (1931) suggested that these lines are the result of 'gastric disturbances'. Schour (1938) suggested that accentuated lines are

caused by pathological conditions that disturb calcium metabolism. Rose (1977, 1979) and Rose et al. (1978) stated that they arise from changes in ameloblast metabolism and movement. Numerous recent reports have suggested that accentuated lines or enamel hypoplasias may provide evidence of stressful conditions during development (reviewed in Goodman and Rose, 1990; Skinner and Anderson, 1990; Bowman, 1991; Macho et al., 1996; Simpson, 1999; Guatelli-Steinberg, 2001; Thomas, 2003; Guatelli-Steinberg, 2004; Skinner and Hopwood, 2004). However, only a few of these studies involve individuals with known case histories (Bowman, 1991; Dirks et al., 2002).

Bowman (1991) reported on the dentitions of several individuals in a captive colony of rhesus macaques, noting that certain events in the life of an infant or juvenile appear to be recorded dental tissues, including social disruptions, poor physical growth, separation from the mother, and the death of the mother. She also suggested that the experience of parturition may leave a mark in the maternal developing M3, although her study subject was also ill at the time of birth, so this is not conclusive. Adding further support, Klevezal and Myrick (1984) reported that accentuated increments in dolphin dentine appeared to correlate with parturition. Dirks et al. (2002) reported on accentuated lines in Awash baboons that appear to correlate with resource seasonality and periods of drought. Additional studies of the position and chronology of these lines in relation to environmental and life history records may provide further support for correlations between the deposition of accentuated lines and the timing of external events (discussed further in Chapter 2).

Concluding remarks

Consideration of the evidence of a relationship between cross-striations and prism varicosities/constrictions suggests that caution should be exercised when counting the number of cross-striation or inferring DSR from changes in the shape of the prism boundary. Risnes' (1986) suggestion that cross-striations represent one half of the undulation wavelength may provide some insight into the nature of this relationship (and is partially supported by Figure 1.10a). Alternatively, if a direct 1:1 relationship is established between varicosities and cross-striations, it may be that intradian lines correspond to a certain part of the wavelength, which is discussed further below. The

effects of acid etching must be considered when characterizing the profile of the prism and the appearance of incremental features (Boyde et al., 1978). Martin (pers. com.) has suggested that possible re-precipitation after etching may complicate attempts to relate prism boundaries with incremental features. However, it has been shown here that prisms in non-etched, naturally fractured teeth do not necessarily demonstrate varicosities/constrictions in areas where incremental lines are seen (Figure 1.10b; see also Smith et al., 2003a, Figure 4b, p. 297). It may be that varicosities correspond to cross-striations in certain developmental conditions, possibly related to the rate of formation and/or the type of prism packing pattern, but in other instances prism varicosities/constrictions are not in sync with incremental features in a 1:1 ratio, or they are not produced at all. The best way to address this issue is with additional documentation of these features in naturally fractured teeth, as well as in teeth sectioned in different planes. As in the case of aprismatic enamel, artifacts of light microscopy are likely to complicate attempts to understand this relationship, thus SEM, TSRLM, or CLSM are advocated.

Despite the models proposed by Boyde and the work done by Okada and colleagues, some recent studies have suggested that the developmental nature of daily lines is still unclear (Dean and Scandrett, 1996; Moss-Salentijn et al., 1997). Explanations for cross-striations proposed by a number of researchers have included differences in the composition or orientation of hydroxyapatite crystals, differences in the degree of calcification, and differences in the organic matrix composition. As Dean (1995a) and FitzGerald (1998) have noted, evidence from the experimental work of Okada and Mimura is in accordance with Boyde's model. It appears that light and dark bands found in enamel and dentine are related to metabolic activity cycles and/or changing acid/base (or pCO_2) levels in the blood, which translate into rhythmic differences in the incorporation of certain minerals into the developing enamel, forming daily lines. Dean (1995a) reviewed a few studies that also showed that pCO_2 levels in sleeping humans may rise up to 13% more than waking levels. Periods of high alkalinity (or CO_2 availability) produce dark-staining lines during sleep, which appear to have a higher level of calcium than light-staining lines (and would appear as light lines under BSE due to higher atomic number contrast relative to the adjacent enamel). Ohtsuka-Isoya et al.

(2001) suggested an additional explanation for the formation of daily and/or sub-daily increments: fluctuating rhythmic hormone levels may influence the activity of enamel and dentine forming cells. This will be reviewed further below and in Chapter 3.

Martin (pers. com.) has suggested that an appropriate test of the metabolic basis for incremental line production may be found through examination of hibernating animals. He predicted that if incremental lines were created by circadian metabolic changes, these lines would not be expected in hibernating animals, which presumably to do not experience circadian metabolic fluctuations. Sarnat and Hook (1942) first examined the effects of hibernation on dentine eruption (extension) and apposition, and found that rates of both were significantly lower than in non-hibernating control animals. They reported that daily incremental features were not evident during hibernation, nor was an area of differential formation (i.e., a 'hibernation mark'). They stated that "in hibernating animals the incremental pattern occurs over a longer period of time" (p. 487). Rinaldi (1999) recently reported that incremental features are formed in the dentine during hibernation in marmots, although it did not appear that these lines were formed every day during hibernation. She tested the possibility that the lines may represent periods of arousal during hibernation, but did not find a relationship.

More recently, Klevezal and Mina (1990) and Klevezal (1996) reported on incremental lines in Russian birch mice after emerging from hibernation. Klevezal and Mina (1990) deductively inferred and identified a 'hibernation mark' in the incisors of rodents that were assumed to have recently come out of hibernation. They described this line as an approximately 10 μm thick band that appeared under transmitted light either as a highly contrasted light band with dark borders, or a dark band with light borders. They related this to the production of annual layers in teeth that do not show continuous development, but did not speculate on the specific process of formation of this mark. Klevezal (1996) reviewed previous work on additional rodents, and suggested that there is variation in the expression and structure of hibernation marks. In several species, a broad zone of contrasting light and dark bands was identified as the 'hibernation zone', with a principle zone of very closely spaced layers (Klevezal, 1996: Figure 61, p. 229). In another species, the hibernation zone is a 'hypochromatic' layer 30 - 100 μm wide, which shows closely spaced layers that became indistinguishable inside the zone. In conclusion,

it appears that certain mammals may show closely spaced incremental layers during hibernation, which may contradict an exclusive metabolic explanation for circadian line formation. However, other species may show a lack of circadian patterning, which supports Martin's suggestion. Additional work on labeled material or teeth that record multiple seasons may shed additional light on this complex issue. The effect of lower body temperature on incremental line formation should be considered as a potential related factor as well.

It appears that there is some confusion regarding the results of the Japanese experimental work discussed above, which may be due to differences in translation. Two different reviews of Okada's (1943) study were presented by Rosenberg and Simmons (1980) and FitzGerald (1998). Regardless, it is clear that certain aspects of cross-striation formation have yet to be conclusively explained, such as how this circadian period originates and is controlled, as well as how the production and rhythm of cross-striations relates to other incremental factors such as laminations, intradian lines, and Retzius lines. This is discussed further in Chapter 3.

The recent re-description of intradian lines in enamel and dentine in the last few years by FitzGerald, Dean, and colleagues has illustrated the need for a more thorough examination of this feature. Of particular significance are the implications for previous studies of incremental features, such as DSR and Retzius lines periodicity, as FitzGerald (1996) noted. It is possible that additional documentation of the variation of cross-striations, both within and between teeth, may allow a distinction between cross-striations and intradian lines based on the repeat interval (or secretion rate). Most recent reports have suggested that cross-striations in primates form at a minimum of ~2.5 μm/day and at a maximum of ~7 μm/day (reviewed in Chapter 2). It may be possible to categorize increments with an interval smaller than a 'taxon-specific minimum daily rate' as intradian lines, although this would not permit a distinction of intradian lines in more fast-forming enamel (Smith et al., 2004). An additional method of establishing the presence of intradian lines would require areas where cross-striations, Retzius lines, and intradian lines can all be seen in association. If the number of cross-striations between adjacent Retzius lines can be established, any area showing a greater number of fine lines between two Retzius lines must contain features with a periodicity of less than a day. As

noted above, experimental studies such as those of Rosenberg and Simmons (1980), Ohtsuka and Shinoda (1995), and Ohtsuka-Isoya et al. (2001) may also provide more insight into this issue.

To date, a model of lamination formation has yet to be proposed, partially due to the fact that the periodicity of these features has not been proven. If they are daily features, Boyde's model of cross-striation formation may not adequately explain their formation, as laminations are structurally similar to Retzius lines, which have a different orientation than cross-striations. It is unknown how a short-period feature may be formed that is parallel to the secretory face of the Tomes' process, although models of Retzius line formation may provide some insight.

The literature on Retzius lines suggests a lack of consensus as to what constitutes a Retzius line, as well as what causes it. Differences in observations relating to the degree of mineralization and the deviation of the prisms have not been adequately explained. Risnes (1990) suggested that prism discontinuities and inferior enamel quality following Retzius line production may explain hypomineralized enamel (as shown in microradiographs). In regards to prism bending, he stated that directional change or prism bending is not associated with staircase type Retzius lines. It remains to be seen if prism bending is associated with the production of (regularly spaced) Retzius lines in different regions of the crown, or if this is more commonly related to the production of accentuated lines. Additionally, the issue of compositional changes along Retzius lines appears to be unresolved. The images shown here have illustrated regions with and without staircase profiles, and have also shown than the profiles can transition into clefts that appear to result from prism discontinuities. As noted above, Risnes (1990, 1998) suggested that interprism expansions at the plane of a Retzius line 'persist' in the inner enamel, while the prism discontinuity (transverse cleft) is 'lost'. This is a plausible suggestion, as staircase or cleft-like Retzius lines are generally not found in the deeper enamel, but it is not obvious why this should be the case developmentally. It is highly possible that Retzius lines represent a variable structural phenomenon that is dependent on the prism packing pattern, the depth within the developing enamel, and/or and the degree of mineralization.

One complication of Risnes' model of Retzius line formation is that it only explains the formation of Retzius lines in pattern 3 enamel. In pattern 1 enamel, there is a discontinuity between prismatic and interprismatic enamel (defining the prism sheath), which would presumably affect the appearance of adjacent interprismatic expansions. They would necessarily be spatially discontinuous, and may be temporally discontinuous as well, due to the relationship between ameloblasts and forming prisms. Given that Retzius lines do exist in pattern 1 enamel, it is possible that, as the packing pattern changes from pattern 3 to pattern 1, the change in the orientation of the Tomes' process/ ameloblast shoulders causes the production of oblique clefts only (rather than the transverse clefts making the staircase profile). This may result in the onion layers seen in aprismatic enamel (see Figures 1.8b and 1.10a above), and/or a potential circadian manifestation, described above as laminations.

Risnes (1998) did relate the production of staircase Retzius lines to the formation of sub-surface aprismatic enamel, suggesting that the constriction of the Tomes' process at the Retzius line may sometimes result in the loss of this process. He suggested that in some areas, the Tomes' process may be lost and then reformed, as the prismatic structure of the layer may appear more defined as prisms reach the upper aspect of the Retzius interval (where the process has presumably reformed). He also suggested that the expansion of interprism enamel during the formation of Retzius lines may be a developmental regression to a primitive enamel aprismatic structure (i.e., parallel crystallites perpendicular to the surface). If this is the case, it is possible that the more extensive, scaly aprismatic layers described by Ripa et al. (1966) and Shellis and Poole (1977) in monkeys and prosimians are a primitive developmental retention. Additionally, the expression of a variably present, thin superficial layer with little definition may be the vestige of an ancestral aprismatic developmental pattern (known from comparative studies of mammals and reptiles). To resolve this further, more extensive surveys should be undertaken in a wide variety of primates and other mammals.

The various theories on the physiological causes of Retzius lines are quite interesting (e.g., Newman and Poole, 1993), although it does not appear that direct evidence of multiple circadian oscillators in enamel has been demonstrated to date. A number of increasingly sophisticated studies from the past decade have brought us closer

to a potential explanation, and additional work on circadian and intradian clocks may shed light on development of incremental features in enamel. Ohtsuka and Shinoda's (1995) study suggesting multiple intradian rhythms may have provided the basis for Dean's theory that Retzius lines may result from an interaction of multiple short-period clocks. However, given the diversity of documented Retzius line periodicities, it is difficult to conceive of a simple 'interaction model' that will explain this range in primates.

As Risnes (2001) noted, additional work is necessary to determine the exact basis of neonatal line formation. This is also true in regards to developmental differences between Retzius lines (*sensu stricto*), neonatal lines, and other accentuated lines. Dean (1987a) noted that it is not clear whether accentuated lines are superimposed on a systemic rhythm, or are merely coincident with one. Despite numerous classifications and descriptions, it is unknown how and if accentuated lines actually differ from one another, especially when considering differences due to preparation and visualization techniques. Given the lack of distinction provided between 'cuspal' Retzius lines and accentuated lines, it unclear how these two classes of features may be distinguished from one another (which provides further support for the use of the strict definition of Retzius lines proposed above).[16] This area will benefit from additional longitudinal studies of individuals with known records of birth, illness, stress, life history events, and environmental factors (e.g., Bowman, 1991; Dirks et al., 2002), as well as additional experimental work.

Factors Influencing Development

Studies of genetic, hormonal, and environmental influences on dental development have benefited from numerous clinical case reports of individuals with several anomalous conditions. These reports have allowed insight into the genetic and physiological basis of dental development. Unfortunately, most of these studies have not distinguished the effects of these disorders on enamel and dentine development

[16] One of the complications in early work was the belief that Retzius lines were not formed with a regular periodicity, and thus could not be distinguished on a temporal basis (e.g., Wilson and Schroff, 1970).

specifically, and very few have examined these effects at the microstructural level. However, recent work on a large population of captive baboons with known pedigrees has allowed the influence of genetic factors on tooth macrostructure to be modeled and quantified. This approach has yet to be applied to studies of the variation of the enamel microstructure, which may shed additional insight into the developmental biology of dental hard tissues.

Genetic component

Lewis, Garn, and colleagues were some of the first to examine the genetic basis of dental development using twin, sibling, and family studies (see Scott and Turner [1997] for a review of early studies). They suggested that variation in dental development is due largely to genetic differences between individuals (e.g., Lewis and Garn, 1960; Garn et al., 1965a,b; Lewis and Grainger, 1967), including variation in crown dimensions, timing of calcification of dental components, eruption timing, and polymorphism in both P_2M_2 formation sequence and in molar size sequences. Others have suggested a genetic basis for missing teeth (and supernumerary teeth), particularly third molars (reviewed in Sofaer, 1975). Garn et al. (1965a) reported that the genetic basis of variation was most apparent in comparisons of female relatives, particularly sisters, implying a sex-linked (X chromosome) aspect. They cited cases of Turner's syndrome (XO genetic makeup) where crown development was normal or often advanced relative to skeletal development and normal dental standards. Zilberman et al. (2000) examined molar crown components in Turner's syndrome, and reported that molars were smaller and enamel was thinner in XO females. They reported that first molars were more affected than second molars, which may be related to the earlier and shorter period of formation and the timing of gene expression. These authors suggested that it would be worthwhile to examine the development of different molars histologically to examine differences in enamel extension and apposition.

Additional support for the sex-linked nature of dental development comes from the work of Kotilainen and Pirinen (1999), who found advanced dental development in boys with fragile X syndrome (X-linked disease) and in females that carried the gene. Sofaer (1975) reviewed studies of abnormal enamel formation, such as amelogenesis

imperfecta of the hypoplastic and hypomineralized types, which appear to be related to X-linked and autosomal genes, respectively. Zilberman et al. (2000) reviewed several decades of work following Lewis and Garn's initial studies, noting that the sex-linked basis of dental development has been well-established. Work by Alvesalo and colleagues on several types of genetic anomalies (reviewed in Zilberman et al., 2000) has demonstrated that both the X and Y chromosomes code for proteins that regulate enamel development, and Y chromosome products also regulate cell division at the forming EDJ.

Scott and Turner (1997) reviewed the development of modern theories on the genetic basis of dental variation. Initial explanations involving simple Mendelian models of inheritance (simple dominance and recessive models) were eventually replaced by models of quasicontinous variation resulting from the influence of polygenetic control (many genes at many loci), although dental development and expression is not believed to be as complex as many other traits that may involve large numbers of loci. Additionally, they reviewed studies that suggested that certain characters may be explained by polygenetic inheritance, while other traits appear to fit a more simple dominance inheritance model. These authors also reviewed the application of quantitative genetics to the characterization of Carabelli's cusp expression, which has been reported to show a wide range of heritability scores (proportion of the phenotypic variance due to additive effects of genes). Scott and Turner (1997) suggested that it is more likely that traits such as Carabelli's cusp and incisor shoveling show high measures of variability, as they are relatively free to vary without deleterious effects. In contrast, genes that code for fundamental developmental processes, such as the initiation of enamel development or the basic morphology of primary cusps, are less likely to vary despite their strong genetic control (resulting in low measures of heritability due to lower variation among relatives).

Recent work by Hlusko and colleagues has recently applied the principles of quantitative genetics to the study of dental character variation, including molar crown dimensions, cuspal areas, cingulum expression, and linear enamel thickness (Hlusko, 2000; Hlusko et al., 2002; Hlusko and Mahaney, 2003; Hlusko et al., 2004). These studies have been based on a large, captive, pedigreed colony of baboons, using dental casts and micro-computed tomography scans. Hlusko and colleagues have begun to quantify the suggestion by Lewis and Garn that a large degree of variation in dental

development is due to genetic factors; conservative estimates of the heritability (resemblance between parents and offspring, or fraction of variance due to additive allele effects) were reported for crown areas: 0.83; mesiodistal width: 0.67; buccolingual width: 0.73; interconulus expression: 0.33 - 0.73; interconulid expression: 0.41 - 0.64; and enamel thickness: 0.32 - 0.44. Additionally, it appears that cingulum expression may be the result of a single gene affecting expression in antimeres, but similarities between maxillary and mandibular teeth are due to multiple shared genes. These studies also suggested that variation in crown area or dimensions may be related to the genetics of body size or length determination, while variation in cingulum expression may be related to genes that code for patterning.

Endocrine factors

Studies examining the relationships between hormones and dental development typically fall into one of two categories: experimental manipulations of hormone levels in rodents or monkeys, or case studies on humans seeking treatment of abnormalities. Typical human disorders examined in the context of dental development include hypopituitarism, hypothyroidism, and delayed puberty or sexual precocity; these are related to levels of growth hormone, thyroid hormones, and sex hormones, respectively. These hormones are known to affect numerous physiological processes, including stimulating bone growth and metabolic functions, increasing calcium deposition in bone (lowering level in blood), and reproductive growth and development (Campbell, 1996). Parathyroid hormone has also been considered in relation to dental development, which is logical given its role in maintaining calcium balance in the blood by promoting calcium release from bone and soft tissue uptake. The general consensus of numerous studies has suggested that abnormal hormonal levels do not affect dental development as dramatically as skeletal development (e.g., Schour et al., 1934; Cohen and Wagner, 1948; van Wagenen and Hurme, 1950; Seipel et al., 1954; Garn et al. 1965b; Edler, 1977; Hansson et al., 1978; Roberts et al., 1985; Pirinen, 1995; Berkovitz et al., 1998; but see Keller et al., 1970). Several studies, including Demirjian et al. (1985), have interpreted this to suggest that dental development is independent of the growth processes that

control somatic development. However, given the result of several studies reviewed below, it is unlikely that these two processes are completely independent.

a) Growth hormone

One of the most common conditions described in the dental literature is hypopituitarism, which causes delayed dental development in growth hormone deficient individuals. However, several authors have presented conflicting reports of the specific effects of hypopituitarism on dental tissues. Several case studies of pituitary dwarves have demonstrated that pituitary hormone deficiency results in eruption delay, impaction, and jaw overcrowding, due to a lack of deciduous root re-absorption, delayed root development, and stunted jaw growth (e.g., Schour et al., 1934; Cohen and Wagner, 1948; Kjellberg et al., 2000). Crown size and development were generally found to be normal in these studies. Symons and Seymour (2000) treated dwarf rats with growth hormone and found that untreated rats show a delay in enamel mineralization (compared to dwarf rats that had received treatment, as well as normal-sized rats), but show relatively normal root development. Schour (1934a,b,c) described the results of a study on rats that had undergone hypophysectomy, noting several changes in dentine formation following surgery, including delayed eruption. After administration of growth hormone, eruption rate increased, which was also recently found in growth hormone deficient human children after treatment (Krekmanova et al., 1999).

Several other studies also found delayed dental development in hypopituitarism, but were not specific about the effects (Garn et al., 1965b; Keller et al., 1970; Edler, 1977), or did not report any differences (Van Erum et al., 1998; Berkovitz et al., 1998 and references therein). Pirinen (1995) suggested that deficiencies affect all aspects of dental development equally. Symons and Seymour (2000) reviewed work that demonstrated that receptors for growth hormone have been found on ameloblasts, odontoblasts, and cementoblasts, which suggests that all dental tissues may be affected by growth hormone deficiency. This issue would benefit from additional histological investigations of enamel and dentine development, as it is likely that this may shed insight into the control of incremental feature production (e.g., Okada, 1963; Yonaga, 1969). Okada (1963) reported that the typical light and dark banding pattern of daily

dentine lines disappeared after the pituitary gland was removed, but it was not clear how this related to changes in the rate of dentine formation (lab animal unknown, possibly rabbit or rodent). Yonaga (1969) did not discuss the appearance of daily increments specially, but demonstrated that dentine extension rate was more reduced by hypophysectomy than dentine apposition rate (which is in contrast to results on thyro-parathyroidectomized rats, discussed below).

b) Thyroid and parathyroid hormones

As in the case of growth hormone, several authors have presented conflicting reports of the specific effects of thyroid and parathyroid hormones on dental tissues. Garn et al. (1965b) suggested that growth hormone is more important than thyroid hormones for dental development, as hypopituitary patients generally show greater delays than hypothyroid patients. In contrast, Hansson et al. (1978) demonstrated that thyroxine administration caused a greater increase in dentine growth than growth hormone in rats, and when administered together they appeared to act synergistically. Ziskin et al. (1940) demonstrated that thyro-parathyroidectomized rats display delayed eruption, dentine formation and root development, but crown size appears normal. Pirinen (1995) also noted similar conditions in humans with hypothyroidism. Noren and Alm (1983) reviewed additional studies of thyroid hormone deficiencies, and suggested that this condition specifically affects enamel production. Their study of infants with hypothyroidism reported frequent sub-surface hypomineralized lesions, which they suggested were a result of changes in matrix deposition and calcification due to reduced thyroid hormones.[17]

Okada (1963) reported that the removal of parathyroid glands caused a strong difference in the calcification pattern of dentine lines, which was restored when transplanted glands were introduced. He concluded that parathyroid hormone specifically influenced the calcium balance of dental tissues and formation of the organic matrix. In a series of elegant experiments, Yonaga (1978a,b,c) histologically examined the effects of parathyroidectomy and thyro-parathyroidectomy on dentine development in rats, using

[17] Noren (1983, 1984) suggested that this condition was also common in normal infants, as well as in low-birth weight infants and those from diabetic mothers.

lead acetate markers to assess growth over a known period of time. He showed that both dentine apposition (secretion) and extension rates were reduced after either surgery, although the reduction was more dramatic after thyro-parathyroidectomy. Subsequent injections of parathyroid hormone helped to restore growth rates to higher than pre-operative levels, which was more dramatic after thyro-parathyroidectomy. After injections, the rate of dentine apposition increased more than dentine extension rate. An examination of the enamel showed that enamel extension changed in an identical manner to dentine, but the change in enamel apposition rate after parathyroid hormone injection was less than the dentine appositional rate. Yonaga (1978a) suggested that this represents evidence that structures derived from the mesoderm (including bone) are more sensitive to changes in parathyroid hormone than ectodermal tissues. In addition, he suggested that the mechanism of bone and tooth apposition responded more to changing levels of parathyroid hormone, rather than cell proliferation (extension), which may be consistent with the role of these glands in regulating calcium balance.

c) Sex hormones

Experimental work has demonstrated that increased testosterone speeds up eruption in macaques, but eventually leads to crowding and impaction due to inhibitory effects on bone growth (van Wagenen and Hurme, 1950; Seipel et al., 1954). Root development appeared to be affected more than crown development. Garn et al. (1965a,b) found that there was a positive relationship between degree of (abnormal) advanced sexual maturation and dental development, which led them to infer a possible relationship between steroid (sex) hormones and movement and completion of the later-forming teeth. Both crown and root development appeared to be advanced. However, Keller et al. (1970) and Roberts et al. (1985) did not typically find advanced dental development with sexual precocity. Keller et al. (1970) and Pirinen (1995) reviewed additional studies that suggested either advanced or normal development in this condition.[18] Studies of children experiencing delayed onset of puberty have shown a delay in dental development (Garn et al., 1965b; Keller et al., 1970; Pirinen, 1995; Gaethofs et al., 1999). This suggests that

[18] It should be noted that several of these studies used different growth standards, which may influence results.

sex hormones are important for the final developmental period of the dentition, but that the factors that lead to advanced sexual maturity do not necessarily have the same impact on all hard tissue growth.

Environmental factors

Garn et al. (1965a) reported that the timing of dental development is positively correlated with thoracic fat, which they believed to be an indicator of caloric excess. They reviewed additional studies that suggested a similar relationship between nutrition and dental development in Latin American children. Niswander (1963) also suggested that dental development in Japanese children may be positively correlated with nutritional status. Other studies have examined the influence of the maternal environment on dental development, such as Noren's (1984) study on deciduous teeth from infants of diabetic mothers. Noren (1984) found that these infants displayed wider neonatal lines and additional defects in the postnatal enamel, which he attributed to calcium deficiencies relating to the maternal condition. It is unknown how or if maternal conditions may influence the development of the permanent dentition, or if other factors such as nutritional differences manifest in the formation of enamel and dentine specifically.

A fair amount of anecdotal evidence exists to suggest that environmental conditions may influence the age at eruption, and, by inference, the crown formation time or rate of root development in non-human primates (Nissen and Riesen, 1945, 1964; Swindler and McCoy, 1965; Marzke et al., 1996, but see Fooden and Izor, 1983). Several of these studies suggested captive chimpanzees that are hand-raised erupt their dentitions faster than those that are raised by their mothers, although the environmental variable appears to have less of an effect on dental development than on weight gain or skeletal development (Marzke et al., 1996). Phillips-Conroy and Jolly (1988) compared wild and captive baboon populations and showed that eruption is consistently earlier in captive animals, however the effects differ with tooth position. M1 and M2 appeared to be less affected than anterior teeth, while M3 was relatively accelerated. They also discussed the influence of diet, citing conflicting studies on humans, and suggested that the laboratory environment may be regarded as 'abnormal' in that it leads to over-nutrition in contrast to the natural setting.

Temporal and chemical changes in enamel

Schour (1936) noted qualitative differences between prenatal and postnatal enamel (distinguished by the identification of the neonatal line, reviewed above). He suggested that prenatal enamel is better calcified than postnatal enamel. Gustafson and Gustafson (1967) also suggested that prenatal enamel is more mineralized, which may be due to fetal growth in a well-protected environment with an adequate nutrient supply. (They also reviewed a few studies that suggested that the converse was true, see also Noren, 1983.) Bowman (1991) also suggested that differences exist in macaques, but was not specific about her observations. Noren (1983) reported a greater frequency of growth disturbances in enamel formed at or after birth than prior to birth, including hypoplasias and sub-surface lesions. If qualitative differences exist, it is unclear if these differences may be related to the identification of incremental features.

Until recently, it had not been entirely clear if incremental features may be detected in prenatal enamel (Komai [1942: in Japanese]: as cited by Boyde, 1964). Several studies have estimated formation times for prenatal enamel, but were not specific about the actual identification of incremental features in this region (e.g., Beynon et al. 1991b; Dean et al., 1993a; Reid et al., 1998a,b). Dirks (1998) noted the presence of both Retzius lines and cross-striations in prenatal gibbon enamel of different teeth from the same individual. FitzGerald et al. (1999) reported counts and measurement of cross-striations in pre- and postnatal enamel in human deciduous canines. These studies contrast with the results of Ohtsuka and Shinoda (1995), who suggested that cross-striations in rats are not formed until 2 - 3 weeks after birth, although intradian lines may be seen earlier. Smith et al. (2002) also reported the presence of incremental features in macaque prenatal enamel. This study suggested that differences may exist in the types and clarity of incremental features present in pre- and postnatal enamel, possibly indicating developmental differences. Additional study of these differences is needed.

Few recent studies of incremental features have considered the effects of changes in mineral composition on the appearance of these features (but see Dean [1998b] regarding dentine). It appears that little consensus exists in regard to the effects of hypo- or hypermineralization on enamel microstructure. Differences in mineral composition are presumed to account for the superior quality of fossilized dental material when compared

to extant material, which is presumably more highly mineralized than extant material. However, partially decalcified enamel may also show clear incremental features (e.g., images in Hals, 1957; Berkovitz et al., 1992: Figure 216, p. 116). It is also apparent that developing material shows a qualitative difference when compared to fully mineralized tissue. As noted above, Schmidt and Keil (1958 [in German]: as cited by Gustafson and Gustafson, 1967) noted that that Retzius lines become more evident after mineralization, while Dean (1987a, 1989) suggested that the maturation of enamel may obscure the clarity of the initial formative event (also discussed further in Chapters 3 and 5). Cross-striations may be more evident in hypocalcified enamel, as shown by their appearance in material that shows signs of pathology, such as carious lesions (e.g., Bell et al., 1991) (Figure 1.19). Incremental features are generally considered to be more visible when associated with these defects (Noren and Alm, 1983; Noren, 1984; Bell et al., 1991).

A few studies have considered the effects of post-mortem changes in enamel quality, particularly in archaeological material (Poole and Tratman, 1978; Bell et al., 1991; Antoine, 2000). Poole and Tratman (1978) examined dental remains from humans excavated from limestone caves in England. They described extensive lesions below the surface of the enamel that extended from the cusp tip to the cervix, as well as into the deeper third or half of the enamel thickness. The appearance of incremental features in these areas was very clear (e.g., Poole and Tratman, 1978: Figure 4, p. 1119). They related this to similarities with the development of caries lesions (where acid dissolution causes demineralization), but suggested that these changes occurred as a result of post-mortem colonization of microorganisms across the enamel, dentine and cementum. Bell et al. (1991) noted that relative to other hard tissues, enamel shows the least diagenetic (post-mortem) changes. They examined bone and dental remains from five different soil-buried contexts and one marine context. In contrast to Poole and Tratman (1978), they did not find evidence of demineralization of the enamel surface, although they did document the appearance of carious lesions, which accentuated the appearance of Retzius lines and cross-striations (illustrated in Bell et al., 1991: Figures 11, 12, p. 178). Studies such as these may permit more accurate understanding of the nature of variation in the quality of archaeological or fossil enamel, and may also lead to advances in preparative techniques.

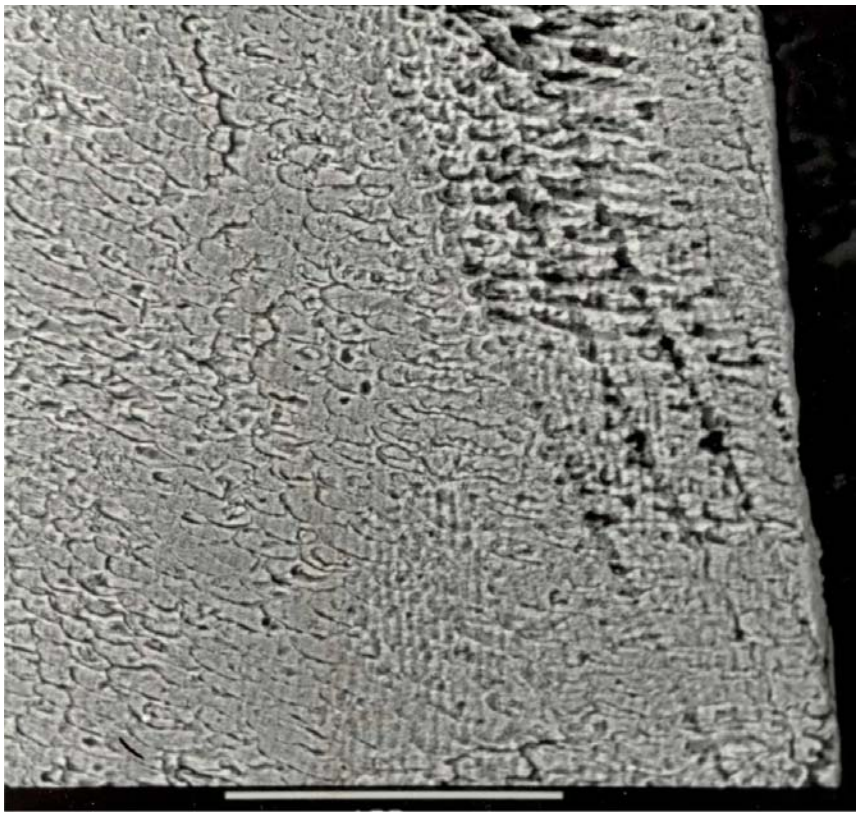


Figure 1.19 Scanning electron micrograph of a potential carious lesion in *Afropithecus turkanensis* (polished and etched section). In this image, prisms run almost horizontally and Retzius lines run to the surface (right side). The dark area appears to be an area of demineralization and prism discontinuity, and is associated with several Retzius lines and an area of marked cross-striations. Scale bar equals 100 μm. Smith and Martin (unpublished image).

Concluding remarks

Several decades of case studies of individuals with various genetic disorders have contributed to our understanding of the genetic control of dental development, as well as studies of sibling, families, and pedigreed populations. Histological studies of teeth from individuals with different anomalous conditions may ideally shed more precise information on the genetic basis of enamel and dentine development. It appears that dental development is also influenced by endocrine factors, most notably growth hormone, thyroid hormone, and parathyroid hormone. Steroid (sex) hormones also appear to influence dental development by speeding up development when increased, as does

cortisone (Kiely and Domm, 1973; Pirinen, 1995). (Okada [1963] also demonstrated that removal of the adrenal gland caused the light and dark banking pattern of dentine to disappear.) Hormone related delays in dental development often appear to be due to changes in root development rather than crown formation, which is not surprising given the embryological similarities between dentine and bone (also noted by Yonaga, 1969, 1978a). Garn et al. (1965b) suggested that it is possible that crown formation is largely independent of hormonal influences, while root development may be more dependent on trophic factors. Multiple hormones may have different effects on aspects of growth within and between dental hard tissues. It may be the case that crown formation is more developmentally 'fixed' in contrast to root development, perhaps due to tighter genetic control (and the precision required by occlusion), although further investigation is needed to substantiate this. Additional aspects of hormonal influences on dental development will be considered in the section on biological rhythms in Chapter 3.

Finally, additional work on the variation of dental development in populations of animals from different environments is warranted, particularly in light of the possibility that differences may exist between captive and wild populations. If this is substantiated, it may have implication for numerous studies that have established growth standard from captive animals. It may also demonstrate the degree to which environmental factors influence dental development. If such developmental plasticity is demonstrated in hominoids, this may have implications for archaeological and paleoanthropological interpretations of dental development and life history.

Chapter 2: The Analysis of Incremental Features of Enamel Microstructure

Analytical Unit Quantification

Incremental Features

Features of the incremental enamel microstructure are defined in this review as structural phenomena that are formed with a consistent periodic repeat interval, traditionally including: 1) cross-striations and 2) Retzius lines, and here, also including 3) intradian lines and 4) laminations. Cross-striations and Retzius lines are also frequently referred to as short- and long-period structures, respectively (reviewed in Chapter 1). They are distinguished from features that may show a regular structural (but not periodic) distribution throughout the crown, such as Hunter-Schreger bands, or from growth disturbances that may manifest as marked accentuations or disturbances of the developing enamel front; features referred to as accentuated lines (i.e., pathological lines, Wilson bands, chevron lines) and enamel hypoplasias, which are believed to have an irregular periodicity thought to conform to an extrinsic form of stress (reviewed below).

Recent analyses of incremental features have sought to characterize several aspects of cross-striations and Retzius lines: 1) daily secretion rate (cross-striation repeat interval) and number of cross-striations between fixed points; 2) periodicity (number of cross-striations between pairs of Retzius lines), number, and distribution of Retzius lines (or their external manifestation as perikymata); and 3) the angle of intersection of Retzius lines with the enamel dentine junction (EDJ). The quantification of these variables yields data that may be used to determine the secretion and extension rates of the crown, as well as the duration of crown formation, which is often the ultimate goal of the analysis. In the following sections, various methods of quantification will be reviewed and critiqued. Subsequently, the application of incremental analyses within the field of anthropology will be reviewed, including a discussion of some of the published criticisms of this type of work. Specific questions for further study will be outlined, and the following two chapters will test the theories upon which these methods are based.

Daily secretion rate

The quantification of daily secretion rate (DSR) is based on the hypotheses that cross-striations represent features that are formed every 24 hours, and may be distinguished from other short-period features (Figure 2.1).[1] Rate determination is generally accomplished by dividing an empirical quantity, such as distance, by a known time; the spacing of a cross-striation is divided by one day (for greater accuracy, a series of cross-striations is generally measured and divided by the same number of days). DSR has been conventionally quantified and reported in four ways: 1) measurements of cross-striation spacing derived from a single area or a number of unspecified areas (e.g., Martin, 1983; Shellis, 1984a,b); 2) regional average values of cross-striations (Beynon et al., 1991a); 3) prism length divided by the known time between intervals (Dean et al., 1993a), and as 4) box plots or cuspal enamel trajectories determined from direct counts of cross-striations between fixed points (e.g., Beynon et al., 1998a; Dean, 1998a; Dean et al., 2001). These methods may produce data on crown growth in different regions and over different periods of development, and the results may not be directly comparable.

Initial DSR data were based on individual or unspecified teeth with little reference to the area of the crown and/or depth of the enamel. As additional work began to suggest that rate was not constant throughout the crown, Beynon et al. (1991a) proposed a standardized method of data collection that divided the enamel crown into eight areas: cuspal inner, cuspal middle, cuspal outer, lateral inner, lateral middle, lateral outer, cervical inner and cervical outer (Figure 2.2). This model was an attempt to organize the crown into zones that represented successive stages of development. However, Dean (1998a) noted that defining rates in discrete inner, middle, and outer zones may mask variation, leading to a simplified categorization of this complex feature. This follows from the fact that by applying the method of Beynon et al. (1991a), individual rate measurements are combined into average values that may obscure periods of markedly slow or fast local development. Because apposition and extension rates change throughout development, an additional problem with this model of crown division is that the spatial divisions proposed by Beynon et al. (1991a) are not likely to correspond to equivalent temporal divisions.

[1] Their periodic nature is examined and discussed in Chapter 3.

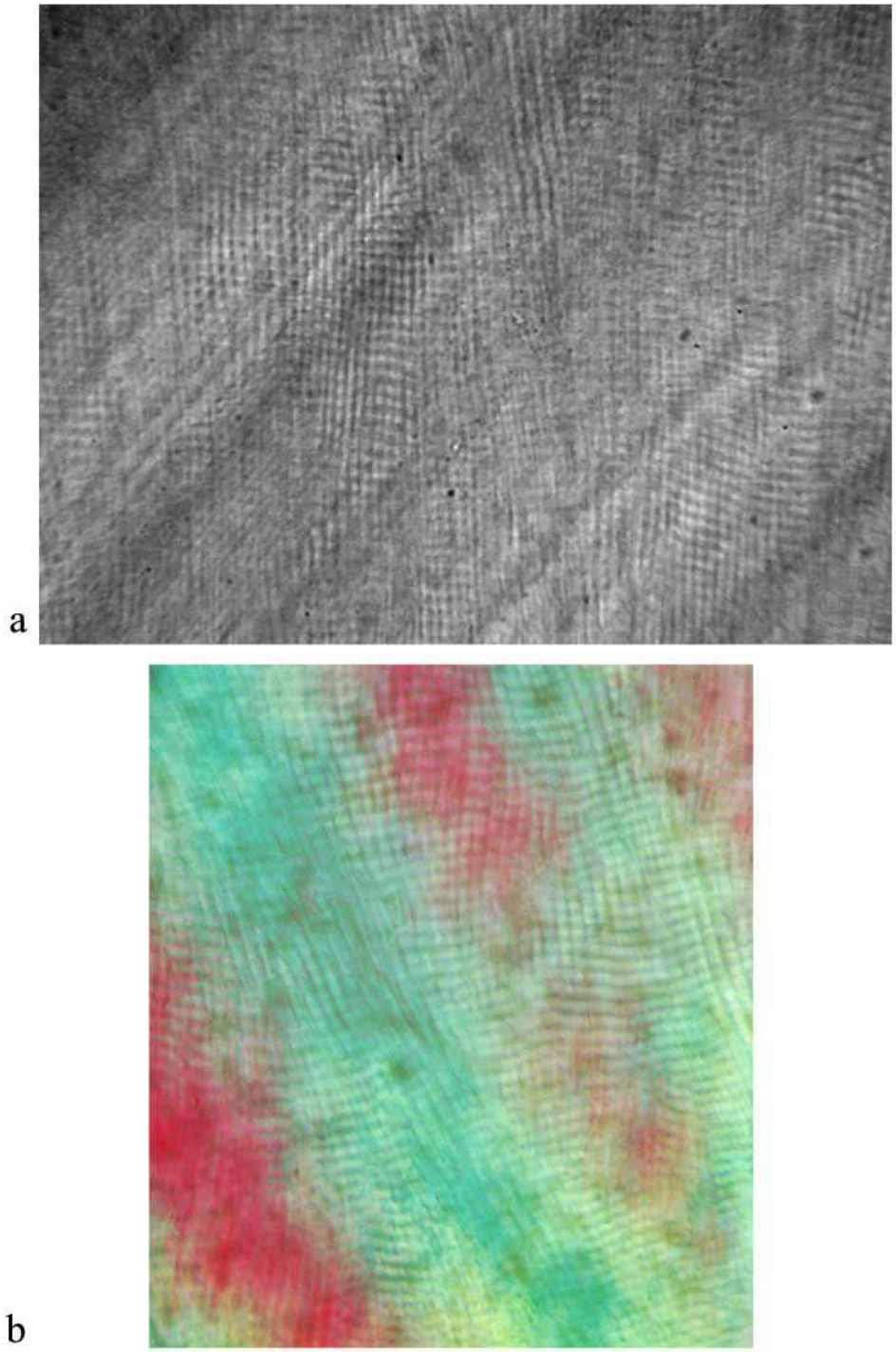


Figure 2.1 (a,b) Transmitted and polarized light micrographs of cross-striations: alternating horizontal light and dark bands. Enamel prisms run vertically in a) *Graecopithecus freybergi* (modified from Smith et al., 2004) and b) *Afropithecus turkanensis.* Smith and Martin (unpublished image).

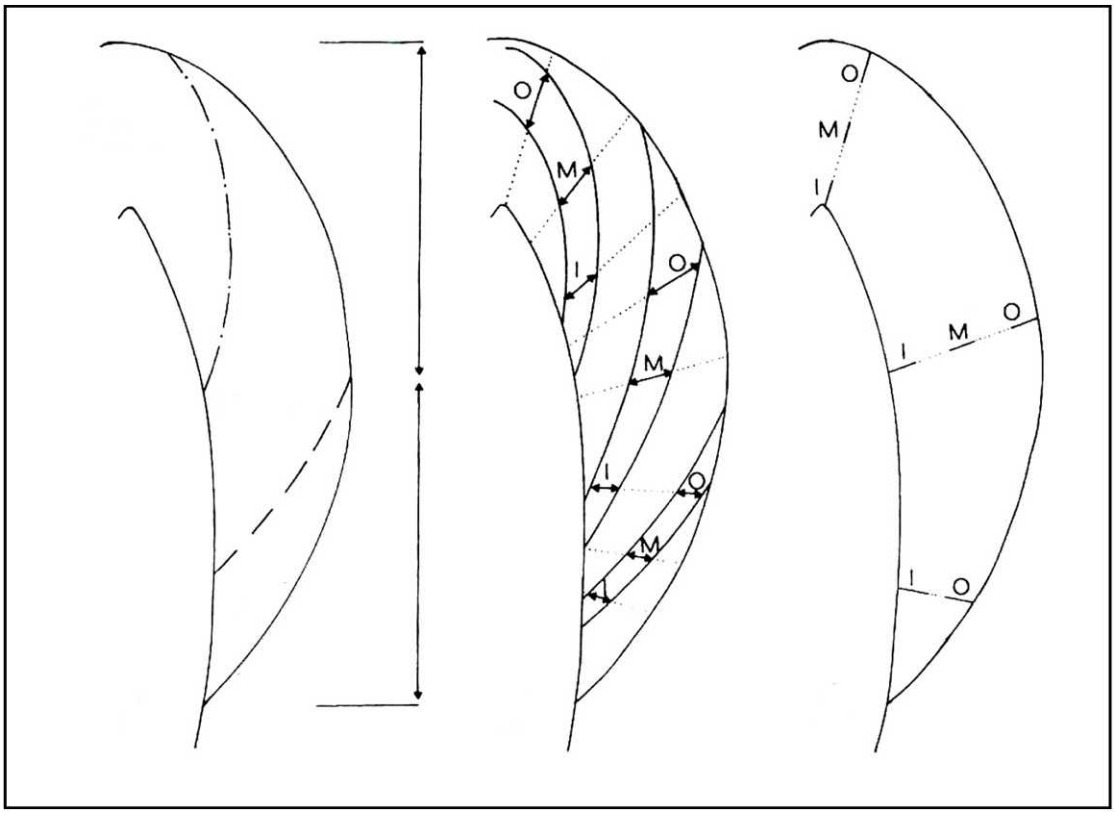


Figure 2.2 An illustration of the method for cross-striation quantification proposed by Beynon et al. (1991a). On the left, the crown is divided into thirds: cuspal, lateral, and cervical from top to bottom. In the middle, the enamel is divided into three depths: inner (I), middle (M), and outer (O). This study suggested that cross-striations should be record and averaged in eight regions for each cusp, as shown on the right: cuspal inner, middle, outer; lateral inner, middle, and outer; and cervical inner and outer regions. Modified and reproduced from Beynon et al. (1991a).

Dean et al. (1993a) presented another method for quantifying rate data, where DSR was determined by measuring the increase in crown height over a known period of time (e.g., Dean et al., 1993a: Figure 5, p. 257). In their study, a montage was prepared from an individual that had been given tetracycline over several years; the time between events was determined, interval length was measured, and length was plotted against time to yield DSR (as the slope). These types of graphs have included data on enamel as well as dentine DSR (Dean 1993; Dean et al., 1993a; Dean, 1995a), and may include information from a series of successive teeth, provided that they are registered with one another (Dean and Scandrett, 1996). There are several advantages of this method. First, it may provide insight into changes in rate throughout the continuous development of the

enamel (and/or dentine), rather than discrete values that represent regional averages. In addition, extreme rates at the beginning and end of crown formation may be apparent. However, for accurate assessment of local DSR, many closely spaced intervals must be utilized. Additionally, this method requires either teeth that have been experimentally labeled, or the identification of a series of accentuations that can be registered with one another in different tissues, cusps, or teeth.

Beynon et al. (1998a) and Dean (1998a, 2000) have also presented DSR data as a series of monthly box and whisker plots of cuspal enamel formation. To collect the data, montages were created at high magnification, cross-striations were counted and measured from the beginning to end of cuspal formation, and the results were grouped into months of formation. This method was considered superior to that of Beynon et al. (1991a), as it revealed more subtle changes in developmental rate, and did not involve the generation of cuspal inner, middle, and outer average values. Dean et al. (2001) illustrated a similar application of this method, where growth trajectories of cuspal enamel were determined from counts of cuspal cross-striations in 100 μm intervals from enamel over the dentine horn to the tooth surface, and the number of days was plotted against increasing enamel thickness (Dean, pers. com.).[2] A serious limitation of this method is that it requires exceptionally high quality sections, which must show an entire series of cross-striations from the beginning to the end of cuspal enamel formation, and sections of such quality are extremely rare (discussed further below). An additional limitation was noted by Smith et al. (2003a, 2004), who pointed out that even in excellent quality material, DSR may be difficult to assess (in cuspal enamel) due to a high proportion of short-period features believed to be intradian lines. These structures are rarely, if ever, used to determine rate or time, due to their variability and uncertain periodicity (considered in Chapter 3).

A serious complication that is apparent from published studies of secretion rate is that different researchers do not agree on the implementation of these methods (originally discussed in Smith et al. 2003a). Beynon et al. (1991a), Dirks (1998), and Reid et al. (1998a,b) have suggested that measurements of cross-striations should not be made in the first 100 μm of enamel at the EDJ, or in the last 100 μm at the surface, because

[2] This is similar to the generation of growth trajectories from known-period intervals, but provides more specific information on rate variation during cuspal enamel formation, as each day is counted.

aprismatic enamel in these regions and the convergence of Retzius lines at the tooth surface may obscure or complicate measurements of daily lines. This disparity may be seen when comparing data from Reid et al. (1998a) with Dean (1998a), as reported values of chimpanzee cuspal DSR in the former do not show the relatively wide range of values reported by the latter.[3] Dean's inclusion of the first and last month of cuspal enamel formation (corresponding approximately to the first and last 100 μm) often increases the minimum and maximum reported range of DSR values (Dean, 1998a; Beynon et al., 1998a). A consistent methodology is needed for determining DSR that reflects the underlying developmental process of crown formation, does not mask variation, and may be applied to the greatest number of available sections (that may not always display excellent quality cross-striations throughout the crown).

Periodicity and distribution of Retzius lines

The determination (and application) of the periodicity of Retzius lines is based on the theory that a consistent number of daily cross-striations is expressed between pairs of Retzius lines in all teeth of an individual's dentition. This has been reviewed and tested by FitzGerald (1996, 1998), and will be considered in the following chapter. Periodicity has been determined traditionally by counting a series of cross-striations between Retzius lines (in areas that preserve them clearly), which is often difficult to do with certainty (Figure 2.3). Dean et al. (1993a) and Swindler and Beynon (1993) suggested that this value could also be determined by measuring the spacing between Retzius lines and dividing this by the average (local) DSR. However, Smith et al. (2003a) noted that this method does not always produce periodicity values that are equivalent to direct counts of cross-striations, as the DSR does not always remain constant between pairs of Retzius lines. Local variation, most commonly resulting from the convergence of Retzius lines at the tooth surface (Figure 2.3b), may influence the secretion rate when cross-striations from only a portion of the interval (between pairs of Retzius lines) are measured. Convergence may cause later-formed cross-striations (closer to the tooth surface) to be more closely spaced than earlier-formed ones.

[3] See Table 15 in Chapter 5 for actual values reported by both sources.

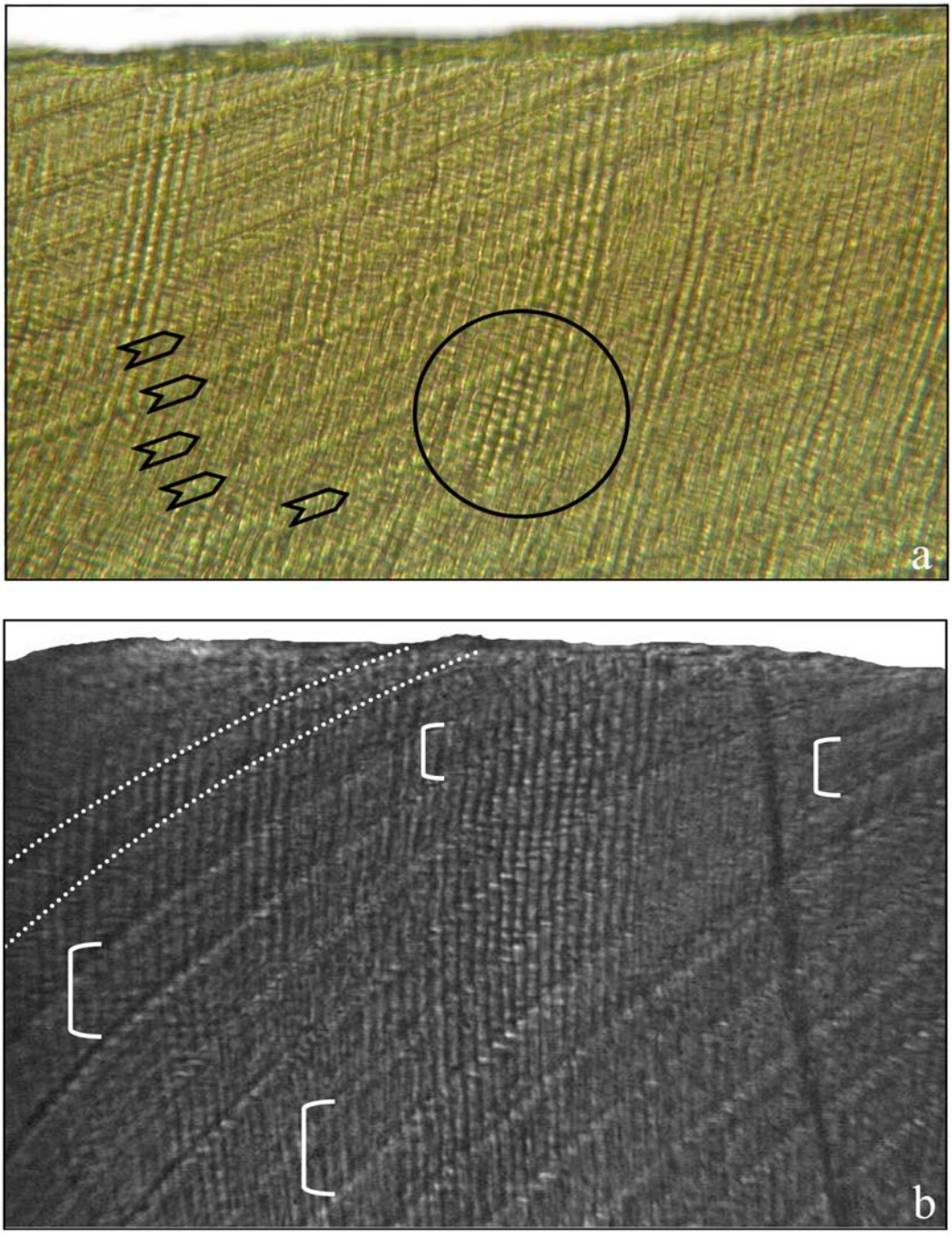


Figure 2.3 (a,b) Polarized light micrographs of chimpanzee outer enamel that illustrate the complicated nature of determining the periodicity of Retzius lines. The tooth surface is at the top of each image, Retzius lines run diagonally from the lower left to the upper right (arrows or brackets), and the cervix is left of the field of view. a) A clear interval with six cross-striations between the pair of Retzius lines can be seen in the center of the circle, which are marked by a dark, stairstep appearance. b) Convergence of Retzius lines towards the tooth surface (shown with dotted lines on the left, and also by the brackets that show a decrease in Retzius line width towards the tooth surface).

This second method for periodicity determination should be applied only in areas that show no evidence of changes in secretion rate, and should ideally be used only to verify direct counts.

Smith et al. (2003a, 2004) have noted several limitations of periodicity determination, including imaging cross-striations, identifying Retzius lines, and distinguishing cross-striations from laminations and intradian lines. An additional complication may relate to the course of enamel prisms in the outer (sub-surface) enamel, as it has been suggested that the prisms may curve as they reach the surface (Beynon, pers. com.). It is possible that this may influence the clarity of cross-striations between Retzius lines (Figure 2.4), particularly under light microscopy, as it is more difficult to recognize these areas. Risnes (1999) illustrated that obliquely-sectioned prisms may still show cross-striations, depending on the plane of section, but the resulting repeat interval will be artificially inflated (Risnes, 1999: Figure 7, p. 319). In areas of prism curvature (or section obliquity) near the surface of the enamel, it is possible that the periodicity may appear to be lower than the actual value, particularly using light microscopy (Figure 2.4b). Analyses of incremental features will benefit from a rigorous examination of the nature of Retzius line periodicity, including the purported regular rhythmicity and the problems associated with its determination.

Counts of Retzius lines are generally made from histological sections viewed under transmitted or polarized light. Given the relatively marked appearance of these lines, this often proves to be straightforward (Figure 2.5a). However, additional features such as accentuated lines, 'chevron lines', or laminations may be found, and these may make identification of Retzius lines more difficult (Figure 2.5b). As noted in Chapter 1, Dean (1987b) suggested that it is important to distinguish Retzius lines that reach the surface as perikymata from other lines that may be superimposed on this pattern. FitzGerald (1996) also noted the confounding effects of additional accentuated features parallel to the Retzius lines, stating that "no decision was harder to make than defining the boundaries of stria of Retzius" (p. 183). He described a class of false striae as 'chevron lines' that divide a true Retzius line into a light and dark half.[4]

[4] According to Martin (pers. com.), the term 'chevron lines' was first used by Boyde to refer to the appearance of the margin of a layer of prisms (end-on). It is used here as described by FitzGerald (1996).

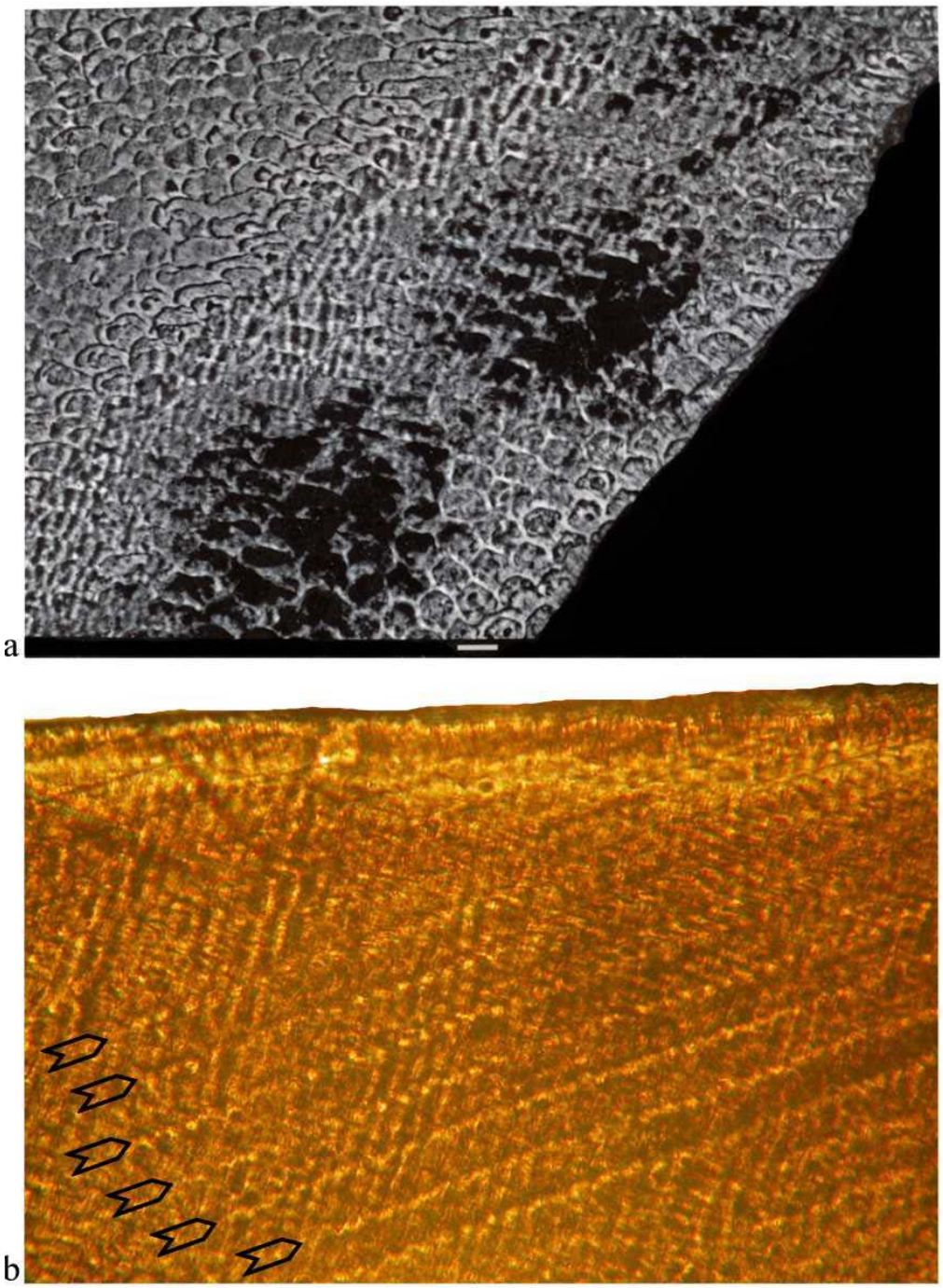


Figure 2.4 (a,b) Scanning electron and transmitted light microscopy of sub-surface enamel. a) Prisms are seen end-on just below the surface (outlined by white interprismatic honeycomb pattern) and obliquely sectioned (upper left) in *Afropithecus turkanensis*. Scale bar equals 10 μm. Smith and Martin (unpublished image). b) Area of (presumably) artificially low periodicity in chimpanzee enamel. Closely spaced Retzius lines are indicated with black open arrows, between which appear 3 - 4 cross-striations (light and dark bands crossing almost vertical prisms). The periodicity of this tooth was determined to be 6 in other areas.

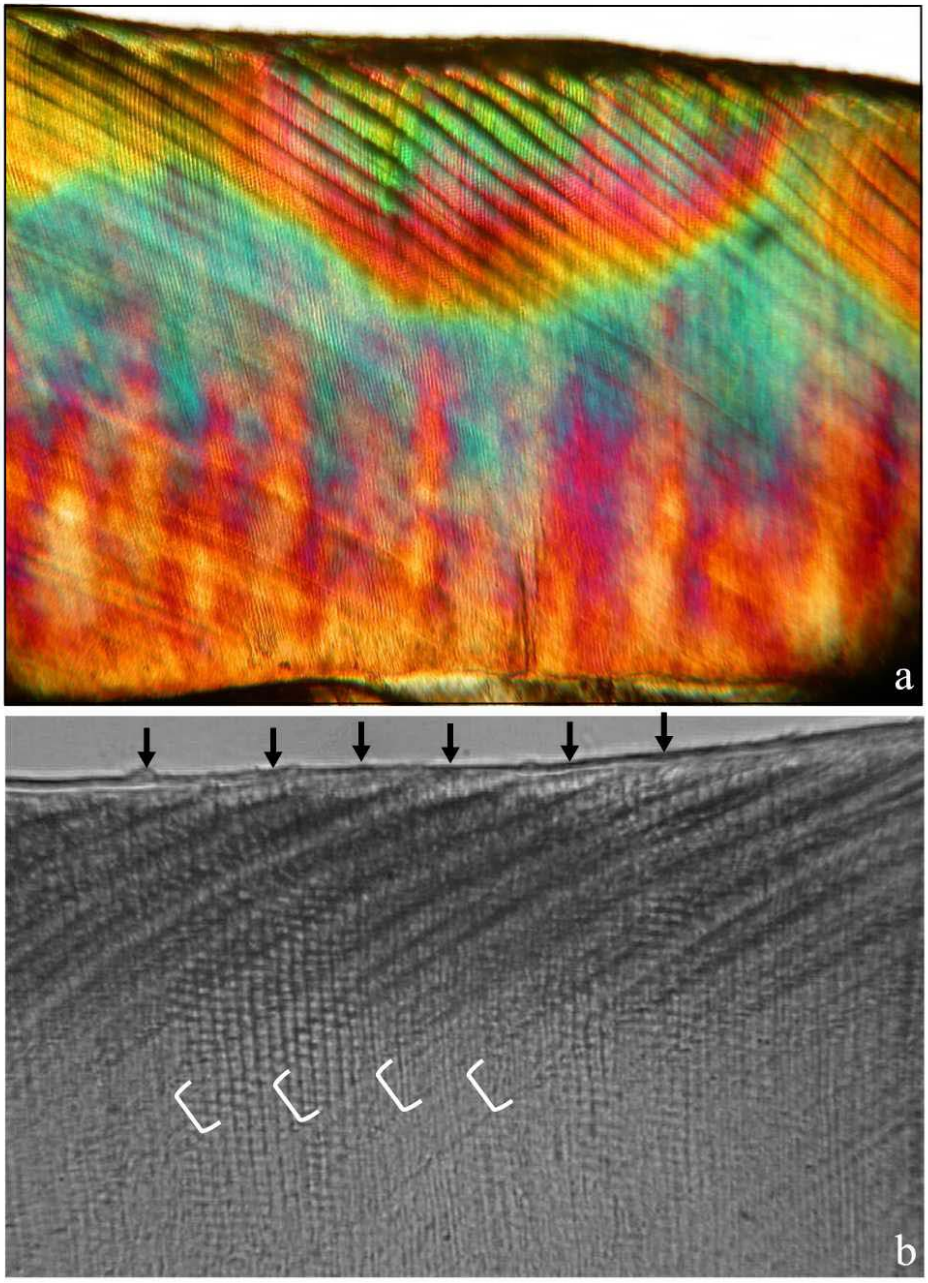


Figure 2.5 (a,b) Polarized and transmitted light micrographs of Retzius lines. a) *Sivapithecus* enamel showing approximately 17 Retzius lines running from the lower right to the surface of the tooth (top of the image). The enamel dentine junction is at the bottom, and the cervix is to the right. Smith and Martin (unpublished image). b) Chimpanzee enamel showing Retzius lines running diagonally from the lower left to the surface of the tooth. The approximate terminal points are indicated with black arrows. Note that the surface is smooth and does not appear to show perikymata. In addition, confounding lines are seen within Retzius intervals (white brackets), which appear to divide each interval into a light and dark half, and may be equivalent to FitzGerald's (1996) description of 'chevron lines'.

Although identification of corresponding perikymata may be a reliable means of discriminating Retzius lines (from other parallel features), it has been shown that the convergence of these lines may result in a smooth enamel surface without obvious terminal points (Figure 2.5b, also reviewed in Chapter 1). It appears that the most reliable method for defining (and counting) Retzius lines may involve the assessment of several characteristics together, including the appearance, spacing, and terminal manifestation on the tooth surface.

In a large sample of human canines, Reid et al. (2002) demonstrated that an inverse relationship exists between periodicity and the number of Retzius lines that reach the surface as perikymata. Their work suggested that these two developmental parameters might co-vary in a way that limits variation in anterior tooth crown formation time. An examination of two *Afropithecus turkanensis* molars did not support this finding, as the tooth with the lower periodicity has fewer Retzius lines than does the tooth with the higher periodicity, resulting in fairly large differences in crown formation time. Examination of data on chimpanzee molars in Reid et al. (1998a) also suggested a variable relationship. It is possible that in taxa such as *Homo*, where there is substantial variation in periodicity, the growth period of imbricational enamel is constrained to produce teeth within a certain period of time, resulting in an inversely proportional number of Retzius lines. In taxa with less variation in periodicity, this relationship may be less constrained. It is also possible that this relationship holds for anterior teeth, but not for multi-cusped posterior teeth, or that it only holds for specific cusps within posterior teeth. This proposed relationship would benefit from an examination of periodicity in a large sample of non-human primates to assess whether periodicity is as variable as in modern humans, and if posterior teeth show an inverse relationship between the number of Retzius lines and periodicity values. If so, this would imply that developmental variables are more tightly constrained than previous work has suggested.

Enamel extension rate

Quantification of enamel microstructure reflects the fact that crown growth in height occurs in two ways: in an appositional manner as ameloblasts move away from the EDJ (represented by the number and spacing of cross-striations), and as a cervical

extension of newly differentiated ameloblasts along the EDJ from the dentine horn to the future cervix (Boyde, 1964; Shellis, 1984a,b). As noted in Chapter 1, Boyde (1964) suggested that the angle of intersection between Retzius lines and the EDJ provides evidence of the differentiation rate of new cells, with smaller angles indicating faster rates. Given the definition of Retzius lines proposed in Chapter 1, it is more appropriate to refer to the angle of 'the developing enamel surface' and the EDJ rather than the angle of 'Retzius lines' and the EDJ, particularly in the cuspal enamel. This would permit the inclusion of accentuated lines that represent the position of the developing front, which are frequently difficult to distinguish from Retzius lines, particularly at the EDJ.

On the basis of Boyde's (1964) model of extension, Shellis (1984a,b) proposed a formula to quantify extension rate (Figure 2.6):

$$c = d\,[(\sin I / \tan D) - \cos I]$$

c = the length of dentine surface covered by enamel in one day (extension rate),
d = the length of enamel prism formed in one day (cross-striation repeat interval),
I = the angle between prisms and the EDJ, and
D = the angle between the developing enamel surface (incremental line) and the EDJ.

Shellis also suggested that the crown formation time could be determined by summing time of extension along the EDJ, which is discussed in the following section.

Smith et al. (2003a, 2004) have noted that several factors influence the assessment of extension (relative measures or calculated values of rate). They suggested that published reports of angles of intersection (developing enamel front and the EDJ) may not be directly comparable in numerous taxa. Because the developing front may begin at a relatively low angle relative to the EDJ and increase rapidly towards the enamel surface, it is not clear if certain studies measured the angle of the overall trend of the line, or the portion immediately adjacent to the EDJ (an example of this curvature is shown in Figure 2.8 below). Additionally, Shellis (1984a, 1998) and Grine and Martin (1988) noted that measurement error must be considered, as it is difficult to determine this curvilinear relationship with precision and accuracy, particularly when angles are small.

Dean (2000) noted that data on this angle in fossil apes and humans are limited, often derived from naturally fractured teeth that have not been sectioned (e.g., Beynon and Wood, 1986; Ramirez Rozzi, 1998b), which may also affect these values.

The second component of Shellis' formula, angle of intersection of the prism and the EDJ, is fairly easy to determine. Prisms show an approximately perpendicular orientation to the EDJ, and do not tend to display much angular change towards the surface of the tooth relative to that of the curved developing enamel front. The final component of Shellis' formula is the local DSR, which is generally difficult to determine from the enamel bordering the EDJ, and may show a rapid increase a small distance away from this position.

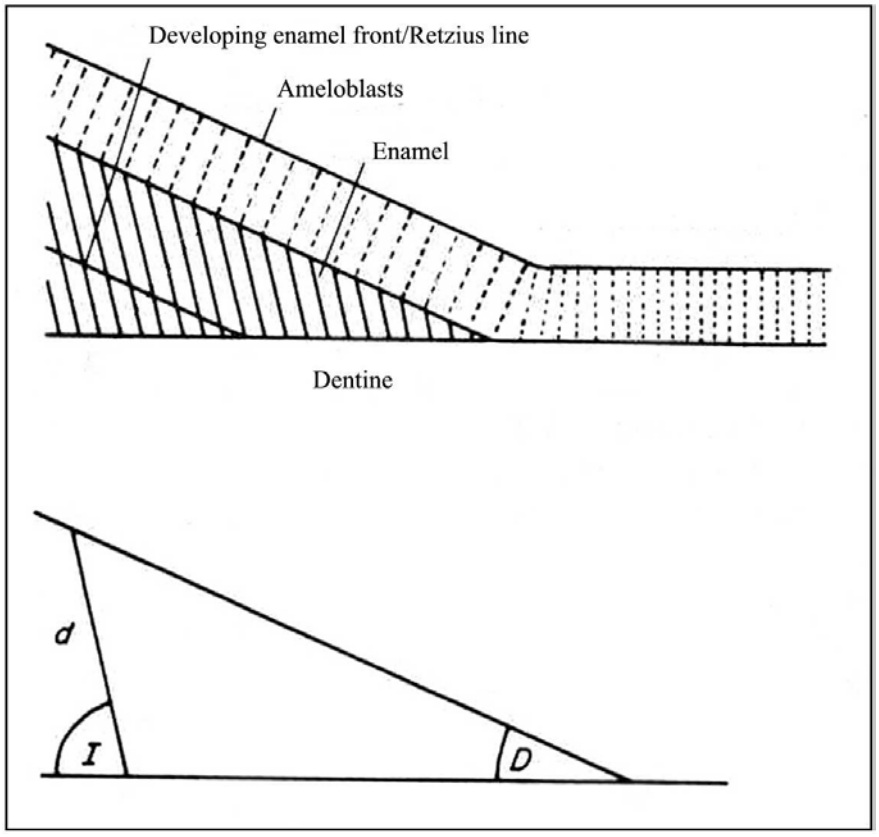


Figure 2.6 The model of enamel extension rate calculation proposed by Shellis (1984a,b). The diagram on the top represents the cervical extension of active ameloblasts, which secrete enamel at an angle that records the position of the developing enamel front. Shellis' (1984a,b) formula is given in the text; the respective components are illustrated here: *d* = length of prism formed in one day (cross-striation repeat interval), *I* = angle between prisms and the EDJ, and *D* = angle between the developing enamel surface and the EDJ. Modified and reproduced from Shellis (1984a).

Based on a suggestion from Jay Kelley, Smith et al. (2003a, 2004) noted that low angles between the developing enamel front and the EDJ appear to represent rapid extension (which is consistent with Boyde's [1964] theory), provided that DSR remains constant. However, because enamel secretion rates have been shown to decrease from the inner cuspal to the inner cervical enamel (along the EDJ), angles between the developing enamel front and the EDJ may not be directly comparable among regions. Smith et al. (2004) illustrated this point in a *Graecopithecus freybergi* molar by showing that enamel under the cingulum formed through rapid extension and secretion, leading to the production of high angles of intersection. However, Boyde's (1964) theory would have predicted a low extension rate from the high angles in this area. This issue is also important to consider when making comparisons among primates that have different rates of enamel secretion in equivalent regions. A thorough examination of extension rate is needed to compare qualitative assessments and calculated values (i.e., Shellis 1984a,b) with known values throughout the crown, and in different sections of the same tooth. This approach would test both the accuracy of this method and the degree of variation throughout the crown and between sections.

Crown Formation Time Estimation

Analyses of incremental features may permit the estimation of crown formation time, which is often divided into cuspal and imbricational components (Figure 2.7). Several methods have been employed: 1) counts of cross-striations from the beginning to the end formation (for cuspal and/or total time); 2) counts of Retzius lines (imbricational enamel time) plus estimations of cuspal enamel time (summed for total time); 3) successive application of the extension rate along the EDJ from the cusp tip to cervix (cuspal and/or total time); and 4) division of the prism path length by the DSR (cuspal and/or total time). In addition, Martin (pers. com.) has suggested an approach based on the relationship between EDJ length and the width of an ameloblast, which may yield the total time of formation. These methods will be reviewed in the following discussion and several will be tested in Chapter 4.

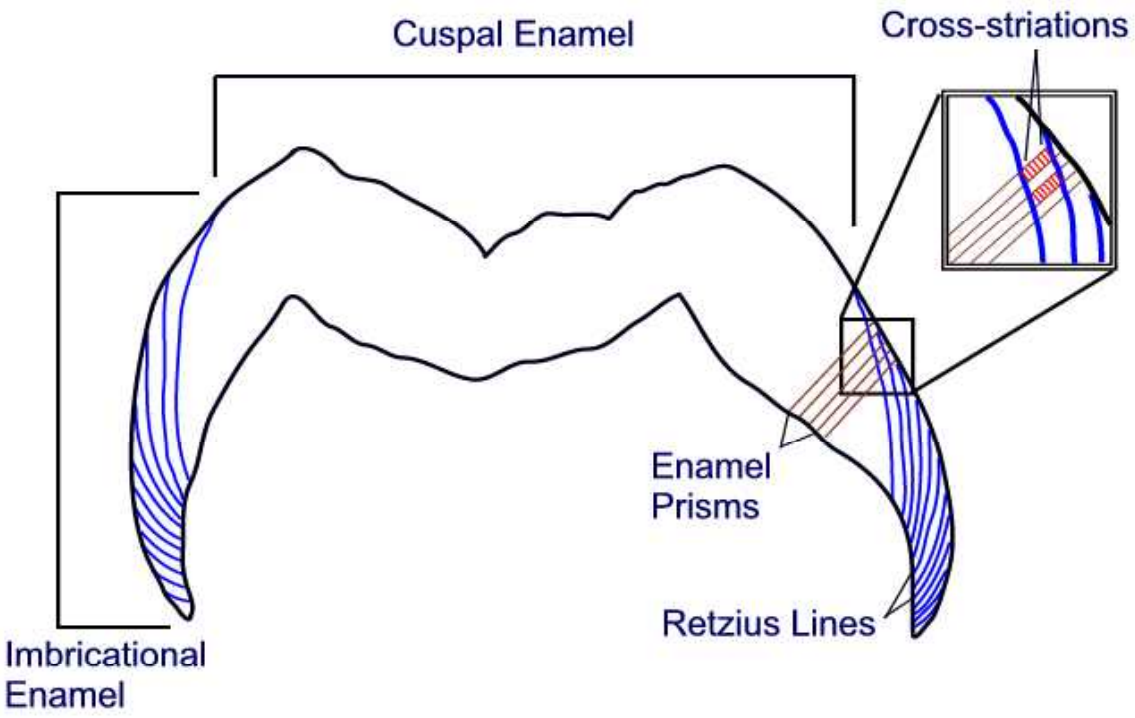


Figure 2.7 Illustration of the components of crown structure used to determine crown formation time. The imbricational enamel (left) is made up of Retzius lines that meet the surface of the enamel. This region is distinguished from the cuspal enamel by the first-formed Retzius line that meets the tooth surface. Retzius line periodicity determination is shown on the right, where a series of cross-striations may be counted between a pair of Retzius lines. Published in Smith et al. (2003a).

Counts of cross-striations

Asper (1916) suggested that counts of cross-striations provide an accurate determination of the period of crown formation, and the method was employed first by Gysi (1931) in his study of human canine development. Boyde (1963) is widely cited as the first to use Asper's method to determine the duration of crown formation and age at death in developing teeth. Boyde made counts of cross-striations between accentuated lines, which permitted him to match teeth and continue the count from the neonatal line in the M1 to the last enamel formed at the cervix of the permanent incisor. Most recently, Beynon and Dean (1987) used this method to determine cuspal formation time in a *Paranthropus* premolar, Dean (1998a) employed this method to derive estimates of cuspal formation time in three hominoid teeth, and Antoine (2000) used it to determine

total crown formation time in a large number of developing teeth. However, this method requires sections of exceptional quality (such as the Spitalfields material used by Antoine), as it is rare that an inclusive series of cross-striations can be counted from the dentine horn to the tip of the cuspal enamel and/or along prisms throughout the crown. Schwartz and Dean (2001a) reported that it was only possible to do this in the cusps of 12 of 115 canines sectioned for their study of hominoid canine development.

Counts of Retzius lines

Several early studies of fossil hominids (reviewed in the following section) have estimated the imbricational aspect of crown formation time from counts of perikymata at the enamel surface or from Retzius lines in naturally fractured teeth, as this is a non-destructive analytical method. One of the major limitations of this method is that the periodicity must be determined from an internal surface of a tooth from the same individual, or an estimated value must be used. A small error in the total number of Retzius lines (which may be common in poorly preserved teeth) may change estimates by a few weeks, but an error in the determination of the periodicity by a single day (cross-striation) may result in a difference of several months.

Few studies of fossil hominids have directly established the periodicity of the material under study (Dean, 1987a; Dean et al., 1993b; Dean et al., 2001). Studies of fossil hominids by Bromage and Dean (1985), Dean et al. (1986), Beynon and Dean (1987), and Dean (1987b) used periodicity estimates of seven to nine days; at the time, these were believed to be the most common values for modern human teeth. Other studies have used an estimate of nine days to calculate formation time (e.g., Dean et al., 2001; Dean and Reid, 2001; Cunha et al., 2004; Ramirez Rozzi and Bermudez de Castro, 2004). A periodicity of nine days was recently reported as the mean and modal value of a large study of great apes and humans (reviewed in Dean and Reid, 2001). However, as noted in Chapter 1, studies of large samples of modern humans have reported a range of Retzius line periodicities from six to 12 days (Beynon, 1992; FitzGerald, 1996; Reid et al., 2002). When single-integer estimates are used instead of range data, the accuracy of these non-destructive methods may be poor (discussed further below). A second limitation of studies that utilize counts of long-period lines for imbricational enamel

formation is the need to estimate the cuspal enamel formation, which often proves to be the most difficult aspect of crown formation time determination.

Cuspal formation time estimation

Several methods have been utilized to estimate the time of cuspal enamel formation: 1) counts of cross-striations in the cuspal enamel (e.g., Beynon and Dean, 1987; Dean et al. 1993b; Dean, 1998a), 2) counts of 'Retzius lines' in the cuspal enamel (e.g., Bullion, 1987; Dirks, 1998; Ramirez-Rozzi, 1998b), 3) counts of Andresen's lines in the corresponding coronal dentine (Smith et al., 2004), 4) cuspal prism path length divided by average cuspal DSR (discussed below) (e.g., Dean et al., 1993b; Dean, 1998a; FitzGerald et al., 1999; Smith et al., 2003a), 5) cuspal enamel thickness (uncorrected or corrected for prism decussation) divided by average cuspal DSR (e.g., Dean et al., 1993b; Reid et al., 1998a,b; Smith et al., 2003a, 2004), 6) axial dentine length divided by dentine DSR (e.g., Dean, 1998a), and 7) cumulative length of cuspal EDJ divided by the local extension rate (discussed below) (Dean [1998a] applied Shellis' [1984a,b] formula). In addition, Schwartz and Dean (2001a) and Dean et al. (2001) presented several predictive equations derived from the regression of enamel thickness against DSR, which they suggested could be used to predict the time of cuspal enamel formation from knowledge of the cuspal enamel thickness.

In a test of four methods of estimating cuspal enamel formation time, Dean (1998a) compared results from: 1) direct counts of cross-striations, 2) corresponding axial dentine length divided by the average dentine DSR, 3) cumulative prism length divided by the average enamel DSR, and 4) cuspal time determined from calculation of the extension rate summed along the corresponding EDJ. The results of all four methods were within 5-10% of one another, and less than 3% from the average of all methods. However, because the actual cuspal formation times were unknown, it was not clear which method was the most accurate. Several of these methods have yet to be tested on known-age material to determine their accuracy.

Enamel extension

As reviewed and illustrated above, Shellis (1984a,b) proposed a formula for the calculation of extension rate that could be used to determine the time of enamel (or dentine) formation, where cumulative formation time is equal to the sum of local times derived from segments of EDJ length divided by local extension rates. Shellis' (1984a, 1998) results for humans and non-human primates yielded several times that were in agreement with the results of previous radiographic studies, but several other times that were shorter and/or longer than published estimates. As noted above, Beynon et al. (1998b) and Reid et al. (1998a,b) showed that radiographic standards tend to underestimate the true time of crown formation, which implies that several of the estimates reported by Shellis are likely to be underestimates, given their similarity with radiographic data. However, as noted above, Dean (1998a) showed that application of this method to cuspal formation estimation did show general agreement with the results of three other methods (although this does not demonstrate the accuracy of this method).

Prism path length

As noted in Chapter 1, Massler and Schour (1941, 1946) first suggested that the length of the prism could be divided by the 'characteristic rate of apposition', or DSR, to determine the time of formation of a specific crown area. Beynon and Dean (1987) applied this theory to estimate part of the imbricational formation time in a *Paranthropus* premolar, as it was not possible to count the entire series of Retzius lines. Work by Risnes (1986) suggested that, due to the three-dimensional orientation of enamel prisms, calculation of enamel formation necessitated the use of a correction factor to adjust the linear enamel thickness (or prism path length). In his study of the lateral enamel of human premolars, he estimated that the prisms deviated by about 1.15 times the linear enamel thickness, and suggested that enamel thickness should be multiplied by this factor before division by the DSR.

Although several early studies applied Risnes' correction factor for cuspal enamel formation time estimation, several others have noted that this correction factor may not be appropriate for all hominoids (reviewed in Smith et al., 2003a, see also Macho et al., 2003). Hominoids with fairly straight prisms paths, such as *Paranthropus* or *G. freybergi*,

may not require a correction factor (Smith et al., 2004). Additionally, little is known about the variation of prism paths in different regions of the crown, different cusps in the same tooth, or different tooth types and positions. To date, only Smith et al. (2003a) have quantified differences in (two dimensional) prism paths between cusps (within a tooth), reporting slight differences between the protoconid and the metaconid of a single tooth of *Afropithecus turkanensis*.

Another promising method of formation time determination was outlined by FitzGerald et al. (1999), which may be used in situations where it is not possible to count or measure an inclusive series of cross-striations (from the EDJ to the tooth surface). They proposed that the apparent prism length should be traced and measured on a high magnification montage, DSR should be determined (opportunistically) within fixed intervals along the length of the prism, interval length should be divided by the average DSR (for each interval), and the number of days should be summed to yield the time of formation. Smith et al. (2003a) found that values derived from this method were similar to the path length tracing method used in their study (8-9% difference).

As noted above, several researchers have suggested that studies utilizing histological methods provide more accurate estimates of crown formation time than previous studies involving radiographic methods (Beynon et al., 1991b; Winkler et al., 1995; Beynon et al., 1998b; Reid et al., 1998a,b). Due to the nature of crown formation, it has been shown that radiographs tend to overestimate the age at crown initiation and underestimate the age at crown completion. This has major implications for studies such as those of Dean and Wood (1981) and Anemone et al. (1991, 1996), which had been considered the best sources for data on pongid crown formation times (discussed further in Chapter 5). Clearly, additional histological data are needed to provide accurate ranges of crown formation time, particularly in larger samples of living hominoids. Although non-destructive studies (e.g., Bromage and Dean, 1985; Ramirez-Rozzi, 1998b) may be the most practical when considering valuable material, data obtained by direct sectioning methods (e.g., Dean et al., 1993a; Reid et al., 1998a,b; Smith et al., 2004) may provide the most accurate values for crown formation time due to the 'faithful recordkeeping' of incremental dental development, as well as the relatively small number of necessary assumptions.

Historical Background and Recent Applications

History

Rapid advances have taken place in the study of incremental features during the past few decades, following the pioneering work of several individuals. As noted above, Asper (1916) was the first to suggest that inclusive counts of cross-striations could be used to estimate the time taken to form the enamel crown, and Gysi (1931) published the first histological estimate of crown formation time in modern humans. This was followed by studies of the secretion rate and circadian nature of cross-striations during the 1930's and 1940's by Schour and colleagues in the US, and Okada and colleagues in Japan. Boyde's (1963) histological estimation of the age at death of developing human dentitions followed, becoming a well-cited example of the forensic potential of studies of incremental development. Fukuhara (1959) and Shellis and Poole (1977) provided the first surveys of enamel microstructure in non-human primates. Dean and Wood (1981) published a seminal radiographic study of developing hominoid dentitions, suggesting that data on incremental development may provide novel information on differences in crown and root formation between apes and humans. These led to histological studies during the 1980's and 1990's that began to provide a new understanding of dental development in hominoids, largely driven by the goal of characterizing the development of fossil hominid dentitions.

Beynon and Dean (1987) stated that prior to several studies in the 1980's, hominid dental development had been assessed by comparisons with human and ape eruption sequences, which yield only relative ages. It was not until studies by Bromage and Dean (1985), Dean et al. (1986), Beynon and Wood (1987), and Beynon and Dean (1987) that actual crown formation times were estimated from studies of incremental features in fossil material. Subsequent studies in the 1990's also began to establish a framework for non-human hominoid dental development, although both sample sizes and number of studies were smaller than similar hominoid studies. Recent work has reported on aspects of incremental development in larger samples of human and primate anterior teeth, as well as in a greater diversity of fossil taxa (e.g., Reid and Dean, 2000; Dean et al., 2001; Dean and Reid, 2001; Kelley et al., 2001; Schwartz et al., 2001a,b; Schwartz and Dean,

2001; Dean et al., 2003; Dean and Schrenk, 2003; Schwartz et al., 2003; Smith et al., 2003a,b; Dean and Leaky, 2004; Ramirez Rozzi and Bermudez de Castro, 2004; Smith et al., 2004).

Applications

Daily secretion rate in hominoids

Many studies of incremental features have provided data on the cross-striation repeat interval, or DSR, in humans and other primates. As noted above, many early studies presented single or average values, or ranges without reference to the location in the enamel or the number of areas measured. This may have perpetuated the erroneous acceptance of single values for a developmental feature that varies throughout the enamel, depending on the depth and region sampled. Some of the earliest reports of DSR in human enamel were derived from the experimental and deductive work of Schour and colleagues, who suggested that rate varied from 1.5 - 8.0 μm/day (e.g., Schour and Poncher, 1937; Massler and Schour, 1946). This range is now considered to be slightly wide; more recent work on hominoids suggests a lower limit of 2 - 3 μm/day and an upper limit of 6 - 7 μm/day. See Dean (1989) for a review early studies reporting human values, Fukuhara (1959) for values in humans and several anthropoid primates, Shellis and Poole (1977) and Shellis (1998) for data on several families of primates, and Martin (1983) for data on extant apes and humans.

As noted above, Beynon et al. (1991a) presented DSR in *Homo*, *Pan*, *Gorilla*, and *Pongo* inner, middle, and outer enamel depths in occlusal, lateral, and cervical zones. They found that average molar values were between 2.3 - 6.1 μm/day, and were fairly similar among hominoids, with *Homo* and *Pan* showing the lowest average values, and *Gorilla* showing higher averages. Reid et al. (1998a) also produced a table of values in these regions and zones for the entire dentition of several individuals of *Pan*, where average molar values ranged from 2.4 - 4.9 μm/day. Dirks (1998) presented a similar table for a second molar of a juvenile *Hylobates lar*, which averaged 1.9 - 4.0 μm/day.

The most commonly studied region of the crown is the cuspal enamel, as it is particularly important for the reconstruction of crown formation time (reviewed above).

Beynon and Reid (1995) presented data on great ape and human molar cuspal enamel formation, with *Pan* showing the lowest average value at 4.0 μm/day, and *Gorilla* showing the highest at 5.7 μm/day. Beynon et al. (1998a) and Dean (1998a) presented DSR data in monthly cuspal enamel zones, which ranged from 2.6 - 5.8 μm/day in *Pan*, 2.7 - 5.4 μm/day in *Pongo*, and 2.5 - 6.4 μm/day in *Homo*. Shellis (1998) reanalyzed the material examined in Shellis and Poole (1977), and reported values for the inner enamel of a number of anthropoid and prosimian primates, including an average for *Pan* at 3.8 μm/day. Schwartz et al. (2003) presented values for mid-outer cuspal enamel in several hominoids, which also appeared to be greatest in *Gorilla* (5.7 - 7.4 μm/day). These studies represent known recent published data sets of specific regional DSR values in extant ape molar teeth, and were generally based on small sample sizes.[5] None of these studies explicitly tested for differences between molar positions.

Boyde (1964) suggested that DSR could be determined in the enamel of fossil mammals by established histological techniques. Martin's (1983) study was the first to report DSR values of 5 - 6 μm/day in the outer enamel of *Sivapithecus* and the Paşalar hominoids (*Griphopithecus*). Several studies have reported cuspal DSR values in Miocene apes, including *Proconsul nyanzae* at 4.2 - 6.7 μm/day (Beynon et al., 1998a), *Dryopithecus laietanus* at 3.0 - 5.9 μm/day (Kelley et al., 2001), and *Gigantopithecus blacki* at 3.8 - 6.1 (Dean and Schrenk, 2003). Schwartz et al. (2003) reported mid-outer cuspal values of 5.1 - 6.2 μm/day for *Lufengpithecus hudienensis* and 5.6 - 6.9 μm/day for the equivalent area of *Lufengpithecus lufengensis*. Smith et al. (2003a) presented average DSR values for two molars of *Afropithecus turkanensis*, which ranged from 3.3 - 5.5 μm/day throughout the crown (cuspal, lateral, and cervical). Smith et al. (2004) reported overall average DSR values of 3.2 - 4.9 μm/day for *G. freybergi*, with individual cuspal values ranging from 3.1 - 5.6 μm/day.

Rates have also been inferred from the spacing of long-period lines in fossil hominid enamel (Beynon, 1992) and dentine (Dean, 1995a). Beynon (1992) reported on Retzius line spacing in different regions of the posterior teeth of *Australopithecus africanus*, *Paranthropus boisei*, and *Paranthropus robustus*. He postulated that these

[5] DSR values for anterior teeth were also reported by Dirks (1998), Reid et al. (1998a), and Schwartz et al. (2001a).

hominids produced enamel faster than modern humans (assuming a periodicity of seven or eight days), and similar to extant apes. This conclusion supported earlier studies suggesting fossil hominids showed an ape-like period of rapid dental development (discussed further below). Dean (1995a) also suggested this on the basis of counts of periradicular bands (dentine equivalent of perikymata) that appeared to show a more rapid period of root growth in *Homo habilis* (OH 16) than in modern humans.

Two general trends have emerged from recent studies of secretion rates: 1) DSR increases from the EDJ to the tooth surface, and 2) DSR decreases (at equivalent depths) from the cusp to the cervix.[6] An additional trend was noted by Komai ([1942: in Japanese]: as cited by Boyde, 1964), who reported that DSR is greater in human deciduous teeth than in permanent teeth. This was also confirmed by Gustafson (1959) and a number of subsequent studies. In a review paper, Schwartz and Dean (2000) noted that DSR tends to be similar in all of the permanent primate teeth studied to date, "despite a fair amount of intra- and inter-specific variation" (p. 214). It is suggested here that the amount of variation due to intra-specific variation, inter-specific variation, or methodological differences remains unclear. Studies of dental development in hominoids would benefit greatly from the application of a standardized methodology that samples analogous regions.

Age at death and crown formation time

Boyde (1963) reported that assessments of dental development are more reliable than assessments of skeletal development for determining age at death of young skeletal material, as dental development is less variable (discussed further in Chapter 1). He noted that by using histological methods, one may avoid making comparisons with 'normal' eruption sequences, which require a large number of individuals and may not encompass the variation within a population. However, he speculated that this time-consuming method was not likely to find widespread application. This proved true until incremental development was applied to the study of hominid evolution in the 1980's, resulting in numerous studies of living and fossil primates.

[6] See Smith et al. (2003a, 2004) for a discussion of exceptions to these trends.

Bromage and Dean (1985) were the first to use enamel microstructure to determine the age at death and crown formation time of several fossil hominids. Using perikymata counts on unworn incisors, they suggested that *Australopithecus afarensis*, *A. africanus*, *P. robustus*, and early *Homo* demonstrated crown formation times more similar to great apes than to modern humans.[7] Dean (1987b) determined the age at death of four *P. boisei* individuals using partially formed incisors, and Dean et al. (1993b) reconstructed the age at death of SK 63 (*P. robustus*), which also conformed to the ape-like trend of early eruption and rapid root growth in early hominids.[8] Dean et al. (1986) and Stringer et al. (1990) also reported on the age at death of the Gibraltar (Neanderthal) child based on a perikymata count of an upper central incisor. Their relatively young age for crown completion was used to infer a pattern of precocious brain growth, a commonly held characteristic of Neanderthals.[9] Similarly, Dean et al. (2001) estimated the age at death of the *Homo erectus* 'Narikotome boy' (KNM-WT 15000) to be close to eight years, also implying a precocious developmental schedule. Counts of cross-striations were also made from a sectioned developing first molar of the Dederiyeh 1 Neanderthal child, which confirmed skeletal assessments of the age at death (Sasaki et al., 2002).

Dean and Beynon (1991) reported on the age at death of a juvenile from the Spitalfields archaeological collection. In a novel approach, they utilized incremental structures and accentuated lines to extrapolate the duration of root growth, which they combined with crown formation time to determine age at death. Beynon et al. (1991b) presented the first use of enamel microstructure for inferring not only the age at death and crown formation time in individual *Pongo* and *Gorilla* specimens, but also the overall chronology of tooth development. They suggested that great apes may require more time for crown formation than humans, but less time for the completion of overall dental development; this is likely due to faster root growth rates and decreased periods of time between calcification of teeth (Dean and Wood, 1981; Beynon et al., 1991b). Using

[7] This was a very dramatic reinterpretation of development in these fossils, particularly of *A. africanus*, which had previously been regarded as similar to the maturational period of modern humans.

[8] Although the age at death for SK 63 was estimated to be slightly older in this study than the age Bromage and Dean (1985) originally reported.

[9] See also Ramirez Rozzi and Bermudez de Castro [2004] who found similar results in a large Neanderthal sample.

accentuated lines in dentine, Dirks (1998) reconstructed the age at death for a juvenile gibbon (*H. lar*), Reid et al. (1998a) reconstructed the age at death of three juvenile chimpanzees, and Dirks et al. (2002) applied this to four baboon dentitions. Kelley and Smith (2003) also estimated the age at death for a juvenile attributed to *Afropithecus turkanensis* (discussed further below).

Since Gysi's (1931) study of human canines, several studies have established crown formation times for modern human populations using histological methods (reviewed in Reid et al., 1998b), and these formation times have been compared to limited data on fossil hominids. Shellis (1984) and Risnes (1986) first utilized extension rates and cumulative path lengths, respectively, to determine formation times. Bullion (1987), FitzGerald (1996), Antoine (2000), and Thomas (2003) completed dissertations on aspects of crown formation in large samples of modern and archaeological human material, which will be discussed further below. Beynon and Wood (1987) reconstructed crown formation times in molars of modern humans, *P. boisei*, and early *Homo* using enamel thickness, DSR, and extension rates in naturally fractured teeth. They concluded that enamel development in *P. boisei* shows a pattern similar to that seen in modern human deciduous teeth, which may imply that there was a strong selective pressure to grow teeth rapidly with very thick enamel (see also Grine and Martin, 1988). Beynon and Dean (1987) reached a similar conclusion for a *P. boisei* premolar; they estimated that it was formed at a more accelerated rate than that of modern humans, and possibly that of great apes. Reid and Dean (2000), Dean and Reid (2001), and Dean et al. (2001) provided histological crown formation times for a large sample of modern and fossil hominid anterior teeth, which further supported the results of previous studies that indicated advanced development in early hominids relative to modern humans.

Crown extension

Fukuhara (1959) presented some of the earliest data on the angle of intersection between Retzius lines and the EDJ, which appeared to be greatest in anthropoids and hominoids, although it is not clear if he explicitly related this to rates of crown extension (as the original is in Japanese). Gohdo (1982) reported regional differences in human incisors using a novel measurement of extension (areas of triangles defined by the angles

of Retzius lines, the EDJ, and prisms), which may correspond to regional differences in DSR as well. A number of recent studies have presented angular data (developing enamel front at the EDJ) for living and fossil apes (e.g., Beynon and Reid, 1995; Reid and Beynon, 1995; Beynon et al. 1998a; Smith et al., 2003a, 2004). The results of Beynon and Reid (1995) suggested an equivalent pattern of variation in crown extension in extant hominoids; angles show an increase from the cuspal enamel to the cervical enamel. This may imply a decrease in extension rate, which also corresponds to the pattern of decreasing DSR cervically.

Ramirez-Rozzi (1998b) presented data on the angle of intersection in hominids from Omo. His data showed two patterns: one in which this angle progressively increased along the EDJ as the cervix is approached, and a second pattern where the angle decreased in the lateral enamel and then increased in cervical enamel. This may imply a progressive slowing of extension in the first case, and a pattern of slowing, speeding up, and finally slowing in the cervix in the second case. This second pattern is similar to the pattern seen in two teeth of *Afropithecus* (Smith et al., 2003a). Reid (pers. com.) has suggested that the different morphology of the cervical enamel in buccal and lingual cusps of lower molars may result in different angles/rates, as 'enamel sleeving' may be more common in buccal cusps. A comparison of average angles between buccal and lingual cusps (cuspal, lateral, and cervical thirds of the crown) in two Miocene hominoids suggested that differences may exist within regions (Smith et al., 2003a, 2004). In both taxa, the protoconid appeared to show lower average angles relative to the metaconid in the cuspal (initial) region, but higher angles in the lateral and cervical enamel. If the initial secretion rate was equivalent within regions between the two cusps, this would imply that the protoconid began enamel formation by extending more rapidly than the metaconid (which is not surprising considering that the protoconid initiated first), and then experienced a greater progressive slowing in lateral and cervical extension, which may explain why the protoconid cervical enamel finished after the metaconid. Because none of these cusps showed dramatic enamel sleeving, this pattern of extension is probably the result of differences in the chronology of cusp formation rather than the morphology of the cervix.

As noted above, Shellis (1984a,b) proposed a formula to predict the actual rate of crown extension (in contrast to studies that reported trends in rate changes inferred from angles of intersection). He presented data on extension rate in human deciduous and permanent teeth, where the former showed a higher rate, likely related to their rapid and brief development. Shellis (1984a) noted that after the initial high rate of extension in the cuspal enamel of permanent teeth, rate decreased and remained constant at about 4 μm/day. He suggested that this corresponds to the differentiation of one row of ameloblasts per day, which is theoretically the minimal unit of enamel extension.[10] Shellis (1998) also calculated average extension rates in a diverse sample of primates, and noted that small primates tend to show a uniform rate of relatively high extension throughout crown formation, while larger primates show a progressive slowing with a lower overall average value. From these rates he predicted crown formation times, which were shorter in the smaller primates, and longer in the larger-bodied primates.[11]

Life history

Several studies have used information from analyses of incremental features for insight into life history traits (Kelley, 1997; Dirks, 2001; Kelley et al., 2001; Macho, 2001; Kelley, 2002; Schwartz et al., 2002; Kelley and Smith, 2003; Smith et al., 2003a,b; Ramirez Rozzi and Bermudez de Castro, 2004; Smith et al., 2004). Macho (2001) suggested that crown formation time is positively correlated with several life history traits across primates.[12] However, Dirks (2001) suggested that the relationship between life history and dental development is complicated; socioecological constraints such as diet and mortality rates should be taken into consideration. Kelley and Smith (2003) also recommended cautionary interpretation of histological data, highlighting published similarities in crown formation time between *Pan* and *Homo*, but differences in eruption

[10] Rates lower than 4 μm/day would indicate that fractions of ameloblasts were differentiating, or that an ameloblast did not extend each day, assuming pattern 1 enamel.

[11] This formula has also been applied recently to estimates of root extension rates, reviewed in Dean (2000).

[12] Her crown formation time data were taken from Shellis (1998), which is discussed further in Chapter 4.

age resulting from different rates of root growth. They noted that additional data are necessary before evaluating the relationship between crown formation time and life history variables across primates, including age at M1 emergence.

Smith et al. (2004) reviewed several studies on Miocene hominoids that have shown evidence of multiple patterns of crown and root formation. They tentatively suggested that evidence from *G. freybergi* and *Sivapithecus parvada* may reveal that hominoids with dental developmental schedules similar to those of chimpanzees evolved more than ten million years ago. However, this hypothesis would benefit from larger analyses of crown and root development. Additional histological studies of enamel microstructure in living and subfossil lemurs are currently underway; their aim is to understand the apparent diversity of dental development and life history strategies in this superfamily (e.g., Schwartz et al., 2002, Schwartz pers. com.).

Dean (1995a) and Dean and Scandrett (1996) suggested that there may be a link between Retzius line periodicity and body size, based on evidence from monkeys, apes, humans, and elephants. Smith et al. (2003b) examined this in 18 living and fossil apes, and found a significant positive relationship between average periodicity and body mass (tested with separate dental and skeletal estimates). However, when considering primates as a whole, this trend may not hold, as large-bodied lemurs show very low values (Schwartz et al., 2002). Further, it is difficult to explain the range of variation within humans and other well-documented hominoids. Additional work is needed to assess the relationships among developmental variables and life history traits, particularly among a broader taxonomic sample of primates.

Phylogeny reconstruction

Numerous studies have attempted to utilize aspects of enamel microstructure to understand the evolutionary developmental biology of specifically primates and other mammals. Several studies have examined the utility of non-incremental features, such as tubule penetration (Carter, 1922), decussation or Hunter-Schreger band appearance and orientation (e.g., Kawai, 1955; Beynon and Wood, 1986; Macho et al., 2003), and prism packing patterns (e.g., Shobusawa, 1952; Boyde, 1964; Poole and Shellis, 1977; Shellis and Poole, 1977; Vrba and Grine, 1978; Martin, 1983; Boyde and Martin, 1984a,b;

Martin and Boyde, 1984; Boyde and Martin, 1987). Kawai (1955), Boyde (1964, 1969b, 1989), and others provided reports and reviews of the various types of prism decussation, noting that broad distinctions may be made between various orders of mammals, but that specific distinction is possible only within certain rodent groups. Shellis and Poole (1977) suggested that primates could be classified into two main groups based on differences in packing pattern, Hunter-Schreger band expression, and incremental features. Macho et al. (2003) recently suggested that there are taxonomic differences in prism path, ranging from the relatively straight three-dimensional path in *Hylobates* to the more complicated courses of *Homo* and *Pongo* prisms.

Martin (1983,1985) argued that hominoid phylogeny may be inferred from a combination of prism packing pattern, secretion rate, and enamel thickness. However, Beynon et al. (1991a) presented data that showed that Martin's (1983) conclusions regarding the rate and pattern of outer enamel formation in apes were flawed. They suggested that enamel thickness, secretion rates, and prism packing patterns may be unrelated characters. Risnes (1998) also suggested that prism packing pattern, decussation, and enamel thickness need to be investigated with more systematic research before taxonomic significance is inferred from these characteristics. As Maas and Dumont (1999) noted in their review paper, recent studies have moved away from the phylogenetic implications of prism packing patterns. They concluded that although enamel organization is adaptive, non-incremental features such as Hunter-Schreger bands and prism packing pattern are not informative for hominoid (or primate) phylogeny.

Several studies have examined the potential phylogenetic or taxonomic utility of long-period features, including Retzius line morphology, perikymata/Retzius line spacing, Retzius line length, and angle of intersection between the developing enamel surface and the EDJ (e.g., Fukuhara, 1959; Shellis and Poole, 1977; Dean and Shellis, 1998; Ramirez Rozzi, 1998b; Ramirez Rozzi and Bermudez de Castro, 2004). Fukuhara (1959) surveyed incremental features of mammalian enamel, and suggested that characteristics of Retzius lines and cross-striations may have some applications in mammalian taxonomy, as anthropoids and humans show much larger angles of intersection between Retzius lines and the EDJ than other mammals. However, Risnes (1998) noted the need for additional data on Retzius line expression in different orders,

genera, and species in order to evaluate the basis or significance of reported variation. Dean and Shellis (1998) reported on the variable presence of 'S-shaped striae' (Retzius lines) in the cervical and lateral enamel of *Pongo pygmaeus*, *Hylobates syndactylus*, and *Proconsul heseloni*. They speculated that this trait may have some phylogenetic significance, but also noted that it may be found in association with enamel hypoplasia. Given the variable nature of 'S-shaped striae' expression within taxa, frequency among a number of African and Asian large and small-bodied hominoids, and existence in various incipient conditions (Figure 2.8), it is argued here that the phylogenetic significance of 'S-shaped striae' is unclear. Macho et al. (2003) suggested that this feature is simply the result of changes in prism orientation, with no phylogenetic significance. Additional study on the developmental basis and variability of this feature within and between groups is necessary before this condition may provide insight into primate phylogeny.

a) Fossil hominid taxonomy and phylogeny

Robinson (1956) was the first to compare perikymata patterns in fossil and modern hominids. He reported that fossil hominids showed more 'regular' spacing of cervical perikymata when compared to a modern sample of postcanine teeth, but he did not observe any difference between *Paranthropus* and *Australopithecus*. Bromage and Dean (1985) suggested that perikymata spacing on incisors may be used to distinguish *Paranthropus* from *Australopithecus*, early *Homo*, and modern humans. They noted that *Parathropus* does not show a marked trend of perikymata 'narrowing and condensation' cervically as shown in these other taxa, implying a more uniform pattern of cervical enamel formation (and possibly a more rapid period of completion) (see also Dean 1987b; Beynon and Dean, 1988; Beynon, 1992; Dean et al., 2001; Dean and Reid, 2001). Ramirez Rozzi (1998b) questioned the use of perikymata spacing or counts for taxonomic distinction, as he found that perikymata spacing patterns did not group the Omo hominid teeth in any fashion that agreed with the taxonomic attribution based on macrostructure (discussed further below). However, Ramirez Rozzi and Bermudez de Castro (2004) recently noted that perikymata patterns may be used to distinguish Neanderthals from Upper Palaeolithic-Mesolithic *H. sapiens*, as the former appeared to show fewer, more widely-spaced perikymata.

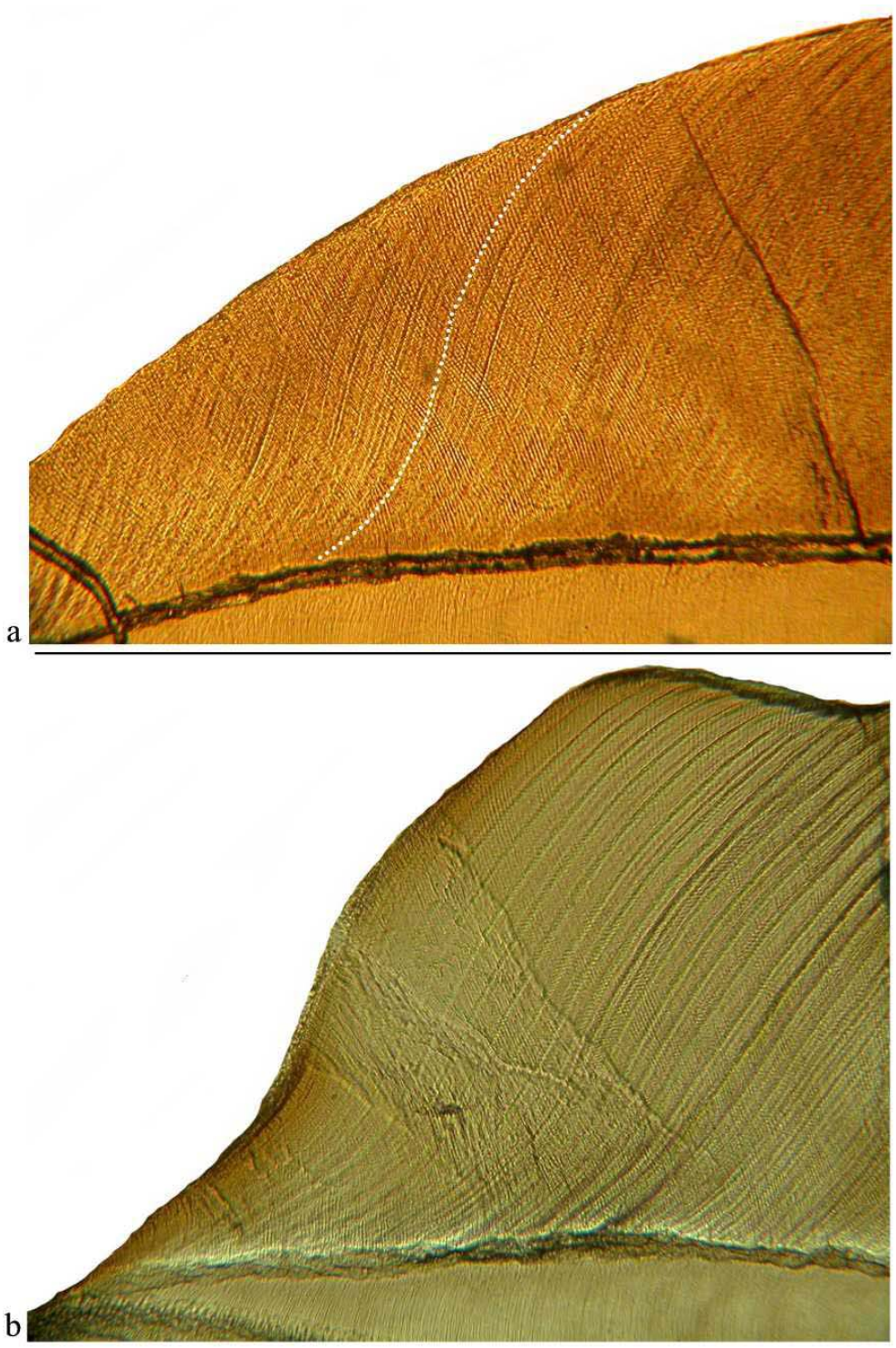


Figure 2.8 S-shaped Retzius lines in chimpanzee lateral/cervical enamel. a) Fully formed tooth showing slight 'S-shape' (white tracing) that does not appear to be associated with pathology. b) Developing enamel showing similar Retzius line shape, which may be related to a hypoplasia on the enamel surface (depression in upper right corner). (The hazy area in the middle of the image is an artifact from fractured enamel.)

Beynon and Wood (1986) presented a study of naturally fractured teeth, where differences in Retzius line morphology, Hunter-Schreger bands, and enamel thickness were used to differentiate 'robust australopithecines' from 'early *Homo*' and an 'unknown' category. Grine and Martin (1988) also reported on enamel development and enamel thickness in sectioned teeth of *A. africanus, P. boisei,* and *P. robustus*. They concluded that enamel thickness and Retzius line morphology may serve to distinguish *Paranthropus* from modern humans and *A. africanus*; *Paranthropus* shows thicker enamel, longer Retzius lines, and more acute angles of intersection of Retzius lines (developing enamel front) with the EDJ. In a review paper addressing overall developmental patterns, Beynon and Dean (1988) reviewed and reaffirmed the idea that developmental differences between *Australopithecus* and *Paranthropus* have lent additional support to generic distinction of these two groups.

A few studies have reported that differences in dental development exist within members of the hominid genus *Paranthropus* (Dean, 1987b; Ramirez Rozzi, 1993a, 1998b; Dean and Reid, 2001). Dean (1987b) and Dean and Reid (2001) suggested that *P. boisei* shows a different pattern of perikymata distribution than *P. robustus*. Dean and Reid (2001) reported that the extremely uniform cervical perikymata distribution in *P. robustus* may represent a derived condition relative to other hominids. Ramirez Rozzi (1993a, 1998b) suggested that *Paranthropus aethiopicus* may show more variation in lateral enamel thickness and cuspal enamel formation than other *Paranthropus* species, including a more rapid period of premolar and molar development than *P. boisei*.[13] His 1992 thesis surveyed features in naturally fractured teeth from the Omo collection, including the number of Retzius lines in different regions, angles of intersection of Retzius lines and the EDJ, Retzius line morphology, Hunter-Schreger band orientation and morphology, lateral enamel thickness, and the position of the dentine horns and first imbricational Retzius lines relative to the cervices. His results did not distinguish the material in any manner that agreed with previous taxonomic attributions, even when traits that did not show intra-individual variation were considered (Ramirez Rozzi, 1998b). While this study may represent evidence that features of enamel microstructure may not be used to distinguish different species or genera, he conceded that this may be a result of

[13] However, Ramirez Rozzi notes that the taxonomic attribution of this material is under debate.

incorrect taxonomic attribution, and concluded that additional studies are necessary to resolve the taxonomic utility of enamel microstructure. It is suggested here that the effects of section plane obliquity (or an uncontrolled plane of section) may have also influenced his results, as it has been shown that obliquity influences assessments of enamel thickness and incremental development (Martin, 1983; Smith et al., 2004).

Dean et al. (2001) published a synthesis of previous work on hominoids and introduced comparisons of 'growth trajectories', which appeared to show differences among modern humans, great apes, australopithecines, Neanderthals, and early *Homo* (Dean et al. 2001: Figure 1, p. 629). The results demonstrated that early hominids showed a more rapid developmental profile than great apes, and early *Homo* showed a very similar profile to great apes, suggesting that modern developmental processes are a relatively recent evolutionary development. The benefit of this type of analysis is that it involves comparisons of analogous regions of the crown, where direct rates are being compared rather than inferential rates based on assumptions of Retzius line periodicity. In addition, this study also included the largest sample of histological sections of fossil hominid material reported on to date.

Archaeological uses and developmental stress

Bullion (1987) completed the first frequently cited dissertation on the application of incremental enamel structures in archaeology, which compared dental development in a large sample of modern humans with a smaller archaeological population. Other studies have focused on the age at death of individuals and the presence of defects of enamel formation, which may provide insight into the mortality rates and living conditions of ancient cultures (e.g., Rose et al., 1978; Hillson, 1992; Antoine, 2000; Thomas, 2003). Recent histological studies of archaeological populations include several on the British Spitalfields collection (Stringer et al., 1990; Dean and Beynon, 1991; Dean et al., 1992; Antoine et al., 1999; Hillson et al., 1999; Antoine, 2000), as well as other medieval British collections (Bullion, 1987; FitzGerald, 1996), medieval individuals from Picardi, France (Reid et al., 1998b), medieval Danish individuals (Reid et al., 2002; Thomas, 2003), and prehistoric Native Americans (Simpson, 1999). Aside from the archaeological implications, these studies provide information about the amount of variation within

modern humans, which is critical for the consideration of individual dentitions or isolated teeth from living or fossil hominids. Two of these studies have also used known-age material to demonstrate the accuracy of histological methods (Stringer et al., 1990; Antoine, 2000) (discussed further in the following chapters).

Several studies have inferred environmental conditions from accentuations in tooth crowns (representing a relatively short period of time in the life of an anthropoid primate). Swindler and Beynon (1993) reported the absence of accentuated lines (or hypoplasias) in a modern day sample of *Theropithecus gelada*, which they interpreted as an indication of environmental stability.[14] Macho et al. (1996) reported the existence of 'regularly spaced' accentuated lines that they believed indicate the effects of seasonality on dental development in *Theropithecus oswaldi*. They also suggested that an older fossil population may have been subject to more stress than more recent populations, based on the greater frequency of accentuated lines in the former. Dirks (1998) suggested that the pattern of accentuated lines in a juvenile *H. lar* dentition may be consistent with the experience of weaning and capture for use in biomedical research, although these results are speculative given the lack of age and capture records. Recently, Bracha (2004) proposed that accentuated lines in human enamel may provide insight into the development of psychological disorders, although there are few empirical data to support this claim. This area of study would benefit from a systematic study of defect expression in individuals from a range of environments, which would be useful for interpreting the significance of stress lines in fossil populations. It would also be worthwhile to examine further the relationship between social factors (e.g., social structure or rank in a dominance hierarchy) and the expression of developmental stress.

A number of studies have used patterns of accentuated lines to match and register different teeth within an individual dentition, which may illustrate the chronology of overall dental development. Gustafson (1955) is widely cited for her study of these lines and patterns of mineralization in contralateral pairs of premolars, where she showed that these lines and patterns were remarkably similar in related teeth of the same type. Boyde (1963, 1964) demonstrated that different teeth forming simultaneously may be registered by accentuated lines as well. Since these initial applications, numerous studies have used

[14] However, the dentine appeared to show evidence of developmental stress (Reid, pers. com.).

these relationships (e.g., Beynon et al., 1991b; Dirks, 1998; Reid et al., 1998a,b; Antoine, 2000; Dirks et al., 2002; Thomas, 2003; Smith et al., 2004), particularly for establishing the developmental chronology of the entire dentition. Reid et al. (1998a,b) and Smith et al. (2004) also used the pattern of accentuated lines to derive sequences of cusp initiation and completion in molar teeth. This aspect of hard tissue registry may allow for more precise estimates of the periods of cusp and crown initiation than is possible using conventional dissection or radiographic techniques (discussed further in Chapter 5).

One noteworthy early study of developmental defects assessed defects on the enamel surface and in the internal microstructure of a number of primate species, including a subset of the chimpanzee material reported on in Chapter 5 (Schuman and Sognnaes, 1956). Simpson (1999) suggested that accentuated lines and enamel hypoplasias have "a different structural and temporal signature, suggesting that they are the products of physiological disruptions with different courses, timings and durations" (p. 259). He suggests that unlike hypoplasias, which may have a number of extrinsic or intrinsic causes, accentuated lines may be a result of acute dehydration caused by dehydration and/or vomiting, although this has yet to be confirmed empirically. Additional work by Skinner and colleagues (e.g., Skinner, 1986; Skinner and Hopwood, 2004) has suggested that enamel hypoplasias in great apes may be related to seasonal resource availability or disease cycles, while Cunha et al. (2004) suggested that these disruptions in fossil hominids were likely related to the metabolic stress caused by weaning. Several recent studies on living and fossil hominoids indicate renewed interest in studies of enamel hypoplasias (e.g., Cunha et al., 2004; Guatelli-Steinberg, 2004; Guatelli-Steinberg et al., 2004; Skinner and Hopwood, 2004).

Kelley and Bulicek (2000) provided an interesting interpretation of enamel hypoplasias in what they believe was a birth cohort of the (presumably) rare species of *Griphopithecus* from the Miocene locality of Paşalar. Based on similar perikymata counts and the presence of an identical pattern of linear enamel hypoplasias on all nine incisors attributed to this taxon, they suggested that the individuals were all at the same maturational stage, and that they experienced the same two stressful episodes simultaneously. From this, they inferred that these animals were part of the same cohort, that they experienced birth seasonality, and that they all died at the same time (based on

identical amounts of wear). This case may be an elegant demonstration of how incremental enamel microstructure may be used to reconstruct not only the "intimate history of the individual," as Gysi (1931) stated, but also the history of a group of organisms and the environmental conditions they experienced. However, studies such as these should be regarded as somewhat speculative without additional information on the relationship between stress and defect expression in populations with demographic and behavioral records (e.g., Bowman, 1991; Dirks et al., 2002).

Criticisms

Studies on dental development and life history in fossil hominids were met with skepticism during the late 1980's and early 1990's. Lewin (1985) and Bower (1985, 1987) were two of the more vocal critics; their conservative stance may have reflected the prevailing lack of acceptance of the incremental (or periodic) nature of enamel microstructure within the paleoanthropological community. Several fundamental aspects of incremental development have been challenged by oral biologists and anthropologists, including the daily nature of cross-striations (e.g., Wilson and Schroff, 1970; Weber and Glick, 1975; Warshawsky and Bai, 1983; Warshawsky et al., 1984; Skinner and Anderson, 1991), and the regular periodic nature of Retzius lines (e.g., Warshawsky et al., 1984; Warshawsky, 1989; Huda and Bowman, 1994). Ramirez Rozzi (1998a) and Reid (pers. com.) have noted that these issues are still under debate within the oral biology community. Additionally, Mann et al. (1990a,b, 1991) suggested that modern human variation in the number of perikymata includes most 'distinctive' values reported for different fossil taxa, casting doubt on the results of early studies. Finally, Macho et al. (2003) recently suggested that prism decussation complicates the determination of DSR, and that the results of histological studies based on these features may be erroneous. Several of these arguments will be reviewed in greater detail below.

<u>Incremental nature of enamel microstructure</u>

Boyde (1989) and FitzGerald (1998) reviewed a number of studies that have suggested that cross-striations do not represent a periodic feature. One of the earliest

studies was by Wilson and Schroff (1970), who examined histological sections prepared by various techniques, including acid etching. They suggested that, due to the lack of cross-striations visible in acid-etched preparations, and the appearance of prisms viewed end-on, previous reports of these incremental lines had misidentified cross-cut prisms. Other studies have also asserted that cross-striations were due to artifacts of preparation, non-rhythmic structural features, or prisms cut transversely (e.g., Weber and Glick, 1975; Warshawsky and Bai, 1983; Warshawsky et al., 1984; Skinner and Anderson, 1991).

A few researchers have also suggested that Retzius lines are not periodic, but are 'vanishing planes of organic material' (Goldberg, 1982 [in French]: as cited by Ramirez-Rozzi, 1993a), or the result of a linear alignment of obliquely sectioned or cross-sectioned prisms (Warshawsky et al., 1984). Warshawsky (1985) conceded that Retzius lines are incremental, but suggested that they arise from the boundary between cohorts of cells of differing ages, and not from a rhythmic deposition during secretion. Boyde (1989), Risnes (1990) and FitzGerald (1996) reviewed these arguments and the nature of evidence that has refuted these criticisms. These issues are considered further in the following chapter.

Consistency of Retzius line periodicity

A review of the literature on incremental development demonstrates that there has also been some debate regarding the consistency of Retzius line periodicity within a single tooth or within the entire dentition of an individual (reviewed in FitzGerald, 1998). Several studies have provided vague or erroneous references to this issue. Bromage and Dean (1985) noted that a report (in German) by Asper (1916) documented variation of five to ten cross-striations per Retzius line in a single tooth. This actually represents a typographical error in the manuscript (Reid, pers. com.). Asper (1916: translated with R. Bloomer) explicitly stated in several places that this relationship is consistent within the same tooth, but varies among individuals, which may show values of five, seven, eight, and ten. Bowman (1991) reported that the periodicity of *Macaca mulatta* is 'usually' four, and did not clarify if she actually observed differences within teeth of the same individual. Beynon (1992) reported different values (eight and nine) in two sections of a lower canine, for which he provides no explanation. Huda and Bowman (1994) explicitly

reported variation in periodicity within a single section from a tooth.[15] Hillson (1992) had previously noted that both intra- and inter-observer error may influence the range of variation reported for periodicity, particularly in modern humans. FitzGerald (1996, 1998) has most recently examined this issue in a modern human sample, which will be discussed in the following chapter.

Assumptions and estimation

Bacon (1989), Mann et al. (1991), and Huda and Bowman (1994) noted the importance of explicitly considering the assumptions that must be made in order to reconstruct crown formation times or ages at death. As noted above, it may not be appropriate to use periodicity values from one population or taxon to infer formation time in another group or taxon. A deviation of one or two cross-striations (per Retzius line interval) may create a substantial amount of error in these reconstructions. It is argued here that, given the inverse relationship between periodicity and perikymata number in modern humans reported by Reid et al. (2002), it is not clear if studies that examine the external manifestation of Retzius lines without knowledge of the specific periodicity are actually demonstrating differences in rate or time. Work in progress by Reid and colleagues on a large sample of modern human teeth will allow better interpretation of data from the external surface of fossil hominids (Reid, pers. com.), and until this work is complete it is suggested that these results should be regarded with caution.

An additional common assumption used to reconstruct age at death is the amount of postnatal delay prior to calcification (discussed for non-human hominoids in Kelley and Smith, 2003). Dean (1987a) explicitly noted that using modern human estimates for certain parameters is likely to cause an overestimate when applied to groups that demonstrate a faster period of overall dental development (such as in Bromage and Dean, 1985; Dean et al., 1986; Beynon and Dean, 1987; Stringer et al., 1990). Mann et al. (1991) also suggested that it may not be appropriate to assume a value of three months for incisors post-natal delay due to the wide range of variation in modern human dental maturation. Recent work on large samples of modern humans (e.g., Beynon, 1992;

[15] However, they considered both intra- and inter-observer error to be a factor in their results. Other more experienced researchers do not feel that their interpretations were valid (Reid, pers. com.).

FitzGerald, 1996; Reid and Dean, 2000) continues to lend support and suggest refinement for some of the assumptions that these early studies were based on. Additional work addressing specific aspects of development in extant apes will allow us to make more appropriate comparisons with living and fossil hominoids. In the following chapters, several of these criticisms will be addressed using an experimental approach. It is clear that the study of incremental development will benefit from a rigorous examination of the periodic nature of incremental features, as well as the methods used to assess the rate and duration of crown formation.

Summary of Specific Questions to be Addressed in the Following Chapters

Theoretical

Q1: Does secretion rate vary among molar positions? What is the nature of intraspecific variation within a tooth type?
Q2: Are intradian lines more frequent in specific areas? How do they relate to cross-striations?
Q3: Do laminations show a circadian rhythm? Are laminations more common near or in 'transition zones' of aprismatic enamel?
Q4: Is there a relationship between periodicity and the number of Retzius lines in non-human primate molars? Could this explain similarities in crown formation time within tooth types?
Q5: How much variation in crown formation time exists in non-human primates? Do differences exist between captive and wild-shot populations?

Methodological

Q1: Does the formula proposed by Shellis accurately quantify the enamel extension rate and predict crown formation time?
Q2: What are the effects of obliquity on analysis on incremental features? How does the quantification of enamel microstructure change in serial sections?
Q3: How accurate are analyses of crown formation time and age at death?

Chapter 3: The Periodicity of Incremental Structures

Specific Aims of the Study

Prior to the fairly recent application of incremental development to studies of primate and human evolution, several fundamental aspects of incremental structure periodicities were called into question (reviewed in Chapter 2, see also Boyde, 1989; Risnes, 1990; FitzGerald, 1998). These criticisms continue to be revisited in recent anthropological literature (e.g., Mann et al., 1990a,b, 1991; Skinner and Anderson, 1991; Ramirez Rozzi, 1998a), as well as in the field of oral biology, including recent editions of standard texts such as Berkovitz et al.'s (1992) *Color Atlas & Textbook of Oral Histology* and *Ten Cate's Oral Histology* (Nanci, Ed., 2003). Several recent studies have attempted to refute criticisms by experimentally or deductively establishing the periodicity of daily features (e.g., Bromage, 1991; Dean et al., 1993a; Antoine, 2000), as well as the regularity and equivalence of long-period features in enamel and dentine (e.g., Dean et al., 1993a; Dean, 1995a; Dean and Scandrett, 1996; FitzGerald, 1996). Despite these studies, it is evident that there is residual skepticism of the daily nature of cross-striations and the regular periodicity of Retzius lines, as well as the methods used to characterize incremental development (e.g., Macho et al., 2003).

Identification of known-period features is the most fundamental aspect of analyses of hard tissue incremental development (Scrutton, 1978; Smith et al., 2003a). The aim of this study is to investigate the development and periodicity of incremental features of enamel microstructure. The most informative material for studies of the nature and relationships of these features involves dental tissues that have been labeled *in vivo* with labels and/or material that is of known age at death. In this study, the repeat intervals of cross-striations, intradian lines, Retzius lines and laminations are examined in relation to multiple, known-period, fluorescent labels in a large sample of developing macaque (*Macaca nemestrina)* teeth. Two classes of incremental features known as intradian lines and laminations have been re-described in the past few years (e.g., Dean 1995a; FitzGerald, 1996; Smith et al., 2003a, 2004), but the periodicity of these structures has yet to be established. Additionally, a model proposed by Shellis (1984) for calculation of the rate of enamel extension is empirically tested, for the first time, against known

extension rates. This study provides important insight into the nature of intradian lines and laminations, which frequently complicate measurements of daily secretion rate and the periodicity of Retzius lines, and provides additional confirmation of the validity of results based on analyses of incremental features.

Background

Pigtailed Macaque Dental Development

Sirianni and Swindler (1985) reported that calcification of the deciduous dentition of pigtailed macaques (*Macaca nemestrina*) begins at approximately 85 gestational days and is complete by the end of the sixth postnatal month, resulting in a total deciduous developmental period of approximately 265 - 270 days (gestation length approximately 170 days). Little has been published about the calcification of the deciduous dentition of this species, nor has the incremental development of deciduous teeth been well characterized. This is due in part to the difficulty of studying prenatal development, as well as the difficulty of imaging incremental features in deciduous teeth. In a preliminary report, Smith et al. (2002) showed that the daily secretion rate (DSR) in the deciduous dentition of this species is lowest in the cervical enamel/dentine, intermediate in the lateral enamel/dentine and highest in the cuspal enamel and axial dentine. Within these regions, the DSR appeared to be fairly uniform, similar to the pattern of rates reported for human deciduous teeth. This study also showed that rates of enamel secretion and extension are higher in deciduous teeth than values reported for permanent teeth.

The permanent dentition of pigtailed macaques begins to calcify between 135 - 140 gestational days, and formation is completed by the end of the sixth postnatal year (Sirianni and Swindler, 1985). The first molar is the first permanent tooth to initiate calcification, beginning before birth and reaching crown completion at about one postnatal year (radiographic estimation), and is also the first permanent tooth to erupt at about 1.4 - 1.5 years. Tarrant (1971) and Swindler and Emel (1990) reported that first molars are generally represented by all four cusps at birth, which may not be evident

from radiographs. Prior to the study of Smith et al. (2002), the only data available on incremental development in this species of macaque were presented by Bromage (1991), who was concerned primarily with confirming the periodicity of daily lines (similar to the present study). Studies on incremental development in rhesus macaques (*Macaca mulatta*) are much more common (e.g., Schour and Hoffman, 1939a,b; Fukuhara, 1959; Molnar et al., 1981; Bowman, 1991; Dean, 1993), due to the frequency with which they have been used as laboratory animals. Pigtailed macaques have become more popular as laboratory animals only recently with the advent of AIDS research (Cleveland, pers. com.).

Three studies have examined experimentally labeled macaque dentitions in the past few decades (Molnar et al. 1981; Newell-Morris and Sirianni, 1982; Dean, 1993). Molnar et al. (1981) injected four juvenile rhesus macaques with a tetracycline series over 175 days. They subjected the animals to heat stress and cortisone injections in an attempt to look at their effects on dentine growth rates, as well as the existence of incremental features in the dentine. Newell-Morris and Sirianni (1982) injected a total of 16 prenatal and postnatal pigtailed macaques with three types of fluorescent labels (discussed in greater detail below) in an attempt to look at rates of bone growth and craniofacial development.[1] Dean (1993) gave four juvenile macaques a tetracycline series to document rates of root growth. For the following study, histological sections of dental tissue were examined from the first two projects. It was decided the second collection would be the most appropriate for this study based on the range of ages and the nature and frequency of the original labeling regime. Observations made on the material and results of Molnar et al. (1981) will be noted in the discussion. The material used by Dean (1993) was too advanced developmentally to be of use for studies of enamel formation, and was not examined.

A number of previous studies have used labeling methods to study enamel and dentine formation in macaques (e.g., Schour and Hoffman, 1939b; Okada, 1943), which will be discussed below. An ideal study of the periodicity of incremental features in enamel requires a labeling protocol that leaves distinct markers (e.g., sodium fluoride or

[1] This material was later used by Bromage (1991) to demonstrate the periodicity of daily lines in enamel and dentine, discussed further below.

lead acetate) over known period intervals. Due to the nature of enamel development, this has proven too difficult to do with antibiotic injections or fluorescent labels, as the markers tend to be lost during mineralization when the organic component is replaced by mineral (reviewed in Bromage, 1991; Dean and Scandrett, 1995). Dean and Scandrett (1995, 1996) have shown that large doses of tetracycline may be detected in enamel, and Bromage (1991) noted this for DCAF. However, it is not practical to use fluorescent labels in enamel as the sole means to determine incremental periodicity, as they commonly appear as diffuse bands equal to or greater than the width of daily lines. Thus, the majority of fluorescent labeling studies have focused on dentine formation due to the relative ease of imaging labels.

Incremental Features

As noted in Chapter 1, cross-striations are defined as light and dark bands that cross enamel prisms perpendicularly with a common interval of about 2 - 6 μm. Antoine (2000) defined them as regularly spaced units that show an increase in 'inter-striation spacing' (light or dark band making up ½ of the cross-striation) from the enamel dentine junction (EDJ) towards the crown surface. This was in contrast to 'artificial striations', which he noted gave the effect of decreased spacing due to a doubling effect (it is likely that he was referring to intradian lines). Risnes (1986, 1998) and Smith et al. (2003a) also distinguished light/dark band units (cross-striations) from prism varicosities/constrictions, which are structural phenomenon visible under SEM that may or may not correspond to cross-striations (reviewed in Chapter 1, see also Li and Risnes, 2004).

Intradian lines have been described as fine bands that divide cross-striations into two or three segments (Gustafson, 1959; Gustafson and Gustafson, 1967; Boyde, 1989; Dean, 1995a; Dean and Scandrett, 1996; FitzGerald, 1996). Smith et al. (2003a, 2004) defined intradian lines as fine bands that appear between square/slightly rectangular light and dark bands (cross-striations). These latter two studies showed conclusively that they are genuine structural phenomena by demonstrating them with scanning electron microscopy (SEM) and tandem scanning reflected light microscopy (TSRLM), methods which are not influenced by optical artifacts that may be present in conventional

transmitted or polarized light microscopy. The ratio of intradian lines to cross-striations is ambiguous; certain studies of enamel and dentine have reported multiple ratios, including ones greater than a 2:1 ratio (Rosenberg and Simmons, 1980a; Ohtsuka and Shinoda, 1995; FitzGerald, 1996), while others have suggested a 2:1 ratio (Kawasaki et al., 1977; Ohtsuka-Isoya et al., 2001).[2] Dean (1995a) noted the empirical difficulty of conclusively documenting evidence for a specific repeat interval, due in part to imaging limitations.

As illustrated in Chapters 1 and 2, Retzius lines are a prominent type of long-period feature found in primate enamel. The consistent periodic nature of this feature has been one of the more contentious topics in the study of enamel microstructure (reviewed in Chapter 2, see also FitzGerald, 1998). Beynon (1992) reported that the periodicity of Retzius lines in 100 modern human teeth, which ranged between 6 - 10, was 'remarkably consistent' within a single tooth. In a careful study involving counts and measurements of periodicity, FitzGerald (1996) also found that the periodicity was uniform within an individual tooth as well as in all the teeth belonging to the same individual, based on analysis of 96 modern human teeth. Similar results have been found in a large sample of medieval Danish individuals (Reid et al., 2002).

Also reviewed in Chapter 1 is the class of incremental feature known as laminations, often found specifically in association with sub-surface enamel and/or aprismatic enamel. Laminations appear parallel to Retzius lines, and have a similar spacing to cross-striations, but they do not appear to cross prisms perpendicularly.[3] It has been unclear how these features relate to cross-striations or intradian lines (Smith et al., 2003a, 2004). Images shown in Bromage (1991) may be interpreted to suggest that these features are equivalent to cross-striations (Bromage, 1991: Figures 2, 4, 6, pp. 207 - 208, 210), which will be discussed below. However, work on the Miocene hominoids *Afropithecus turkanensis* and *Graecopithecus freybergi* has suggested that laminations have a periodicity greater than cross-striations, as fewer laminations may be seen between pairs of Retzius lines than cross-striations (Smith et al., 2003a, 2004).

[2] Dean (1998b) supported the existence of intradian lines in dentine and enamel, yet presented data to suggest that the inferred 12-hour rhythm of dentine lines is not supported by Kawasaki et al. (1977).

[3] Save for in the cervical enamel, where prisms may bend in a cervical direction.

Finally, Shellis (1984a,b) suggested that the extension rate of enamel formation may be inferred from the angle of the Retzius line at the EDJ, the angle of the prism at the EDJ, and the daily secretion rate at the EDJ (reviewed in Chapter 2). From these parameters, Shellis provided a model and trigonometric formula that purportedly yields the daily length of extension along the EDJ, and may be used to determine crown formation time. However, Shellis (1998) noted that this model may not be as accurate in small or rapidly formed crowns, and suggested that results from this formula should be tested against those obtained by different methods.[4]

Methods

Material

A total of seventeen immature pigtailed macaques (*Macaca nemestrina*) were used in this study. The dental material included 88 previously prepared histological sections of mandibular teeth (dc, dp3, dp4, M1) and six recently prepared sections of maxillary teeth (dp4, M1) of 16 macaques (Appendix 1). These individuals represent various developmental stages from birth to 443 days old, and were acquired on loan from Dr. Joyce Sirianni (University of Buffalo). Approximately 100 thin sections of the 16 embedded mandibles were generated in the late 1970's/early 1980's as part of study on bone growth (Newell-Morris and Sirianni, 1982; Sirianni, 1985). The right mandibular halves were embedded and cut in several approximately coronal orientations, producing 115 μm thick sections that were reportedly ground to approximately 80 μm in thickness. The left mandibular halves were also embedded, cut in transverse planes, and sections were ground to a similar thickness. Cover slips were mounted with an unknown media. The cover slips were removed in 2002 from the majority of the collection with the assistance of Dr. Reid at the University of Newcastle (Newcastle upon Tyne, UK). In order to improve the quality, each section was immersed in xylene, its cover slip was

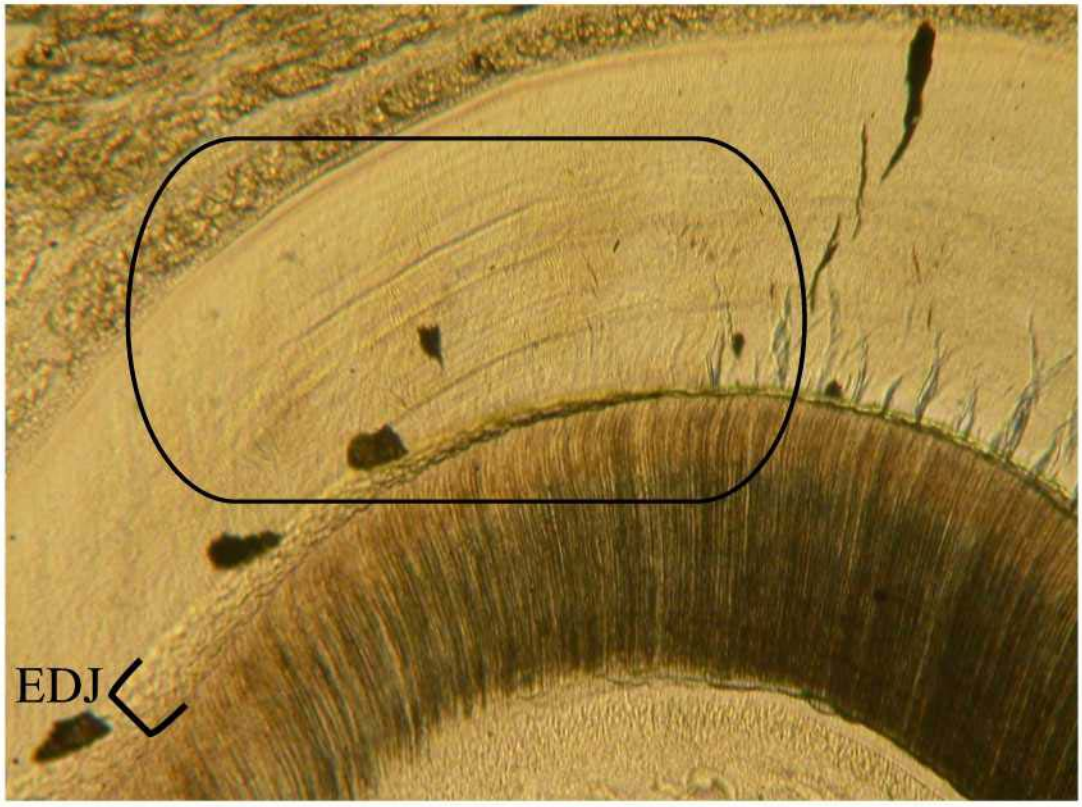
[4] This had yet to be done prior to the current study, yet the results of his 1998 work have been used as the basis of Macho's (2001) study on the relationship between crown formation time and life history traits.

removed, and the section was lifted from the slide (removing water and air trapped underneath). Section thickness was measured for each, excess embedding media was trimmed away, sections were cleared in xylene, and new cover slips were added using DPX mounting media. This substantially improved the quality of the material. Six new sections were generated from the left upper dp4 and M1 of individual 336 according to standard histological procedures (described in the following chapter), which were dissected from the maxilla and prepared at Stony Brook University.

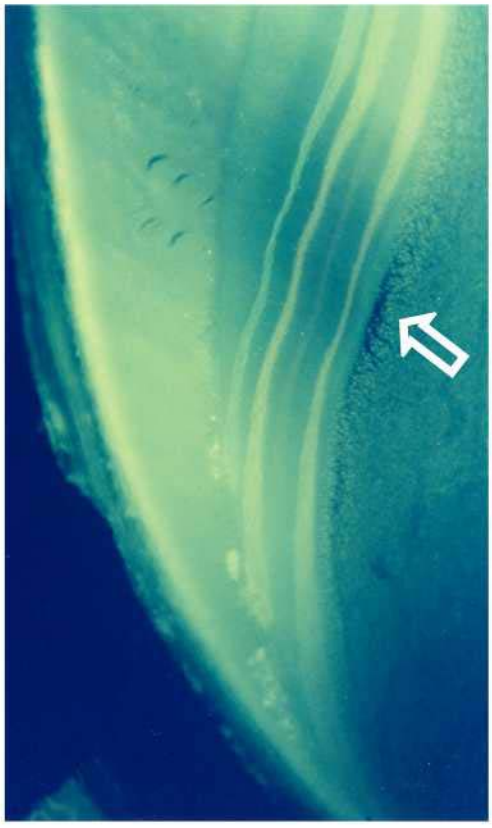
In addition, four sections of a mandibular first molar from a 374 day old individual were acquired (on loan to Dr. Donald Reid from Dr. Daris Swindler), which did not undergo fluorescent labeling.[5] Thin sections representing the mesial and distal cusps were prepared by Dr. Reid according to procedures described in Reid et al. (1998a,b). Although this individual was not part of the original labeling study, these four sections were included as the preparation quality was superior to a majority of the original material from Newell-Morris and Sirianni (1982) (illustrated in Chapter 4, Figure 4.1). Because the original study focused on bone growth rather than dental development, many of the sections were cut oblique or perpendicular to an ideal plane across the mesial cusps. Although this permitted imaging of features in a number of different planes, it reduced the number of sections that could be used for reliable imaging of incremental features. The transverse sections showed variable planes of section across the roots of anterior teeth and the developing posterior dentition, which forms in a rotated or tilted position relative to the occlusal plane. It was possible to discern which sections were cut obliquely by assessing the outline of the crown, the presence of dramatic prism decussation, and also by the appearance of the accentuated lines and fluorescent labels, which became doubled in appearance or poorly defined when oblique (Figure 3.1). In some instances, half of a tooth would appear to be cut properly, while the other half would be very oblique (e.g., 324 b29 shown in Chapter 4, Figure 4.1a). Areas within sections that were determined to be oblique were not used to assess the periodicity of incremental features, due to the likelihood of interference artifacts.

[5] This individual may be from material described by Swindler and Emel (1989), but could not be confirmed.

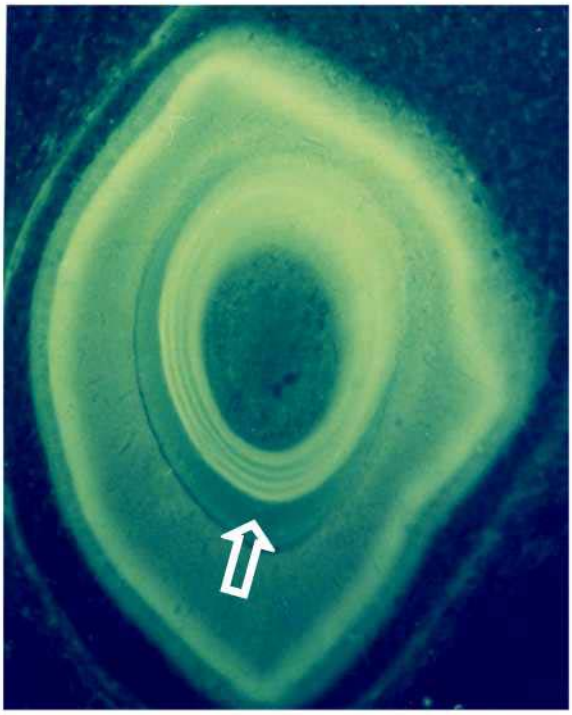
Figure 3.1(a-c) Light micrographs illustrating the appearance of obliquity. a) Transmitted light microscope image showing the variable appearance of curvilinear accentuated lines (black circle) and the enamel dentine junction (EDJ- labeled with black bracket), appearing as hazy, indistinct lines in this transversely cut section (slide CF 325 t 3). b) Obliquity illustrated under fluorescence microscopy in the cervical enamel. Note the hazy appearance of the four fluorescent labels, which become distinct (white arrow), and then hazy as they approach the tip of the cervical dentine (slide CF 324 b 29). c) Obliquity in a transverse section of a cusp tip under fluorescence microscopy, where fluorescent labels can be seen encircling the dentine horn with variably poor and sharp definition (white arrow) (slide CF 327 t 6).



a



b



c (above)

Treatment

During the original study on bone growth rates, all individuals were injected three to five times with one to three fluorescent labels (minocycline hydrochloride, xylenol orange, and DCAF 2,4-Bis [N,N' Di (carbomethyl) aminomethyl] fluorescein during the final two months of life (Newell-Morris and Sirianni, 1982; Sirianni, 1985).[6] Intravenous injections were given to pregnant females at 20 mg to 50 mg/kg pregnant weight, and postnatal injection doses were 35 mg/kg (Newell-Morris and Sirianni, 1982). Dates of conception, injections, birth, and sacrifice are shown in Table 1. Although the three labels in the original study were chosen because of their minimal effect on bone growth, they are all apparent in the dentine when viewed under fluorescent light microscopy. It appears that the minocycline injections may have caused a marked disruption in enamel growth that appears as an accentuated line due to a break or bend of the prisms (or change in composition), which is visible under light microscopy as a line parallel to the developing front (also observed by Bromage, 1991). Xylenol orange and DCAF also frequently appeared to cause disturbances in enamel growth, although the accentuated lines were not as well defined as those presumed to be caused by minocycline. The exact physiological effect of these labels on hard tissue formation is unknown. When matched and confirmed under fluorescent microscopy, these accentuated lines/labels permit identification of known-period intervals in the enamel, with which incremental features can be related, and increment periodicities may be determined.

[6] One of the individuals (CF 285) was reported to not show fluorescent labels in bone, and was not included in the original publications, although labels were found in dental tissues in the present study.

Table 3.1. Material and treatment record of *Macaca nemestrina* used in this study.

Prenatal				Postnatal			
CF	**Event**	**Age[1]**	**Interval**	**CF**	**Event**	**Age[2]**	**Int.**
285	XO	129	-	317	M	2	-
	DCAF	136	7		XO	4	2
	M	143	7		DCAF	6	2
	M	153	10		M	8	2
	Birth	170	17		Sacrifice	8.7	0.7
	XO	181	11				
	M	193	12	319	M	6	-
	Sacrifice	199	6		DCAF	8	2
					XO	10	2
296	DCAF	146	-		M	12	2
	XO	157	11		Sacrifice	13	1
	M	167	10				
	Stillborn	170	3	324	DCAF	14	-
					M	16	2
300	DCAF	142	-		XO	18	2
	XO	151	9		M	20	2
	M	159	8		Sacrifice	20.9	0.9
	Birth	178	19				
	Sacrifice	186	8	325	XO	10	-
					M	12	2
302	DCAF	127	-		DCAF	14	2
	XO	136	9		M	16	2
	M	144	8		Sacrifice	16.4	0.4
	Stillborn	145	1				
				326	XO	18	-
303	DCAF	122	-		M	20	2
	XO	131	9		DCAF	22	2
	M	139	8		M	24	2
	Birth	160	21		Sacrifice	24.7	0.7
	Sacrifice	166	6				
				330	DCAF	22	-
320	M	146	-		M	24	2
	M	153	7		XO	26	2
	M	159	6		M	28	2
	M	167	8		Sacrifice	28.7	0.7
	Birth	172	5				
	M	174	2	336	M	57	-
	Sacrifice	177	3		XO	59	2
					DCAF	61	2
327	M	146	-		Sacrifice	62.6	1.6

	M	153	7
	M	160	7
	Birth	166	6
	M	167	1
	M	171	4
	Sacrifice	174	3
333	M	159	-
	M	166	7
	Birth	not recorded?	
	M	172	6
	XO	183	11
	DCAF	197	14
	Sacrifice	200	3

337	M	57	-
	XO	59	2
	DCAF	61	2
	Sacrifice	65.4	4.2
M6898	no treatment		

CF indicates the code of each individual in the original study. **Event** indicates administration of one of the three labels: XO= xylenol orange, M= minocycline hydrochloride, DCAF= 2,4-Bis [N,N' Di (carbomethyl) aminomethyl] fluorescein, or birth, stillbirth or sacrifice. **Age[1]** is post-conception days. **Age[2]** is weeks after birth. **Interval/Int.** is interval between events, in days for prenatal individuals and in weeks for postnatal individuals. (A bar graph of these injection records has been published in Newell Morris and Sirianni [1982] and Sirianni [1985], although the ages at injections for individuals 336 and 337 appear to be premature by one week in the latter publication.)

Analysis

The sections were first examined under light microscopy and observations were recorded on: 1) the presence and appearance of four types of incremental features (cross-striations, intradian lines, Retzius lines, laminations) and accentuated lines (putative labels or neonatal line), 2) the potential for determining the extension rate between labels, and 3) the overall quality of the initial preparation. Incremental features were distinguished from accentuated lines by their frequency and regular spacing, whereas accentuated lines were less common and were typically only found in a particular area of the crown. Overviews were initially generated using a 1.25 X objective and 35 mm film with a camera mounted on a Zeiss polarizing light microscope, and subsequently with a Nikon Coolpix 4500 digital camera. Several sections were also initially examined and photographed under laser confocal microscopy at the University Microscopy and Imaging Center (Stony Brook University) and with several fluoroscopes at the Analytical Microscopy and Imaging Center in Anthropology (AMICA), Hunter College.

The sections were then reprepared and reexamined under polarized, transmitted, tandem scanning reflected, and fluorescent light microscopy (Zeiss Universal Photomicroscope with a mercury discharge lamp and Zeiss Axioskop Fluorescope). During 2002 and again in March 2003, 35 mm and digital photographs were taken using transmitted light, partial, and full fluorescence. The sections that showed clear growth disturbances in the enamel were identified, and the disturbances were matched to labels in the dentine using fluorescent light microscopy. Regions showing clear incremental features in association with confirmed labels were selected and imaged under high magnification light microscopy. The number of each specific feature (cross-striations, intradian lines, Retzius lines, laminations) was determined between markers, and then divided by the injection interval (in days) to yield a periodicity (number of features per day). Features were also related to one another to provide additional confirmation.

In several sections, measurements were also taken on the angle of intersection of the enamel prisms with the EDJ (*I*), the angle of the either the developing enamel front or the labels with the EDJ (*D*), the spacing of the cross-striations (*d*), and the width of the enamel prisms using NIH Image software. This permitted calculation of the extension

rate using Shellis' (1984a,b) formula: extension rate (c) = d [(sin I/tan D)- cos I] (illustrated in Chapter 2, Figure 2.6). Average extension rate was determined as the average of upper and lower values (boundaries) of each segment along the EDJ. To test the accuracy of this formula, the length of the EDJ was measured between labels or lines, which was then divided by the injection interval or known time of formation. The predicted extension rates and local formation times from Shellis' formula were compared with the empirically derived extension rates and known times of extension.

Results

General Observations

Due to the ages of individuals in the original study, only specific developmental stages were represented. In the youngest individuals, deciduous canines were almost crown complete at sacrifice (or stillbirth), while in the oldest individuals the deciduous dentition had erupted, p3 and p4 had just begun formation beneath their deciduous counterparts, and M1 was crown complete but had not reached gingival emergence at the time of sacrifice (Figure 3.2). The original research design attempted to provide a longitudinal perspective of macaque development, with individuals ranging from 122 days post conception at first injection to 61 weeks at final injection (approximately 62 weeks at sacrifice). This period included the entire development of the M1 crown, which was observed to begin calcification a few weeks before birth and complete crown formation at approximately one year (discussed further in Chapter 4).

The three labeling agents minocycline, xylenol orange, and DCAF were apparent in the dentine of developing teeth using UV fluorescence microscopy and laser confocal microscopy as orange, red, and green lines, respectively (Figure 3.3).[7] Additionally, DCAF, which showed the strongest fluorescence in dentine, was also apparent in the enamel of several postnatal labeled individuals, although it generally appeared as a hazy band rather than as a distinct line (Figure 3.4).

[7] The colors of each varied slightly depending on the type of illumination used.

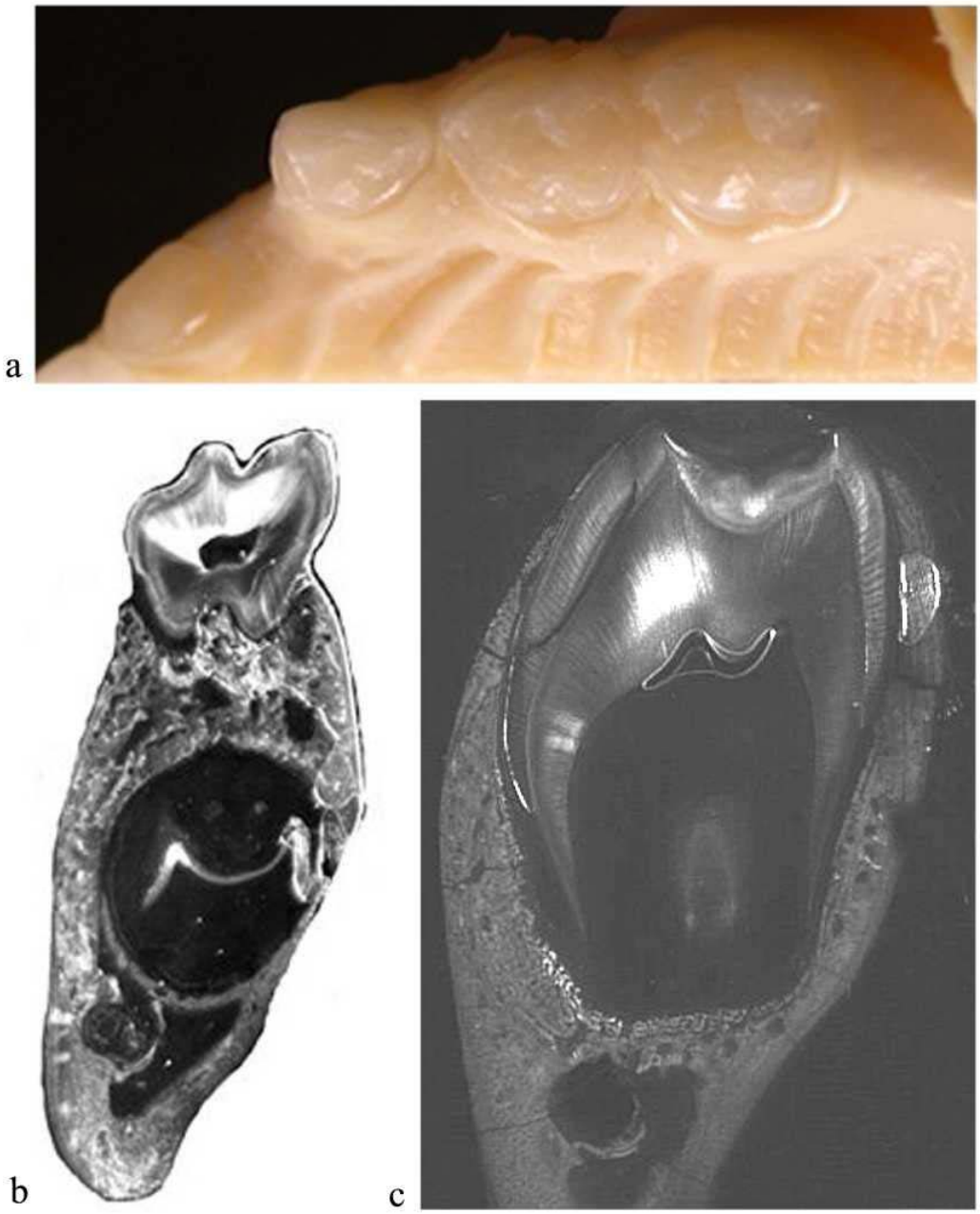
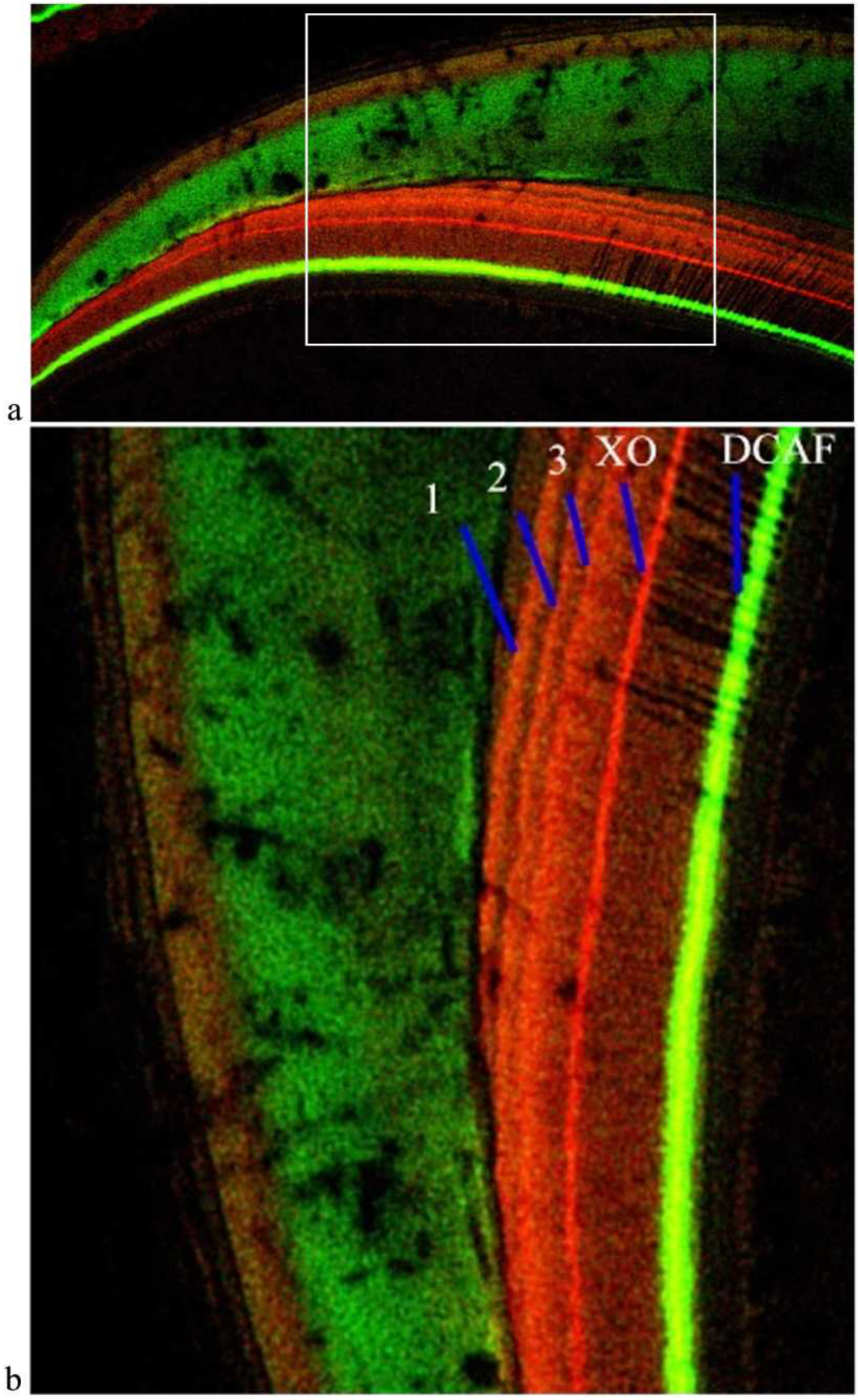


Figure 3.2 (a-c) Overviews of individual CF 337, the oldest study subject sacrificed at 458 days old. a) Left maxillary deciduous dentition (di2, dc, dp3, dp4 - not examined in this study). b) Histological section of the mandibular dp4/developing p4 (slide CF 337 b 13). c) Histological section of the mandibular M1 prior to gingival emergence (slide CF 337 b 20). Not enlarged to the same scale.

Figure 3.3 Laser confocal scanning microscope images of a deciduous third premolar (slide CF 333 b 8). a) Overviews of the lateral and cervical enamel (cervix to the left), showing the area enlarged in 3.3b (white box). b) Enamel is the faintly illuminated green tissue on the left, and the dentine (on the right) shows five fluorescent lines that correspond to the series of five injections given to this individual (CF 333). Line 1 (red/orange) represents the first minocycline injection, followed seven days later by a second minocycline injection (Line 2), six days later by a third minocycline injection (Line 3), eleven days later by a xylenol orange injection (Line XO, red) and 14 days later by a DCAF injection (Line DCAF, bright green). This animal was sacrificed three days later.


a
b
1
2
3
XO
DCAF

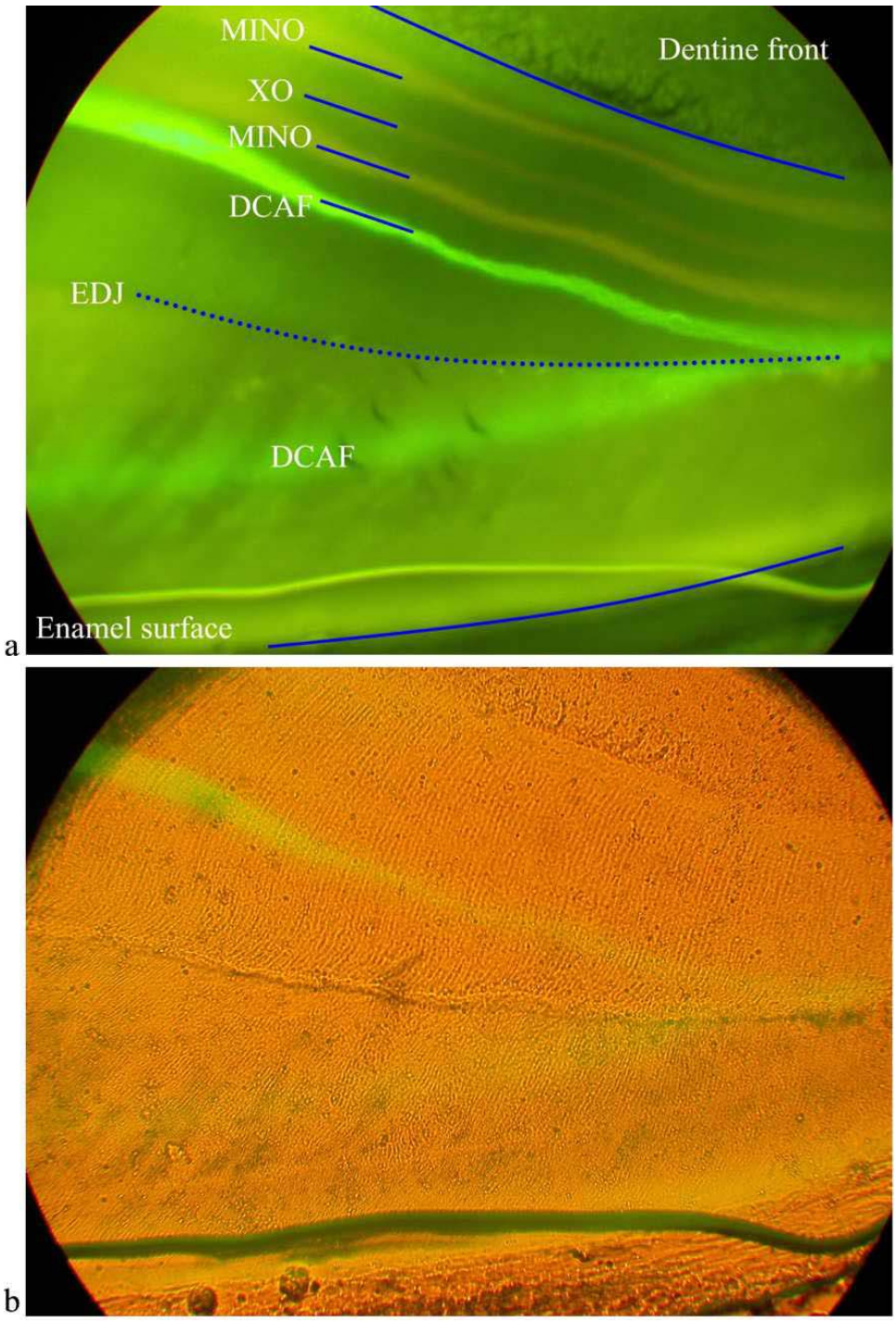


Figure 3.4 (a,b) DCAF fluorescence in enamel and dentine under a) full fluorescence and b) partial fluorescence (slide CF 324 b 29). Each label (minocycline, xylenol orange, and DCAF) is indicated in the top image, along with the dentine front, enamel dentine junction (EDJ), and enamel surface. Note how the DCAF label can be matched from the dentine to the enamel, which allows the label to be related to an accentuated line in the enamel. The cervix is to the right of the images.

Minocycline appeared to cause the greatest growth disturbance in both the enamel and dentine (Figure 3.5), although in these doses, these labels did not appear to disrupt the production of enamel increments for long, as daily increments within the injection intervals were present as expected (see below). Xylenol orange and DCAF did not appear to cause growth disturbances as frequently as did minocycline. Additional accentuated lines were found in some teeth that resembled the proposed effects of minocycline, but when examined under fluorescent light, it was shown that these accentuated lines were formed prior to the administration of labels (Figure 3.6). A similar series of accentuated lines of unknown etiology appeared in the developing first molar of multiple individuals (e.g., CF 324, 325, 326), which appeared to have been caused by a stressor unrelated to the fluorescent labels.

Incremental features, including cross-striations, intradian lines, Retzius lines, and laminations, were observed in teeth from all ages and labeling conditions, including deciduous and permanent teeth. Prenatal enamel frequently showed cross-striations and laminations. The two stillborn individuals did not show incremental features as clearly as those that were sacrificed several days after birth. When a neonatal line could be identified between labels, it did not appear to show an associated temporal delay (e.g., CF 333). Ameloblasts (enamel-forming cells) were found attached to the developing enamel surface in many sections (e.g., Figure 3.7), and were observed at various secretory stages (tall columnar cells, secretory cells, reduced enamel epithelium). Differences were also noted in the clarity of incremental features in immature enamel as compared to mature (well mineralized) enamel, which often showed more clearly defined Retzius lines than the former. Retzius lines were more apparent in (permanent) first molars than in deciduous teeth, possibly due to differences in mineralization.

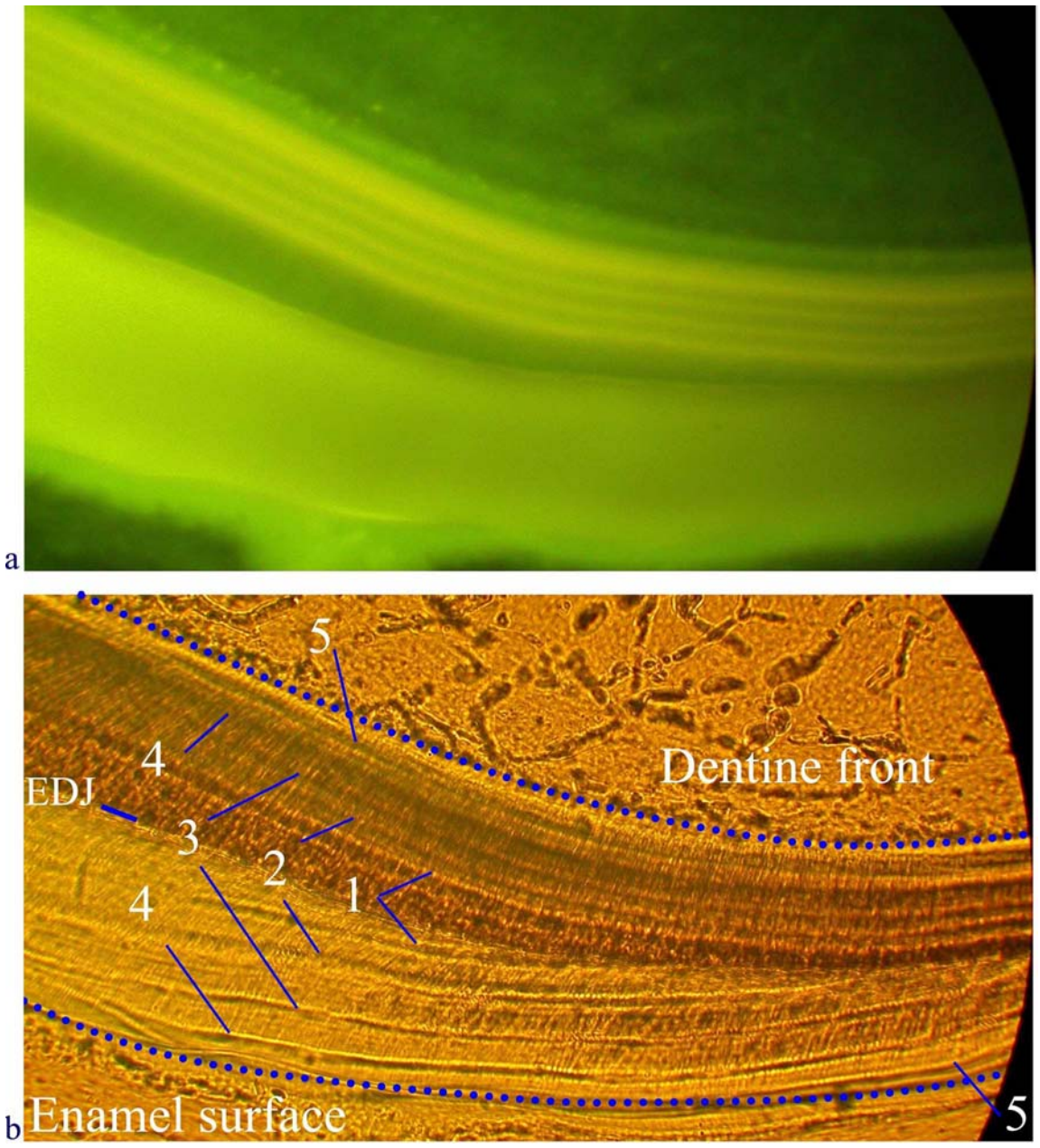


Figure 3.5 (a,b) Fluorescence and transmitted light microscope images showing a series of accentuated lines (growth disturbances) related to minocycline injections (slide CF 327 t 4). a) Fluorescence microscopy, and b) light microscopy of the same area showing five labels (numbered) in the dentine (above EDJ), which correspond with the same five labels in the enamel (below EDJ). The enamel dentine junction (EDJ) is labeled on the left, and the cervix is to the right of the images.

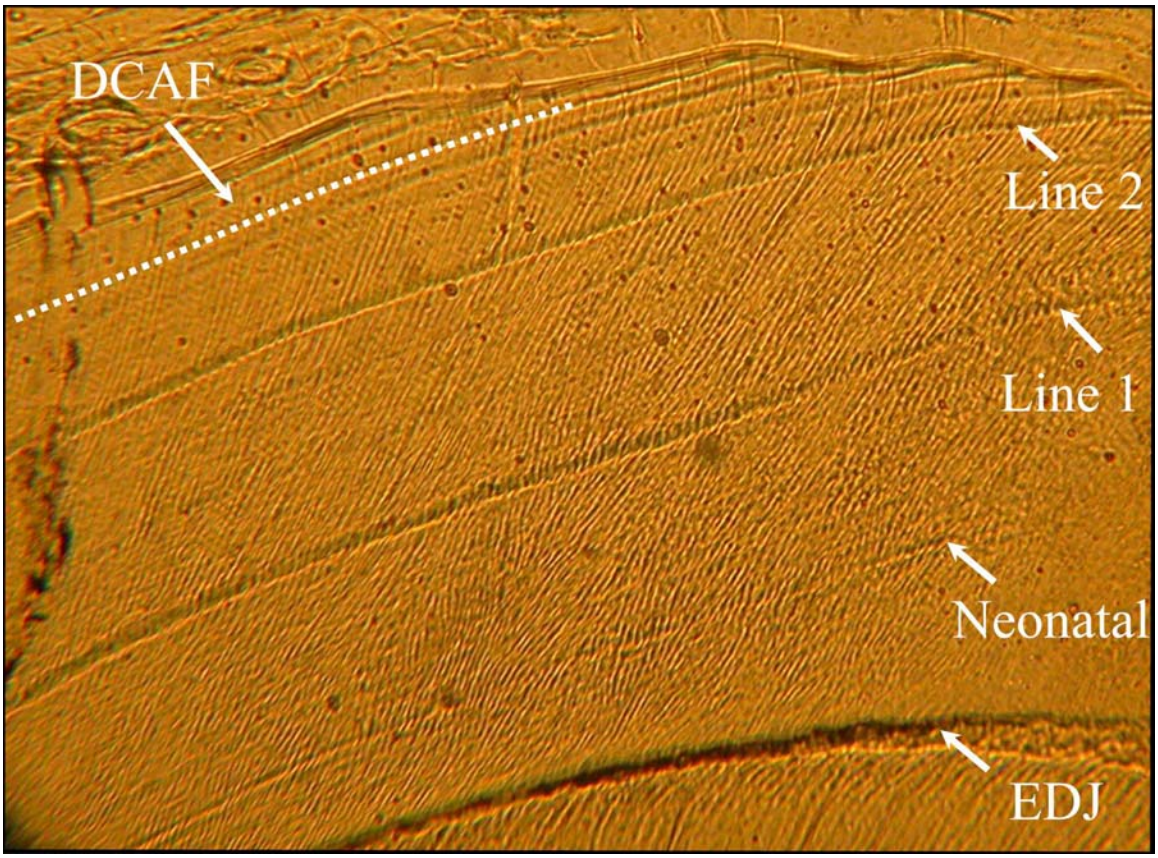


Figure 3.6 Transmitted light micrograph showing accentuated lines that could be mistaken for treatment effects (labels) in the enamel (slide CF 324 b 29). A likely neonatal line was identified above the enamel dentine junction (EDJ- labeled on right), and two lines (Line 1 and Line 2) were identified that were not the result of fluorescent labels. The position of the first fluorescent label is indicated by the DCAF label (top left), which was matched with dentine labels under fluorescence for confirmation.

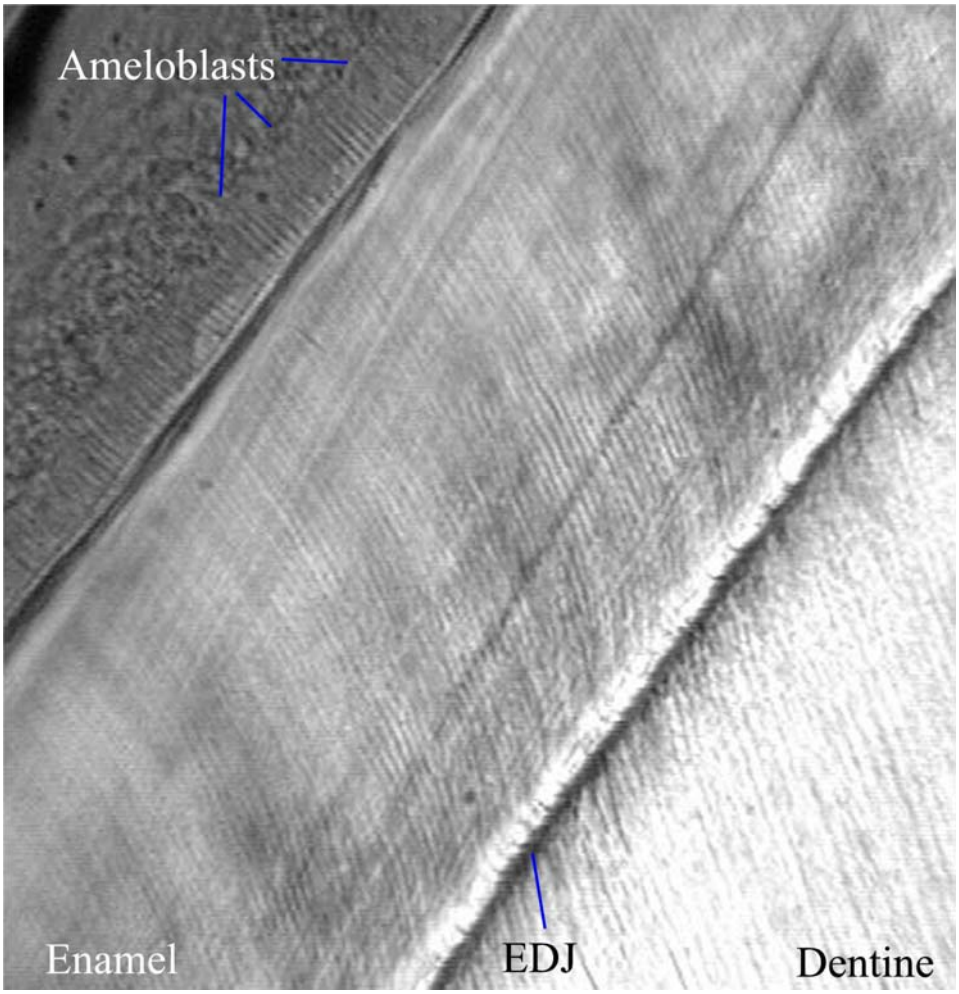


Figure 3.7 Polarized light micrograph of the cervical enamel of a deciduous canine (slide CF 285 a 3). Ameloblasts (enamel-forming cells) are visible in the upper left as tall columnar cells on the surface of the developing enamel. The cervix is towards the lower left.

Incremental Features

Cross-striations

This study provides additional support for the theory that features identified as cross-striations, light and dark bands that cross prisms perpendicularly, are the result of a 24-hour rhythm. Lines in the enamel matched to labels in the dentine show the same number of cross-striations between them as days between respective injections (Figure 3.8). This relationship was observed in sections of prenatal labeled enamel of multiple

individuals and sections (e.g., 300 b 11, 303 b 15, and b 19). Additional evidence of their circadian (daily) nature is discussed in the following chapter, where counts and measurements of cross-striations were used to estimate the age at death from a developing tooth (324 b 29), which predicted the known age at death accurately.

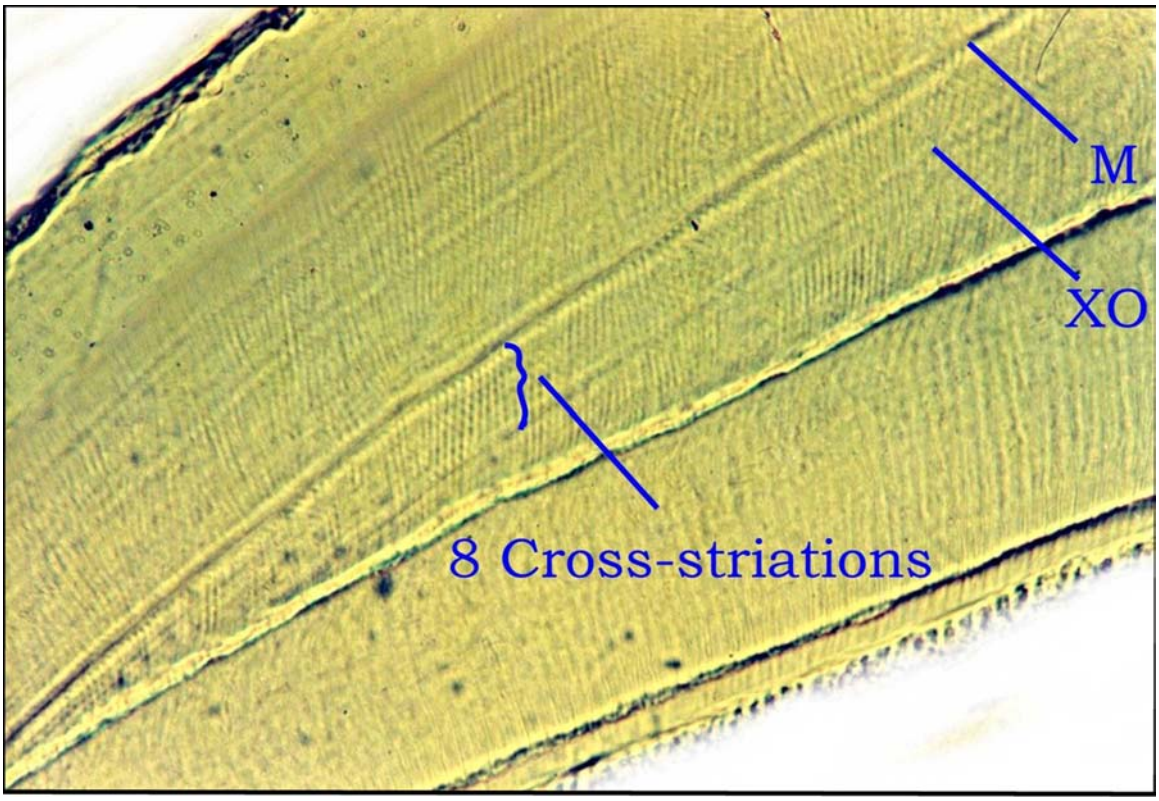


Figure 3.8 Light microscope image of cross-striations in cervical enamel (300 b 11). In this individual, a xylenol orange injection (XO) was followed eight days later by a minocycline injection (M). Eight cross-striations (paired light and dark bands) can be clearly distinguished between these two lines, confirming the daily nature of this feature. The cervix is to the lower left.

Intradian lines

This study provides evidence that intradian lines, defined here as thin bands that sub-divide daily lines, are the result of a 12-hour rhythm in enamel production (Figure 3.9). Intradian lines are generally difficult to resolve clearly, as they are very closely spaced and require high magnification, which results in poorer definition of features. In

some cases, it appears that there are either two or three intradian lines between cross-striations (Figure 3.9b); information from underlying layers may give the impression of additional structures in the focal plane. As one manually focuses through the section, features tend to shift position and number. When viewing this region under lower magnification with more distinct resolution (Figure 3.9c), it is clear that there is single subdivision located in the center of the interval between cross-striations (implying two evenly spaced 12-hour increments). Images from confocal light microscopy confirm the presence of closely spaced bands that are not due to interference from adjacent layers (Figure 3.10), although it is difficult to relate these bands to cross-striations with this method.

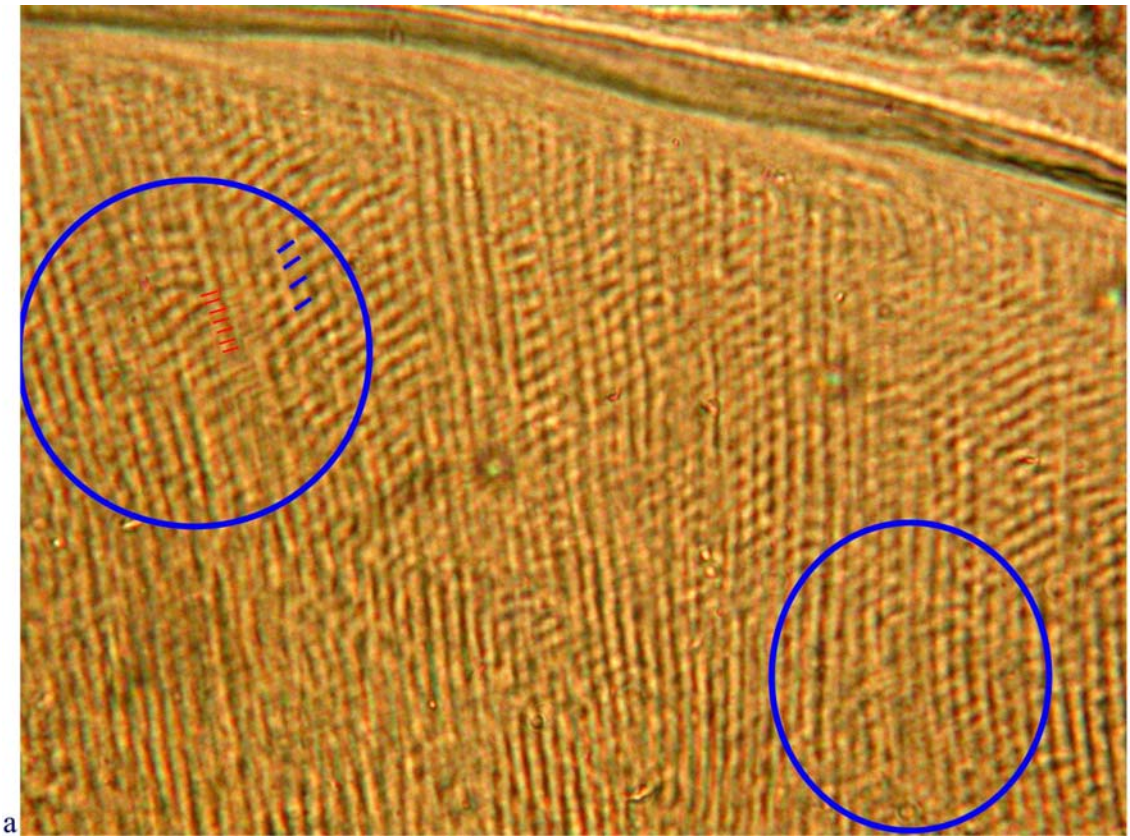
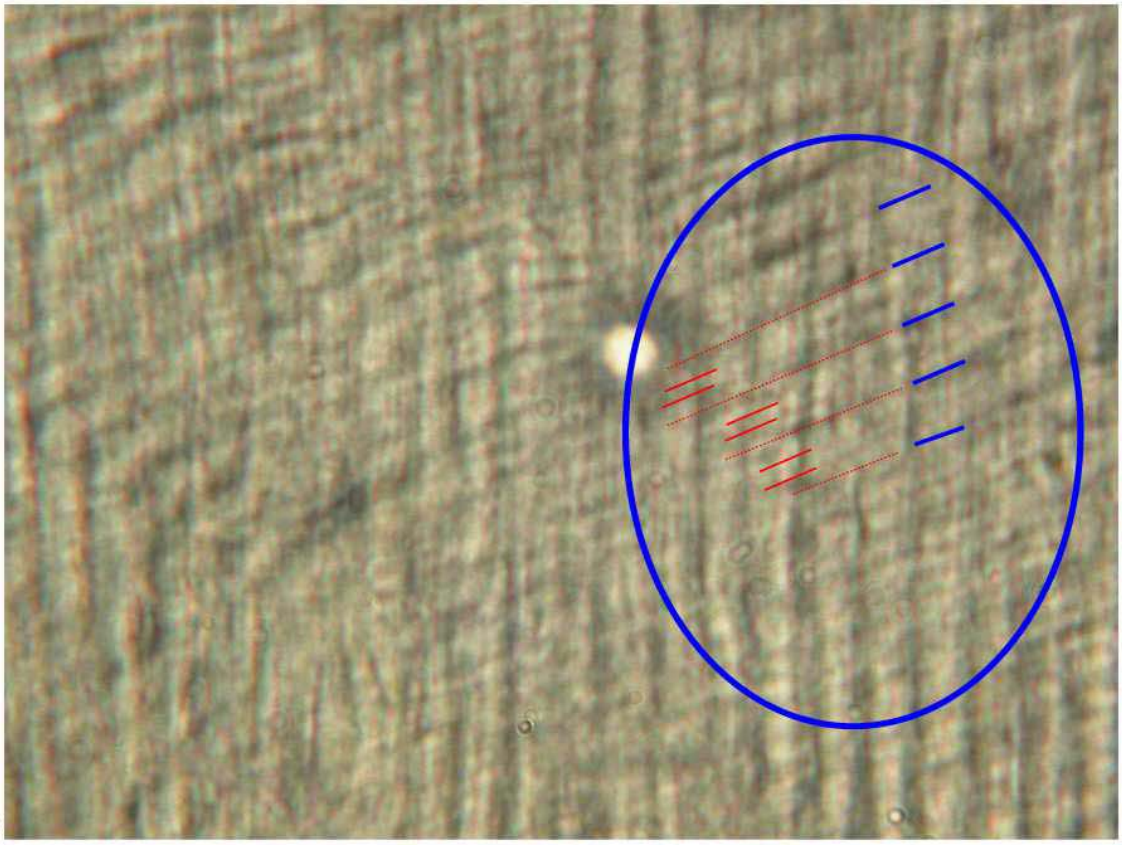
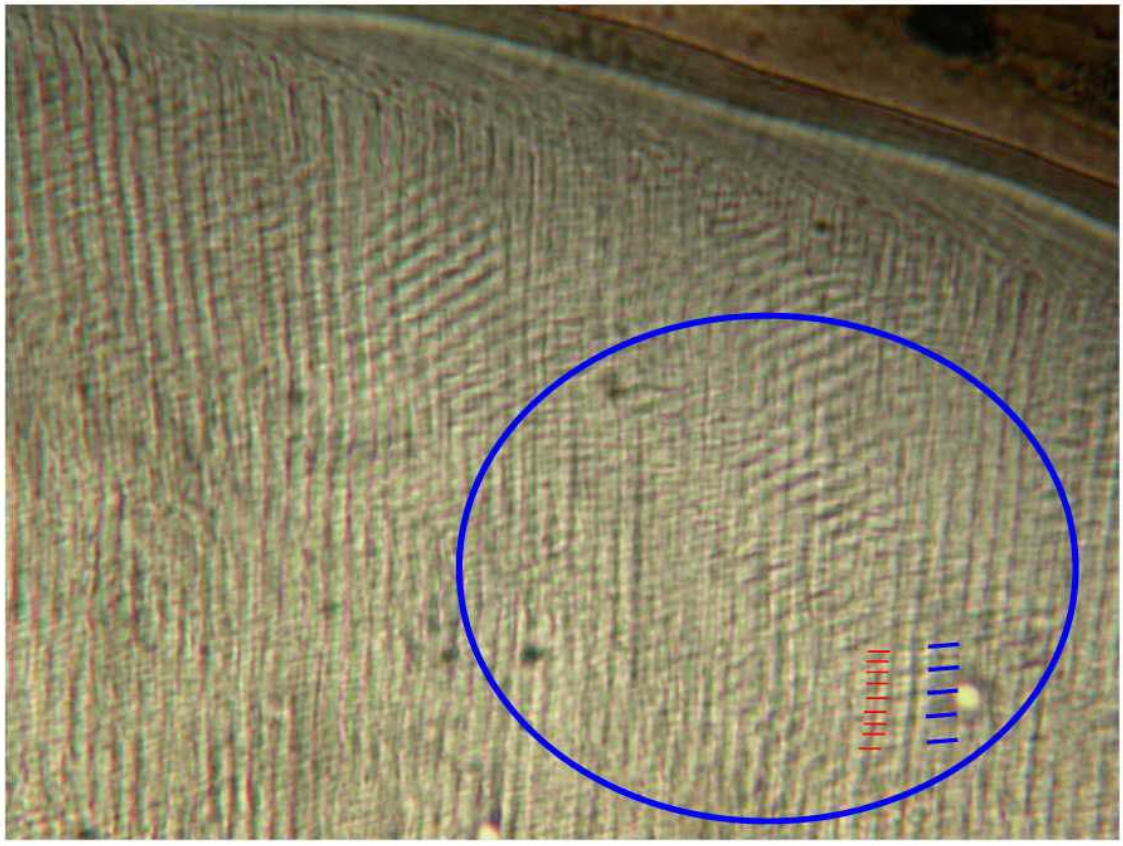


Figure 3.9 (a-c) Intradian lines between cross-striations (slide CF 336 b 24).
a) Transmitted light image showing several areas (circled) where twice as many intradian lines (red lines on left) can be seen relative to cross-striations (in blue). b) High magnification polarized light image of an area where 2 - 3 intradian lines (red) are visible between cross-striations (blue), and c) the same area at lower magnification showing pairs of intradian lines between cross-striations. Enamel surface is at the top. (Figures [b-c] are on the following page.)



b



c

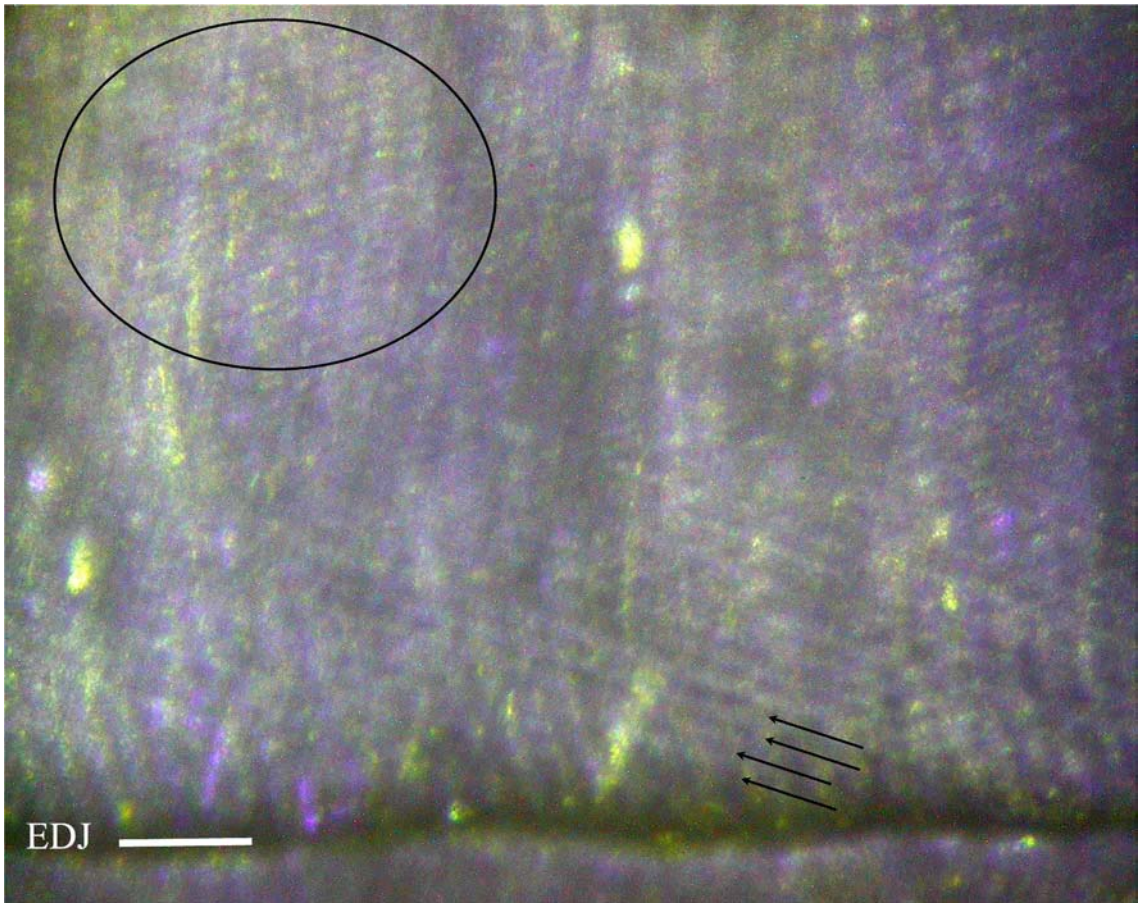
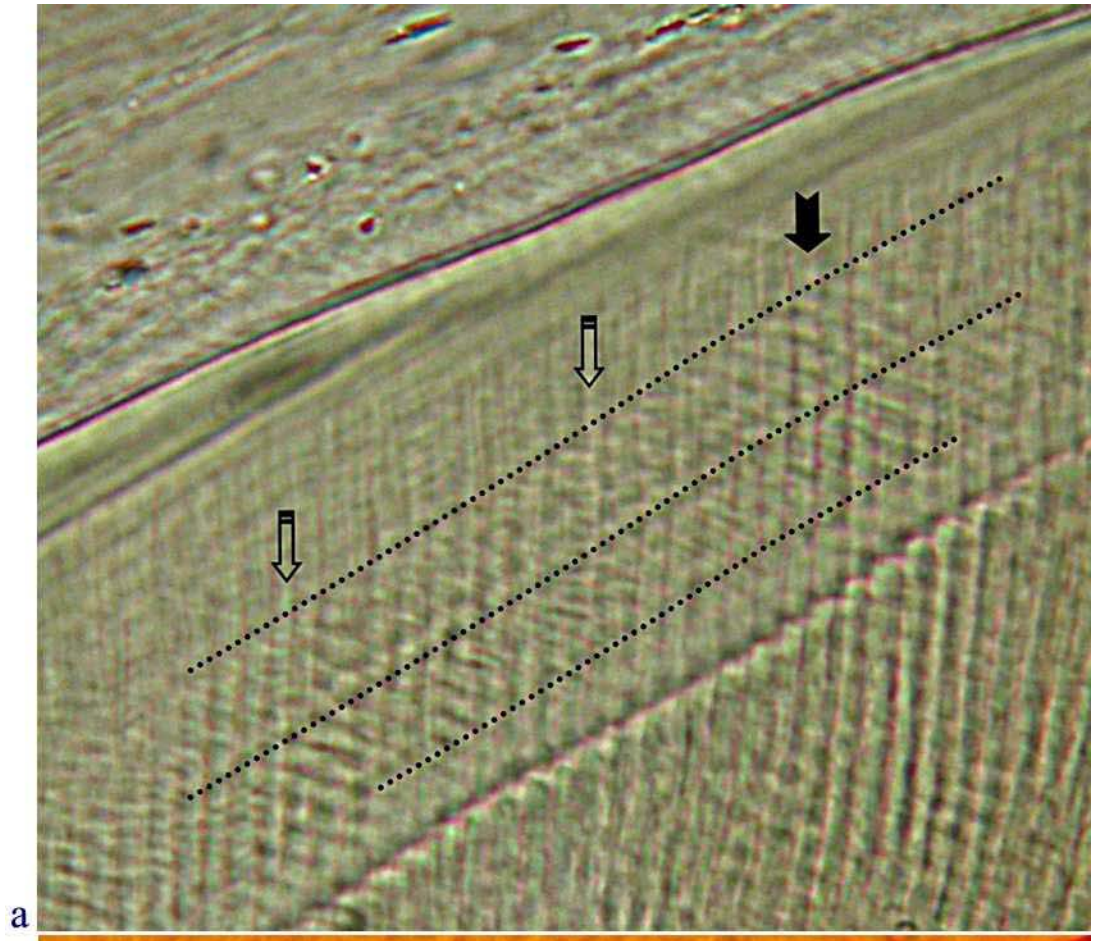


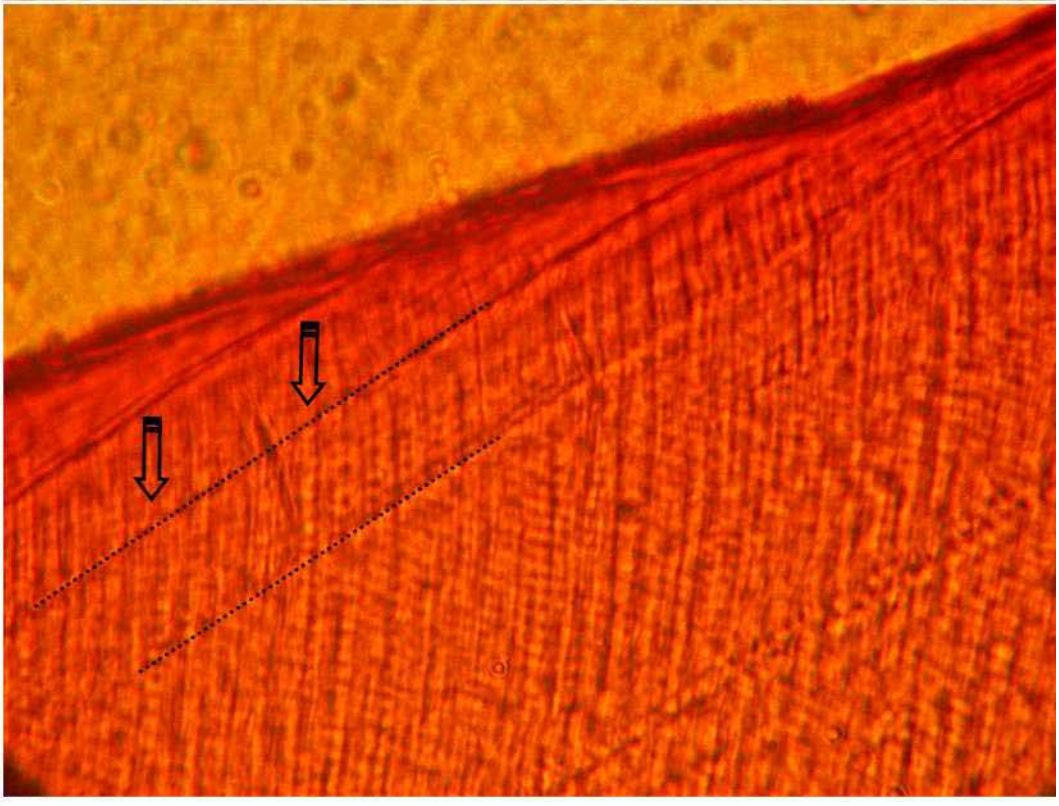
Figure 3.10 Tandem scanning reflected light microscope image of intradian lines (light and dark lines in the black circle at the top of image) (slide CF 337 b 20). Laminations may also be seen at the bottom of the image (small black arrows) running diagonally from the enamel dentine junction (EDJ) towards the surface of the tooth (which appear to be in register with neighboring cross-striations, seen to the upper right of the arrows).

It was not possible to count intradian lines between pairs of labels in any section. Intradian lines are generally very localized, not commonly appearing clearly in long runs over time. Also, they appeared most frequently in outer immature enamel in the prenatal labeled group after the final label (e.g., 300, 303), or between Retzius lines near the surface in the postnatal labeled group (e.g., 330). However, it was possible to show an inclusive series of 8 intradian lines between a pair of Retzius lines in two teeth with 4 day Retzius line periodicities (determined in several areas), representing additional evidence that these features have a 12-hour periodicity (Figure 3.11).

Figure 3.11 (a,b) Transmitted light micrographs of intradian lines between Retzius lines. a) Four cross-striations are evident along a prism (solid arrow) between a pair of Retzius lines (dotted lines), and eight intradian lines are present within the same interval (open arrows pointing down prisms) (slide CF 326 b 26). The dark line below the third Retzius line is an accentuated line related to a minocycline injection, also shown in Figure 3.12. b) Eight intradian lines (open arrows) between a pair of Retzius lines (dotted lines). This tooth is stained red due to unrelated Alizarin red S staining (slide M6898 m2). In both images the enamel surface is the dark diagonal line, and the cervix is to the lower left.



a



b

Retzius lines

Retzius lines in deciduous or immature enamel of first molars were only visible in the outer enamel (just below the tooth surface), often in association with aprismatic enamel (e.g., CF 324, 325, 326). They were less apparent in the developing cervical regions than in the earlier-formed lateral enamel, although the plane of section may have influenced the appearance of these features. More developmentally advanced first molars (CF 330, 336, 337, M 6898) showed well-defined Retzius lines that extended deeper towards the EDJ and had clearly defined boundaries.

It was difficult to determine experimentally the periodicity of Retzius lines, as few sections showed Retzius lines between labels. Retzius lines were often best defined near the enamel surface, while the labels were often clearest near the EDJ, and the two generally did not extend far enough into the mid-thickness of enamel to be related to one another. Labels were also most frequently found in the cervical-most immature enamel, which generally did not show well-developed Retzius lines.

It was possible to relate Retzius lines to a known-period interval (determined from the timing of injections) in one section (Figure 3.12). This individual received a minocycline injection, followed two weeks later with a DCAF injection, two weeks after that with a minocycline injection, and was sacrificed shortly after the final injection. The minocycline lines were identified in the cervical enamel and traced to the surface, representing 28 days of enamel formation between them. Six complete Retzius line intervals (7 total lines) can be seen between these two labels, plus 2-3 days between the first label and the first Retzius line, and 1-2 days between the 7th Retzius line and the last label, totaling approximately 28 days. This suggests that each Retzius line interval represents 4 days on average, which is consistent with observations made on cross-striations between individual pairs of Retzius lines in this individual.

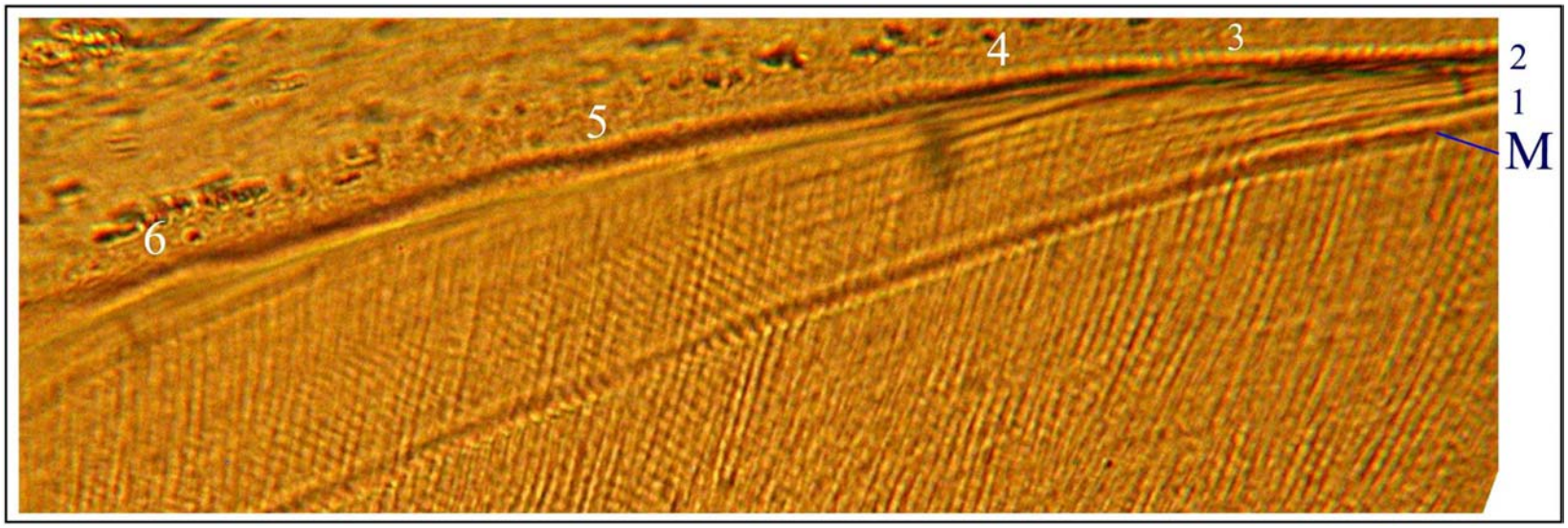


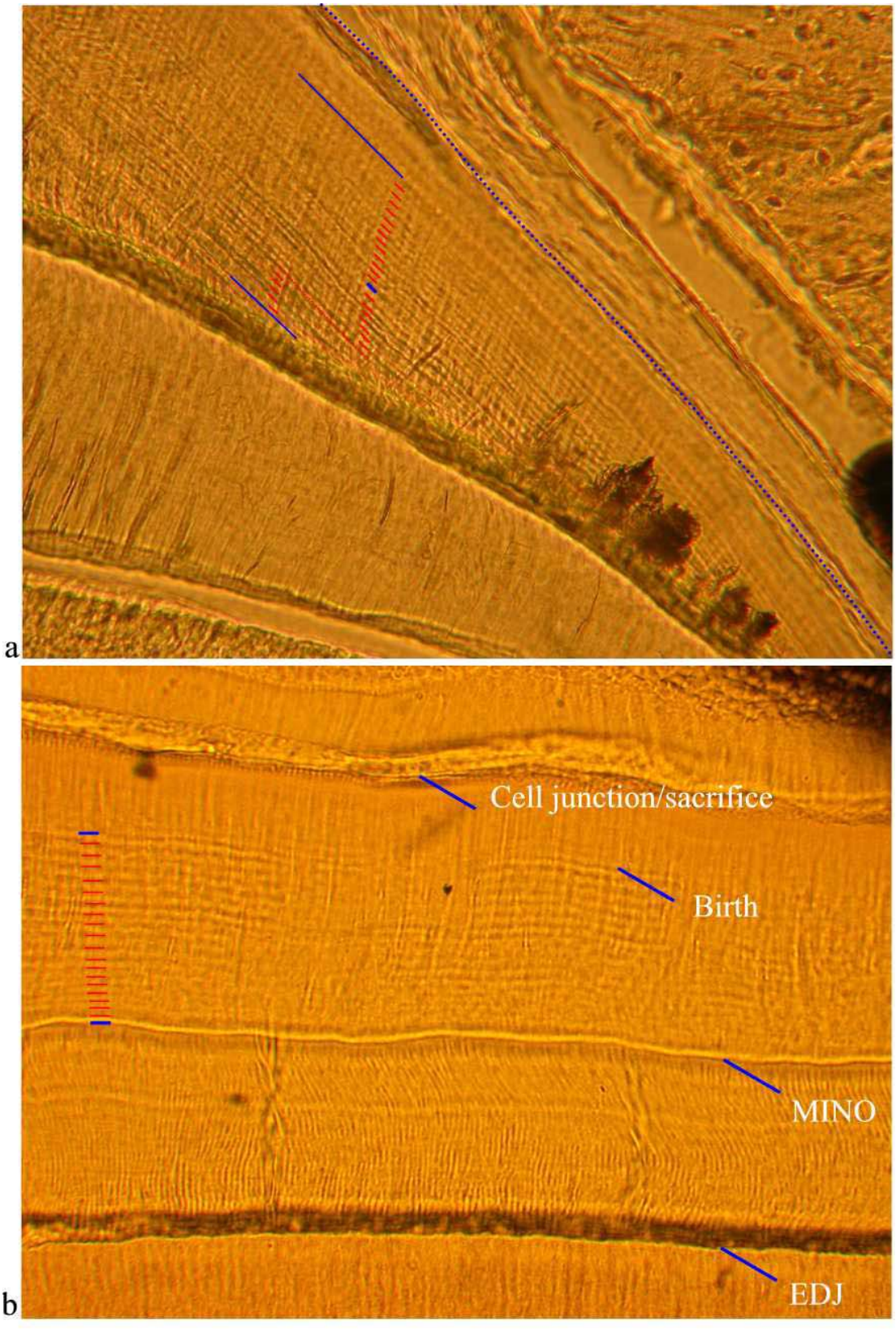
Figure 3.12 Transmitted light micrograph of Retzius lines formed after a minocycline injection (labeled M) (slide CF 326 b 26). Two to three cross-striations are seen between the label and the first Retzius line above it (dark line near l), followed by five Retzius lines (numbered 2 - 6). (The final interval and subsequent minocycline label discussed in the text are not seen in this image.) The periodicity of the Retzius lines in this tooth appears to be four days, as also seen in the intervals between Retzius lines 1 - 2 and 2 - 3.

Six (postnatal) individuals were found to have a Retzius line periodicity of 4 days as determined by counts of cross-striations between Retzius lines (CF 324, 326, 330, 336, 337, M 6898). This was frequently difficult to determine, and was complicated by several factors such as intradian lines, laminations, and accentuated lines. No irrefutable evidence was found in this study to suggest that Retzius lines do not have a regular periodicity. Where it was possible to make counts, the number of cross-striations between pairs of lines was consistent within a section, tooth, and/or individual. Retzius lines were also infrequently related to laminations in the cervical enamel (e.g., CF 326, M 6898), and counts of laminations at the EDJ also suggested a regular periodicity between Retzius lines. However, counts of laminations at the surface of the tooth did not consistently yield the expected Retzius line periodicity, and should not be used to determine or test for a uniform Retzius line periodicity (discussed further in the following section).

Laminations

This study provides strong evidence that laminations, closely spaced bands that follow the course of the developing enamel front, show a daily periodicity (Figure 3.13). This relationship is also seen between minocycline labels shown at low magnification in Figure 3.5. Additional evidence is present in several regions of cervical enamel, which show a number of laminations at the EDJ that is equivalent to the number of corresponding cross-striations (Figure 3.14). This correspondence has also been seen under confocal light microscopy (faintly visible in the lower right corner of Figure 3.10). The documentation of these features under confocal light microscopy confirms that they are not an artifact of transmitted light microscopy.

Figure 3.13 (a,b) Transmitted light micrograph showing the daily nature of laminations. a) Laminations (labeled with red lines) between injections (labeled in blue) at the cervix (to the right) (slide CF 326 b 26). This individual received a minocycline injection, followed two weeks later with a DCAF injection, two weeks after that with a minocycline injection, and was sacrificed shortly after the final injection. Twenty-eight laminations can be seen between the first and third labels, which are equal to 28 days of formation. b) Laminations in the occlusal enamel of a mandibular dp4 cut transversely (slide CF 300 t 4). In this individual, DCAF, xylenol orange, and minocycline prenatal injections were given, which was followed by birth nineteen days later, and sacrifice eight days after that. This image shows the enamel dentine junction (EDJ) at the bottom, a growth disturbance caused by minocycline (MINO), 19 laminations (red lines on the left) between this disturbance and the likely neonatal line (Birth), and eight laminations (not labeled) between this disturbance and the end of enamel formation (sacrifice).


a
b
Cell junction/sacrifice
Birth
MINO
EDJ

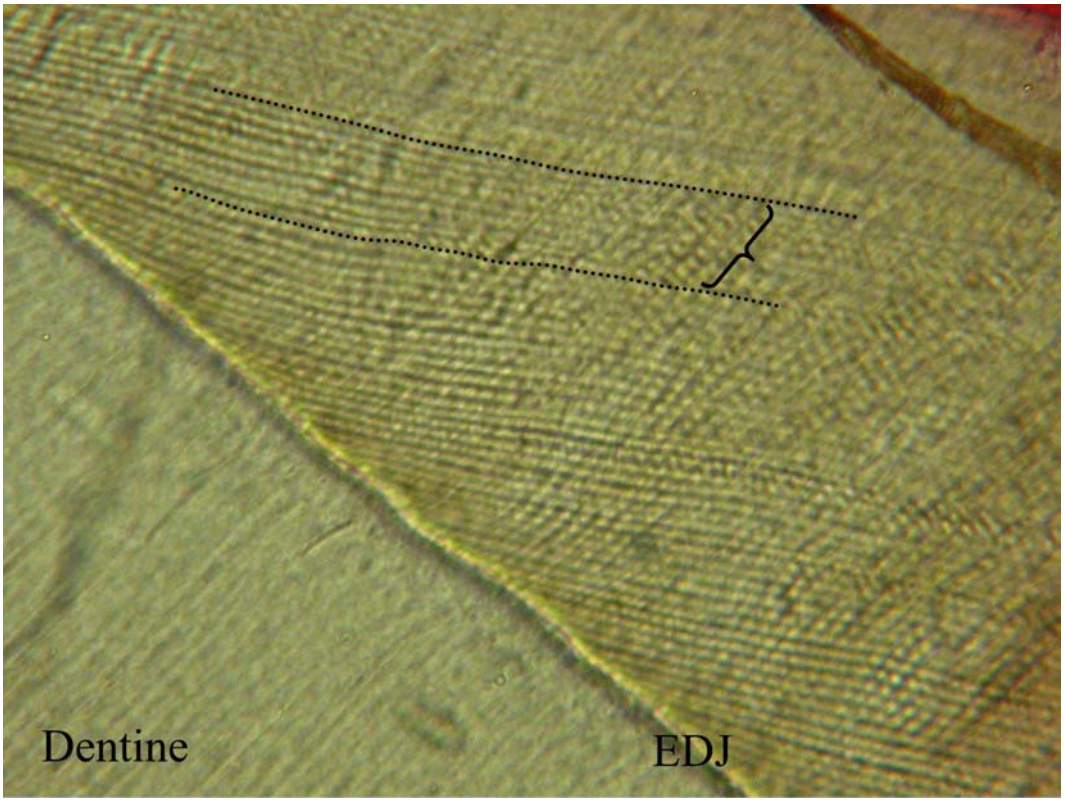


Figure 3.14 Transmitted light micrograph showing nine laminations in register with nine cross-striations (between the two dotted isochronous lines), confirming that these features are of equal periodicity (slide M6898 d2).

Laminations were observed to be most common in the enamel over the dentine horn, along the EDJ in the cervical region, and in the outer enamel in association with aprismatic enamel as well as between Retzius lines. As shown in Figure 3.15, the number of laminations is equal to the number of cross-striations between Retzius lines, providing further confirmation of their daily periodicity. It is critical to note that when determining the periodicity of these features, care must be taken to avoid the effects of optical superimposition with adjacent laminations or with Retzius lines. This is illustrated in Figure 3.16, and suggests that the number of laminations may be underestimated without careful imaging, and Retzius lines near the surface may appear wider and more pronounced when laminations from adjacent planes are not clearly separated.

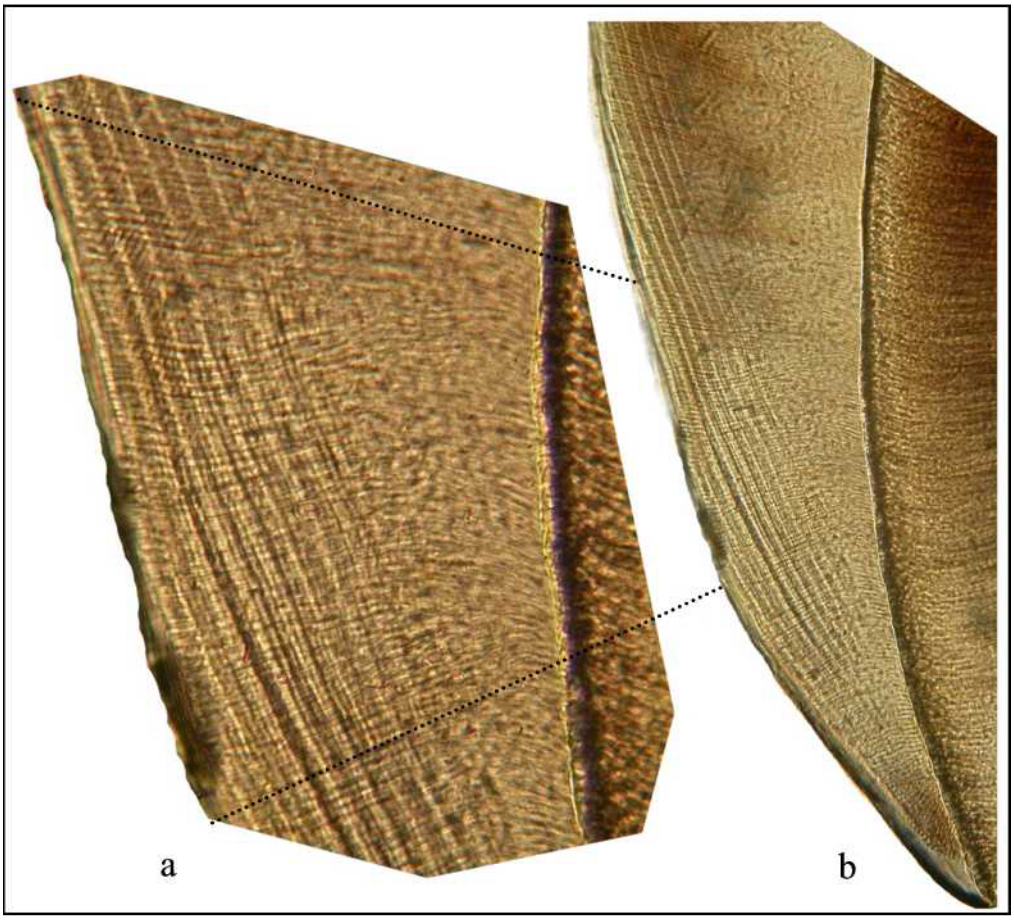


Figure 3.15 (a,b) Transmitted light micrographs of laminations (slide CF 336 b 24). a) High magnification of the region in b, showing a periodicity of four cross-striations (upper left) and laminations between Retzius lines (although in some areas only three laminations are evident between Retzius lines). b) Overview of cervical enamel of this unusual first molar, showing extensive laminations in the deeper enamel.

Laminations were also occasionally observed to show a sub-daily division (Figure 3.17). This was seen in several sections between features in the cervical and lateral enamel at the EDJ. When it was possible to image these subdivisions, it consistently appeared that there was a single subdivision, representing additional evidence that a class of sub-daily feature is produced every 12 hours. In addition, fine lines parallel to the developing enamel surface were also observed in the sub-surface aprismatic enamel (Figure 3.18), but it was not possible to establish their periodicity in this area.

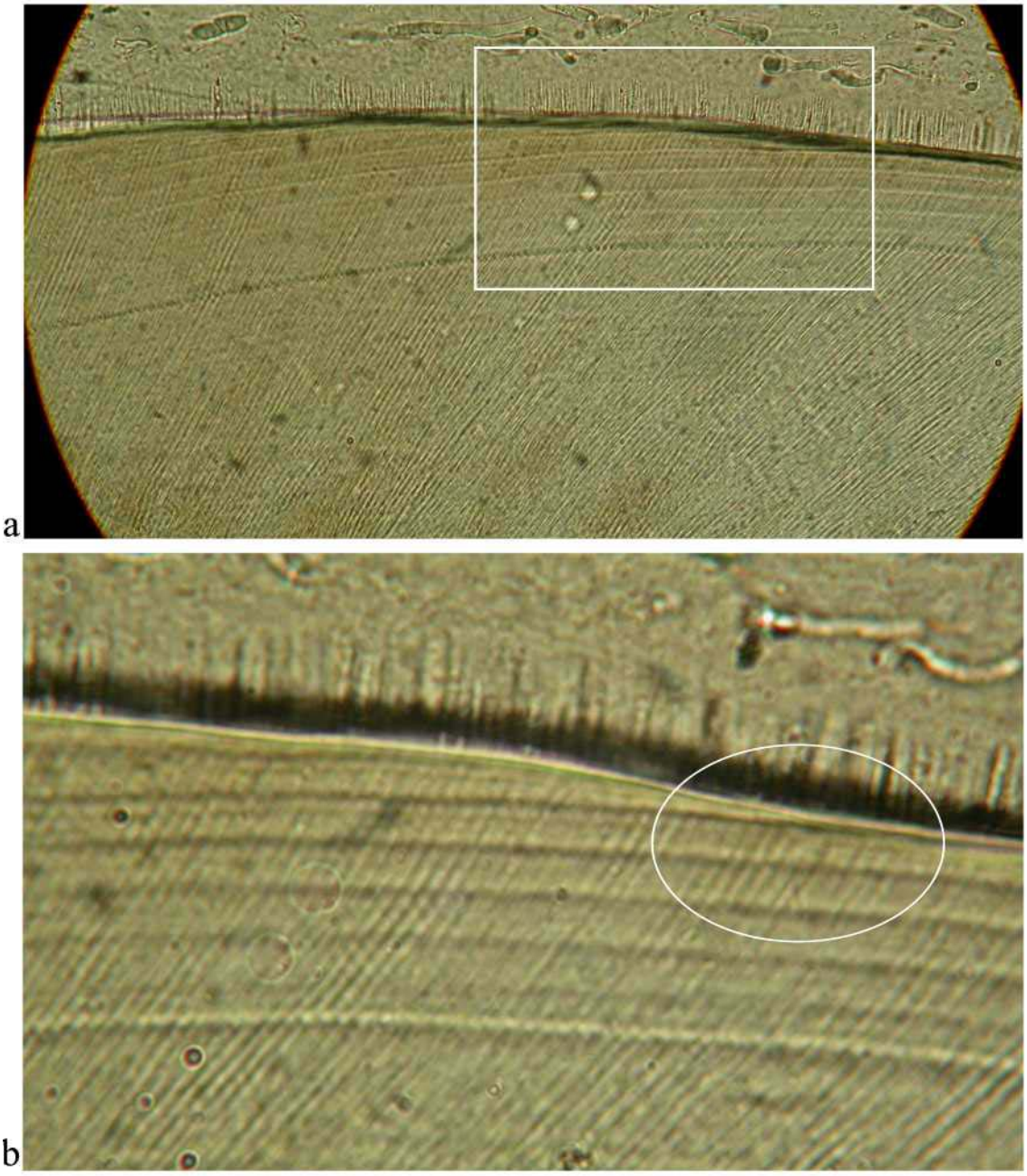


Figure 3.16 (a-f) Series of transmitted light micrographs demonstrating optical superimposition of laminations (slide CF 330 b 19). a) Overview of the lateral enamel region showing the area of interest (white box). b - f) Succession of focal planes showing the changing relationship between the Retzius lines and laminations (within white circle). In the early figures, the laminations (blue lines) appear as three hazy lines between thick dark Retzius lines. By the focal plane shown in e), four laminations can be seen between thin light Retzius lines, demonstrating that superimposition may influence attempts to count these lines near the tooth surface. (Figures [b-f] are on the following page.)

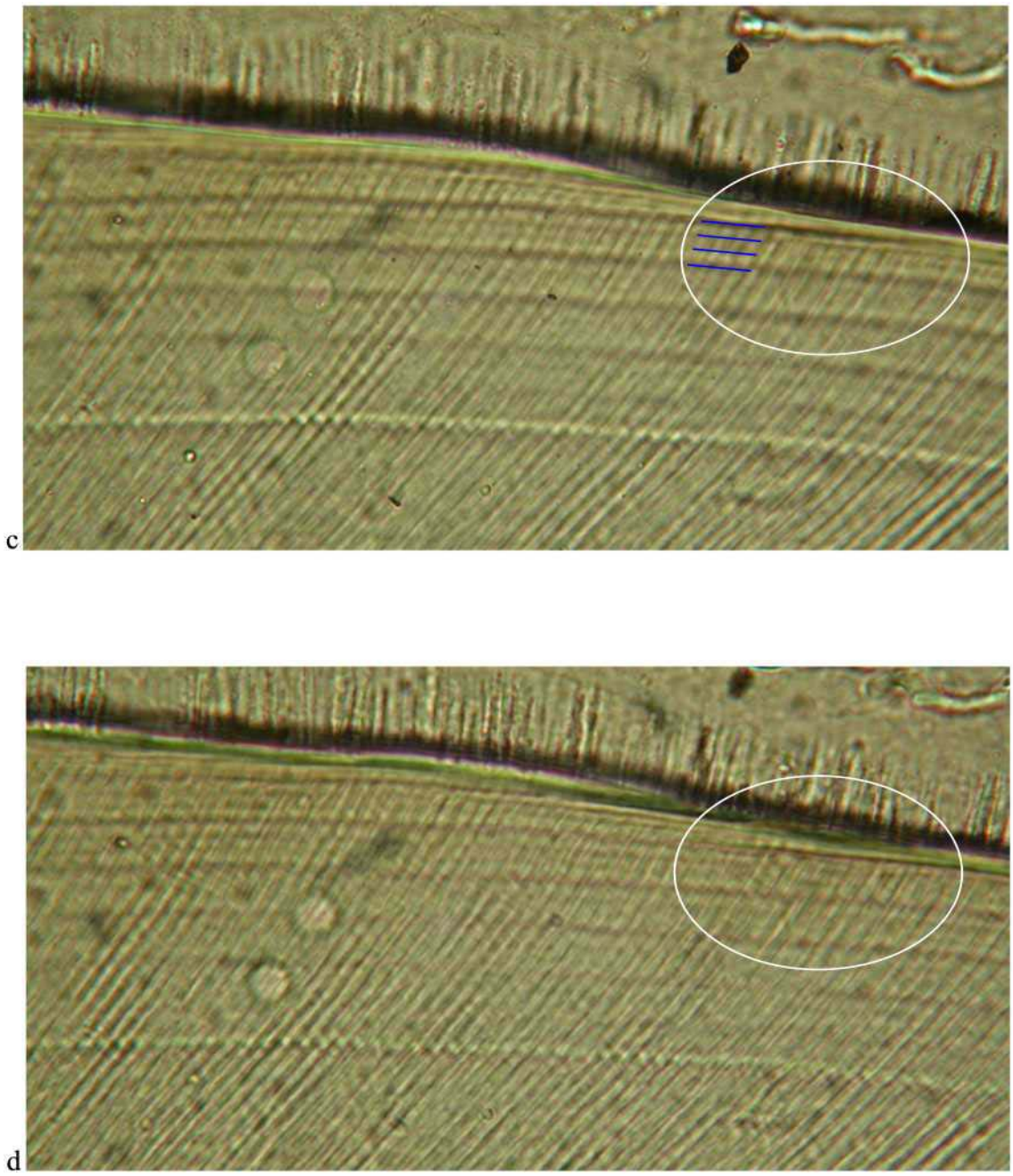
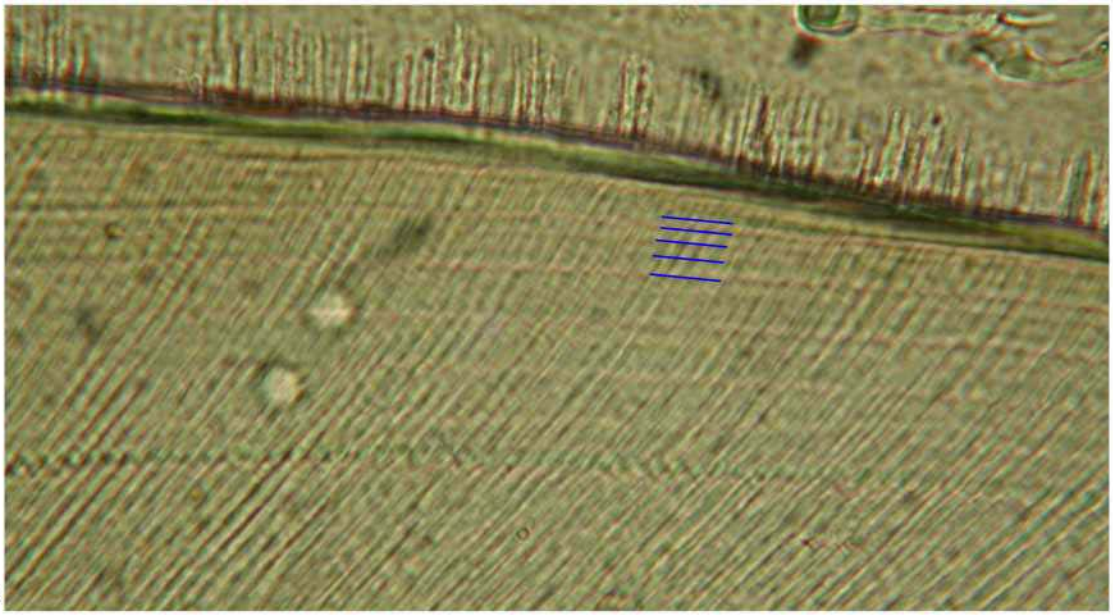


Figure 3.16 (c,d) See caption above.

e



f

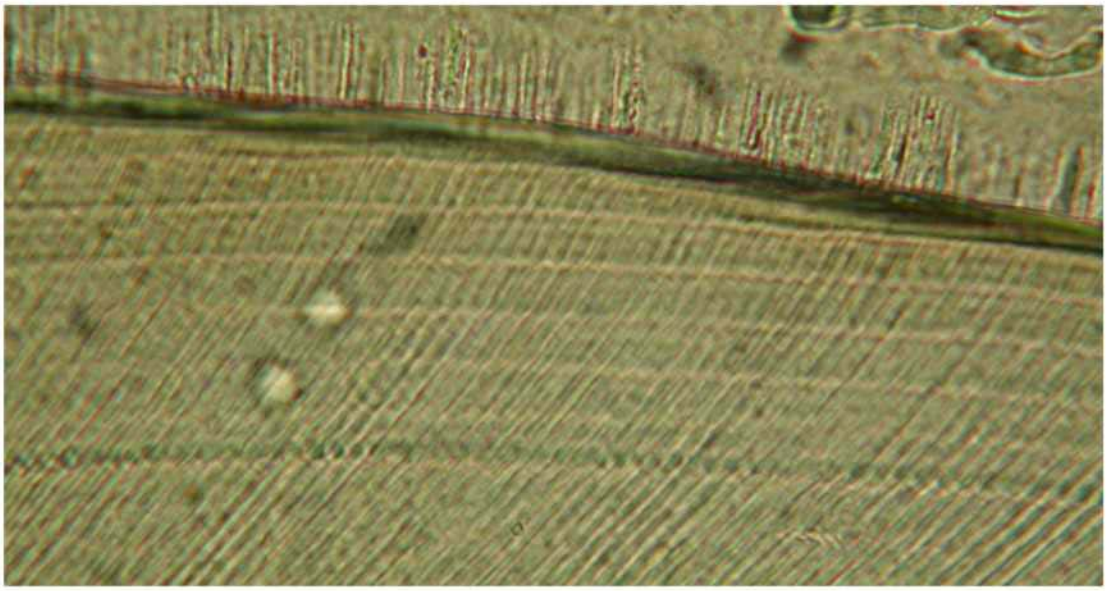


Figure 3.16 (e,f) See caption above.

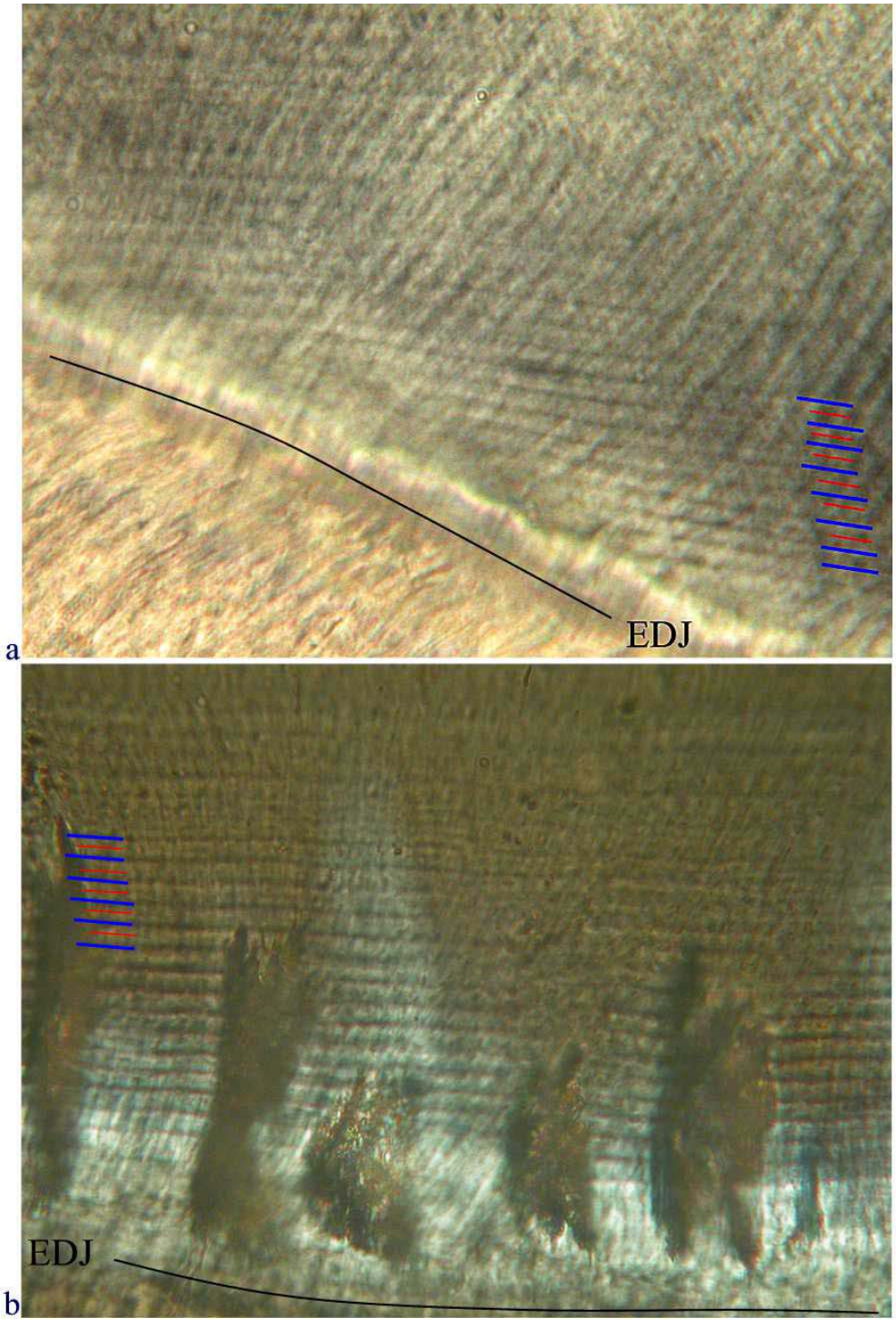


Figure 3.17 (a,b) Transmitted light micrograph showing sub-daily lines (red lines) between laminations (blue lines) in a) lateral enamel at the enamel dentine junction (EDJ) (slide CF 336 b 24), and b) cervical enamel of a transverse section (slide CF 324 t 5).

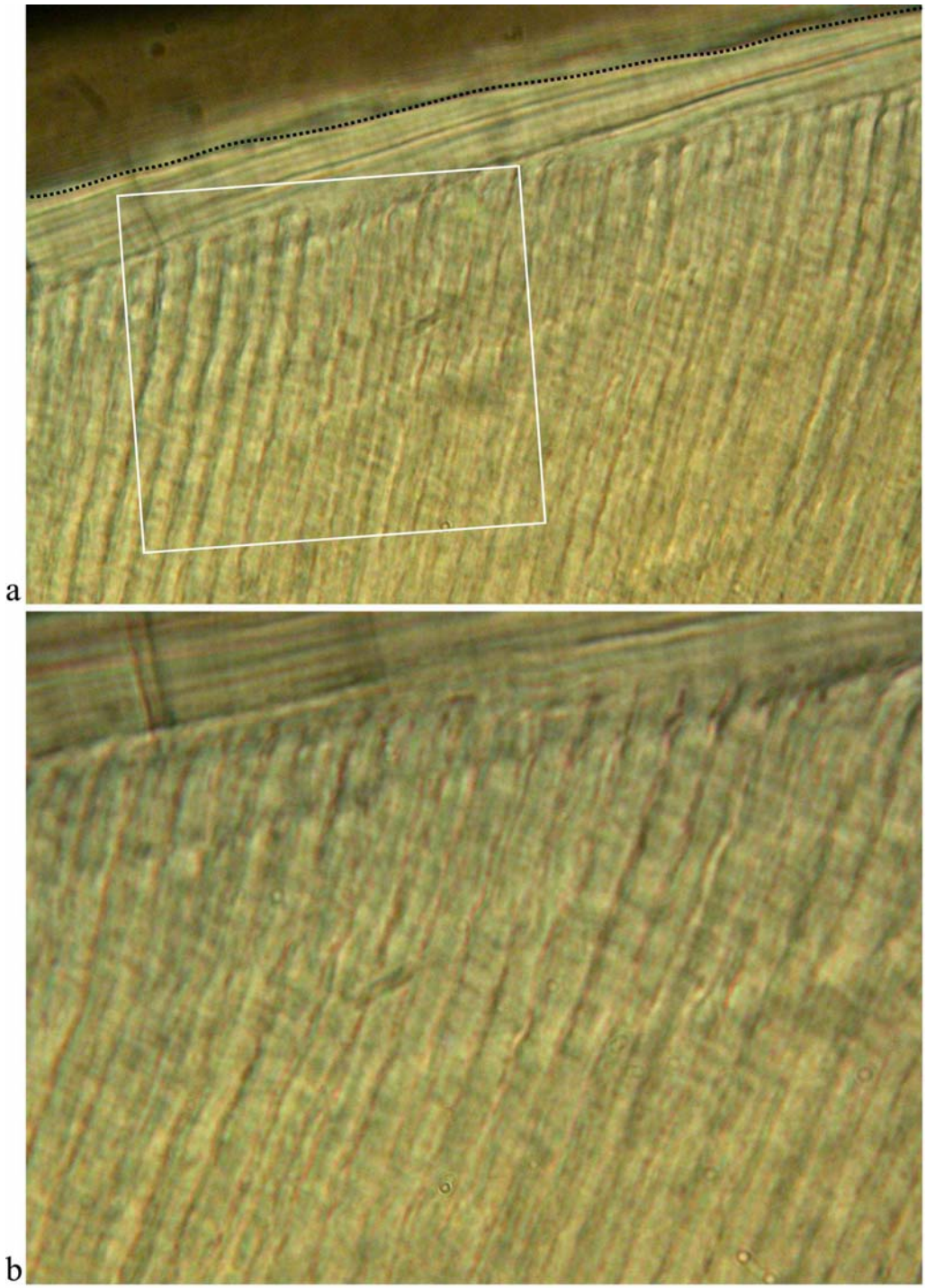


Figure 3.18 (a,b) Transmitted light micrographs of sub-divided aprismatic laminations (slide CF 336 b 24). a) Lower magnification image with enamel surface at top (dotted line), white box showing lower image area. b) High magnification image showing many sub-daily lines.

Extension rate

The results of the calculated and measured extension rates at various positions in a single deciduous canine, four deciduous premolars and two permanent molars of several individuals are presented in Table 2. Known interval length varied from six days to 28 days. The extensions rates in deciduous teeth calculated from Shellis' formula tended to yield a lower value than the actual local extension rates, which resulted in significant overestimates of formation time (Wilcoxon signed ranks test: Z= -2.778, n= 19, P=0.005). Estimates in the two molar areas were fairly accurate. Absolute differences between the calculated and known formation times ranged from 2 - 174%, with an average difference of 40%. Average time differences were: 74% (*s.d.*=70, n=4 areas) for the dc areas, 36% (*s.d.*=20, n=13 areas) for dp4s, and 3% (n=2) for M1s. Only three of 19 areas were predicted with more than 90% accuracy, two of which were 28-day intervals in the lateral/cervical enamel of developing first molars (one these is shown in Figure 3.13a). In the two molars, the angle of intersection of the developing enamel front (D) was greater than in most of the deciduous teeth, which was easier to measure and may have resulted in more accurate determination of the local extension rate.

Table 3.2. Measured and calculated extension rate in *Macaca nemestrina*.

				Known				Shellis							Difference	
CF	**Slide**	**Tooth**	**Area**	**Event**	**Dist**	**Time1**	**Rate**	**Min *d***	***I***	***D***	***c***	**Int *c***	**Dist**	**Time2**	**2-1**	**Ratio**
300	b 11	dp4 m	lat/cer	XO				4.6	66.3	11.4	19.04					
				Mino	260	8	32.50	4.8	76.8	13.0	19.15	19.09	260	13.6	5.62	0.70
				Birth	633	19	33.32	3.9	74.1	6.0	34.62	26.88	633	23.5	4.55	0.24
303	b 15	dp4 m	lat/cer	DCAF				5.5	61.2	7.1	36.05					
				XO	260	9	28.89	5.4	59.4	7.8	31.18	33.61	260	7.7	-1.27	-0.14
				Mino	284	8	35.50	5.0	70.6	9.1	27.78	29.48	284	9.6	1.63	0.20
				Birth	521	21	24.81	5.2	66.4	7.0	36.73	32.25	521	16.2	-4.85	-0.23
	b 19	dp4 d	lat/cer	DCAF				4.5	59.1	6.2	33.23					
				XO	287	9	31.89	5.1	58.1	8.3	26.98	30.11	287	9.5	0.53	0.06
				Mino	274	8	34.25	4.9	59.8	8.7	25.21	26.10	274	10.5	2.50	0.31
320	a 3	dc lab	lat/cer	Mino				5.6	42.5	10.3	16.69					
				Mino	417	7	59.57	5.1*	46.0	6.9	26.77	21.73	417	19.2	12.19	1.74
				Mino	267	6	44.50	4.6	48.7	4.8	38.12	32.45	267	8.2	2.23	0.37
				Mino	421	8	52.63	4.3	39.4	5.6	24.51	31.32	421	13.4	5.44	0.68
				Mino	263	7	37.57	4.3	43.5	4.0	39.21	31.86	263	8.3	1.25	0.18
	b 12	dp4 m	lat/cer	Mino				4.3	54.9	9.2	19.25					
				Mino	244	7	34.86	4.7	56.4	7.0	29.28	24.27	244	10.1	3.06	0.44
				Mino	194	6	32.33	3.9	49.1	6.3	24.15	26.71	194	7.3	1.26	0.21
				Mino	299	8	37.38	4.4	52.6	7.4	24.24	24.19	299	12.4	4.36	0.54
				Mino	208	7	29.71	3.7	52.3	9.1	16.01	20.13	208	10.3	3.33	0.48

325	b 17	M1 m	lat/cer	Mino				3.7	77.2	16.8	11.17					
				Mino	409	28	14.61	4.0*	74.0*	11.0	18.68	14.92	409	27.4	-0.59	-0.02
326	b 26	M1 m	lat/cer	Mino				3.5	83.4	16.8	11.11					
				Mino	396	28	14.14	3.6	82.8	10.7	18.45	14.78	396	26.8	-1.21	-0.04
333	b 18	dp4 d	lat	Mino				5.2	57.9	10.5	21.00					
				Mino	175	7	25.00	4.5	49.1	11.2	14.23	17.62	175	9.9	2.93	0.42
				Mino	148	6	24.67	4.5	54.9	11.6	15.35	14.79	148	10.0	4.01	0.67

CF= individual macaque; Slide= slide designation (see Appendix 1 for a complete list of individuals and slides); Tooth= tooth type, m- mesial section, d- distal section, lab- labial aspect; Area= region of the enamel where the measurements were made, either the approximate border of the lateral and cervical thirds or the lateral enamel third; Event= birth or injection, XO- xylenol orange, Mino- minocycline, DCAF- (2,4Bis) N,N' Di aminomethyl fluorescein; Dist= length of the interval along the EDJ in µm; Time1= known time between labels in days; Rate= actual extension rate in µm/day, determined by division of the distance by the known time; Min *d*= minimum (measured) average local cross-striation spacing in µm/day; *I*= angle of intersection of the enamel prisms with the enamel dentine junction (EDJ); *D*= angle of the developing enamel front/labels with the EDJ; *c*= calculated extension rate using the formula proposed by Shellis (1984a,b): extension rate (*c*) = *d* [(sin *I*/tan *D*)- cos *I*] in µm/day; Int. *c*= average extension rate of the upper and lower interval boundaries in µm/day; Dist= length of the interval along the EDJ in µm; Time2= predicted time of formation in days, determined by division of the distance by the average calculated extension rate; 2-1= Shellis' calculated time (Time 2) minus known time (Time 1); Ratio= 2-1 difference divided by known time.

* = estimated value

Discussion

Previous studies have suggested that incremental features are not present or are difficult to see in prenatal or deciduous enamel (Komai [1942: in Japanese], as cited by Boyde, 1963). However, FitzGerald et al. (1999) and Smith et al. (2002) demonstrated that features are evident in both human and macaque deciduous enamel. The latter study found short- and long-period features in both pre- and postnatal deciduous and permanent enamel, which has implications for the ontogeny of these features. In the following discussion, the results of the current study will be considered in the context of several other labeling studies, followed by a discussion of the periodicity of each feature and the quantification of extension rate. In the closing, studies on mammalian biological clocks are reviewed and related to incremental feature development and periodicity.

Labeling Studies

Building upon work from the 1930's, this study represents the most comprehensive examination of incremental features in labeled primate material. Schour and Smith (1934) conducted a laboratory rat study to illustrate the effects of sodium fluoride as a tool for investigation of dentine and enamel formation.[8] Schour and Poncher (1937) published the first experimental study of primate enamel and dentine development, as they injected a terminally ill human infant with sodium fluoride regularly until death, and measured the secretion rate (between labels) in several deciduous and permanent teeth. Schour and Hoffman (1939b) similarly injected sodium fluoride and alizarin red into several different types of animals (including 27 rhesus macaques) and quantified enamel and dentine secretion rate. However, neither of the latter two studies related secretion rates to incremental features, despite images that show cross-striations between labels (e.g., Schour and Poncher, 1937: Figure 7, p. 771).

[8] This study also inadvertently demonstrated the daily nature of incremental features in dentine by showing and describing a section with an equivalent number of incremental features and days of formation (see their Figure 34, Plate XVI), which was not emphasized in the text.

In 1938, the Japanese researchers Mimura and Okada developed a 'time-marking procedure' that involved injecting a number of animals (including macaques) with lead acetate and sodium fluoride over certain intervals, which illustrated the daily periodicity of incremental features in enamel and dentine (Okada, 1943; Okada, 1963).[9] Okada experimented with several manipulations designed to identify the cause of daily features (reviewed in Chapter 1). More recent experimental work has examined the periodicity, secretion rate, and development of incremental features in dentine (Miani and Miani, 1971; Kawasaki et al., 1977; Yilmaz et al., 1977; Kawasaki et al., 1980; Rosenberg and Simmons, 1980a; Klevezal, 1981; Molnar et al. 1981; Klevezal and Myrick, 1984; Shinoda, 1984; Klevezal and Mina, 1990; Dean, 1993; Dean et al., 1993a; Ohtsuka and Shinoda, 1995; Dean, 1995a; Dean and Scandrett, 1995, 1996; Erickson, 1996; Klevezal, 1996; Dean, 1998b; Ohtsuka et al., 1998; Rinaldi, 1999; Ohtsuka-Isoya et al., 2001).

Molnar et al. (1981) labeled juvenile rhesus macaque dentitions with tetracycline to examine the rate of dentine secretion and the periodicity of incremental features (as well as the effects of heat stress and cortisone injections). Observations of some of their original material (loaned by Dr. David Gantt) during the present study showed that the tetracycline labels could not be seen in the enamel, nor was it possible to relate markers in dentine to enamel accentuations (Figure 3.19). The original study concluded that dentine formation proceeded along a gradient, but did not show any relationship to incremental lines, which were interpreted to be structural phenomena with no regular nature (but see Dean, 1995a). However, consideration of the treatment record and careful examination of their Figures 1 and 2 (p. 444 and 446), which are overviews of lines and labels in the dentine, show that 20 long-period increments can be counted between the (very marked) 6^{th} and (final) 14^{th} injections, which are 80 days apart. This suggests an average of four days per increment, which is consistent with the periodicity of long-period lines in six of the macaques in this study. Additionally, the regular spacing of these features represents further support for a consistent repeat interval (see their Figure 1, p. 444).

[9] See Dean (1995a, 1998b) and FitzGerald (1998) for a more complete listing of the work of Okada and colleagues, which is mainly published in Japanese.

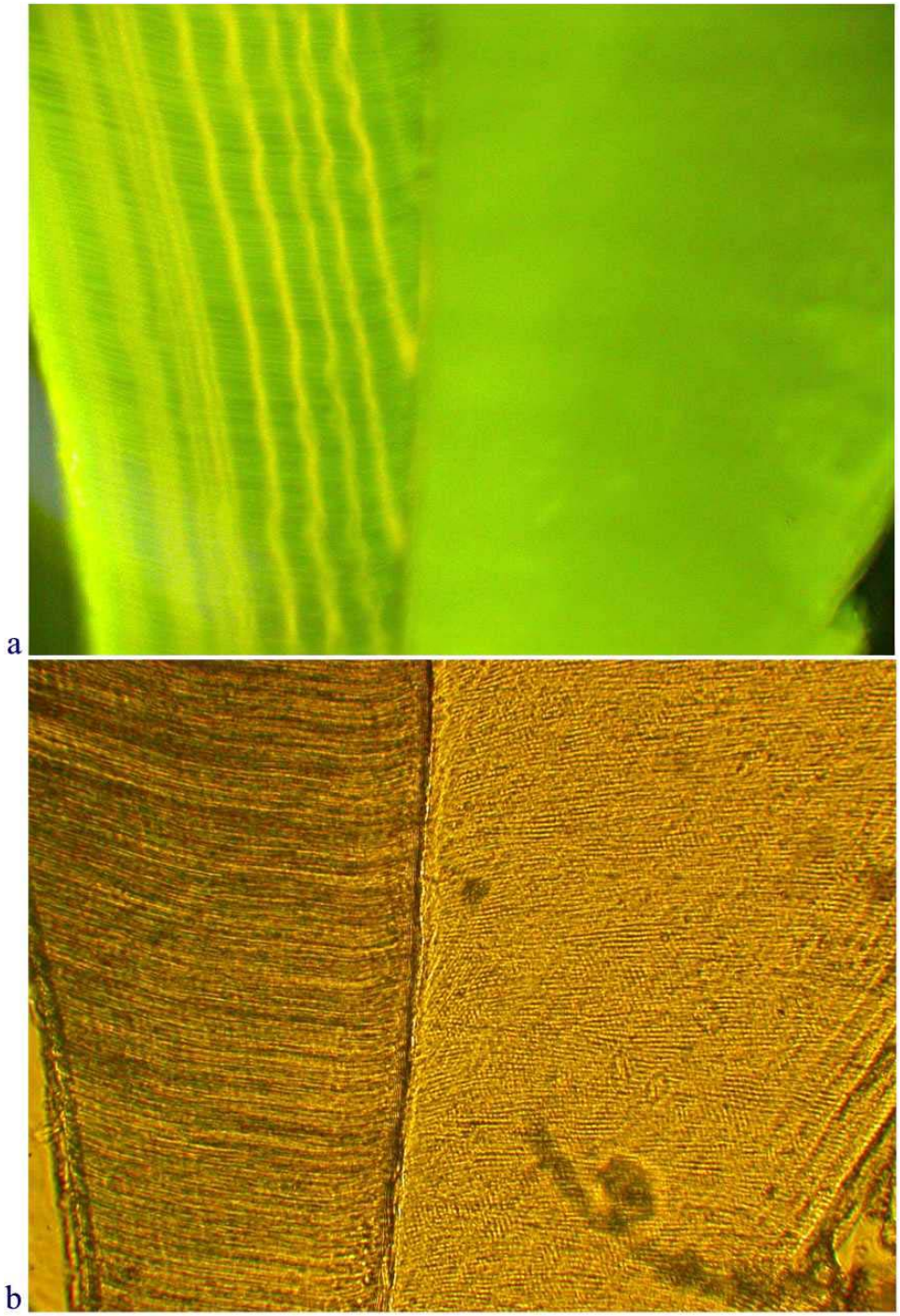


Figure 3.19 (a,b) a) Fluorescent and b) transmitted light images of tetracycline labels in the dentine (left side) of material reported on by Molnar et al. (1981) (slide 915-19-5). Note that dentine labels do not show a strong correspondence with accentuated lines in the enamel or dentine.

Recently, several studies have yielded important information about periodicity and developmental rates. Based on a suggestion from Alan Boyde, Bromage (1991) prepared histological sections of two developing maxillary first molars from two individuals that were part of Newell-Morris and Sirianni's (1982) bone labeling study.[10] Bromage identified accentuated lines in the enamel and related them to markers in the dentine (as in the current study), which provided a known-period interval over which the daily nature of features was demonstrated. He also demonstrated the daily nature of dentine increments between labels. Following this, Dean (1993, 1995a) conducted an experimental study on rates of dentine formation in labeled juvenile macaque dentitions and an opportunistic study on dental development in an unidentified modern human who had received numerous tetracycline doses over 12 years (Dean et al., 1993a; Dean and Scandrett, 1995, 1996). These represent some of the most convincing evidence of incremental periodicities, as well as the correspondence of short- and long-period features between enamel and dentine.

Incremental Features

Cross-striations

This study demonstrates that cross-striations have a 24-hour repeat interval. This is consistent with experimental studies by Okada (1943) and Bromage (1991). As noted in Chapter 2, the incremental nature of cross-striations has been controversial, with a few studies suggesting that these features are an artifact of sectioning with no true periodic nature, or that they are due to the optical phenomenon of cross-cut prisms viewed end on (e.g., Wilson and Schroff, 1970; Weber and Glick, 1975; Warshawsky and Bai, 1983; Warshawsky et al., 1984; Skinner and Anderson, 1991). Boyde (1989) listed several of the strongest counter-points to these criticisms: experimental evidence, regular density differences along prisms, their appearance in fractured preparations, and their appearance

[10] Based on injection records, Bromage's Specimen 1 and 2 may be CF 337 and 330. However, the early developmental stage of his Specimen 1 (M1) is surprising given that it was reported to be 455 days old at death, which is much longer than the expected crown formation time. See Chapter 4 for information on first molar development.

in confocal microscopy (or TSRLM). The density differences are best demonstrated with micromilled sections viewed in back-scattered electron SEM mode, which show regular light and dark bands (due to atomic number contrast) on prisms that may be regarded as topography-free surfaces. FitzGerald (1998) also reviewed the lines of evidence supporting the incremental nature of cross-striations, listing a number of studies that provided direct experimental evidence, credible function explanations, regularity of circaseptan intervals, and development inferences tested against known standards. Recent work by Antoine (2000) on crown formation time in known-aged material has also provided convincing evidence of the daily nature of cross-striations in human dentitions.

This study also suggests that features that are similar in appearance and spacing to 'cross-striations *sensu stricto*', yet are found in slightly oblique orientations to prisms, do appear to be equivalent structures (e.g., Fig 3.9a left circle). In this material there was variation in the orientation of these features, suggesting that the traditional 'perpendicular definition' of cross-striations is too narrow. These bands of acutely oriented cross-striations are common in other primates, such as the Miocene hominoid *G. freybergi*, and have previously complicated measurements of daily features. It is not clear how these are formed; the traditional model of cross-striation formation proposed by Boyde (1979) postulated that the secretory surface of the Tomes' process is perpendicular to the long axis of the forming prism, which registers the cross-striation parallel to the process (but see Risnes, 2001). The presence of cross-striations in what appear to be varying angles to the prism long axis may be explained by either prism decussation in an adjacent plane that is not apparent under transmitted light, or by variation in the morphology of the secretory face of the Tomes' process. Additional work on developing enamel surfaces may provide greater resolution of this issue.

Finally, previous criticisms that suggested cross-striations result from prisms cut end-on, knife chatter, or optical superimposition have been shown to be unsubstantiated through the use of multiple forms of microscopy (Dean, 2000; Smith et al, 2003a; Dean, 2004; Smith et al., 2004). Criticisms that postulated that these features do not show a temporal nature have been refuted with experimentally labeled material such as in the

present study. Given the consistent appearance and periodicity of cross-striations, it is clear the counts and measurements of this feature may be used to establish the periodicity of other incremental features, as well as the rate and duration of enamel formation.

Intradian lines

This study provides evidence that intradian lines are the result of a 12-hour rhythm in enamel secretion. Although this feature had been mentioned and debated in the literature since Gustafson's (1959) description (reviewed in Chapter 1, Smith et al., 2003a), the periodicity had yet to be demonstrated in enamel. As noted above, FitzGerald (1996) provided the most definitive statement about the existence and periodicity of intradian lines, reporting instances where two, or rarely three, intradian lines were found between cross-striations (FitzGerald, 1996: Figure 7-4A), which he interpreted to suggest that intradian lines have an eight- and/or 12-hour rhythm. As shown in Figure 3.9b, it is possible to image more than two subdivisions between cross-striations using high magnification, but these can be 'focused through' to yield pairs of cross-striations. The majority of evidence in this study supports the existence of a single 12-hour subdivision of cross-striations, as intradian lines generally appear to subdivide cross-striations into even halves, which implies that they occur as a factor of two. Further, images of intradian lines between daily laminations also show a single division (discussed in the following section). To date, there is no conclusive published evidence from either confocal or scanning electron microscopy to suggest more than one division per cross-striation. If it is the case that there is a periodicity in enamel of less than 12 hours, a plausible explanation may be that the structures are generally obscured by the width of the dark half of the cross-striation, in a similar manner to the phenomenon of lamination superimposition near the border of Retzius lines. However, this does not explain why three intradian lines per cross-striation are not evident under confocal or SEM, nor why they do not appear divide cross-striations into thirds more often under light microscopy.

As noted above, experimental work in dentine has confirmed the presence of sub-daily features (Kawasaki et al., 1977; Rosenberg and Simmons 1980a; Ohtsuka and

Shinoda 1995; Ohtsuka-Isoya et al., 2001), which will be referred to here as intradian lines. Work by Rosenberg and Simmons (1980a) and Ohtsuka and Shinoda (1995) showed two to three intradian lines formed in dentine per day. However, in the former study, daily and intradian lines were typically identified in different preparations, and were not illustrated together in the same section. These authors contrasted the ratio of the average sub-daily repeat interval (~10 μm) to the average daily repeat interval (~20 μm, sometimes up to 30 μm) to argue for the presence of an eight- or 12-hour rhythm, but did not illustrate sub-daily lines between labels or daily lines. Ohtsuka and Shinoda (1995) presented more convincing evidence for intradian lines using rats injected with lead salt markers. They showed several images of transverse thin sections where more than two and less than three intradian lines were formed per day (14 lines/5 days, 7 lines/3 days, 14 lines/5 days). They also contrasted the average 16 - 24 μm daily line spacing with the sub-daily 6 - 8 μm spacing, which they regarded as additional evidence for an eight-hour rhythm.[11] Ohtsuka-Isoya et al. (2001) recently illustrated intradian lines that showed a 12-hour periodicity, but did not report finding lines with an eight-hour periodicity (Ohtsuka-Isoya et al., 2001: Figure 3b, p. R1366).

Qualitative differences in the appearance of intradian lines in this study may be due to differences in the degree of local mineralization. In certain sections, it was noted that the immature outer enamel shows a higher frequency of intradian lines and a lower frequency of cross-striations than fully formed enamel. As noted in Chapter 1, the developing outer layer is less well mineralized than the earlier-formed inner layer of enamel. Traditionally, the illustration of intradian lines in the enamel of crown complete teeth is often accomplished using hypomineralized areas (e.g., Gustafson and Gustafson, 1967: Figure 6, p. 85; Smith et al., 2003a: Figure 5, p. 300). An additional area reported to show a high frequency of intradian lines is the outer cuspal enamel in fossilized or archaeological material (Smith et al., 2003a, 2004a; Antoine, pers. com.), which may be prone to postmortem demineralization (discussed in Chapter 1). Further evidence for a

[11] However, Dean (1998b) provided evidence that demineralized sections (such as the preparations of Kawasaki et al., 1980 and Ohtsuka and Shinoda, 1995) may show tissue shrinkage, and cautioned against accepting absolute values obtained from these types of preparations.

relationship between the visibility of intradian lines and degree of mineralization comes from the use of acid-etching or demineralization to enhance the visibility of incremental features (e.g., Hinrichsen and Engel, 1966; Boyde et al., 1978; Boyde, 1989; Li and Risnes, 2004). A number of figures published on similarly prepared material illustrate lines more closely spaced than typical cross-striations (e.g., Whittaker, 1982: Figure 7, p. 392; Martin, 1983: Figure 5.4e, p. 332; Berkovitz et al., 1992: Figure 216, p. 116). If intradian lines appear more commonly in areas that are less mineralized, or have undergone demineralization, it is likely that they could be defined by a change in either (or both) chemical or structural composition, which is less evident in fully mineralized enamel.

Retzius lines

The current study provides no evidence to reject the hypothesis that Retzius lines have a consistent periodicity within an individual, shown here to be four days per line in six of the individuals examined.[12] If the periodicity was not consistent within a tooth, it would be very difficult to demonstrate this conclusively due to a number of imaging issues (e.g., resolution limitations, optical artifacts, section obliquity, prism curvature, Retzius line convergence, presence of irregular accentuated lines). It is theoretically possible that the periodicity may vary by +/- 1 day, where the mean equals the modal value and shows low variance. However, given the meticulous study of FitzGerald (1996), which determined the periodicity in several areas of the same tooth and individual, as well as the observations of several other accomplished researchers, it is unlikely that Retzius line periodicity is variable. The most rigorous test of this theory would necessarily involve counting every cross-striation between each Retzius line from the first to the last imbricational line in every tooth from an individual dentition. However, it is highly unlikely that this could be accomplished given variation in the quality of cross-striations and Retzius lines within a section, as well as among sections.

[12] This study does suggest that the periodicity is difficult to demonstrate conclusively and consistently, as discussed in Chapter 2.

Laminations

This study suggests that laminations are the result of a circadian rhythm in enamel production. Bromage (1991) also inadvertently provided similar evidence by diagramming and counting a series of laminations between labels that corresponded to the number of days of formation (Bromage, 1991: Figures 4 and 6, p. 208 and 210). He did not distinguish cross-striations from laminations, although each type of structure was distinct from one another in his Figure 4. Additional evidence for their daily nature comes from observations in the current study that cross-striations and laminations may be seen in register with one another, also implied by Whittaker (1982), Risnes (1998), and Li and Risnes (2004). In addition, laminations may be counted between accentuations at the EDJ, traced to the surface, and related to Retzius lines in a manner that is equivalent to the corresponding number of Retzius lines multiplied by their periodicity.

Despite this finding, laminations should not be primarily used to determine the periodicity of Retzius lines, particularly near the enamel surface. It is suggested that the common appearance of Retzius lines as broad, dark bands are often the result of the optical superimposition of laminations. When images are optically focused through, the 'Retzius line' may shift position and split into two lines. This problem may result in underestimation of Retzius line periodicity when laminations are counted as a proxy for cross-striations. This is likely the reason that counts of laminations between Retzius lines in two Miocene hominoids did not agree with counts of cross-striations (Smith et al., 2003a, 2004). A partial solution to the problem of interference (or superimposition) of features in light microscopy involves determining the periodicity in several areas of a tooth by 'focusing through' Retzius lines until the interval to be counted is well defined from the upper and lower bounding Retzius lines.

The geometric relationship between laminations and Retzius lines in enamel appears to be similar to the relationship between von Ebner's lines and Andresen lines in dentine. In both instances, the daily feature runs parallel to the long-period feature. Given the potential for interference and the difficulty of accurately resolving series of daily lines in dentine, enamel cross-striations are generally used to determine the periodicity of long-

period lines in both tissues (Dean et al., 1993a; Dean and Scandrett, 1996).[13] The relationship between cross-striations and Retzius lines is ideal, as cross-striations are oriented at an angle to the Retzius line (excluding the case of staircase Retzius lines), and may be more easily distinguished from the optical effects of Retzius line superimposition than laminations. Thus, cross-striations are the better choice for the determination of the periodicity of Retzius lines.

The finding of daily features parallel to Retzius lines requires an explicit definition in order to distinguish one feature from another. As noted in Chapter 1, Dean (1989) suggested that Retzius lines should be defined as only those features that meet the surface of the tooth and form perikymata. It is suggested here that laminations should be contrasted with Retzius lines, as the latter are generally more defined under light microscopy, terminate as perikymata, extend farther toward the EDJ, and show a consistent number of cross-striations between successive pairs. Laminations should be defined as closely spaced features that show a similar repeat interval to nearby cross-striations, run parallel with the developing enamel front, and do not terminate as perikymata at the tooth surface. Caution should be used when distinguishing parallel features from one another, particularly in aprismatic sub-surface enamel (as shown in Figure 3.18a). As noted in Chapter 1 and in Smith et al. (2003a, 2004), the development of laminations in specific regions may be related to aprismatic enamel production or to a certain type of prism packing pattern (i.e., Pattern 1), but this requires further study.[14] Finally, laminations may also show fine bands between them, which appear similar to intradian lines seen between cross-striations. This has also been seen in a number of other taxa, including several Miocene hominoids (reviewed in Chapter 1). The developmental basis of these sub-daily features between cross-striations or laminations is unknown.

[13] Dean (1998b) published some of the most convincing images of von Ebner's lines between Andresen lines in dentine, but these were not as clear as numerous images of cross-striations between Retzius lines.

[14] Like aprismatic enamel, this class of feature may be more common in certain groups of primates, although the study of this is beyond the scope of this project.

Extension rate

The results of the empirical test of Shellis' extension rate model suggest that this model should not be applied to deciduous teeth. Shellis (1998) suggested that faster-forming teeth may be more difficult to assess with accuracy, which was confirmed in the current study. The deciduous canine showed the greatest difference, followed by the deciduous second molar, and lastly the permanent first molar. It is likely that several factors affect the accuracy of this formula. Shellis (1998) noted that it is more difficult to measure small angles with accuracy, which are found in fast forming teeth. In addition, it was sometimes more difficult to find clearly defined daily lines in the deciduous teeth, which may have introduced additional error into the formula. As reviewed in Chapter 2, Shellis (1998) applied his extension rate formula to determine crown formation time in a number of primates. The accuracy of this formula for determination of crown formation time will be assessed in the following chapter.

Biological Clocks

The study of biological clocks is a multidisciplinary field with a vast body of literature, including several dedicated journals. Recent advances in molecular biology have facilitated a dramatic increase in the number of studies on circadian rhythms in particular. The intent of this review is to provide a general summary of the field and to highlight certain aspects that may shed light on the periodicities seen in dental hard tissues, most specifically the circadian, intradian, and circaseptan periodicities seen in enamel and dentine development. Lunar and annual cycles are briefly mentioned, but are not considered in detail.

Biological rhythms have been identified in both plants and animals, including single-cell organisms and fungi, and appear to be ubiquitous in eukaryotes (Hastings, 1997). Rhythm periodicities may range from a few hours to a yearly interval. The control of rhythm is related to intrinsic (endogenous) factors (formally referred to as *biological clocks*), as well as extrinsic (exogenous) factors. These factors may take on different roles

by entraining/synchronizing a rhythm (known as a *Zeitgeber* or "time-giver"), or by maintaining a rhythm (Halberg et al., 1959). The study of intrinsic factors is a dynamic field, largely due to breakthroughs in molecular biology, which have shed light on the genetic component of rhythm (reviewed below). In addition, studies of hormonal, neurological, and behavioral aspects of biological rhythms have begun to provide information on the signaling processes and anatomical basis of rhythmic control (reviewed in Hastings, 1997). Extrinsic factors include commonly noted phenomena such as the environmental temperature cycle, light/dark cycle, rotation of the earth, and tidal rhythm/lunar phase, which may often work in combination with one another (Scheving and Pauly, 1974; Scrutton, 1978). When exogenous influences are experimentally removed, rhythms often shift slightly, and are then termed 'free-running'. This implies that the maintenance of rhythms involves an interplay between intrinsic and extrinsic factors. An understanding of biological clocks, particularly in mammals, has been complicated by the complexity of rhythms from the cellular to organismal level. Pittendrigh (1960) noted that "the organism comprises a population of quasi-autonomous oscillatory systems" (p.165), which may have implications for a system that demonstrates multiple rhythms such as dental hard tissue formation. Roenneberg and Merrow (2001) recently reviewed this topic, describing networked chains of oscillators that may feedback or modulate this network within an organism, which will be discussed in greater detail below.

Structural and physiological aspects

Common examples of structural evidence for biological rhythms include annual rings in the trunks of trees, or the daily or annual ridges in shells of marine organisms. Structural evidence in dental enamel includes short-period cross-striations and intradian lines and long-period Retzius lines. These features are known to have dentine analogues, and an annual ring is present in cementum as well. Scrutton (1978) suggested that any organism with 'preservable hard parts' formed by a 'continually additive mode of growth' may provide evidence of rhythms. He reviewed studies that suggested a range of

sub-daily to annual rhythms in marine organisms, including sub-daily increments in bivalves; possibly related to tidal rhythms and to the opening and closing of the shell during a 24-hour period. Neville (1967) and Scrutton (1978) reviewed structural examples of daily layers such as in insect skeletons, caused by regular changes in chitin orientation; layers in crayfish gastroliths, caused by changes in calcium-salt deposition; stripes in cuttlefish shells; layers in clam shells, caused by changes in protein concentration; layers in fossil corals, possibly due to changes in calcification at the growing edge of the calyx or epitheca; layers in cotton fibers, possibly due to changes in microfibril orientation; starch grain layers, possibly due to crystallization differences in the basic molecular organization; and several types of daily growth layers in fungi.[15] Neville (1967) and Dean (1987a) also reviewed studies of fossil corals that showed high numbers of daily striations between annual layers, which were suggested to represent the greater number of days per calendar year in the past (due to shorter day lengths) (see also Scrutton [1978] for a review of paleontological applications). Simmons (1992) reviewed studies on the circadian nature of bone and cartilage formation.

The physiological basis of biological rhythms has been extensively studied; both the eye and two specific regions of the forebrain (diencephalon) are considered to be important for the production and maintenance of these rhythms: the pineal gland and the suprachiasmatic nucleus (SCN). Hastings (1997) reviewed evidence that suggests that there is a photoreceptive clock that is based in the eye, which produces melatonin with a circadian rhythm. Numerous studies have shown that this is not the only source of rhythm maintenance, as animals that have been experimentally blinded continue to demonstrate circadian rhythms. The pineal gland is located near the center of the brain, posterior to the hypothalamus (which houses the SCN), and has been frequently noted for its potential role in maintaining circadian rhythms (reviewed in Hastings, 1997; Haus and Touitou, 1997). Historically, this gland has been regarded as sharing some similarities with the eye, which is due to its neurological connections that respond to the perception of light, as well as some analogous cellular components (Young, 1973; Hastings, 1997).

[15] Scrutton's review included descriptions and illustrations of circadian increments in bivalves that appear to be very similar to daily lines in enamel and dentine (e.g., Scrutton, 1978: Figure 4, p. 163).

The main secretory product of the pineal gland is melatonin, which shows a circadian (and possibly circaseptan) production rhythm during the night, and is known to effect the production of a number of hormones as well as the maintenance of several physiological cycles (Vollrath et al., 1975; Haus and Touitou, 1997). Haus and Touitou (1997) noted that melatonin indirectly controls a number of circadian rhythms by interacting with serotonin and many endocrine products, including growth hormone. Hastings (1997) described melatonin production as 'an endocrine representation of darkness.' Melatonin production is known to peak in early development and decline during the life of an individual. It appears to have a positive effect on slowing the aging process, due in part to its role as a free radical scavenger (reviewed in Haus and Touitou, 1997). The cells of the pineal gland that produce melatonin continue to do so even in isolation in some vertebrates, suggesting a parallelism with unicellular organisms (Hastings, 1997). Hastings (1997) reviewed studies that have shown that the mammalian pineal gland is ultimately controlled by the SCN; in contrast to lower vertebrates, this gland does not contain an endogenous oscillator.

Experimental studies have demonstrated that the mammalian control center of biological rhythms is in the SCN within the hypothalamus (e.g., Reppert, 1995; Jin et al., 1999; Yamazaki et al., 2000; Ohtsuka-Isoya et al., 2001). Hastings (1997) reviewed several decades of studies that have led to this conclusion, including SCN transplantation studies that have proven that the donor SCN is actively determining the periodicity in the recipient. The SCN may entrain the organism via direct nerve inputs from the retina (retinohypothalamic tract). Experimental evidence suggests that changing light-dark cycles may trigger the production of specific proteins that may induce a chemical response in the hypothalamus, which releases hormones via the pituitary that affect a number of physiological activities. Additional evidence has shown that behavior may also induce a shift in the phase of the SCN though serotonin input, which is produced as a result of stimulation. Ohtsuka-Isoya et al. (2001) have presented convincing evidence that the SCN is responsible for the production of daily lines in dentine, as rats in which the SCN is fully or partially lesioned experience respective permanent or temporary loss of

circadian dentine increments. These authors noted that, given that there is no direct neurological connection between the SCN and the odontoblasts, it is likely that there is a hormonal cue that triggers or maintains the production of daily increments. Several hormones that target odontoblasts include corticosterone, growth hormone, thyroxines, and parathyroid hormone, which may play a role in the network from the SCN to the odontoblasts (reviewed in Chapter 1, Ohtsuka-Isoya et al., 2001). Future work will hopefully examine specific hormonal influences on dental hard tissue formation.

The study of the genetic basis of biological rhythms has its roots in Konopka and Benzer's (1971) discovery of 'clock mutants' of *Drosophila*, which included flies that cycled with 19-hour, 28-hour, and arrhythmic cycles, resulting in the identification of the first gene responsible for the clock (the period gene *per*). Young (2000) reviewed subsequent developments, including the isolation of additional genes from mutants of *Drosophila*, *Neurospora*, and mammals including mice and hamsters. These genes affect the production of certain proteins, which may interact with one another and influence the SCN, which in turn sends out information to the rest of the body. Several of these genes products are affected by light, which may affect the formation of protein complexes that act on the SCN, and may ultimately autoregulate the expression of the original gene as a feedback loop (Young, 2000). Lowrey et al. (2000) reported on a lineage of hamsters that have mutant circadian clock genes, which has allowed additional insight into the mechanism of genetic control. It appears that primary gene products produced in the morning turn on secondary genes, which produce other proteins that build up to certain levels outside the cell. Once the concentrations reach a certain point, the proteins pair up and return to the nucleus in the evening, where they turn off the primary genes that make the initial morning proteins. Once these inhibitory products are degraded in the cell, the primary genes resume protein production and begin the cycle again. These processes appear to be conserved across animals and appear to extend to plants as well. (Reviews of the molecular biology of biological clocks may be found in Roenneberg and Merrow, 1997; Dunlap, 1999; Zordon et al., 2000; Young, 2000; Morre et al., 2002; and Panda et al., 2002).

Development of biological rhythms

Simmons (1992) and Reppert (1995) recently reviewed experimental work on the development of circadian rhythms in rodents. Reppert (1995) reported that the fetus is entrained by a unidirectional signal from the mother to the fetal SCN. In rodents, this region of the brain begins to develop at approximately 13 - 16 post-conception days (gestation length ~22 days), which shows synapses by day 19, and circadian rhythms by approximately 21 days. Experimental manipulations have shown that prenatal circadian rhythms do not require maternal control for timekeeping, although there appear to be several redundant maternal signals that naturally entrain the fetus to a light-dark cycle, including food consumption and melatonin levels. Reppert (1995) reviewed studies that examined the relationship between melatonin and the SCN, noting that there appear to be receptors for melatonin in the fetal SCN that may modulate the release of dopamine, which ultimately leads to the production of a phase shift. Hastings (1997) reviewed studies that suggest that in the fetus, dopamine and melatonin may complement one another, maintaining the respective circadian day and night. However, the dopamine receptors are lost shortly after birth, which is probably related to the functional replacement of light as the entraining signal. He also noted that although melatonin has the potential to regulate the SCN beyond the fetal period, the SCN is minimally sensitive to it during its typical nocturnal production.

Ohtsuka and Shinoda (1995) demonstrated that daily lines in rat dentine become apparent two to three weeks after birth, the same time other circadian rhythms including sleeping-waking, pituitary-adrenal, and pineal-melatonin cycles become apparent. However, they also noted the presence of a sub-daily increment with an 8 - 12-hour periodicity in the first week after birth, which was dominant before circadian increments and continued after their appearance. This study also cited unpublished work noting that sub-daily increments have been found in rabbit dentine formed prior to birth. There is also evidence of incremental lines in primate prenatal enamel from both deciduous teeth (FitzGerald et al., 1999; Smith et al., 2002) and permanent teeth (Dirks, 1998; Reid et al., 1998a). However, given the generally poor quality of increments in the first-formed

enamel, and the difficulty of labeling enamel, it has been difficult to determine which type of increment appears first in primates. The present study suggests that laminations may be the first prominent incremental feature in macaque enamel, as they are common in the first-formed enamel over the dentine horn. However, this would benefit from additional study.

Several studies have examined the developmental basis of biological rhythms in humans. Tenreiro et al. (1991) examined the records of a number of premature babies (24 - 29 weeks post-conception), and found that daily and sub-daily physiological rhythms were variably present, which did not appear to stabilize during the 6 - 17 weeks of the study. They related this to the poor development of the SCN prior to birth, and suggested that the oscillating rhythms may couple through time, come into phase with one another, and produce a dominant frequency (beyond the period of development sampled in this study). The pioneering work of Hellbrügge (1960) examined the development of the circadian heart rate rhythm in almost 100 infants and the circadian rhythm in urine excretion in 50 infants. He found that circadian cycles of heart rate appeared during the sixth week after birth and urine excretion in the second to third weeks after birth, both of which continued to become more marked (greater oscillations) with advancing age. He also reviewed studies that demonstrated that body temperature begins to show a circadian rhythm after three weeks, sleep cycles appear between the third and sixth weeks, and electrical skin resistance (related to sweat production) begins to cycle during the first week of life. All of these became more marked with age. Hellbrügge (1960) also showed that relative to full term infants, premature infants developed circadian rhythms in sleep patterns and heart rate later, which he interpreted to suggest that exogenous environmental factors are less important in rhythmic development than developmental stage. He concluded that circadian (monophasic) rhythms originate from free-running (polyphasic) rhythms that have been entrained.

Circadian rhythms

Pittendrigh (1960) listed several defining aspects of circadian rhythms: 1) the free running cycle is an approximation of the period of the earth's rotation; 2) they are ubiquitous, endogenous, and innate in living systems; 3) they have self-sustaining oscillations; 4) they appear at different levels of cellular and organismal organization; 5) they are largely temperature independent, and 6) they are generally light-intensity dependent. Mammalian circadian rhythms have been documented in many biological activities, including sleep cycles, vital signs, locomotion, body temperature, metabolism, blood sugar, enzymes, cellular organization, blood cell count, adrenal activity, RNA and DNA synthesis, cell division, and psychological state of mind (Halberg et al., 1959; Scheving and Pauly, 1974). Scheving and Pauly (1974) reviewed a number of studies that suggested susceptibility to drugs or poisons may also fluctuate with a circadian rhythm (see also Simmons, 1992). These systems may show phase differences between species, from the obvious example of activity patterns to rhythms of temperature and blood cell counts (Halberg et al., 1959). Numerous studies have examined the influence of lighting conditions on rhythms, including subjecting subjects to constant light and/or constant dark conditions (e.g., Lobban, 1960; Pittendrigh, 1960; Shinoda, 1984; Ohtsuka-Isoya et al., 2001). Circadian rhythms frequently maintain their periodicity for some time following the beginning of the experimental manipulations, eventually showing a slight deviation or dampening after a month or more. Humans may readjust to a circadian cycle when external cues are removed, but variation is apparent within individuals and within specific cycles (Lobban, 1960; Campbell, 1996). Other studies examined rhythms in blind animals, and showed that light perception is not critical to the development or maintenance of circadian rhythms (Halberg et al., 1959).

Newman and Poole (1974) noted that ameloblasts must have a daily cycle of activity, causing enamel quality to change on a regular basis, which results in cross-striations. They suggested that this rhythm may be endogenous, and that a number of physiological factors show circadian oscillations that may relate to enamel formation. Some of these factors may include levels of plasma phosphate, amino acids, blood sugars,

enzymes, electrolytes, and hormones. Gasser et al. (1972) provided evidence to suggest that rat inner enamel epithelial cells show a circadian rhythm of cellular division, although they did not relate this to incremental feature production specifically. Boyde (1989) hypothesized that circadian rhythms in ameloblast metabolism may be responsible for changes in pCO2, which lead to regular differences in the mineral composition of enamel that manifest as cross-striations. The majority of studies on circadian rhythms in secretory cells and hard tissue development have been conducted on odontoblasts. Ohtsuka et al. (1998) demonstrated that there is a circadian rhythm in collagen synthesis and secretory activity in odontoblasts, which they suggest may be the proximate mechanism of incremental line formation in dentine. Several studies have also shown a circadian rhythm in radiolabel (^{3}H-thymidine or ^{3}H-proline) uptake in dental tissues (mainly odontoblasts) (Gasser et al., 1972; Kiely and Domm, 1973; Ohtsuka et al., 1998). Due to the similarities between incremental features in enamel and dentine, it is likely that these results may be extended to the process of enamel formation, but this area will benefit from future work.

Other rhythms

Rosenberg and Simmons (1980a) reviewed a number of well-documented sub-daily physiological rhythms, including REM sleep, heart rate, body temperature, and many hormone concentrations (including growth hormone). As noted above, experimental work has also demonstrated the presence of two to three intradian increments per day in dentine, possibly suggesting that fluctuating hormone levels may play a role in the development of these lines (Rosenberg and Simmons, 1980a; Ohtsuka and Shinoda, 1995; Ohtsuka-Isoya et al., 2001). Rosenberg and Simmons (1980a) reported that fluctuating levels of calcium and sulfur in the dentine were found to correlate with incremental features, although these levels were not always in phase and demonstrated complex relationships. Ultimately, they suggested that intradian structural features were the result of hormonal and/or metabolic cycling. Additionally, Miani and

Miani (1971) demonstrated that feeding cycles may influence the phase of dentine production in dogs.

Neville (1967) and Dean (1987a) reviewed several examples of circaseptan periodicities, including studies that suggested approximately weekly rhythms in the formation of cuttlefish 'bones', dental 'lamella', and periosteal bone. Pöllmann (1984) presented evidence and reviewed studies that suggested that in humans, wounds heal with a circaseptan periodicity, and kidney transplants tend to show circaseptan peaks of rejection rates. Haus and Touitou (1997) reviewed work that suggested that human infants show circaseptan rhythms in cardiovascular activity. Vollrath et al. (1975) demonstrated that the activity of the pineal gland in rodents demonstrated a circaseptan periodicity in melatonin production.

Examples of potential circannual rhythms include striations on clam shells, fossil corals (which also show a lunar periodicity), tree rings, sheep's horns, cementum annulations, and seasonal growth spurts in bone formation (Neville, 1967; Scrutton, 1978; Kay et al. 1984; Simmons, 1992). Campbell (1996) reviewed circannual behaviors such as breeding, migration, and hibernation that appear to have internal and external cues. He gives an example of fat deposition in ground squirrels, which continues in preparation for hibernation even when individuals are kept under constant light conditions. Several researchers have examined the effects of hibernation on dental development, which appears to produce a 'hibernation mark' in the dentine similar to an annual ring in cementum (Klevezal and Mina, 1990; Klevezal, 1996). It does not appear that dental development ceases during this period, although it is possible that the rate of formation and/or eruption decreases (Rinaldi, 1999) (discussed further in Chapter 1).

A few studies have suggested a potentially adaptive component of biological clocks. Reppert (1995) suggested that maternal entrainment might provide coordination with the environment, which would facilitate efficient transition to a temporal niche and promote survival. Neville (1967) suggested that, due to the necessity of synchronizing with the fluctuating annual day length, as well as the changing day-length through geological periods, a flexible, light entrained clock would prove more adaptive than an

inflexible system. Cloudsley-Thompson (1960) reviewed the relationships between rhythms and activity cycles, ecological factors, and life history, suggesting that it is difficult to identify the adaptive function of circadian rhythms due to the complexity of interactions, as well as the dissociation between ecological and environmental factors which entrain rhythms.

Interplay between rhythms

An understanding of central and peripheral oscillators may explain the periodicity of incremental structures in dental tissues, from the level of intradian lines to cementum annulations. Roenneberg and Morse (1993) documented the presence of two circadian oscillators in one cell (of a unicellular organism). They showed that a minimum of two separate oscillators control circadian rhythms relating to bioluminescence and locomotor behaviors, which has potentially dramatic implications for the regulation and development of rhythms in multicellular organisms (see also Roenneberg and Merrow, 2001). Yamazaki et al. (2000) hypothesized that a central circadian pacemaker in the SCN maintains phase control of circadian oscillators in the periphery, and that this control may be temporarily lost during large shifts in the environmental light cycle. Their work showed that certain systems such as the lungs, muscles, and liver have their own intrinsic rhythms; these organs require a longer period of time to readjust their cycle than does the central oscillator. In one of the few studies on the physiological basis of periodicities in dental tissues, Ohtsuka and Shinoda (1995) reported the presence of two short-period lines in rat dentine that developed at different times after birth. The authors also suggested that the co-existence of these two lines may result from two independent oscillatory mechanisms. This was further supported by the recent study of Ohtsuka-Isoya et al. (2001), who found that SCN obliteration correlated with cessation of daily lines, but not intradian lines in rodent dentine.

As reviewed in Chapter 1, Newman and Poole (1974, 1993) postulated that the existence of two circadian rhythms in enamel production that may account for the relationship between Retzius lines and cross-striations. They suggested that a precise 24-

hour rhythm and a free-running circadian rhythm may run in tandem, regularly producing a Retzius line when the two cycles were most offset from one another. This theory of multiple physiological circadian cycles is supported by the experimental work of Roenneberg and Morse (1993). The latter study noted 'phase jumps' roughly every seven days, which occurred when a faster circadian rhythm corrected itself relative to a slower rhythm. They suggested that separate but coupled (approximately circadian) oscillators may produce a rhythm that appears to be controlled by a seven-day clock. Additionally, Roenneberg and Morse (1993) noted that an infradian (greater than a day) rhythm operated in this system, which provides support for Newman and Poole's (1974) theory of a 24-hour and a 27-hour rhythmic interplay. FitzGerald (1996) explained Dean's (unpublished) theory that circaseptan rhythms may result from interactions between the complicated intradian and circadian rhythms.[16] Given the diversity and complexity of cellular-levels rhythms (reviewed in Roenneberg and Merrow, 2001), it is entirely possible that multiple, independent rhythms are responsible for the production of different incremental features within a developmental system. Additional work is needed to determine if and how interactions among incremental features relate to their periodicity and structural manifestations.

Summary and Conclusions

1) Vital labeling of dental hard tissues is a useful technique for examination of the periodicity of features of the enamel and dentine microstructure.
2) Cross-striations are the result of a daily rhythm in enamel secretion.
3) Intradian lines appear to be the result of a 12-hour rhythm in enamel secretion.
4) Retzius lines appear to have a regular periodicity within individual dentitions. This periodicity has been shown to be 4 days in six individual macaques.

[16] FitzGerald (1996) also suggested that long-period line periodicity may be explained by chaos theory, a complicated non-linear theory of mathematics, which he suggested may relate to the interaction of multiple physiological rhythms that result in a long-period rhythm that is a 'chaotic strange attractor'.

5) Laminations are the result of a daily rhythm in enamel secretion. They also appear to show 12-hour sub-divisions. These features should not be used to determine the periodicity of Retzius lines, as they may be obscured by the superimposition of Retzius lines, which run parallel, and may be formed in a similar manner.
6) Identification of the periodicity of intradian lines and laminations within multiple individuals represents the first empirical evidence of these incremental features in primates. These results will allow important insight into features that frequently complicate precise measurements of secretion rate and the periodicity of Retzius lines, key components for the determination of total crown formation time.
7) The extension rate formula proposed by Shellis (1984a,b) should not be used in deciduous or fast-forming teeth. Limited data suggest that it may yield fairly accurate estimations of local extension rate in permanent teeth.
8) Research on biological clocks within mammals demonstrates a large number of rhythmic cycles, ranging from a few hours to a year in frequency. Structural evidence for these rhythms is common across both invertebrates and vertebrates. It is possible that long-period incremental features in dental tissues result from interactions between short-period rhythms. However, this does not explain the known range of periodicities among primates or within humans. Additional genetic, neurological, and hormonal work may provide more insight into the physiological and structural basis of incremental feature formation.

Chapter 4: Testing Histological Assessment of Dental Development

Introduction

The periodic nature of short- and long-period incremental features in enamel and dentine has been well established (Chapter 3; FitzGerald, 1998). However, few studies have tested the histological methods used to determine crown formation time (Stringer et al., 1990; Dean, 1998a; Antoine, 2000; Smith et al., 2004). Because it is very rare for histological material to preserve a complete, clearly visible succession of increments from the beginning to the end of enamel formation, some estimation is generally required. Two main types of methods have been proposed for crown formation time reconstruction: 1) assessment of the cuspal and imbricational components of appositional growth from counts and measurements of short- and long-period increments, and 2) estimation of the duration of extension from measurements of the short-period features, developing enamel front, and prisms at the enamel dentine junction (EDJ). Appositional growth occurs as ameloblasts progressively secrete enamel from the EDJ to the surface of the tooth, while extension occurs by the activation of secretory ameloblasts along the EDJ from the cuspal portion to the cervix.

The first method requires histological sections from teeth that are not missing enamel, which should ideally be derived from the plane of section preserving the tips of the dentine horns (non-oblique section). The second method may be used on partially worn material, as long as the entire EDJ is preserved. The effect of obliquity on this method is unknown. There are three independent ways of determining the accuracy and/or precision of these methods: comparison to a known period of formation in teeth that were developing at the time of death, comparison to enamel in other cusps or teeth forming simultaneously, or comparison to a corresponding amount of dentine formation. Of these, only the first method may yield a direct assessment of accuracy. It is proposed here that studies of incremental development would benefit from an empirical test of these methods, as well as an examination of the effects of obliquity, which may serve to counter recent criticisms of the validity of results derived from these types of analyses (e.g., Macho et al., 2003).

Specific Aims

The aims of this chapter are to test: 1) the accuracy of the 'standard methodology' for determining the crown formation time (recently reported in Smith et al., 2003a, 2004) and age at death, and 2) the accuracy of a method of crown formation time estimation proposed by Shellis (1984a,b, 1998). This may provide justification for the choice of methods employed in subsequent work, including the following chapter, where a large sample of histological sections of worn and unworn chimpanzee teeth are examined, and crown formation time is determined when possible. The first aim is accomplished using first molars from five individual pig-tailed macaques (*Macaca nemestrina*). Three aspects of dental development are quantified to assess crown formation time and age at death: cuspal formation time, imbricational formation time, and duration of root formation prior to death. Estimated age at death is then compared with known age at death to determine the degree of methodological accuracy. Following this, the second aim is to employ adjusted crown formation times to assess the accuracy of Shellis' method. In addition, a new potential method of crown formation time estimation involving the relationship between crown formation time and EDJ length is considered. The results of this study may permit assessment of the nature and degree of error in histological analyses of dental development, as well as greater confidence in estimates of crown formation time and/or age at death in material that is of unknown age.

Background

Crown formation time determination

Crown formation time is the product of cuspal and imbricational (lateral and cervical) enamel formation, which are generally determined individually and then combined to yield a total time of formation (reviewed in Chapter 2). Assessment of cuspal enamel formation, which occurs as ameloblasts move in a three-dimensional fashion away from the dentine horn, frequently depends on identification and counts or measurements of daily cross-striations. Work on *Graecopithecus freybergi* suggested that calculations of daily secretion rate (DSR) and cuspal formation time may be affected by the unintentional inclusion of intradian lines, causing rates to be underestimated and

cuspal formation time to be overestimated when intradian lines are confused with cross-striations (Smith et al., 2004). In addition, this study showed that section plane obliquity may cause an overestimation of formation time derived from cuspal enamel, due to artificially inflated cuspal thickness, and an underestimation of time from axial dentine due to the underestimate of dentine in the dentine horn (see also Martin, 1983). Thus, it is postulated that estimates of cuspal crown formation time based on counts or measurements of cross-striations, as well as estimates based on knowledge of the cuspal enamel thickness, may sometimes represent overestimates. The degree of overestimation may be related to the clarity of daily features as well as the degree of section plane obliquity. Imbricational enamel formation is typically quantified by counting Retzius lines, or the external manifestations of these lines known as perikymata, and then by multiplying this by the Retzius line periodicity (either determined from thin sections or estimated). Provided that both a complete series of Retzius lines (or perikymata) and an area of cross-striations facilitating periodicity determination are clear, imbricational enamel formation is typically easier to determine than cuspal formation time. If these conditions are satisfied, it is predicted to be highly accurate.

Macho et al. (2003) recently suggested that, due to the three-dimensional course of prisms from the EDJ to the surface, measurements of cross-striations may not accurately represent the true growth processes of the tooth, and estimates of crown formation time derived from counts and measurement of cross-striations may be invalid. They concluded that "several aspects of dental growth studies are problematic for heuristic reasons; such investigations give the impression that the results are empirically derived, the histological methods are well-proven, and the sectioning of fossil teeth is a worthwhile pursuit. Based on our work, none of these seems to be the case" (p. 89). Although the conclusions of this study are problematic for a number of reasons (reviewed in Dean, 2004), the sentiment of skepticism regarding histological analyses is worth noting from a historical perspective. Numerous papers criticizing the basis of incremental development followed Boyde's (1963) seminal assessment of dental development in human remains over 40 years ago (reviewed in Chapters 2 and 3). By 1989, Boyde (1989) noted that the original criticisms had been sufficiently refuted with evidence from several different forms of microscopy. Additional work by FitzGerald (1996) and

Antoine (2000) on the nature of short- and long-period increments carefully reaffirmed the theoretical basis of histological analyses. The recent publication of another attack (Macho et al., 2003) on these techniques underscores the value of additional empirical work, as well as the need for greater awareness of previous work within the anthropological community.

Root formation and age at death estimation

Root formation is represented by dentine that forms after completion of the cervical enamel, which grows by extension and apposition, resulting in progressive lengthening and thickening of the root (towards the pulp). Dentine formation is also characterized by short- and long-period incremental lines, which represent the regular secretion of the odontoblasts, similar to the process of enamel formation (reviewed in Chapter 1). Short-period lines are known as von Ebner's lines, which are equivalent to cross-striations; long-period lines are known as Andresen's lines, which are equivalent to Retzius lines (Dean et al. 1993b; Dean, 1995a; Dean and Scandrett, 1996). The duration of root formation may be assessed by several methods: counting Andresen's lines, which are multiplied by the periodicity to yield the time in days; division of the length of the root along the cementum enamel junction by the extension rate; or division of the thickness of the root (parallel to the dentine tubules) by the dentine DSR. In teeth that are still developing, this information may be used to assess the age at death.

Several aspects of dental development are critical for assessment of age at death: a neonatal line must be identified, crown formation time must be determined (or the corresponding dentine formation), and the duration of root formation must be established. If a tooth cannot be related to an individual's developmental chronology, such as the time of birth, or if the tooth has completed root formation, it is not possible to assess age at death histologically. The first permanent tooth in hominoids to begin crown formation prior to birth is the first molar, which often registers the event as an accentuated line known as the neonatal line (reviewed in Chapter 1). The neonatal line permits developmental time to be registered in teeth that have yet to complete crown or root formation at the time of death; subsequent formation time can be added for estimation of chronological age. Teeth (or individual cusps) that do not show a neonatal line must be

registered with other teeth (or cusps) in order to relate formation time to chronological age. This is generally accomplished by matching a series of accentuated lines in different cusps or teeth. When matched accurately, they allow demarcation of periods of formation, and may permit counts to be continued between teeth (or cusps), although their crown positions may differ depending on differences in initiation time (e.g., Boyde, 1963; Antoine, 2000; Ferrell & Reid, 2004).

Enamel extension and formation time determination

Shellis (1984a,b) suggested that the extension rate of enamel formation may be understood as a trigonometric model of enamel development related to the angle of the developing enamel front at the EDJ, the angle of a prism at the EDJ, and the daily secretion at the EDJ (reviewed in Chapters 1 - 3, see Figure 2.6). From knowledge of this growth parameter, he suggested that it is possible to determine the crown formation time by dividing the length of the EDJ by the extension rate in successive increments. He also noted several limitations of this method: it was not possible to use in teeth that preserved few incremental lines, was less accurate in areas with low angles of intersection between the developing enamel front and EDJ, and most importantly, that it is essential that measurements of extension rate are derived from all levels of the crown as extension rates vary from cusp to cervix. For example, he noted that using the extension rate derived from the cervical enamel would result in an underestimation of the average rate, and overestimation of the total formation time. Recently, he noted that additional investigation is necessary to determine the accuracy of this formula for determination of total crown formation time, particularly in teeth that are relatively fast-forming (Shellis, 1998).

Martin (pers. com.) has recently suggested that a predictive relationship may exist between the length of the EDJ and crown formation time. This is based on the idea that, in a two-dimensional sense, if one prism were formed along the EDJ per day from the beginning to the end of formation, the total time taken to form the crown should be equivalent to the EDJ length divided by the average width of a prism (which yields the number of prisms or days). He also suggested that multiple prisms could be formed daily, which would necessitate dividing the EDJ by the width of all prisms formed in a day.

Recent work has shown that macaque extension occurs at a rate several times greater than the width of a prism, which is approximately 4 - 5 μm (Chapter 3). Additionally, extension rate appears to decrease progressively from cuspal to the cervical regions in a number of primates (Shellis, 1998), implying the number of prisms formed daily also decreases. This suggests that, as Shellis (1984a,b) proposed, calculation of crown formation time requires that the EDJ length must be divided by the average extension rate (unless it is equivalent to the average prism width). However, it appears that accurate estimation of the extension rate is difficult, and a different method of estimating this is necessary, particularly in deciduous teeth (Chapter 3).

Theoretically, a regression of crown formation time against EDJ length should yield the average extension rate (as the slope). Given a large enough sample, and provided that extension rate is not highly variable between teeth, it is possible that a predictive regression equation could be derived that would yield crown formation time without requiring extension rate determination throughout the crown. This could be applied to large samples of moderately worn teeth, or to tooth crowns imaged with the use of high-resolution micro-CT. To test this hypothesis, crown formation time from the cusps of animals that were of known age at death will be regressed against the length of the cusp- specific EDJ, and the relationship will be tested for significance. If is successful, it will be possible to assess this relationship in chimpanzee cusps, where crown formation time will be calculated from standard histological techniques. This may prove to be a novel method of histological assessment of crown formation time.

Materials and Methods

Material

Five unerupted first molars belonging to five macaque (*Macaca nemestrina)* dentitions were examined in this study, all of known age at death (Figure 4.1). Four individuals were part of the labeling study described in the previous chapter: CF 324, 326, 336, and 337. One first maxillary molar was examined from CF 336, and first

mandibular molars were used from the other three individuals. An additional first mandibular molar was obtained from a developing macaque dentition (M 6898) previously dissected out and stained with Alizarin red S. Basic information on each tooth is given below, including details of the sections that were prepared during previous studies (CF 324, 326, 337, M 6898), and material prepared during the course of this study (CF 336).

Specimen 1 (CF 324)

Individual CF 324 was sacrificed at 146 days of age, at which time the first molar had yet to complete crown formation. A single histological section of the mesial cusps of the mandibular first molar was prepared during the original study (described in Chapter 3). Partial crown formation time/age at death was determined from the metaconid only, as the protoconid appeared to have been cut obliquely (Figure 4.1a). Because the tip of the cusp was missing in the metaconid, accentuated lines were identified that were assumed to represent the neonatal line and the approximate end of cuspal formation, which allowed this period to be estimated from counts and measurements of cross-striations. Imbricational enamel formation time was assessed as described below.

Specimen 2 (CF 326)

Individual CF 326 was sacrificed at 173 days of age, at which time the first molar had yet to complete crown formation. A single histological section of the mesial cusps of the mandibular first molar was prepared during the original study. Only the protoconid was used for determination of partial crown formation time/age at death, as the metaconid appeared to have been cut obliquely (Figure 4.1b). The cuspal enamel thickness of the protoconid also appeared to be exaggerated due to obliquity, which was apparent from the pronounced degree of curvature of Hunter-Schreger bands near the dentine horn. Cuspal enamel formation time was estimated using accentuated lines in the lateral enamel that appeared to represent the neonatal line and the approximate end of cuspal formation, which allowed this period to be estimated from counts and measurements of cross-striations. Care was taken to avoid areas that showed dramatic prism decussation. Imbricational enamel formation time was assessed as described below.

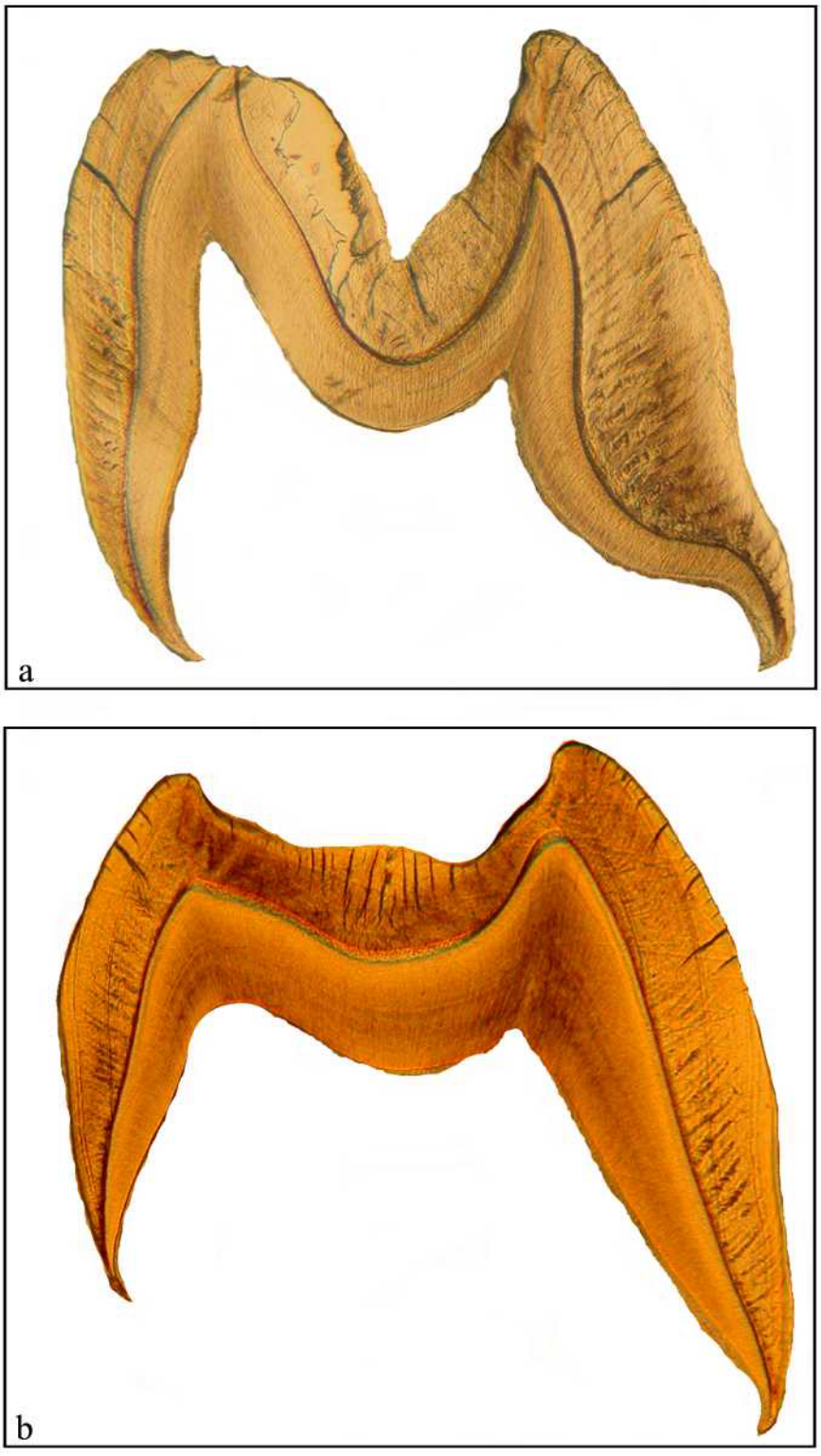


Figure 4.1 (a-j) Transmitted light microscope overviews of the histological sections used in this analysis. a) Mesial section of Specimen 1, metaconid on left, protoconid on right. b) Mesial section of Specimen 2, metaconid on left, protoconid on right.
(Figures [c-j] are on the following pages.)

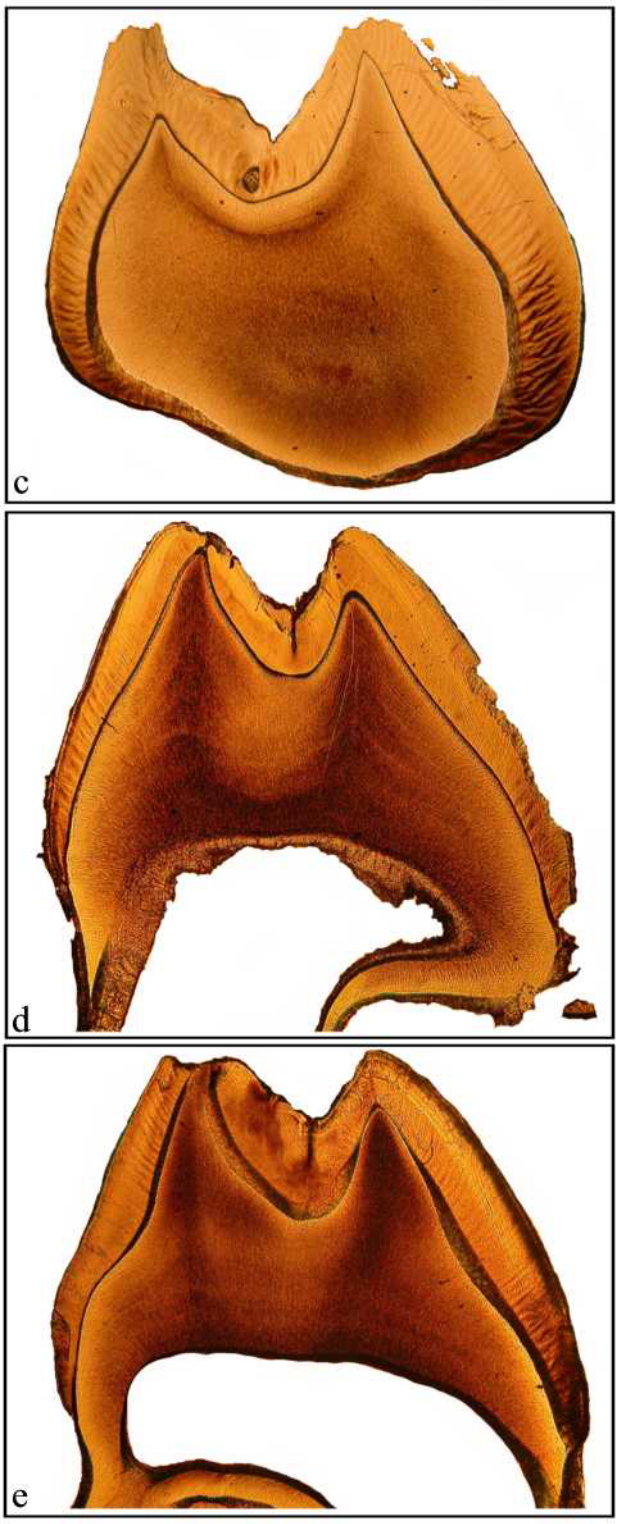


Figure 4.1 (c-e) Protocone on left, paracone on right for all three sections. c) Most mesial section of Specimen 3- Section 3.1. d) Mesial section of Specimen 3- Section 3.2. e) Mesial section of Specimen 3- Section 3.3.

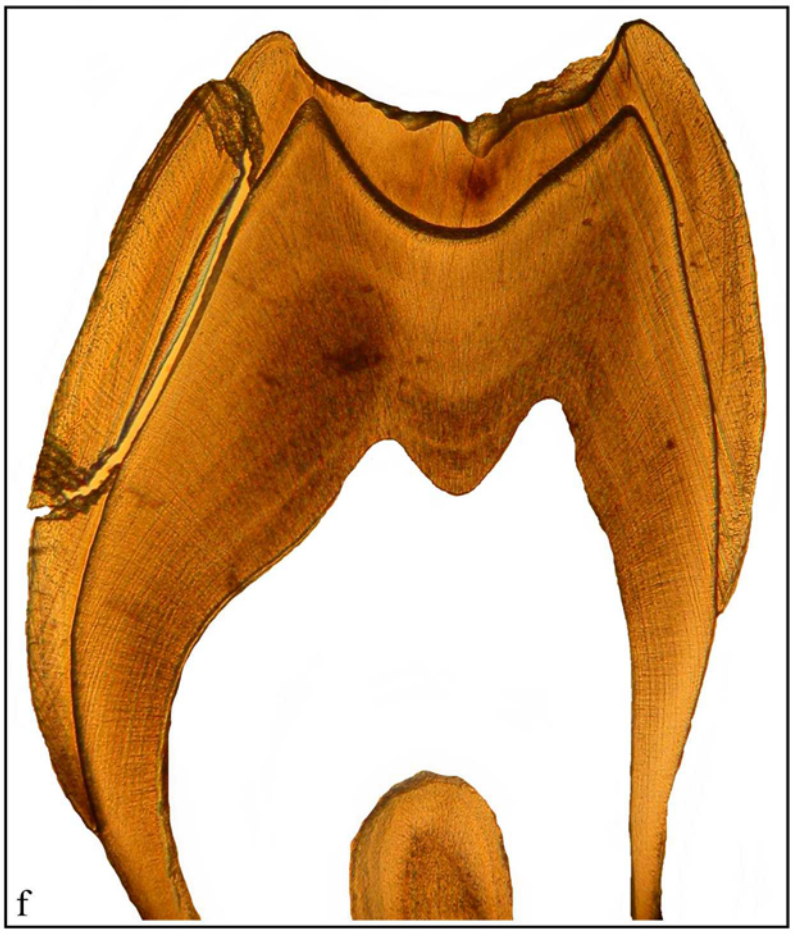


Figure 4.1 f) Mesial section of Specimen 4, protoconid on left, metaconid on right.

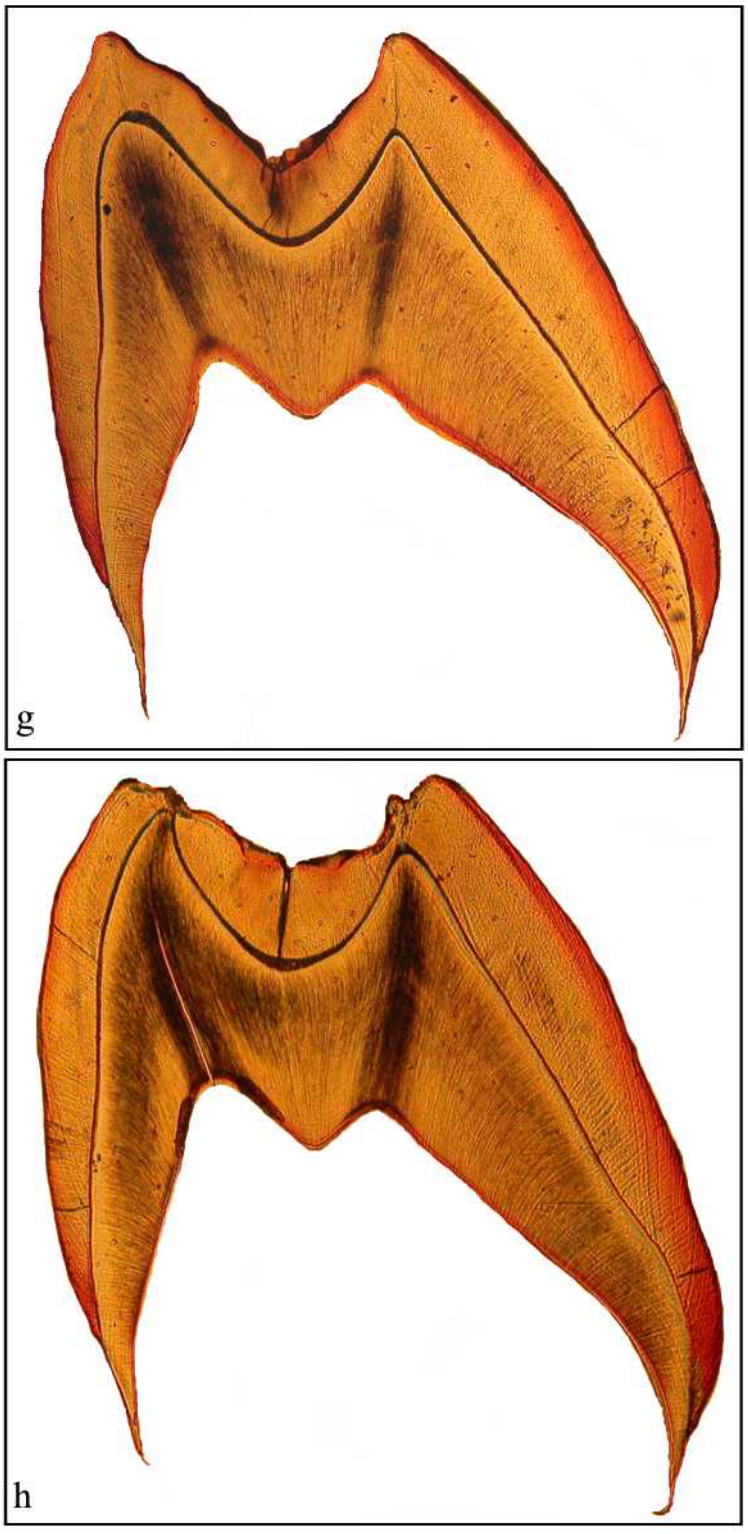


Figure 4.1 (g-h) Mesial sections of Specimen 5, metaconid on left, protoconid on right. g) Section 5.1. h) Section 5.2.

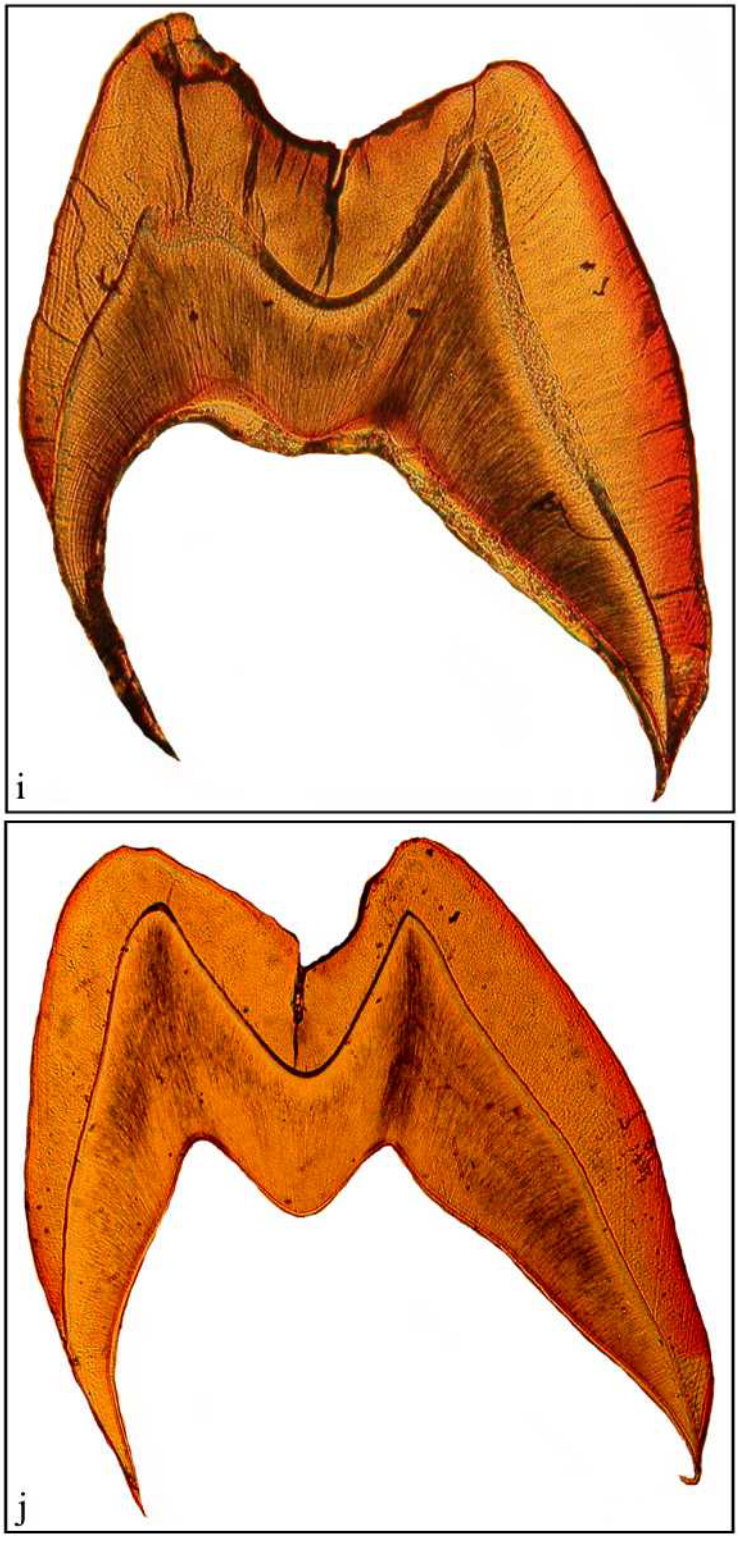


Figure 4.1 (i-j). Distal sections of Specimen 5, entoconid on left, hypoconid on right. i) Section 5.3. j) Section 5.4.

Specimen 3 (CF 336)

Individual CF 336 was sacrificed at 438 days of age, at which point the maxillary first molar had completed crown formation and formed approximately 3.4 mm of root beyond the protocone cervix and 1.5 mm of root formation beyond the paracone cervix. The crown complete unerupted maxillary left first molar was first extracted from a hemi-maxilla preserved in 70% ethanol during the course of this study (Chapter 3, Figure 3.2a). The tooth was dissected out, refluxed in methanol and chloroform for several days, and embedded in methyl methacrylate according to procedures described in Boyde (1989). Longitudinal cuts were made in a buccal-lingual plane through the mesial cusps using a diamond wafering blade mounted on a Isomet low speed saw, and two methods were used to produce a total of three histological sections. Initially, a single cut was made, and the two resulting faces of the mesial cusps were polished, cleaned, and mounted to slides using two-part epoxy resin. After curing, each slide/block was mounted on the saw, and the blade was advanced several hundred microns from the slide prior to cutting off the block. This produced a slightly 'thick section' that was then polished down to approximately 80 - 100 μm and finished with 1 μm alumina. However, because the quality of the bond between the section and the slide was not ideal, the two sections were later removed, cleaned, remounted, and a cover slip was added using DPX mounting media (with the assistance of Dr. Donald Reid at the University of Newcastle). During repreparation, some of the enamel was lost from the outer lateral region of the paracone of Section 3.2.

A third section was later generated by mounting the remaining block on the Isomet saw, and cutting a section several hundred microns thick. The face that was determined to be closer to the ideal plane through the tips of the dentine horns was polished with successively finer grades of silicone paper and finished with 1 μm alumina paste on a polishing cloth. This face was then bonded to a slide using a UV curing resin under pressure. After curing, the section was then lapped down to approximately 100 μm thickness on a Buehler grinder polisher, polished with 1 μm alumina paste, cleaned, cleared, and a cover slip was mounted with DPX mounting media.

Observation of the three sections shows that Section 3.1 is the most mesial section, which is clearly more oblique than Sections 3.2 and 3.3 (Figure 4.1c-e).

However, due to the orientation of the original cut, it appears to preserve the best plane for the cuspal enamel of the paracone (but not of the protocone, possibly due to asymmetry in occlusal crown position). It is unfortunate that the tip of this cusp was broken off during preparation, preventing a reliable assessment of the cuspal thickness. Obliquity is also clearly indicated in Sections 3.1 and 3.3 by the appearance of an indistinct EDJ. When focused through, the position of the EDJ appears to be running obliquely through the 100 μm thick section. Cross-striations and Retzius lines were not apparent, or were very difficult to define near several of these areas. Section 3.2 represents a good plane of section, but is missing some outer enamel, therefore Sections 3.2 and 3.3 were both used for determination of paracone and protocone crown formation time and age at death, as described below.

Specimen 4 (CF 337)

Individual CF 337 was sacrificed at 458 days of age. The crown complete unerupted mandibular right first molar had formed approximately 2.5 mm of root beyond the protoconid cervix and 3.5 mm of root beyond the metaconid cervix at the time of death. This tooth appeared to have begun alveolar emergence, but had not completed gingival emergence. A single histological section of the mesial cusps was prepared during the original study (Figure 4.1f). Both the protoconid and metaconid were used for determination of crown formation time and age at death as described below, although they both appeared to be slightly oblique, and were not expected to give highly accurate estimates *a priori*.

Specimen 5 (M 6898)

The known age at death of this individual was 374 days. This tooth had just completed crown completion, and had formed approximately 1.1 mm of root formed beyond the metaconid cervix, 0.6 mm of root formed beyond the protoconid cervix, 1.8 mm of root formed beyond the entoconid cervix, and 0.2 mm of root formed beyond the hypoconid cervix. Two thin sections were cut for each of the mesial and distal cusps of the lower left first molar (M 6898) by Dr. Donald Reid, following procedures described in Reid et al. (1998a,b) as part of a separate study on dental development in macaques

(Figures 4.1g-j). Section 5.2 is the least oblique section of the two mesial sections. Cuspal enamel thickness and daily secretion rates are lower than those of Section 5.1, thus, it was used in determination of crown formation and age at death. Of the two distal sections, Section 5.4 is much less oblique than Section 5.3, and was used for crown formation and age at death. In addition, the Andresen's lines in this individual were very clear (Figure 4.2), which permitted estimation of root formation.

Methods

Crown formation time

Due to the relatively poor quality of cuspal enamel in this material, it was not possible to count an inclusive series of cross-striations representing cuspal formation, nor was it possible to track prism paths. Cuspal formation time was generally determined by dividing the cuspal enamel thickness (measured from the dentine horn to the position of the first imbricational Retzius line) by the average cuspal DSR, determined from an average of measurements of cross-striations in inner, middle, and outer cuspal enamel. When possible, the neonatal line was identified, and formation times were determined separately for the enamel formed prior to and after this line. When multiple planes of section were available, the minimum cuspal thickness (not corrected for prism deviation from a straight path) and minimum DSR were used, as it is likely that these may be inflated by section obliquity (Risnes, 1999; Smith et al., 2004).

Imbricational formation time was determined by counting Retzius lines from the cervix to the last line at or near the cusp tip, and multiplying this number by the periodicity, or number of cross-striations between Retzius lines (determined opportunistically in each section). In certain areas of the cervical enamel, it was possible to count daily laminations at the EDJ and compare this to the number of Retzius lines as a cross-check (see Chapter 3). This was done to confirm Retzius line counts, as several sections showed accentuations that complicated determination of the last-formed Retzius lines (Specimen 4 and 5) (Figure 4.3). Additionally, well marked accentuated lines were identified in the enamel and dentine of each cusp in Specimen 3 - 5, which allowed

registration and assessment of the consistency of estimates. Total crown formation time was estimated by combining cuspal and imbricational enamel formation times.

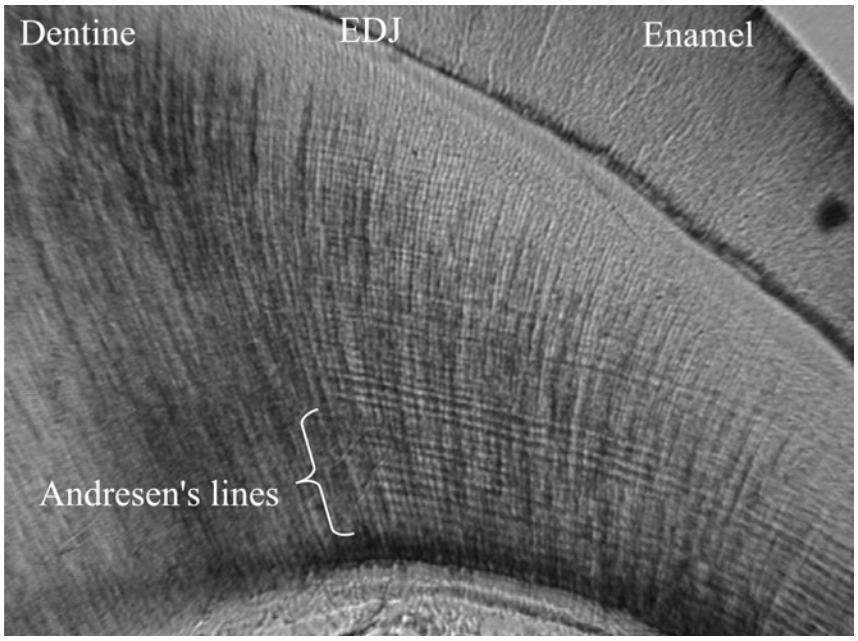


Figure 4.2 Polarized light micrograph of long-period (Andresen's) lines in the dentine of Specimen 5, showing the beginning of root formation prior to death.

Root formation and age at death

Estimation of the period of root formation prior to death was accomplished by two methods when possible. First, an accentuated dentine line[1] parallel to the Andresen's lines was chosen at or near the point of crown completion, and Andresen's lines were counted between this line and the end of dentine formation. This number was multiplied by the periodicity (determined between Retzius lines in enamel) to yield the time of root formation. When this was not possible, the distance along a dentine tubule was measured from the EDJ at the tip of the cervix to the end of dentine formation, and this was divided by the average local dentine DSR. The DSR was determined in two ways: from measurements of several successive Andresen's lines divided by the number of days of formation (number of lines times the periodicity), or by measurements of the daily von Ebner's lines under high magnification (50 X objective).

[1] Often termed the contour lines of Owen (Dean et al., 1993a).

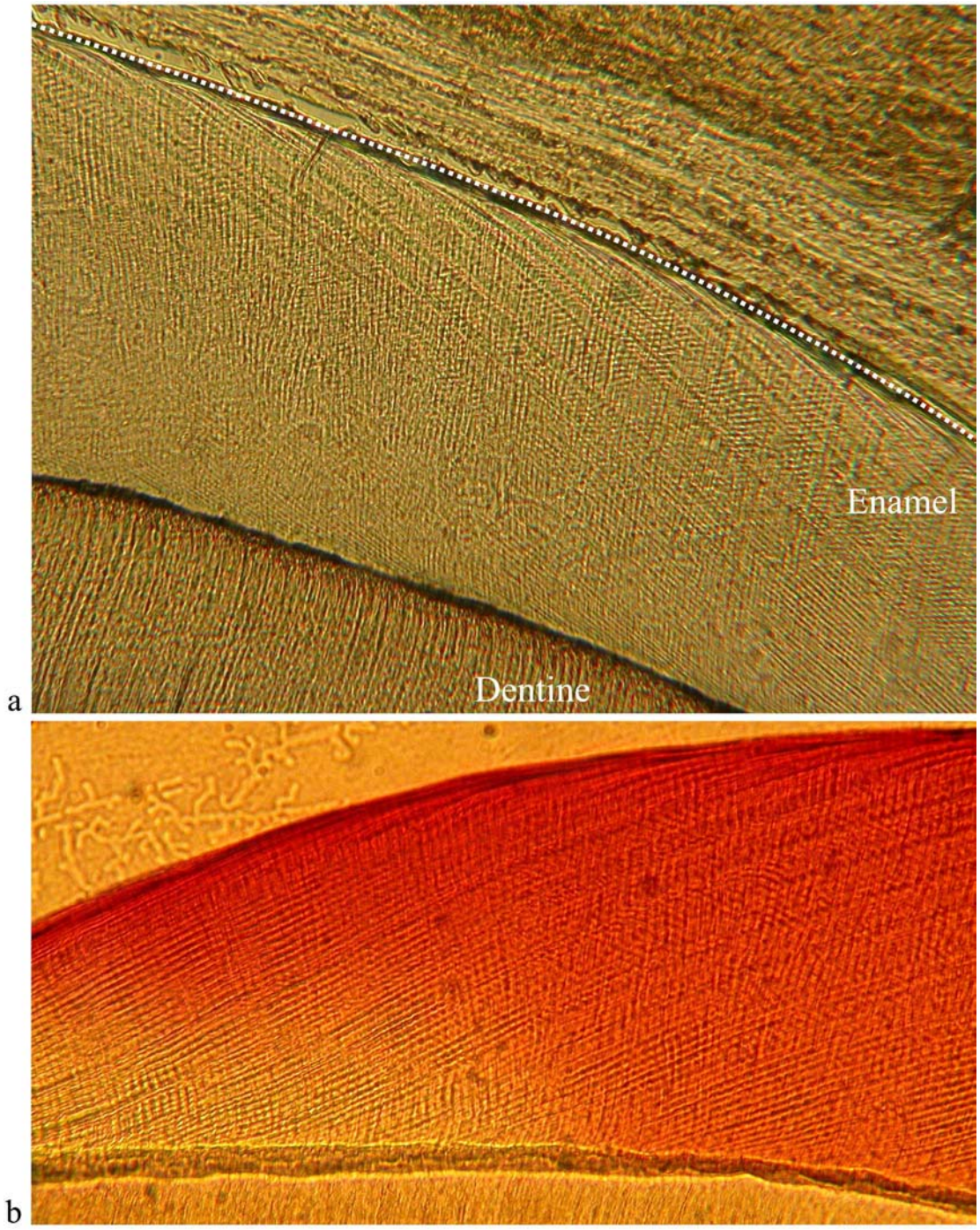


Figure 4.3 (a,b) Transmitted light micrographs of cervical enamel showing accentuations running parallel to Retzius lines near the enamel surface. a) Accentuated lines are seen running from the enamel dentine junction towards the surface of the tooth (dotted line) in Specimen 4; cervix is to the right. b) A similar area in Specimen 5, which also shows laminations that were used to confirm counts of Retzius lines; cervix is to the left.

These methods sometimes yielded different estimates due in part to the variable quality of the dentine, as well as the difficulty of imaging short-period incremental features (prompting the use of two different rates for estimates of root growth in the paracone of Specimen 3). The age at death was determined by combining the time of postnatal crown formation and the duration of root formation for each individual cusp. This estimate was compared to the known age at death to determine the accuracy of the methods.

Extension rate

The application of Shellis' (1984a,b) extension rate formula for crown formation time calculation was tested against the actual or adjusted crown formation time of each tooth using the Wilcoxon Signed Ranks test. Shellis' extension rate formula, (c) = d [(sin I/tan D)- cos I], required quantification of the following: angle of intersection of the enamel prisms with the EDJ (I), angle of the developing enamel front with the EDJ (D), and the spacing of cross-striations (d). In Specimen 1 and 2, adjusted formation time was determined as the sum of the known age at death and estimated prenatal enamel formation. In Specimen 3 - 5, the average root formation times were subtracted from the known age at death, and the prenatal enamel estimate was added to yield crown formation time. From this, the average crown extension rate was determined by dividing the cusp-specific EDJ length (from the tip of the dentine horn to the tip of the cervix) by the adjusted time of formation (total crown formation time inclusive of prenatal enamel). The length was derived from the section of each tooth that showed the longest EDJ, assumed to indicate minimum obliquity (excluding Section 3.1, which was very oblique). Lengths were measured from overviews with Sigma Scan software and a SummaSketch III digitizing tablet. Finally, the relationship between crown formation time and EDJ length was assessed using simultaneous linear and hierarchical curvilinear regression analysis with SPSS software (v. 11.5).

In the following section, results are presented as age at death estimates registered by the position of the presumed neonatal line and contrasted with the known age at death. Following this, information from additional accentuated lines in the enamel and dentine is used in conjunction with the known age at death to examine the nature and degree of under- or overestimation. Finally, EDJ lengths divided by adjusted crown formation time

estimates are used to calculate average enamel extension rates, which are compared to results derived from the application of Shellis' formula.

Results

Initial Assessments of Crown Formation Time and Age at Death

The estimated prenatal, cuspal, and imbricational enamel formation times, root formation time, and age at death of each first molar are shown in Table 4.1. Total crown formation time represents the sum of prenatal and postnatal enamel formation, which are listed separately under prenatal time and age at crown completion (Age CC). Specimen 1 & 2 were still forming their crowns at the time of death, so data on age at crown completion or duration of root formation were not available. The estimated chronology of cuspal development and age at death for Specimen 3 - 5 are shown in Figure 4.4. In general, calculated ages at death tended overestimate the actual age (in four of five teeth or in six of nine cusps), which suggests that crown formation time was also overestimated (assuming that dentine formation time was assessed accurately). The average percent error was 3.28 % more than the known age, and the average absolute percent error was 8.03 % (in either direction). A few of the cusps with the highest overestimates were known to be oblique *a priori* based on the morphology of the dentine horns, which did not appear to come to a true 'horn' in the available planes of section, and these sections were expected to yield inflated values.

Table 4.1 Estimated crown formation and age at death in *Macaca nemestrina* first molars.

	Prenatal			**Postnatal (Cuspal)**			**Imbricational**		**Age**	**Dentine**				**Age at Death**			
Cusp	Thick	Rate	Time	Thick	Rate	Time	Ret*Per	Time	CC	Thick	Rate	Time	Andr	Min-Max	Known	Diff	% Error
Specimen 1																	
Meta	170	4.50	38	460[1]	5.92[1]	78	19 * 4	76	n/a	n/a				154	146	8	+5.48
Specimen 2																	
Proto	~200	4.30	47	575[1, 3]	5.78[1]	99	25 * 4	100	n/a	n/a				199	173	26	+15.03
Specimen 3																	
Proto	145[2]	4.24[2]	34	300	4.79[2]	63	62 * 4	248	311	620	3.40	182	43	483-493	438	50	+11.42
Para	150	3.90	38	580[3]	5.13	113	59 * 4	236	349	390	3.40	115		464			
											3.80	103		452		20	+4.57
Specimen 4																	
Meta	150	4.70	32	505[3]	5.45	93	46[4] * 4	184	277	765	3.45	222	47	469-499	458	26	+5.68
Proto	245	5.25	47	395[3]	4.83	82	70 * 4	280	362	550	3.20	172	25-30[4]	462-534		40	+8.73
Specimen 5																	
Meta	110	4.52	24	335	4.75	71	50[4] * 4	200	271	280	3.50	80	20	351	374	-23	-6.15
Proto	185	4.35	43	400	5.12	78	58[4] * 4	232	310	140	3.65	38	10	348-350		-25	-6.68
Ento	n/a			570[3]	5.03	113	30[4] * 4	120	233+	360	3.40	106	26	337-339+		n/a	
Hypo	155	4.45	35	460	4.98	92	60[4] * 4	240	332	25	2.12	12	2-3[4]	340-344		-32	-8.56

Cusp values for lower first molars: meta- metaconid, proto- protoconid, ento- entoconid, hypo- hypoconid; for the upper molar (Specimen 3), proto- protocone, para- paracone. For **prenatal** enamel, values were derived from enamel formed prior to the presumed neonatal line; Thick= maximum linear thickness from dentine horn to neonatal line in μm, Rate= daily secretion rate in this area in μm/day, Time= thickness divided by rate in days. **Postnatal** values are given for the cuspal formation after birth; Thick= maximum linear thickness from neonatal line to cusp tip in μm, Rate= daily secretion rate in this area in μm/day, Time= thickness divided by rate in days. For **imbricational** enamel, Ret= number of Retzius lines, Per= periodicity of Retzius lines, Time= number of lines multiplied by the periodicity in days. For **age**, CC= age at crown completion in days, determined by adding the postnatal (cuspal) time and the imbricational time. For **dentine**, Thick= maximum linear thickness along a tubule from the enamel dentine junction to the end of dentine formation in μm, Rate= daily secretion rate in this area in μm/day, Time= thickness divided by rate in days, Andr= number of Andresen's lines in the same area, which was multiplied by the periodicity for a separate estimate of dentine formation time (not shown but included in the following column). For **age at death**, the Min-Max= range of times of estimated age

at death in days, calculated by adding the age at crown completion to the minimum and maximum estimates of root formation (from two methods- Time and Andr), Known= actual age at death, Diff= difference score, calculated by subtracting the average calculated age at death from the actual age at death, % Error = difference score divided by the known age * 100. Direction of estimation is indicated as + = overestimate, - = underestimate.

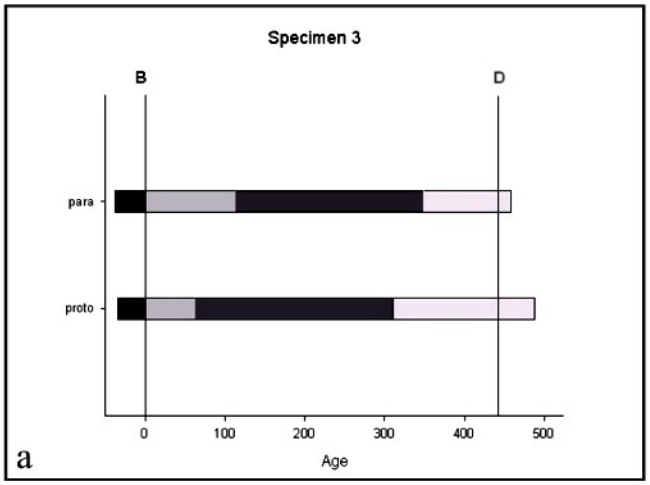
[1] Derived from the lateral enamel corresponding to the approximate period of cuspal formation. Values are likely to be higher than the actual cuspal thickness and rate, but it was not possible to calculate these due to missing enamel (Specimen 1) or section obliquity and heavy decussation (Specimen 2).

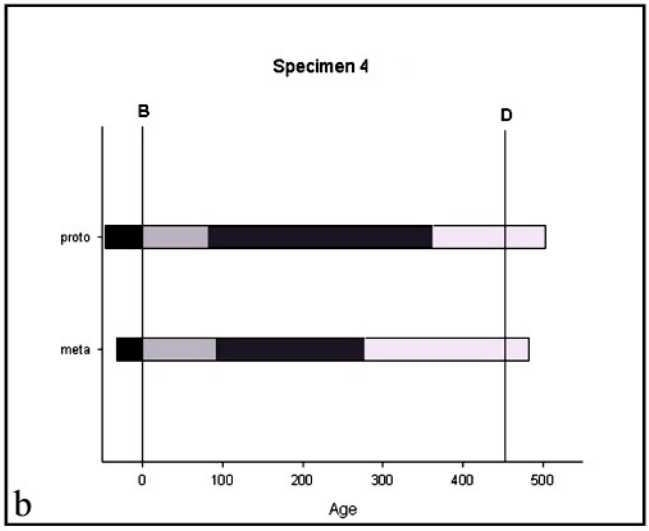
[2] Represents an average of two sections, due to missing cuspal enamel, which was estimated in Section 3.3.

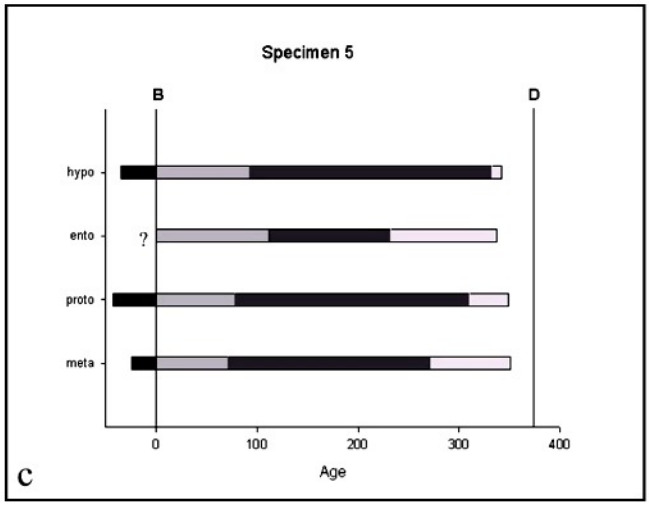
[3] Believed to be slightly oblique, which may result in inflated cuspal formation time.

[4] Includes slight estimate due to the lack of clarity of a few long-period lines.

Figure 4.4 (a-c) Developmental chronology of individual cusps of three first molars. Prenatal enamel formation is shown in black, followed by cuspal formation in light gray, imbricational formation in dark gray, and root formation in light gray. Both birth (B) and age at death (D) are approximated by vertical lines. a) Specimen 3, para and proto represent the paracone and protocone, b) Specimen 4, proto and meta are represent the protoconid and metaconid, c) Specimen 5, hypo, ento, proto, and meta represent the hypoconid, entoconid, protoconid, and metaconid respectively. In the entoconid, a neonatal line could not be found, thus it was not possible to determine the age at initiation (indicated by '?') or age at death. Note that the scales are different on the three graphs. Data are from Table 4.1.


Specimen 3
B
D
para
proto
0
100
200
300
400
500
Age
a


Specimen 4
B
D
proto
meta
0
100
200
300
400
500
Age
b


Specimen 5
B
D
hypo
ento
?
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meta
0
100
200
300
400
Age
c

The presence of accentuated lines in Specimen 3 - 5 permitted a secondary chronology of development to be determined through comparisons of simultaneously forming regions of enamel. In Specimen 3, an accentuated band was identified in the cuspal enamel of the paracone and in the imbricational enamel of the protocone, which allowed a comparison of estimates of formation time between the neonatal line and this line. The estimates of these two cusps were approximately 17 days apart from one another, as the paracone was estimated to have produced this band at approximately 92 days after birth, while the protocone was estimated to show this line approximately 75 days after birth. This implies that the cuspal formation time of the paracone was overestimated, the cuspal formation time of the protoconid was underestimated, and/or that the neonatal line in either cusp was incorrectly identified. In Specimen 4, an accentuated line was identified in the imbricational enamel of the metaconid and protoconid, which showed the estimations were approximately nine days different from one another (the metaconid was estimated to have produced this line at 117 days after birth, while the protoconid showed this line at 126 days after birth). Given that both cusps are slightly oblique, it is not clear if one or both cusps were either over- or underestimated, or if the neonatal line was identified incorrectly.

In Specimen 5, a pair of accentuated lines was identified in the enamel; initial calculations suggested that the first line was formed at approximately 139 days of age in the metaconid, 138 days in the protoconid, and 128 days in the hypoconid, showing differences of one to eleven days between estimates. The most marked accentuated line (E_1) in Specimen 5 was then matched to a marked line in the dentine in each cusp, which permitted an iterative chronology to be determined from the cessation of dentine formation (at death) to the formation of the accentuated line (Figure 4.5). Based on counts of Andresen's lines, the marked line was determined to have occurred 53 increments, or 212 days, before death. When the time of formation was adjusted from the known age at death, the iterative chronology suggested very little prenatal enamel formation in the metaconid and hypoconid. Given the 23 - 32 day average underestimate of these two cusps when compared to the known age at death, it is likely that one or all of the following are true: the neonatal lines were not correctly identified, the cuspal formation time was underestimated, and/or the number of earliest-formed Retzius lines

was underestimated. Considering the difficulty of imaging Retzius lines near the cusp tips, it is likely that the last reason was the cause of the underestimates, particularly in the hypoconid. In addition, the presence of numerous accentuated features in the cervical enamel also may have contributed to the inaccuracy of Retzius line counts.

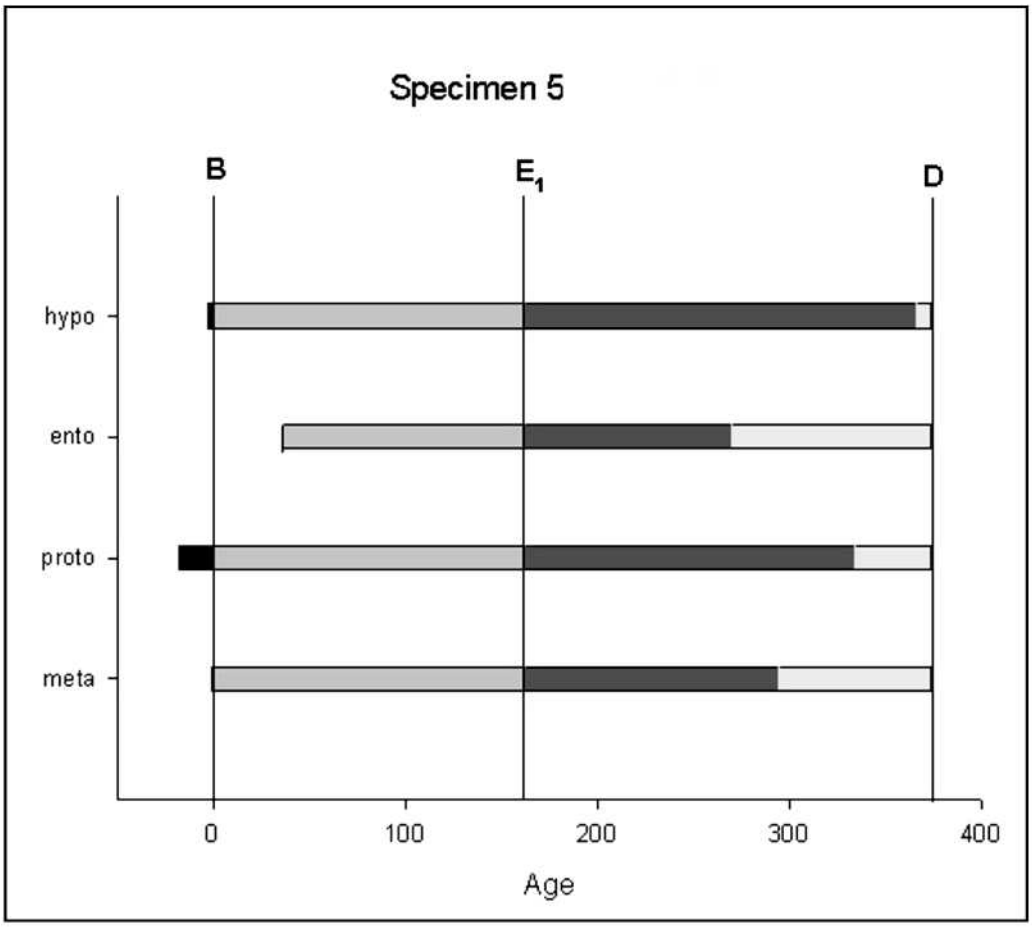

Figure 4.5 Adjusted developmental chronology in Specimen 5. Information derived from long-period lines formed after a marked accentuated line (E_1) is used to adjust developmental time by iteratively working from death to birth (see text for details). Codes are given in Figure 4.

Enamel Extension and Crown Formation Time

The adjusted crown formation times derived from knowledge of the actual age at death and cross-registry of the cusps were compared to times derived from the

application of Shellis' formula (Table 4.2). Shellis' formula yielded estimates of crown formation times that are significantly greater than the actual or estimated times (Wilcoxon signed ranks test: Z= -2.805, n=10, P=0.005). These ranged from 5% to 84% more than the actual time, and were 31% greater on average. This was due to underestimation of extension rate, revealed by comparison to the actual extension rate (determined by dividing cusp-specific EDJ length by crown formation time).

Table 4.2 Adjusted crown formation time and extension in *Macaca nemestrina*.

			Shellis'		**Known**			
Spec	Cusp	EDJ	Time	Rate	Time	Rate	Diff	% Error
1	Metaconid	4210	228	18.46	184[1]	22.88	44	23.91
2	Protoconid	4689	326	14.38	220[1]	21.31	106	48.18
3	Protocone	5470	427	12.81	295	18.54	132	44.75
	Paracone	5433	415	13.09	367	14.80	48	13.08
4	Metaconid	4280	366	11.69	285	15.02	81	28.42
	Protoconid	6380	476	13.40	374	17.06	102	27.27
5	Metaconid	4596	335	13.72	318	14.45	17	5.34
	Protoconid	5603	466	12.02	378	14.82	88	23.28
	Entoconid	3660	429	8.53	233[2]	15.71	196	84.12
	Hypoconid	5091	447	11.39	399	12.76	48	12.03

Spec= specimen numbers as described in the text. Cusp= derived from first molars. EDJ= enamel dentine junction length in μm, **Shellis'** values derived from the application of his formula using measurements from a minimum of four areas along the EDJ (see text for formula), Time= predicted formation time in days, Rate= predicted extension rate in μm/day, determined by dividing EDJ by time. **Known** values derived from actual age at death, from which root formation was subtracted and prenatal time was added, Time= prenatal plus postnatal enamel formation in days, Rate= extension rate in μm/day, determined by dividing EDJ by time, Diff= difference score, calculated by subtracting the calculated crown formation time from the actual formation time, % Error = difference score divided by the actual time * 100.
[1] Incomplete crown. [2] Original estimate not adjusted due to lack of neonatal line.

Cuspal crown formation time was regressed against cusp-specific EDJ length in ten mixed cusps (mesial and distal cusps from maxillary and mandibular molars), which showed a significant positive linear relationship (r^2=0.541, $F_{1,8}$=9.43, P=0.015, adjusted r^2=0.484) (Figure 4.6). Log transformation, second, or third order transformations did not appreciably increase the correlation. The two developing teeth were subsequently excluded from the analysis, due to their higher extension rates relative to fully formed crowns (which may not be comparable to average rates from fully formed crowns). Cuspal crown formation time was then regressed against cusp-specific EDJ length in the

eight mixed cusps, which also yielded a significant positive linear relationship (r^2=0.579, $F_{1, 6}$= 8.24, P= 0.028, adjusted r^2=0.508). Because it was not clear if the predictor variable and the independent variable were normally distributed, both were log-transformed, which increased the strength of the relationship (r^2 =0.650, $F_{1, 6}$=11.161, P=0.016, adjusted r^2= 0.592). A hierarchical polynomial regression analysis was then conducted with log of EDJ length as the predictor variable. Adding a quadratic term did not add a significant further increment (r^2=0.085, $F_{1, 5}$=1.600, P= 0.262), although the overall model was still significant despite the reduction in degrees of freedom (r^2=0.735, $F_{1, 5}$= 6.938, P= 0.036, adjusted r^2=0.629) (Figure 4.7).

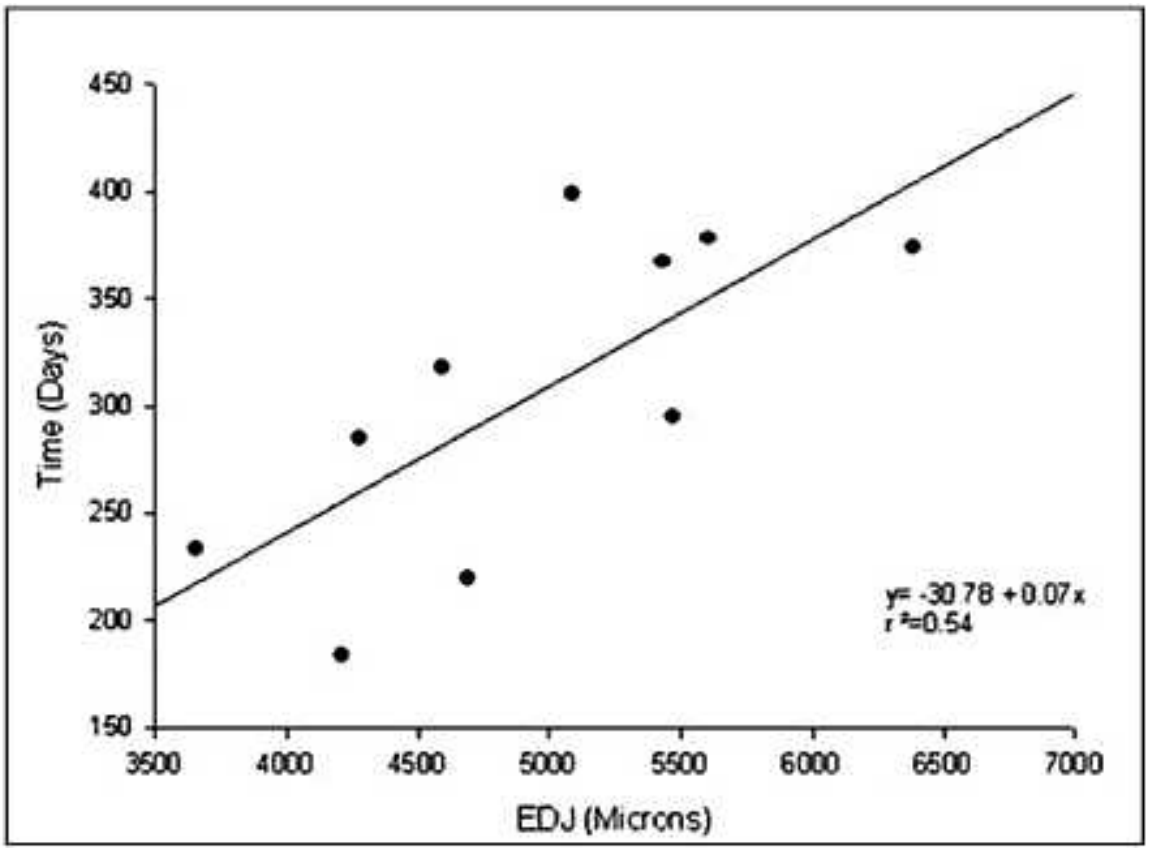

Figure 4.6. Regression of cusp specific enamel dentine junction (EDJ) length on cuspal crown formation time for all ten cusps, with linear best-fit line shown. Data are from Table 2. Note that data are derived from different cusps of upper and lower first molars.

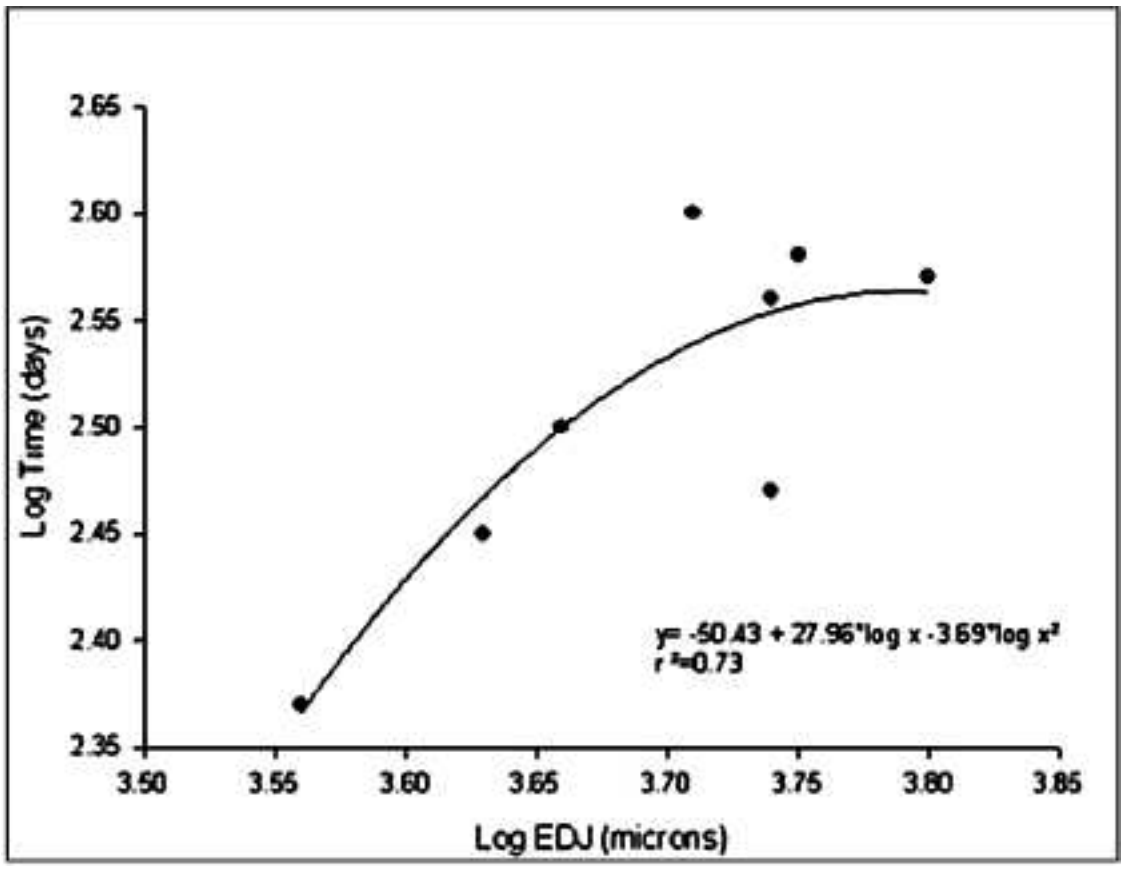

Figure 4.7 Regression of cusp specific enamel dentine junction (EDJ) length on cuspal crown formation time for eight fully formed cusps, with the quadratic best-fit line shown. Data are from Table 2 (Specimen 3 - 5).

Discussion

Crown Formation Time/Age at Death

Accuracy

Radiographic information indicates that pig-tailed macaques initiate first molar crown formation prior to birth, completing mandibular first molar formation at 341 days of age and maxillary first molars at 372 days on average (both sexes averaged from

Sirianni & Swindler, 1985). This is in general agreement with the results of this study.[2] Crown formation time may be estimated histologically with a high degree of accuracy, particularly in sections that preserve well-defined incremental features, and are not cut oblique to the ideal plane of section (discussed further below).

Few studies have examined material of known age at death (Stringer et al., 1990; Huda & Bowman, 1995; Antoine, 2000; Schwartz et al., 2001b), or made comparisons with an equivalent amount of dentine (Dean et al. 1993a; Beynon et al., 1998a; Dean, 1998a; Smith et al., 2004). Earlier studies suggested that counts of incremental features are accurate to about +/- 10%, but did not test this empirically (Boyde, 1963; Dean & Beynon, 1991). Antoine (2000) estimated the age at death in several individuals from the Spitalfields collection and compared his estimates to known ages at death, yielding an accuracy of 94% or greater. He determined crown formation time in several teeth developing simultaneously, checked results among teeth, and demonstrated a high level of consistency in most cases. However, his study utilized material of exceptional quality, which permitted counts of cross-striations throughout the crown. Antoine did not test the accuracy of more common methods that involve counts of short- and long-period increments, which are most often applied to material of 'average' quality. Regardless, the similarity between the accuracy of Antoine's study and the current one suggests that histological methods in general (direct counts of increments and/or estimation of appositional growth) may yield accurate estimations of crown formation time and/or age at death, provided that the material is well prepared and that incremental features are clearly defined.

Sources of error

It is not surprising that poorer quality sections yield less accurate estimations of crown formation time and/or age at death, as both short-period and long-period features may be difficult to image. In the current study, it was difficult to assess DSR where there was a lack of clarity of cross-striations, which made it difficult to assess cuspal enamel formation. In addition, if Retzius line periodicity is not clear, this may introduce an

[2] But see Beynon et al. (1998b) for a discussion of differences between radiographic and histological analyses.

additional source of error into imbricational enamel formation time. For example, had the periodicity of any of the (crown complete) molars been five instead of four days/line, 30 - 70 additional days of imbricational formation time would have be added, as well as up to 47 additional days of root formation. As noted in Chapter 2, in longer-forming primate dentitions (such as extant hominoids), a difference of a single day (in the periodicity) may affect molar crown formation time estimation by approximately 80 to 140 or more days. Smith et al. (2003a, 2004) noted two additional potential confounding factors that may also complicate determination of the periodicity: the appearance of prominent laminations throughout the enamel, and the presence of intradian lines (discussed in Chapter 3). Laminations, as well as numerous accentuated lines, may also complicate determination of the total number of Retzius lines in certain areas (e.g., Figure 4.3).

Sub-optimal quality material may also show poor quality long-period lines. In this instance, Retzius lines are often obscured in the first-formed enamel near the cusp tips, or in the last-formed cervical enamel. Errors in counting these structures are related to the periodicity; teeth with low periodicity, such as the macaques in this study, show a smaller number of days per line than hominoids.[3] (The exclusion of a few Retzius lines would represent less time than in hominoids with higher periodicities.) Identification of the neonatal line presents an additional complication. As shown above, this may result in underestimations of prenatal enamel formation (as well as overestimations of the [postnatal] cuspal enamel formation) if it is not identified when present. Alternatively, incorrect identification may cause either over- or underestimation of the prenatal and postnatal cuspal enamel formation times. Unfortunately, it is difficult to validate estimations of prenatal enamel, as there is no (previously formed) 'bounding event' against which the day of birth can be checked (save for experimentally labeled prenatal material).

The issues of section obliquity and its potential confounding affects on histological analyses have been considered only recently (Risnes, 1999; Antoine, 2000; Dean and Schrenk, 2003; Smith et al., 2003a, 2004). There are several potential problems with section planes that do not represent the true axial thickness of cuspal enamel and the

[3] Long-period lines in the dentine may be miscounted as well, which is particularly easy given the difficulty of distinguishing accentuated features (contour lines of Owen) from regular long-period lines (Andresen's lines).

profile of the dentine horn: exaggerated cuspal thickness, exaggerated prism path length, exaggerated daily secretion rate and/or number of cross-striations, and poor definition of Retzius lines. Obliquely-sectioned cusps with inflated enamel thicknesses may yield overestimated cuspal formation time when estimated from enamel, and underestimated time when estimated from dentine (Smith et al., 2004).[4] Because the ideal plane of section preserves the thinnest cuspal enamel, which may show the slowest secretion rates (Risnes, 1999), deviations from this plane will necessarily increase both. It was difficult to confirm this in the present study, as the quality of cross-striations in the cuspal enamel was generally poor. However, the cusps with thicker enamel often did yield higher daily secretion rates than other sections from the same individuals (these higher rates were not used in the final calculations of cuspal formation time). Some evidence from serial sections of *G. freybergi* supports also this (Smith et al., 2004). Additional work on a larger collection of serial sections may shed more light on the degree to which secretion rates are affected by plane of section. In addition, this study suggests that obliquity results in an underestimation of EDJ length, as sections that showed the thinnest cuspal enamel and the most well-defined dentine horns generally had the longest cusp-specific EDJ lengths (see also Martin, 1983).

A final area that may influence the accuracy of crown formation time estimation is the three-dimensional course of prisms, which may lead to underestimation of cuspal formation time when linear thickness is used (Risnes, 1986). However, given the degree of overestimation found in this study without correction, it may not be necessary to apply a correction factor for accurate determination of cuspal formation time. As noted in Chapter 2, although many early studies relied on Risnes' (1986) correction factor for cuspal enamel formation time in humans, others have suggested that this correction factor may not be an appropriate factor for other fossil hominids or other primates (e.g., Beynon & Wood, 1987; Dean et al., 1993a; Dean, 1998; Dirks, 1998; Reid et al., 1998a; Macho et al., 2003; Smith et al., 2003a, 2004). Smith et al. (2004) showed in *G. freybergi* that it was not necessary to correct the cuspal enamel thickness due to the relatively straight course of enamel prisms, which was confirmed by a separate estimate of cuspal enamel

[4] A similar problem exists for the determination of prism path length, but due to the three-dimensional nature of enamel prisms, it is not clear if the exaggeration of linear thickness is proportional to the inflated prism path length.

formation time obtained independently from corresponding dentine. Antoine (2000) also showed that the complication imposed by decussation in human teeth could be minimized when cross-striations were counted along prisms that did not show marked decussation. The degree of accuracy of this method showed that, *contra* Risnes (1986) and Macho et al. (2003), decussation does not prohibit accurate determination of formation time from cross-striations. In summary, there is substantial variation in the course of prisms through the cuspal enamel, and appropriate corrections appear to be specific to taxa, or even cusps within taxa, but these corrections are not always necessary for accurate assessment.

Extension rate and crown formation time

It is clear from the present study and the results of Chapter 3 that the application of Shellis' extension rate formula generally overestimates the local and total time of enamel formation in this sample of macaque teeth. Shellis (1998) noted that estimates of extension rate are more likely to be prone to error in rapidly-formed teeth, such as those examined in this study. There are several possible reasons for this. In taxa that show a progressive reduction in extension rate from crown initiation to completion, the earliest-formed enamel (which is prenatal in M1) will have the lowest angles of intersection between the forming front and the EDJ (due to relatively fast extension). These acute angles often prove to be difficult to measure accurately (Shellis, 1984a), but more importantly, less 'intersections' are available to measure due to the lack of clarity of the earliest-formed enamel and the rapid extension rate. In this study, it was not possible to sample the initial 1000 - 2000 μm of EDJ length in any cusp. If successive measurements taken along the EDJ do not sample the initial period of rapid extension, enamel formation will be overestimated, as a lower extension rate calculated from the subsequent enamel must be used as a proxy for the initial rate (Shellis, 1984a). This is illustrated in Figure 4.8, where the slope of the curve represents extension rate and the numbered lines signify the regions measured. The length of EDJ is divided by the rate at point 1, which is a minimum value; all successive lengths are divided by an average value of the bounding points, which may or may not be appropriate given the slope of the line. Unless numerous areas are sampled, particularly during the initial period of formation (as in Dean, 1998a),

artificially low extension rates result in an overestimation of time (Shellis, 1984a), which explains the results found in the current study.

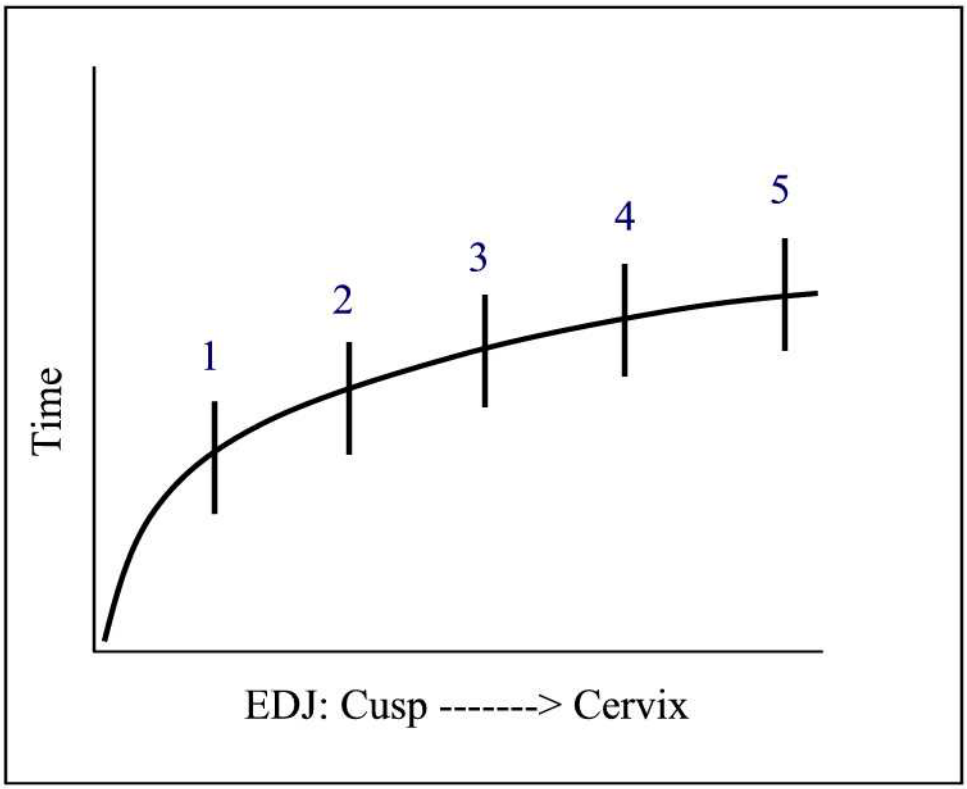

Figure 4.8 Model illustrating the relationship between crown formation and enamel dentine junction (EDJ) length, with the sampling strategy advocated by Shellis (1984a,b). At each numbered point the extension rate was calculated using Shellis' formula, and crown formation time was determined by dividing the EDJ length by the average extension rate. Note that for the first increment, the rate is derived from point 1, which is necessarily an underestimate of the interval-specific rate, and will result in an inflated period of formation.

It is possible that Shellis' formula may alternatively result in an underestimation of crown formation time in certain situations (although this did not occur in the present study). It is well established that the DSR in hominoids varies based on the position of measurement within the crown (Chapter 2, see also Beynon et al., 1991a,b; Reid et al., 1998a,b; Smith et al., 2003a, 2004). In keeping with Shellis' original theory, the secretion rate needed to predict the extension rate is the speed of the first daily increment produced.

This is essentially impossible to sample in practice, as aprismatic enamel is common near the EDJ, and the clarity of daily lines is generally poor in this area (as well as in the dentine). Shellis (1998) recommended measuring rate from the inner 30 μm of enamel, which may give a fairly accurate estimate of the rate of secretion. However, this may be greater than the initial secretion rate in taxa that show a rapid increase, such as *Pan*.

In his early reports, Shellis (1984a,b) suggested the average DSR is 4.8 μm/day in the inner enamel among primates, which he revised and re-reported as 3.9 μm/day in 1998. However, Shellis (1998) failed to note that the inner enamel secretion rate decreases from the cuspal to cervical enamel. Thus, the use of a single value for inner enamel daily secretion rate is not appropriate for calculating extension rate along the entire length of the EDJ. If the secretion rate determined from the inner cuspal enamel was used for the lateral and cervical enamel extension calculations, this may result in an inflated extension rate, and a decrease in calculated crown formation time. It is possible that the low values for human crown formation times reported in Shellis (1984a) (relative to recent data on human crown formation time from Reid et al., 1998b) were due to the use of an inflated inner DSR. It is also likely to be true when one considers some of the non-human primate values reported in Shellis (1998). For example, he gives an average daily secretion rate of 3.8 μm/day for *Pan* inner enamel, which is greater than most published reports for the inner enamel secretion rates in *Pan* (Beynon et al., 1991a: 3.1, 2.6, and 2.7 μm/day for cuspal, lateral, and cervical inner enamel, respectively; Dean, 1998a: 2.6 μm/day for cuspal inner enamel; Reid et al., 1998a: [for M_1] 3.7, 3.8, 3.3 μm/day for cuspal, lateral, and cervical inner enamel, respectively, but these were taken 100 μm from the EDJ). By his calculation, Shellis (1998) reported that one *Pan* M_1 formed in 1.46 years, which is much lower than the values for this tooth type reported by Reid et al. (1998a). However, if extension rate is recalculated using inner enamel secretion rates of 3.0 or 2.5 μm/day, *Pan* crown formation time increases from 1.46 years to 1.85 or 2.22 years, respectively. These times are closer to the average values for a large sample of mesial cusps of lower first molars of *Pan* (Chapter 5).

An additional problem with Shellis' determination of crown formation time in this specimen of *Pan* relates to the plane of section. It appears that the section he used was cut distal to the ideal plane of section, and does not preserve the entire EDJ (Figure 4.9).

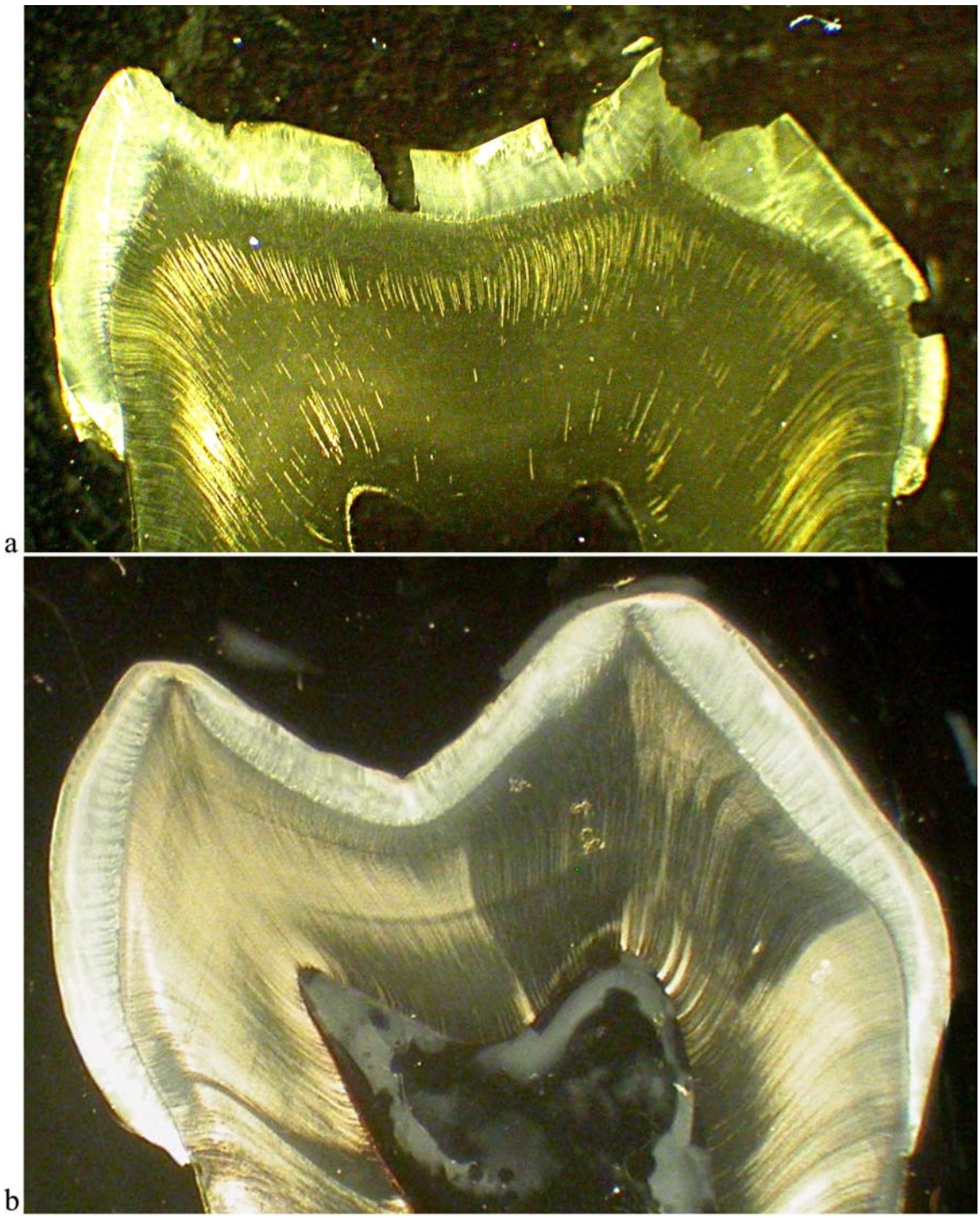

Figure 4.9 (a,b) Overviews of chimpanzee mandibular first molar cross-sections. a) Oblique section believed to be analyzed by Shellis (1998) (Reid, pers. com.), reported to show a crown formation time of 1.46 years. b) Specimen from a different individual showing a more ideal plane of section. Note the difference in dentine horn morphology and enamel dentine junction length between the two sections.

It is has been suggested in the current study that oblique sections (including those cut mesial or distal to the ideal plane of section) do not preserve the complete profile of the EDJ, which will yield underestimates of crown formation time using Shellis' method, as the entire period of extension is not represented.

Finally, due to the fact that average macaque first molar extension rate (shown in this study to be about 15 μm/day in a mixed cusp sample) is greater than the width of an enamel prism, it is not possible to divide the EDJ length by a single prism width to derive crown formation time. It appears that it is necessary to determine the average extension rate as a divisor for EDJ length, as Shellis (1984a,b) originally suggested. Additionally, it appears that there may be potential applications for a predictive regression equation based on the relationship of crown formation time and EDJ length, which may be approximated by a linear or quadratic relationship. Some of the variation seen in the graphs may be due to the fact that values from different cusps were used in this analysis. An analysis of a single cusp type may show a stronger relationship, and may potentially yield a method for determining crown formation time in material that cannot be analyzed by other methods. It is interesting to note the similarity of the curvilinear best-fit line (Figure 4.7) to the model of enamel extension with a tooth (Figure 4.8), which may imply that extension occurs along a continuum among cusps. Additional work on material of known formation time is necessary to substantiate this claim.

Summary and Conclusions

1) The age at death of five individual pig-tailed macaques (*Macaca nemestrina*) was estimated using histological analysis of the dental development of the cusps of first molars, and compared to the known age at death. Estimates of age at death ranged from underestimations of 6% to overestimations of 15%, with an average overestimate of 3% and an absolute difference of 8%. This degree of error is comparable to results derived from a study of developing human dentitions (Antoine, 2000). These results demonstrate that histological analyses of dental development involving counts and measurements of short-period features may yield fairly accurate estimations of crown formation time (*contra* Macho et al., 2003).

2) The estimated crown formation times were found to be similar to the results of a radiographic study of this species (Sirianni and Swindler, 1985). It appears that, in mandibular first molars, the protoconid initiates enamel production first, followed by the metaconid and/or hypoconid, and finally the entoconid. The protoconid and hypoconid appear to be the longest-forming cusps, finishing after the metaconid and entoconid. In maxillary first molars, the paracone appears to begin formation before the protocone, and completes crown formation later. (Data were not available on maxillary distal cusps.) These results are similar to those on macaque and hominoid cuspal initiation and completion sequences (Swindler and Emel, 1990; Reid et al., 1998a; Smith et al., 2004).

3) A number of sources of error in crown formation time estimation were identified relating to preparation quality and section obliquity. Estimates of cuspal formation time may be influenced by erroneous daily secretion rate determination or enamel thickness assessment. Imbricational enamel formation may be influenced by poor definition of Retzius lines, poor definition of cross-striations, or by the presence of accentuated lines or laminations, which may complicate determination of the long- and short-period features. The most serious complications are from section obliquity, which may impact cuspal enamel thickness, prism path length, daily secretion rate, definition of Retzius lines, and estimation of EDJ length. Estimates of dental development derived from oblique sections (or most naturally fractured teeth) should be regarded with caution.

4) A method of crown formation time estimation proposed by Shellis (1984a,b, 1998) was tested in material of known age at death, which overestimated crown formation time by 5 - 84% in different cusps. It is suggested that this method should not be applied to ‘fast-forming teeth’, such as the ones in this study. Without additional verification on ‘slow-forming teeth’, results on crown formation time derived from this method should be regarded with caution.

5) The relationship between crown formation time and enamel dentine junction length was examined, and it was shown to be a significant positive relationship that may best be explained by a polynomial regression. Additional work on a larger sample of individual cusps is recommended to investigate further the predictive value of this relationship.

Chapter 5: Incremental Development in Chimpanzees

Introduction

Studies of dental development, eruption, morphology, and wear have played significant roles in the field of anthropology, often underlying theories of evolutionary relationships among primates and within the human lineage. Assessments of the skeletal and dental development of chimpanzees have been underway since before the beginning of the 20th century (Keith, 1899; Selenka, 1899), and continue today in both captive and natural environments (e.g., Kraemer et al., 1982; Goodall, 1986; Marzke et al., 1996; Zihlman et al., 2004). Hominoid dental development and tooth emergence have historically been valued for insight into theories of life history and phylogeny (Zuckerman, 1928; Krogman, 1930; Schultz, 1935; Bennejeant, 1940; Clements and Zuckerman, 1953; Gavan and Swindler, 1966; Gavan, 1967), as well as for insight into the absolute ages of individuals that are still developing their dentitions (e.g., Garn et al., 1959; Bailit, 1976; Dean and Wood, 1981; Smith et al., 1994).

In the following review, the results of studies on chimpanzee dental development will be presented (and other hominoids where noted) to provide a background for the consideration of specific issues that are addressed in the current study. Following this, aspects of chimpanzee crown formation will be reported from a large sample of molar teeth. This will increase the amount of published histological data and will permit the examination of variation in molar development within and between individuals. Additionally, several unresolved aspects of chimpanzee cusp development will be investigated during the course of this study.

Dental Development in Chimpanzees

Eruption and crown formation

Several early studies of tooth eruption in chimpanzees took advantage of small numbers of captive animals or museum specimens of known age (reviewed in Zuckerman, 1928; see also Bingham, 1929; Schultz, 1935; Bennejeant, 1940; Schultz, 1940). It was not until the studies of Nissen and Riesen (1945, 1964) that longitudinal data became available on gingival eruption in more than a few individuals. In 1945, they

reported on the eruption of the deciduous dentition of 16 captive individuals, followed by a 1964 report on the eruption of the permanent dentition in 15 of the original 16 chimpanzees. More recently, Kraemer et al. (1982) and Conroy and Mahoney (1991) reported age at eruption in captive animals, and the latter study presented longitudinal data from intraoral exams on 58 chimpanzees over ten years, yielding the largest known dataset on age at emergence in captive individuals. Published eruption data for molars are shown in Table 1. These studies suggest that M1 eruption occurs between 32 - 49 months of age, M2 eruption is between 65 - 101 months, and M3 eruption is between 108 - 163 months. (The sequence, or order, of anterior and posterior tooth eruption will not be considered in this review, save for a brief discussion of sequence polymorphism in the section on variation below.)

Data on crown formation time in chimpanzees did not become available until recently (Anemone et al., 1991, 1996; Kuykendall, 1996; Reid et al., 1998a; Shellis, 1998). In 1981, Dean and Wood published a radiographic study of pongid dental development based on a large sample of juvenile museum specimens, which has stimulated several studies over the past two decades (including studies examining enamel and dentine microstructure). Given the lack of data on great ape crown formation at the time, they made several assumptions that have been subsequently challenged: they assumed that there was no developmental overlap in crown formation between molars, that molar crown formation began at birth, that molar crown formation time was equal among molars, and that this time was approximately 2.5 years. Anemone et al. (1991) revisited the radiographic records of Nissen and Riesen's subjects, and provided the first report of crown formation time from a longitudinal radiographic study. This was followed by larger longitudinal and cross-sectional studies by Anemone et al. (1996) and Kuykendall (1996) respectively. Reid et al. (1998a) presented the first histological estimates of crown formation time in several chimpanzee dentitions, which were substantially longer than previous reports. Shellis (1998) also presented a histological estimate for M1 crown formation time using a different method, which was discussed and questioned in Chapter 4. The results of these studies of age at molar crown calcification, completion, and total formation time are shown in Table 2.

Table 5.1 Published estimates of age at molar emergence in primarily captive chimpanzees (*Pan troglodytes* sp.).

Tooth (*n*)	**Age at Eruption**	**Source**
LM1 (75)	38.4±5.7	Conroy and Mahoney (1991)
LM1 (30)	32-45	Nissen and Riesen (1964)
LM1 (8)	~33-36*	Schultz (1935)
LM1 (?)	34-39.6	Kraemer et al. (1982)
UM1 (74)	40±5.5	Conroy and Mahoney (1991)
UM1 (30)	33-45	Nissen and Riesen (1964)
UM1 (8)	~33-36*	Schultz (1935)
UM1 (1)	49.2#	Zihlman et al. (2004)
UM1 (?)	39.6-48	Kraemer et al. (1982)
U/LM1 (2)	>~42	Oka and Kraus (1969)
U/LM1 (2)	<<45	Bennejeant (1940)
Consensus	**~32-49**	
LM2 (6)	~73-79*	Schultz (1935)
LM2 (17)	75.8±8.9	Conroy and Mahoney (1991)
LM2 (30)	67-88	Nissen and Riesen (1964)
LM2 (?)	70-96	Kraemer et al. (1982)
UM2 (6)	~73-82*	Schultz (1935)
UM2 (16)	74.4±9.7	Conroy and Mahoney (1991)
UM2 (30)	68-94	Nissen and Riesen (1964)
UM2 (2)	<98.4-100.8#	Zihlman et al. (2004)
UM2 (?)	70-77	Kraemer et al. (1982)
Consensus	**~65-101**	
LM3 (6)	~104-121*	Schultz (1935)
LM3 (28)	108-157	Nissen and Riesen (1964)
LM3 (?)	96-142	Kraemer et al. (1982)
UM3 (6)	~118-134*	Schultz (1935)
UM3 (28)	117-163	Nissen and Riesen (1964)
UM3 (2)	>148.8-165.6#	Zihlman et al. (2004)
UM3 (?)	126-168	Kraemer et al. (1982)
Consensus	**~96-168**	

Tooth position sample sizes (*n*) may include right and left analogues from same individual. Age at eruption is given in months, followed by the standard deviation when reported. *Ages of individuals in Schultz (1935) were not precisely known. # Known to be from a wild population.

Table 5.2 Published ages at calcification, crown formation, and root formation, as well as crown formation time in chimpanzees.

Tooth (*n*)	Age Calc.	Age C.C.	CFT	Age Root	Source
Radiographic Studies					
LM1 (4)	n/a	21-27	~24	~60	1
LM1 (23)	n/a	20.5±3	<24	53.3±1.5	2
LM1 (9)	1.6	20.3±3.6	~18.7	99.6±13.4	3
Consensus	1.6	~17-27	~19-24	~52-99	
LM2 (3)	15-18	48	30-33	~96-108	1
LM2 (14)	15.7±3.3	41.6±4.6	~26	n/a	2
LM2 (7)	16.1±1.6	55.1±7.1	~39	~128.8	3
Consensus	~12-19	~37-62	~26-39	~96-128	
LM3 (2-3)	42-48	84-96	36-54	~132-156	1
LM3 (12)	43.7±3.9	n/a	n/a	n/a	2
LM3 (8)	42±7.7	87.4±10.6	45.4	n/a	3
Consensus	~34-50	~77-98	36-54	~132-156	
Histological Studies					
LM1 (3)	-1.8- -0.6	>28.8-36.6	34.2±3.1*	n/a	4
LM1 (1)	n/a	n/a	17.5	n/a	5
UM1 (2)	-1.8	>27-27.6	32.8±3.7*	n/a	4
Consensus	~-2- -1	>27-36.6	17.5-37	n/a	
LM2 (2-3)	20-23.4	>54.2->67.3	44.3±3.4*	n/a	4
UM2 (1)	16.8	55.2	42.7*	n/a	4
Consensus	~17-23	~55-67	41-48	n/a	
LM3 (2)	43.2-43.4	83.2-84	48.5±5.4*	n/a	4
UM3 (1)	46.1	n/a	41.8	n/a	4
Consensus	~43-46	~83-84	42-54	n/a	

Tooth position sample sizes (*n*) may not be equal to the number of individuals, as some studies combined data on left and right analogous. **Age Calc.** and **Age C.C.** are ages at crown calcification and crown completion respectively in months, followed by the standard deviation (when reported). **CFT** is crown formation time, or the period from calcification to completion. **Age Root** is age at root completion (apical closure). **Sources**: 1- Anemone et al. (1991), 2- Anemone et al. (1996), 3- Kuykendall (1996), 4- Reid et al. (1998a), 5- Shellis (1998). * In this source, the age at initiation and crown formation times do not consistently add to age at crown completion; pooled data from their Tables 5 and 6 are not consistent with one another. Determining the sample size and time of crown formation from this source is difficult due to the way the results were presented.

Radiographic studies have commonly suggested that M1 formation is complete in 24 months or less, while Reid et al. (1998a) reported crown formation times of approximately 29 - 37 months using histological methods. Similarly, the former studies suggested M2 formation required approximately 26 - 39 months, while Reid et al. (1998a) reported approximately 41 - 48 months. For M3 formation, radiographic methods suggested between 36 - 54 months, while Reid et al. (1998a) reported 42 - 54 months.

Prior to the work of Reid et al. (1998a), several studies demonstrated that radiographic determination of crown formation time underestimated the actual duration of development, as the initial period of crown formation is not visible in radiographs for several months after initiation, and the end of cervical crown formation appears to be completed on a radiograph prematurely (e.g., Hess et al., 1932; Winkler, 1995; Beynon et al. 1998a). Beynon et al. (1998a) reviewed this problem and illustrated that it results from low contrast due to the properties of the dental tissues, and the fact that the buccal and lingual cusp tips initiate sooner and grow longer than the mesial and distal approximal surfaces. The latter are used to judge crown development in conventional lateral radiographs. In addition, the accuracy of radiographic data must be considered. Levesque and Demirjian (1980) noted that inter-observer error is +/- one stage in 20 - 25% of all cases of radiographic assessments of the eight traditional stages of dental development proposed by Demirjian et al. (1973) (the method used by Anemone et al. [1991, 1996] and Kuykendall [1996]). In summary, radiographic studies are not directly comparable to histological studies, and the latter should provide more accurate assessments of crown formation time.

Individual cusp development

Although numerous studies have reported on the timing and sequence of cuspal calcification in human first molars, only a few studies present these data in small numbers of chimpanzees (Oka and Kraus, 1969; Tarrant and Swindler, 1972; Moxham and Berkovitz, 1974; Siebert and Swindler, 1991; Winkler, 1995; Reid et al., 1998a). These studies suggest that mandibular and maxillary first molars begin to calcify about one to two months before birth in chimpanzees. The general consensus of these sources is that two to three cusps begin formation prior to birth, and sometimes a fourth cusp has

also begun. However, there is some disagreement between radiographic/dissection-based studies and a histological study concerning the order of cuspal initiation in maxillary teeth, which may be due to methodological differences or intra-specific variation.

Dissection-based studies show that the first cusp to calcify in both mandibular and maxillary molars is the mesiobuccal cusp (protoconid or paracone), which is generally followed by the mesiolingual cusp (metaconid or protocone), followed by the distobuccal cusp (hypoconid or metacone). The distolingual cusp (entoconid or hypocone) is consistently reported to be the fourth cusp to begin calcification (followed by the hypoconulid in mandibular molars). Reid et al. (1998a), however, presented histological data on a maxillary first molar that suggested an order of mesiolingual, mesiobuccal, distolingual, and distobuccal, which they related to the fact that 'principle maxillary cusps' (mesiolingual and distolingual) are generally larger, rounder, and have thicker enamel. Several studies have reported variation in the initiation order of the second, third, and fourth cusps, as well as differences in the number of cusps present at birth (Oka and Kraus, 1969; Moxham and Berkovitz, 1974; Winkler, 1995), but the study by Reid et al. (1998a) represents the only data to suggest that maxillary mesio- and distolingual cusps precede the mesio- and distobuccal cusps in chimpanzees.

Data on other primates are relatively rare. Swindler and McCoy (1965) noted that in human and rhesus macaque maxillary and mandibular molars, the first cusp to calcify is the mesiobuccal and the last is consistently the distolingual, and that the mesiolingual generally preceded the distobuccal. They demonstrated that the distobuccal cusp is the most variable. Oka and Kraus (1969) reported on the status of neonatal crowns in great apes and humans, which showed a consistent order of initiation in both upper and lower molars beginning with the mesiobuccal and ending with the distolingual. The second and third cusps were variable in sequence. A histological study of the cusps of a human first and second mandibular molar by Reid et al. (1998b) showed the order to be mesiobuccal, distobuccal, mesiolingual, and distolingual, but data on maxillary teeth were not available.

The only study that provided data on the duration of specific cusp formation in chimpanzee molars is Reid et al. (1998a), who reported that in maxillary molars, mesiolingual and distolingual cusps take the longest to form, followed by the

mesiobuccal and distobuccal cusps. They reported that in mandibular molars, the mesiobuccal and distobuccal cusps take the longest time to form, followed by the mesiolingual and distolingual cusps (Reid et al., 1998a: Table 8, p. 445). In a similar study on human dentitions, Reid et al. (1998b) reported a different order for the duration of maxillary molar formation, with buccal cusps generally forming for a longer duration than their lingual analogues (Reid et al., 1998b: Table 4, p. 471). It is not clear if this represents a discrete difference between chimpanzees and humans, or if maxillary cusps are more variable within a taxon. Resolution of the order of molar cusp initiation, and assessment of how this relates to the duration of individual cusp development will clearly benefit from the study of a large sample of mesial and distal cusps of maxillary and mandibular molars.

Previous Work on Incremental Development in Chimpanzees

During the past two decades, several studies have reported on the incremental development of chimpanzee molar teeth (e.g., Martin, 1983; Beynon et al., 1991a; Chandrasekera et al., 1993; Beynon and Reid, 1995; Beynon et al., 1998b; Dean, 1998a; Reid et al., 1998a; Shellis, 1998). Martin (1983) examined six chimpanzee molars with scanning electron microscopy, and suggested that the cross-striation repeat intervals showed a rapid inner enamel secretion, while cross-striations in outer regions showed a slower secretion rate.[1] Beynon et al. (1991a) presented the first systematic polarized light microscopy data from five chimpanzee molar teeth and demonstrated that, *contra* Martin (1983), cross-striation spacing tends to increase from inner to outer enamel, as shown by measurements of cross-striations and the width of Retzius lines (discussed in Chapter 2). Several other studies have followed, reporting additional data on daily secretion rate (DSR) and Retzius line spacing, distribution and periodicity, which are summarized below. The majority of these studies were exploratory in nature, and did not explicitly examine the variation within and between individuals. Reid et al. (1998a) presented the most complete data on partial and full dentitions of four individuals and three isolated

[1] This conclusion was based on assessment of the prism packing pattern, distribution of Hunter-Schreger (HSB) bands, and a limited number of measurements of cross-striations. Martin incorrectly argued that secretion rate could be predicted from packing pattern and HSBs.

teeth of unknown origin. At the time, that represented the largest dataset on non-human hominoid incremental development, providing the first histological estimates of total crown formation time in chimpanzees (see Table 2 above).

Recent work by Reid, Dean, and colleagues has provided insight into the development of a large sample of chimpanzee anterior teeth, as well as variation of aspects of the enamel microstructure (Schwartz and Dean, 2000; Dean and Reid, 2001; Schwartz et al., 2001a). Dean and Reid (2001) demonstrated that anterior teeth are characterized by a large number of closely packed perikymata (surface manifestations of Retzius lines), and are formed over a relatively longer period than equivalent human teeth. Schwartz and Dean (2000) and Schwartz et al. (2001a) examined canine development in 12 - 20 chimpanzee teeth, which showed significant differences in crown formation time and DSR between male and female canines. Crown formation times were one year longer in males on average. The DSRs for both sexes were reported to be similar to previously published values for molar teeth (generally less than a 0.5 μm difference), and significant differences were found between inner and outer enamel. The Retzius line periodicity was reported to range from 6 - 9, similar to the degree of variation seen in other hominoids (save for humans). From two ideal sections, regression equations were also calculated to predict the time of cuspal formation from a given enamel thickness.[2] Despite these studies, several questions remain regarding variation within a species. Little is known about species-level variation in the rate and time of formation within molar cusps or within molar types (Smith et al., 2003a, 2004). In the absence of data on variation at this fundamental taxonomic level, interpretations of incremental development in small samples of fossil and living hominoids are necessarily speculative.

Summary and Aims

Scrutiny of previous studies has illustrated a contradiction regarding the ages at eruption for first and second molars from longitudinal and cross-sectional studies, which overlap with histological estimates of the age at first and second molar crown completion

[2] Dean et al. (2001) presented a similar regression equation derived from a sample of 20 chimpanzee and gorilla teeth. Dean (pers. com) suggested that this equation is appropriate for application to chimpanzee molar formation, despite the fact that it was based on a mixed sample of *Gorilla* and *Pan* anterior teeth.

(from Reid et al., 1998a). In this scenario, there is little time available for root growth prior to eruption, which is not a reasonable developmental model. It is unlikely that this disparity can be explained solely by methodological differences, as is the case for estimates involving the radiographic determination of crown calcification and completion. Given that the majority of the estimates for age at eruption were derived from animals of known age using radiographs or oral exams, it is unlikely that eruption ages would be markedly underestimated. One of the aims of this study was to increase data on crown formation time and age at crown completion in a large sample of chimpanzee molar teeth (by inference or directly). This analysis may resolve this disparity between previously published radiographic and histological growth standards.

Additionally, the chimpanzee material examined by Reid et al. (1998a) was re-evaluated and compared to the previously published data. Aside from this single histological study, relatively little is known about molar cusp development in chimpanzees, including the timing of initiation and the crown formation time of specific cusps. Given the reported disparity in cuspal initiation order for maxillary molars, an additional aim of the present study was to examine the order of cuspal initiation and completion. Observations were also made to determine whether the pattern of mandibular cuspal initiation is consistent with dissection-based observations.

Several aspects of incremental development were examined in this sample, which is several times greater than the sample size of previous studies. The cuspal DSR was calculated for inner, middle, and outer enamel, and the average rate was tested among cusps within molar types, and within cusps among molar types for potential variation. Secretion rate from inner to outer enamel zones was also examined to test previous reports of a regional gradient within cuspal enamel. Retzius line periodicity was determined in multiple teeth of each individual when possible. Retzius line number was determined within cusp types, and the average number was tested for variation among cusps within molar types, and within cusps among molar types. Molar crown formation was also calculated from knowledge of the DSR, Retzius line periodicity, Retzius line number, and cuspal enamel thickness. Values were tested for differences among cusps within molar types, and within cusps among molar types. In addition, the relationships between several developmental variables were considered, including an investigation of

the relationships between enamel dentine junction (EDJ) length and crown formation time. These results were related to data on other living and fossil apes and humans, as well as to previously published work on chimpanzees.

Material and Methods

Histological Collections

Harvard collection

Material acquired on loan from the Peabody Museum (Harvard University) included 122 molar teeth (almost entirely upper M1s and M3s) from 67 Liberian chimpanzees (*Pan troglodytes verus*). Little is known about the collection except that the individuals were wild-shot from a population in northeastern Liberia (Schuman and Brace, 1954). Histological preparation for primary study presumably took place in the 1940's and 1950's, and included 179 teeth from 78 individuals (Schuman and Sognnaes, 1956). The teeth were originally molded, cast, embedded in methyl methacrylate resin, and sectioned according to the techniques described in Sognnaes (1947). They were the subject of a metric and morphological investigation by Schuman and Brace (1954), and a study of microscopic developmental defects by Schuman and Sognnaes (1956).

The original histological sections from these studies have yet to be recovered, but the majority of the embedded blocks were found recently associated with crania in the Peabody Museum. The collection was examined at the museum in August 2001 and loaned to Stony Brook University in December 2001. Teeth from 20 individuals had been initially quartered (cut in a mesial-distal plane and a buccal-lingual plane, yielding four embedded quarters), and these teeth were excluded from the current study. Re-preparation of the remaining material took place in February 2002 at the University of Newcastle, as detailed below. The new histological collection consists of 104 sections from 72 molars of 47 individuals (Appendix 2). The large majority of teeth were from adult individuals, and many were assumed to be from older adults, evidenced by the extreme degree of wear and large calculus deposits. The sex of the majority of the

individuals is unknown. This collection represents the largest sample of sectioned non-human hominoid teeth presently known from a single population. However, after preparation it was clear that, due to the advanced state of wear and obliquity of the available section planes, it was not possible to determine crown formation time for most teeth. Data were collected opportunistically on features of the enamel microstructure, particularly Retzius line periodicity where possible.

Martin's thesis collection

The original material loaned to Lawrence Martin from the Natural History Museum (London) included 23 molar teeth from six wild-shot individuals of *Pan troglodytes* (subspecies unknown). Specimens were intentionally chosen from individuals of unknown provenience that showed damaged or incomplete dentitions (in order to minimize the impact of semi-destructive study on the collection). Histological sections were prepared from several molars, including 29 sections prepared prior to the present study, and 12 new sections that were recently prepared in the Newcastle histology lab. The complete histological collection now consists of 41 sections from 15 teeth, representing five individuals (Appendix 2). The sex of these five individuals is known. This collection also contains a number of teeth from adult (or sub-adult) individuals that are worn or were sectioned obliquely, which precludes crown formation time reconstruction. Data were collected opportunistically on features of the enamel microstructure.

Ashton collection

This material was also acquired by Lawrence Martin, who originally radiographed and dissected out the developing mandibular dentitions from 11 infant/juvenile chimpanzees (*Pan troglodytes* sp.) donated to the Natural History Museum (London), from the collection of Eric Ashton at the University of Birmingham (United Kingdom) in 1982. Little is known about the collection, including the subspecific distinction of the material or if it was obtained from a captive or wild-shot collection, although it is likely to be from individuals that were raised in captivity. The age of the material, based on radiographic and histological appearance, is estimated to range from approximately 2 - 3

years old in several individuals to approximately 4 - 5 years old in two older individuals (based on the information in Tables 1 and 2). In the two older individuals, M1 had emerged (full occlusion), M2 had just completed crown formation, and M3 had been forming for a year or more. Prior to this study, 12 molar teeth from ten individuals were embedded in MMA and sectioned as described below, which produced 15 very thin, but generally damaged sections. Recently, 40 new sections of the mesial and distal cusps of 12 fully formed teeth and five additional developing teeth were prepared following the procedures described below. The entire histological collection now consists of 55 sections from 17 teeth representing ten individuals (Appendix 2). The sex of the individuals is unknown.

Newcastle collection

This material was acquired by Donald Reid, Chris Dean, and David Beynon from a number of sources, including the Royal College of Surgeons, the Powell Cotton Museum, Cambridge University and University College London. Little is known about this material, including subspecific designations, but it likely that most of the individuals were from research facilities and/or zoos (Dean, pers. com.). The collection was prepared prior to this study, and includes 67 sections from 29 molar teeth of 13 individuals. A subset of this material was examined and published in Reid et al. (1998a), including data from 21 sections of 15 molar teeth from seven individuals. The material ranges in age from an infant with an unerupted M1,[3] to adult individuals with moderately worn third molars. The sex of the majority of the collection is unknown.

In summary, the entire sample of chimpanzee material represents an uneven distribution of molar types from different collections. The majority of data from the Harvard collection are derived from upper M3s, the Ashton collection consists primarily of lower M1s, the Martin collection is mixed, and the Newcastle collection is mainly lower M1 - M3. The least-represented tooth positions are upper M1, upper M2, and lower M3 (Appendix 2).

[3] Some confusion exists regarding this specimen (59/89- originally loaned to David Beynon by Daris Swindler), which was changed from an M1 to an M2 and designated as an M2 in Reid et al. (1998a).

Methods

Preparation

Three main preparative techniques were employed by several individuals at different times and institutions. Most teeth had been embedded and cut prior to the present study, when 156 new sections were generated. In the following section, each process is described and the material prepared by this technique is indicated at the conclusion.

The majority of the preparation work was conducted at Dr. Donald Reid's lab in the Department of Oral Biology, University of Newcastle (Newcastle-upon-Tyne, UK). Preparation consisted of a number of steps, which are described in Reid et al. (1998a,b) and are briefly described here. Thin sections were cut from embedded blocks (or whole teeth) using a Microslice 2 annular saw, which produced approximately 200 μm thick sections (saw cut thickness approximately 200 - 300 μm). The sections were mounted to a microscope slide with dental sticky wax, and the more ideal (less oblique) face was lapped on a Logitech PM2 lapping machine with 3 μm alumina (to remove saw marks), ultrasonicated, and finished with a 1 μm diamond suspension. This face was then fixed to a microscope slide with Logitech ultraviolet (UV) curing resin under pressure and UV illumination. After curing, the section was lapped to an approximate 100 μm thickness, ultrasonicated, and finished with a 1 μm diamond polishing suspension. Sections were then ultrasonicated, dehydrated in an alcohol series, cleared in xylene, and cover slips were mounted with DPX mounting media (Fluka Chemicals). The material prepared in this fashion includes 103 of the Harvard sections, 12 of the Martin sections, 37 of the Ashton sections, and the entire Newcastle collection (67 sections).

Preparation of a subset of the material prior to the present study took place at Stony Brook University according to ‘less than ideal’ procedures described in Smith et al. (2003a). Teeth were first refluxed in a 50/50 methanol chloroform bath and embedded in methyl methacrylate according to procedures described by Boyde (1989). Longitudinal cuts (buccal-lingual) were made across the mesial and distal cusps using a Unipress wire saw (ideal cut thickness approximately 50 μm). The cut face was polished and mounted to a microscope slide with cyanoacrylate, and a second cut was made with the wire saw,

freeing the remaining tooth block and leaving a thin section attached to the slide. The target thickness of the remaining section was approximately 100 μm. However, given the unreliability of wire saw preparations, sections were often much thinner. These were ground and/or polished to a 40 - 60 μm thickness using a Buehler Metaserv grinder-polisher and decreasing grades of diamond paste. They were then ultrasonicated and finished with a 0.3 μm alumina suspension on a polishing cloth. The material originally prepared in this fashion includes 29 of the Martin sections and 15 of the Ashton sections. During the current study, these sections were ultrasonicated, cleaned with alcohol, cleared in xylene, and cover slips were mounted with DPX mounting media. Although remounting and additional polishing may have improved their quality, the relatively thin and fragile condition prohibited more substantial re-preparation.

A third technique was applied at Stony Brook University for the preparation of a small number of sections during the present study. In each case, an embedded block was cut across the mesial and/or distal cusps with a diamond wafering blade on a Buehler low speed saw (minimum cut thickness approximately 250 - 300 μm), the cut face was ground and polished with 3 and 1 μm alumina, ultrasonicated, dehydrated, and fixed to a slide with UV curing resin as described above. After curing, the block was cut from the slide, leaving an approximately 200 μm thick section, which was ground to approximately 80 - 100 μm on abrasive paper, ultrasonicated, and finished with 1 μm alumina. Sections were ultrasonicated, cleaned with alcohol, cleared in xylene, and cover slips were mounted with DPX mounting media. The material prepared in this fashion includes one Harvard section and three Ashton sections.

Overviews of all sections were generated with a stereo microscope and a black and white Hitachi CCD camera or a Nikon Coolpix 4500 digital camera, cropped with either Microsoft Photo Editor or Microsoft PhotoDraw software, and printed with a Hewlett-Packard inkjet printer.

Data collection

The thin sections were examined with a Zeiss (Jena) polarized light microscope (PLM) housed in the Department of Anthropology, Stony Brook University. Images were captured digitally with a Hitachi KP-C553 CCD color camera and a Nikon Coolpix 4500

digital camera, and NIH Image and Sigma Scan Pro 5 (SPSS Science, Inc.) software were used for measurements. Each section was examined, and data were recorded on a standard data sheet (Appendix 3). Qualitative information was recorded on: presence of pathology, accentuated features, order of cuspal initiation/completion, cuspal cross-striation quality, presence of laminations, presence of intradian lines, and dentine quality. The preservation of each crown was qualitatively assessed, along with the degree of obliquity, and sections that were clearly oblique or moderately/heavily worn were only used for periodicity determination.[4] Several aspects of the enamel microstructure were quantified: (1) cross-striation repeat interval (DSR), (2) periodicity of Retzius lines (number of cross-striations between), (3) total number of Retzius lines, and (4) cuspal enamel thickness. From these data, the total crown formation time (5) was established. These were determined as noted below.

(1) The DSR was determined in three zones for each unworn cusp in the cuspal inner, cuspal middle, and cuspal outer zones with a 50X objective, and recorded on a separate data sheet (Appendix 4). Although previous studies have collected data on DSR in cuspal, lateral, and cervical enamel, it was decided that the most readily identifiable internal crown landmark is the (maximal) cuspal prism path. Measurements were made along an axis that approximated the path of a prism from the tip of the dentine horn to the position of the first imbricational Retzius line. This facilitated the collection of data from analogous regions in different cusps and molars, and may permit a more refined analysis of rate variation. A minimum of three cross-striations was measured in each area, and a minimum of two areas was measured per zone (generally in stepped intervals from near the EDJ to near the surface of the enamel). Additional measurements were made when the quality of the sections permitted. As stated in Chapter 2, cross-striations were defined as light and dark bands that cross enamel prisms perpendicularly, and intradian lines were defined as closely spaced, fine bands dividing the cross-striations, which were avoided. Cross-striations were not counted from the EDJ to the enamel surface, as intradian lines, poor incremental feature definition, and marked cuspal decussation precluded accurate counts in the majority of this material. When a neonatal line was identified in the cuspal

[4] Unfortunately, due to the state of wear and original preparation of the material, few unworn or lightly worn cusps were available for assessment of formation time. The approach taken in this study was intentionally conservative, as worn or missing enamel was generally not estimated.

enamel of first molars, the prenatal DSR was quantified from a minimum of three cross-striations in three areas between the dentine horn and the farthest point on the line (maximum prism path).

(2) Periodicity was determined between Retzius lines that clearly met the tooth surface. However, in the majority of the material examined, Retzius lines converged or did not display clear cross-striations in the subsurface enamel, necessitating periodicity determination in slightly deeper enamel. Whenever possible, cross-striations were counted over multiple Retzius line intervals and average periodicities were determined (which is believed to give a more accurate estimate than counts within single intervals). Measurements were not made of the average Retzius line spacing and local cross-striation repeat intervals as an alternative to direct counts; it was determined that DSR was not constant within Retzius line intervals, and this method may not consistently yield reliable periodicity estimations. In some instances, when a single integer could not be determined with confidence, a range was recorded.

(3) The total number of Retzius lines that met the surface of the enamel was counted directly from the cervix to the cusp tip using 25X and 50X objectives. Slight corrections were made when cervical tips were broken or when light wear obscured the first-formed Retzius lines in cusp tips. For each cusp, Retzius lines were counted three or more times and an average was calculated. When missing enamel was moderate or substantial, Retzius lines were determined only for the enamel preserved, and were recorded as incomplete counts. In developing material, Retzius lines were counted from the beginning of imbricational enamel formation to the point of death, and were recorded as incomplete counts. In the latter two instances, Retzius lines were only counted one time in each cusp.

(4) The cuspal (linear) enamel thickness was measured from the tip of the dentine horn to the approximate point where the first imbricational Retzius line was identified at the tooth surface (in unworn teeth only). Measurements were usually made with a 12.5X objective. When possible, the neonatal line was identified in first molar cusps, and the maximum linear thickness (prism path) was recorded from the dentine horn to the maximum position of the line (usually directly under the cusp tip). A rough estimate of

the degree of prism decussation (deviation from a straight line) was also made for each cusp based on the prism path from the dentine horn to the tooth surface.

(5) Total crown formation time represents the development of two regions of the crown: cuspal and imbricational enamel. Cuspal enamel formation was determined using three methods, minimum and maximum estimates were determined, and the average of these two was used to calculate crown formation time. First, the linear cuspal enamel thickness was divided by the average cuspal DSR to yield a conservative estimate of cuspal formation time in days. This value was multiplied by an estimated correction factor (1.05 - 1.30) to compensate the three-dimensional curvature of enamel prisms. Finally, the enamel thickness was entered into a regression equation (derived from molar data on African apes), which predicts the time of cuspal formation in days (Dean et al., 2001). This equation was also used as the only method of assessing cuspal enamel formation in two cusps where DSR could not be determined due to poor cross-striation quality. In addition to sections where total formation time was determined, cuspal enamel formation was also determined in 20 cusps where it was not possible to determine imbricational enamel formation due to damage or incomplete development. These were included in the calculation of average cuspal formation time.

The imbricational formation time, or lateral plus cervical enamel, was determined by multiplying the total number of Retzius lines by the periodicity. As noted above, total (cusp-specific) crown formation was generally determined from the sum of cuspal and imbricational enamel. However, a modified approach was taken due to light cuspal wear or missing enamel in ten cusps. Retzius lines were counted to the edge of the wear facet or break, the enamel thickness was measured from this point to the dentine horn (along a prism path), DSR was determined along this measurement, and the thickness was divided by the average rate for this region. Additionally, prism decussation was assessed, a corrected estimate of cuspal formation was determined, the two were averaged, and the cuspal and imbricational regions were combined to yield total crown formation time. Because a similar approach yielded fairly accurate estimates of crown formation in macaque material (Chapter 4: Specimen 1 and 2), it was assumed to be appropriate for these ten lightly worn cusps.

Additional data were recorded on the length of the EDJ from the dentine horn to the cervix of each cusp, as well as the bi-cervical diameter of each tooth using stereo microscope overviews and Sigma Scan Pro 5 software.

Analysis

Data analysis consisted of nonparametric statistical tests, which were performed using SPSS software (v12.0, SPSS Science, Inc.). Rank-based statistical methods are appropriate given the relatively small sample sizes for specific cusps and molar positions, which nearly guarantees a failure to meet the assumptions of parametric analyses. Non-parametric tests are also deemed appropriate because many of the data collected are discrete (non-continuous) (Conover, 1999).

(1) For DSR, means, ranges, and standard deviations were computed for inner, middle, and outer cuspal enamel zones, as well as for the overall average of each individual cusp and molar type. Kruskal-Wallis tests for rate differences between samples were performed using both cusp type and molar type as the factors, testing all six tooth types separately and all eight cusp types separately. When significance was achieved, the multiple comparisons technique described by Conover (1999) was performed in order to determine which cusp types or molar types differed from one another significantly.[5] The Mann-Whitney U test was used to test for differences between buccal and lingual analogues (within mesial or distal pairs), and between upper and lower molar analogues for each cuspal zone and for the overall average cuspal value. Conover's (1999) adaptation of the Jonckheere-Terpstra test for trends was used to test for a gradient in rate from inner to outer cuspal enamel. Spearman's Rho is the statistic of choice for assessing the level of significance of the Jonckheere-Terpstra test statistic, and is a more appropriate test for trends than the parametric ANOVA model (discussed further in Smith et al., in review).

(2) Retzius line periodicity was determined from each section when possible, including multiple sections per individual, to confirm the uniformity of this feature and

[5] This was accomplished using a computer program written and executed by Anthony J. Olejniczak, as it is not available in standard statistical computer packages.

the accuracy of the count.[6] Values are presented in a frequency table, and the mean, mode, and range were calculated.

(3) For Retzius line number, means and ranges were computed for individual cusp and molar types. Kruskal-Wallis tests for group differences were performed using both cusp type and molar type as the factors, testing all six tooth types separately and all eight cusp types separately. When significance was achieved, the multiple comparisons technique described above was used. The Mann-Whitney U test was used to test for differences between buccal and lingual analogues, and between upper and lower molar analogues. The Jonckheere-Terpstra test for trends was used to test for trends in Retzius line number from M1 to M3.

(4) Cuspal enamel thickness was measured, and means and ranges were computed for individual cusp types and molar types. Kruskal-Wallis tests for group differences were performed using both cusp type and molar type as the factors, testing all six tooth types separately and all eight cusp types separately. When significance was achieved, the multiple comparisons technique described above was used. The Mann-Whitney U test was used to test for differences between buccal and lingual analogues, and between upper and lower molar analogues. The Jonckheere-Terpstra test for trends was used to test for cuspal enamel thickness trends from M1 to M3.

(5) For crown formation time, cuspal and imbricational components were determined, and means and ranges were computed for individual cusp types and molar types for each component and for (total) crown formation time. Kruskal-Wallis tests for group differences were performed using both cusp and molar type as the factors, testing all six tooth types separately and all eight cusp types separately. When significance was achieved, the multiple comparisons technique described above was used. The Mann-Whitney U test was used to test for differences between buccal and lingual analogues, and between upper and lower molar analogues. The Jonckheere-Terpstra test for trends was used to test for trends in cuspal enamel formation, imbricational enamel formation, and crown formation from M1 to M3. Prenatal formation times are also presented for each cusp type, including mean values and ranges.

[6] The periodicity was re-examined in a subset of corresponding anterior teeth from the Newcastle collection after molar analysis, which led to revised estimates in some instances due to the greater clarity of features.

To examine the relationships between developmental variables, a correlation matrix was constructed describing the relationships of 12 variables: periodicity; Retzius line number; imbricational crown formation time; cuspal enamel thickness; cuspal inner, middle, outer, and overall average DSRs; cuspal formation time; crown formation time; bi-cervical diameter; and EDJ length. Data on bi-cervical diameter may serve as a surrogate for body size (Martin, 1983; Schwartz, 2000a; Grine, 2002; Smith et al., in review), and EDJ length may serve as a predictor for crown formation time (Chapter 4).

Results

General Observations

Some evidence for developmental stress was observed in each collection (Figure 5.1), although the frequencies appeared to differ among sources. The Harvard material represents a population of chimpanzees that appeared to have experienced marked and prolonged stress during dental development. Enamel hypoplasias, accentuated bands, and dentine disturbances were commonly observed.[7] Several individuals from Martin's thesis material also showed accentuated lines and hypoplasias. The material from the Ashton collection is striking in terms of general morphological and developmental uniformity. In general, fewer accentuations and growth disturbances were seen in this material than in the first two collections. Hypoplasias were observed less frequently than in the Harvard collection.[8] The Newcastle collection also contained fewer individuals with dramatic accentuations and hypoplasias; however, this material did show some variation. Given that first two collections are derived from wild populations, and the likelihood that the Ashton collection and most (or all) of the Newcastle collection are from captive animals, it is possible that the frequencies of accentuated lines and developmental pathology may relate to more stressful ecological and/or social developmental environments in the wild.

[7] This was also noted in the original macroscopic and microscopic investigation of pathology in this collection by Schuman and Sognnaes (1956)

[8] However, it is possible that hypoplasia expression varies for different molar types; thus, is it is not clear that comparisons of hypoplasias on upper third molars and lower first molars are appropriate

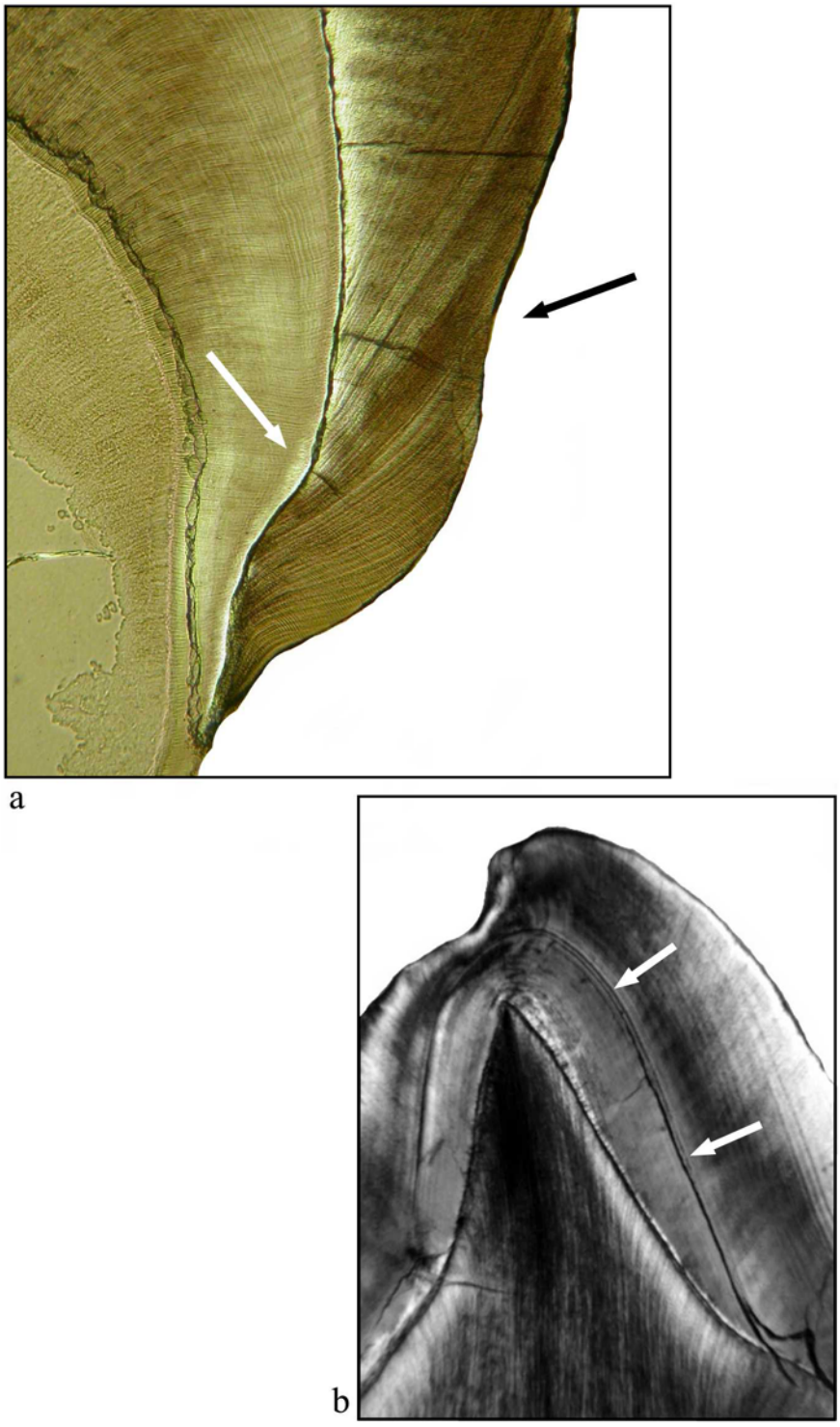

Figure 5.1 (a-b) Transmitted and polarized light images of suspected pathology in chimpanzee enamel. a) Disturbance in enamel and dentine formation in a developing lower M1 hypoconid; arrows denote the bend in the enamel dentine junction and the corresponding enamel accentuations and hypoplasia (depression) at the enamel surface (slide 112-1). b) Strongly accentuated line over the dentine horn in an upper third molar (slide 6911.222). These areas did not generally affect incremental feature quantification.

Quantitative Information

Daily secretion rate (DSR)

The DSR was quantified for the molar enamel of 69 unworn cusps, and the average values and ranges for each cusp and tooth type are shown in Table 3[9]. The grand mean of 207 average regional cuspal measurements was 4.17 μm/day (*range* 2.78 - 5.77 μm/day, *s.d.*=0.62 μm/day). No significant difference in inner, middle, outer, or overall rates was found among cusps within a molar type, nor within cusps among molar types. No significant difference was found in inner, middle, outer, or overall rates between buccal and lingual analogues or between mandibular and maxillary analogues. Average values for inner, middle, and outer enamel zones are shown in Table 4. Zones differences were tested using the Jonckheere-Terpstra test for trends, which revealed a significant increase in rate from inner to outer enamel ($P<0.001$, $n= 207$), despite considerable overlap between middle and outer zones.

Periodicity

The periodicity was found to range from 6 to 7 (days between pairs of Retzius lines) in 61 chimpanzees (Table 5). The average value was 6.4 days, and the mode was 6 days. Values for each individual are listed in Appendix 5. Values of either 5 or 8 could not be ruled out in three individuals. No conclusive evidence was found to suggest that periodicity varied within or among teeth of the same dentition. However, given the highly variable appearance of cross-striations and Retzius lines, it was not possible to demonstrate that this value was identical within and between all teeth belonging to individual dentitions.

Retzius line number

The average and range of Retzius line numbers of each cusp and molar type are shown for 59 cusps in Table 6. Significant differences were found among cusps within lower M1 only ($P=0.013$, $df=3$). Post hoc tests showed that mesiobuccal cusps had greater numbers of Retzius lines than mesiolingual and distolingual cusps, and

[9] Averages of each enamel zone for each cusp and tooth type are given in Table 15, where they are compared to other published values.

distobuccal cusps showed greater numbers of Retzius lines than both mesiolingual and distolingual cusps. Significant differences were not found within cusp types among molar types using the Kruskal-Wallis test, but a significant increasing trend in Retzius line number was found in lower molar distobuccal cusps from M1 to M3 using the Jonckheere-Terpstra test for trends (P=0.033, n=9). In lower molars, mesial and distal buccal cusps showed significantly greater Retzius lines than their lingual analogues using the Mann-Whitney U test (mesial: P=0.006, n=20; distal: P=0.023, n=11), but upper molars did not show significant differences. Upper and lower cuspal analogues did not show significant differences except for mesiolingual cusps, where the upper cusp showed significantly greater numbers of Retzius lines than the lower mesiolingual cusp (P=0.027, n=16). In general, Retzius line numbers appeared to be greater in mesial cusps than in distal cusps within a tooth type, and greater in second molars relative to first and third molars, but these differences were not significant. (Additional indirect data on Retzius line number in lightly worn or moderately broken material are presented in Table 9, where this is considered in light of the variation in imbricational enamel formation time.)

Cuspal enamel thickness

Cuspal enamel thickness is reported for each cusp type and molar type for 72 unworn cusps in Table 7. Significant differences were found among cusps within the lower M2 only (P=0.031, df=3). Post hoc tests showed that mesiobuccal cusps demonstrated greater cuspal enamel thickness than mesiolingual and distolingual cusps, and distobuccal cusps showed greater cuspal enamel thickness than both mesiolingual and distolingual cusps. Significant differences were found within cusp types among molars types only in distobuccal cusps among lower molars (P=0.02, df=2).[10] Post hoc tests showed that distobuccal cusps showed significantly greater cuspal enamel thickness in M2 than in M1, in M3 than in M1, and in M3 than M2. In general, upper lingual cusps were equal to or thicker than upper buccal cusps, and the opposite pattern was found in lower molars, but neither of these trends was found to be significant with the Mann-Whitney U test.

[10] The mesiobuccal and mesiolingual cusps showed nearly significant differences in thickness among lower molar types ($0.05<P<0.10$, df=2).

Table 5.3 Cuspal enamel daily secretion rate averages and ranges.

Tooth	mb	range (n)	ml	range (n)	db	range (n)	dl	range (n)
UM1	3.74	3.49-4.00 (2)	3.99	---	4.23	---	3.32	---
UM2	4.17	4.10-4.24 (2)	4.66	---	4.36	---	n/a	
UM3	4.06	3.72-4.33 (4)	4.22	4.19-4.24 (2)	3.80	3.48-4.13 (6)	4.12	3.86-4.38 (2)
LM1	4.18	3.78-4.52 (5)	4.39	3.96-4.62 (5)	4.44	4.0-4.79 (4)	4.44	3.93-4.74 (4)
LM2	4.20	3.69-4.70 (6)	4.16	3.84-4.57 (8)	4.35	3.8-4.76 (4)	4.37	3.85-4.89(2)
LM3	4.17	3.83-4.37(3)	4.11	3.60-4.55 (3)	3.77	---	3.70	-

Average values represent the mean of several measurements of cross-striations in inner, middle, and outer cuspal enamel in μm/day. Cusp types indicated for upper molars: mb= mesiobuccal cusp (paracone), ml= mesiolingual cusp (protocone), db= distobuccal cusp (metacone), dl= distolingual cusp (hypocone). For lower molars: mb= mesiobuccal cusp (protoconid), ml= mesiolingual cusp (metaconid), db= distobuccal cusp (hypoconid), dl= distolingual cusp (entoconid). Range data indicate the minimum and maximum values and the number in parenthesis indicates the number of cusps sampled. Sample sizes are one when range data are not indicated. Data were not available for the distolingual cusp of UM2, indicated by n/a.

Table 5.4 Inner, middle, and outer chimpanzee cuspal enamel daily secretion rate.

Position	N	Min	Max	Mean	Std. Dev
Inner	69	2.78	4.72	3.62	0.42
Middle	69	3.01	5.38	4.28	0.48
Outer	69	3.64	5.77	4.61	0.49

Position indicates the zone within the cuspal enamel where measurements of cross-striations were derived from. N indicates the total number of cusps sampled, min and max indicate the minimum and maximum average values, mean indicates the average of all 69 cusps, and the standard deviation is also in μm/day.

Table 5.5 Retzius line periodicities of 75 individual chimpanzee molar dentitions.

	Unknown	5-6	6	6-7	7	7-8
Frequency	7	2	41	4	20	1

Frequency ranges are from individuals where a single value could not be determined conclusively, and are not intended to imply that the periodicity was variable in these individuals. Values are in number of cross-striations (or days) between successive Retzius lines.

Table 5.6 Average Retzius line number in chimpanzee molar teeth.

Tooth	mb	range (n)	ml	range (n)	db	range (n)	dl	range (n)
UM1	113	99-127 (2)	99	91-107 (2)	86	---	77	---
UM2	129	---	131	---	193	---	n/a	
UM3	119	106-130 (4)	128	125-130 (2)	112	101-126 (6)	96	84-107 (2)
LM1	116	90-147 (5)	86	74-103 (6)	102	89-117 (7)	80	67-98 (4)
LM2	128	110-143 (3)	103	72-130 (3)	128	---	116	---
LM3	123	---	90	73-107 (2)	121	---	90	82-97 (2)

Headings are the same as in Table 3.

Table 5.7 Average linear cuspal enamel thickness in chimpanzee molars.

Tooth	mb	range (n)	ml	range (n)	db	range (n)	dl	range (n)
UM1	445	190-700 (2)	445	---	445	---	475	---
UM2	588	565-610 (2)	670	---	860	---	n/a	---
UM3	705	675-740 (4)	740	685-795 (2)	732	560-1015 (6)	860	855-865 (2)
LM1	644	420-875 (5)	598	480-815 (6)	672	615-785 (6)	726	665-825 (4)
LM2	932	670-1075 (6)	772	635-910 (8)	1002	950-1050 (4)	812	730-895 (2)
LM3	705	470-920 (3)	697	625-820 (3)	1055	---	700	---

Headings are the same as in Table 3. Values are in μm.

No significant differences were found in cuspal thickness between upper and lower molar analogues. In general, cuspal thickness appeared to increase from M1 - M3. The Jonckheere-Terpstra test for trends showed a statistically significant increasing trend in cuspal thickness from M1 - M3 in the lower distobuccal cusp only (P<0.001, n=11).[11]

Crown formation time

Cuspal and imbricational enamel formation times were determined individually and combined to yield the cusp-specific crown formation time (shown separately in Table 8). Cuspal enamel formation time was determined for 81 cusps. Significant differences in cuspal times were found among cusps only within lower M2 (P=0.026, df=3). Post hoc tests indicated that mesiobuccal cusps shower longer cuspal formation time than mesiolingual and distolingual cusps, and distobuccal cusps showed longer cuspal formation time than both mesiolingual and distolingual cusps. Significant differences were found among lower molars within mesiobuccal cusps (P=0.034, df=2), mesiolingual cusps (P=0.046, df=2), and distobuccal cusps (P=0.015, df=2). Post hoc tests showed that within mesiobuccal cusps, M2 showed longer cuspal formation time than both M1 and M3, but there were no significant differences between M1 and M3. Within the mesiolingual cusp, M2 showed longer cuspal formation time than M1, and M3 showed longer cuspal formation time than M1, however M2 and M3 could not be statistically distinguished. Within the distobuccal cusp, M3 was significantly greater than M2 and M1, and M2 was significantly greater than M1. In general, upper lingual cusps showed longer times than buccal cusps, and the opposite pattern was found in lower molars, but neither of these trends was found to be significant with the Mann-Whitney U test. No significant differences were found in average cuspal enamel formation time between upper and lower molar analogues. In general, cuspal enamel formation time appeared to increase from M1 - M3. The Jonckheere-Terpstra test for trends showed a statistically significant increasing trend in cuspal enamel formation time from M1 - M3 in the lower mesiolingual cusp (P=0.047, n=18) and the lower distobuccal cusp (P<0.001, n=12).

Imbricational enamel formation time was calculated for 61 cusps. Significant differences in imbricational times were found among cusps within lower M1 only

[11] A similar trend in the upper mesiolingual cusp also approached significance (P=0.051, n=4).

(P=0.014, df=3). Post hoc tests showed that the mesiobuccal cusp showed a longer imbricational formation time than mesiolingual and distolingual cusps, and the distobuccal cusp showed a longer imbricational formation time than both mesiolingual and distolingual cusps. Significant differences were not found within cusps among molar types. Upper cusps showed a variable pattern of imbricational times between lingual and buccal cusps, while lower molars consistently showed longer imbricational times in buccal cusps than in lingual analogues. This was found to be significant for lower mesial cusps (P=0.018, n=21).[12] Significant differences in imbricational enamel formation times between upper and lower molar analogues were found in mesiolingual cusps only (P=0.009, n=17); upper cusps showed greater values. In general, imbricational enamel formation time appeared to increase from M1 - M2 and decrease from M2 - M3. The Jonckheere-Terpstra test for trends showed a statistically significant increasing trend in imbricational formation time from M1 - M3 in the lower distobuccal cusp only (P=0.033, n=9).[13]

Additional imbricational formation times for are shown in Table 9 for 55 cusps that were not used in the calculation of crown formation time, due to the lack of data on cuspal formation. Statistical analyses were not performed on this sample, as these data should be regarded as minimum estimates only. However, it is worth noting similar trends when compared to the imbricational data in Table 8. These additional data also extend the range of several cusps and tooth types, most notably the lower M1 and M2 mesiobuccal cusps, the upper M1 and M2 mesiolingual cusps, and the upper M2 distolingual cusp. Several of these additional cusps show <u>minimum</u> imbricational formation times that are equal to or longer than three years.

Crown formation time is shown for each cusp type and molar type in Table 10, and for each individual cusp in Appendix 6. Significant differences in crown formation time were found among cusps within lower M1 only (P=0.036, df=3).[14] Post hoc tests

[12] Lower distal cusps showed nearly-significant differences in imbricational formation times between buccal and lingual cusps (P=0.071, n=16).

[13] Lower mesiolingual cusps showed a nearly-significant increasing trend in imbricational formation time from M1 to M3 (P=0.078, n=12).

[14] Upper M3 showed nearly-significant differences in crown formation time among cusps (P=0.085, df=3).

showed that mesiobuccal cusps showed a longer crown formation time than mesiolingual and distolingual cusps, and the distobuccal cusp showed a longer crown formation time than both mesiolingual and distolingual cusps. No significant differences were found within cusps among molar types.[15] In general, maxillary lingual cusps showed slightly longer crown formation times than buccal cusps, but these differences were not significant. In lower molars, buccal cusps showed longer crown formation times than lingual cusps, which was significant between mesial analogues (P=0.034, n=21). Significant differences were only found between upper and lower mesiolingual cusp analogues (P=0.019, n=17), where upper cusps showed longer crown formation times than lower cusps. Crown formation time generally appeared to increase from M1 to M2 and decrease from M2 to M3, and the Jonckheere-Terpstra test for trends showed a statistically significant increasing trend in crown formation time from M1 - M3 in lower mesiolingual cusps (P=0.018, n=12) and in lower distobuccal cusps (P=0.033, n=9).

Prenatal crown formation times are shown in Table 11. In maxillary molars, the order of initiation appears to be variable, as the paracone initiated before the protocone in one individual, while the opposite pattern was found in a second individual. The metacone followed both mesial cusps in a single individual, and no evidence was found to suggest that the hypocone had begun formation before birth. Among mandibular first molars, the protoconid initiated before the metaconid in three of four comparisons. The hypoconid was the second cusp to initiate in two individual comparisons with the metaconid, and it consistently followed the protoconid in three individual comparisons. No evidence was found for prenatal formation in the entoconid. Prenatal secretion rates ranged from 2.64 - 4.00 μm/day, with a mean of 3.53 μm/day. Cross-striations were visible in prenatal enamel, and laminations were very common in the first-formed enamel over the dentine horn (Figure 2).

[15] Crown formation time differences were almost significant in lower mesiolingual cusps among molars (P=0.054, df=2).

Table 5.8 Average cuspal and imbricational enamel formation times in chimpanzee molars.

Tooth	mb	range (n)	ml	range (n)	db	range (n)	dl	range (n)
Cuspal								
UM1	118	60-176 (2)	148	123-172 (2)	110	---	170	153-186 (2)
UM2	151	146-156 (2)	154	---	202	---	n/a	
UM3	178	160-194 (5)	193	170-216 (3)	167	60-266 (7)	205	197-216 (3)
LM1	162	118-199 (5)	148	124-198 (6)	169	143-204 (7)	173	154-207 (4)
LM2	230	179-254 (6)	191	150-237 (9)	239	215-264 (4)	194	192-196 (2)
LM3	181	140-221 (3)	179	151-200 (3)	274	---	194	186-202 (2)
Imbricational								
UM1	791	693-889 (2)	693	637-749 (2)	602	---	514	490-539 (2)
UM2	903	---	917	---	1351	---	n/a	
UM3	742	636-833 (4)	830	750-910 (2)	670	606-756 (6)	626	504-749 (2)
LM1	695	540-882 (5)	516	444-618 (6)	630	534-763 (7)	502	402-588 (4)
LM2	805	770-858 (3)	688	504-910 (4)	896	---	696	---
LM3	861	---	630	511-749 (2)	847	---	626	574-679 (2)

Headings are the same as in Table 3.

Table 5.9 Additional minimum estimates of imbricational enamel formation time (not used for crown formation time estimation).

Tooth	mb	range (n)	ml	range (n)	db	range (n)	dl	range (n)
UM1	700	540-882 (4)	980	---	735	594-1029 (3)	n/a	
UM2	n/a		1022	---	n/a		1015	---
UM3	820	780-875 (3)	798	702-930 (3)	648	624-684 (3)	672	582-810 (3)
LM1	700	570-861 (6)	551	426-721 (5)	705	570-900 (6)	460	330-693 (6)
LM2	950	756-1120 (3)	882	---	480	---	659	618-686 (3)
LM3	1225	---	742	---	n/a		567	---

Data represent counts of Retzius lines from the cervix to the edge of the cuspal enamel, frequently representing inclusive counts, or estimates that are close to the total number of Retzius lines, which have been multiplied by the periodicity to yield a time in days. Headings are the same as for Table 3.

Table 5.10 Average cusp-specific crown formation time in chimpanzee molars.

Tooth	mb	range (n)	ml	range (n)	db	range (n)	dl	range (n)
UM1	909	753-1065 (2)	840	809-872 (2)	712	---	684	676-692 (2)
UM2	1059	---	1070	---	1553	---	n/a	---
UM3	921	818-993 (4)	1035	966-1104 (2)	821	761-938 (6)	826	701-951 (2)
LM1	857	734-1081 (5)	664	601-816 (6)	798	709-967 (7)	674	556-796 (4)
LM2	952	804-1037 (3)	860	713-1086 (4)	1160	---	888	---
LM3	1001	---	823	697-949 (2)	1120	---	820	776-865 (2)

Times represent the sum of imbricational and cuspal enamel formation in days. Headings are the same as for Table 3.

Table 5.11 Average prenatal crown formation time in chimpanzee molars.

Tooth	mb	range (n)	ml	range (n)	db	range (n)	dl	range (n)
UM1	33	29-36 (2)	37	25-49 (2)	n/a		14	---
LM1	49	26-70 (8)	36	23-44 (5)	35	28-49 (4)	n/a	

Values are in days. Headings are the same as for Table 3.

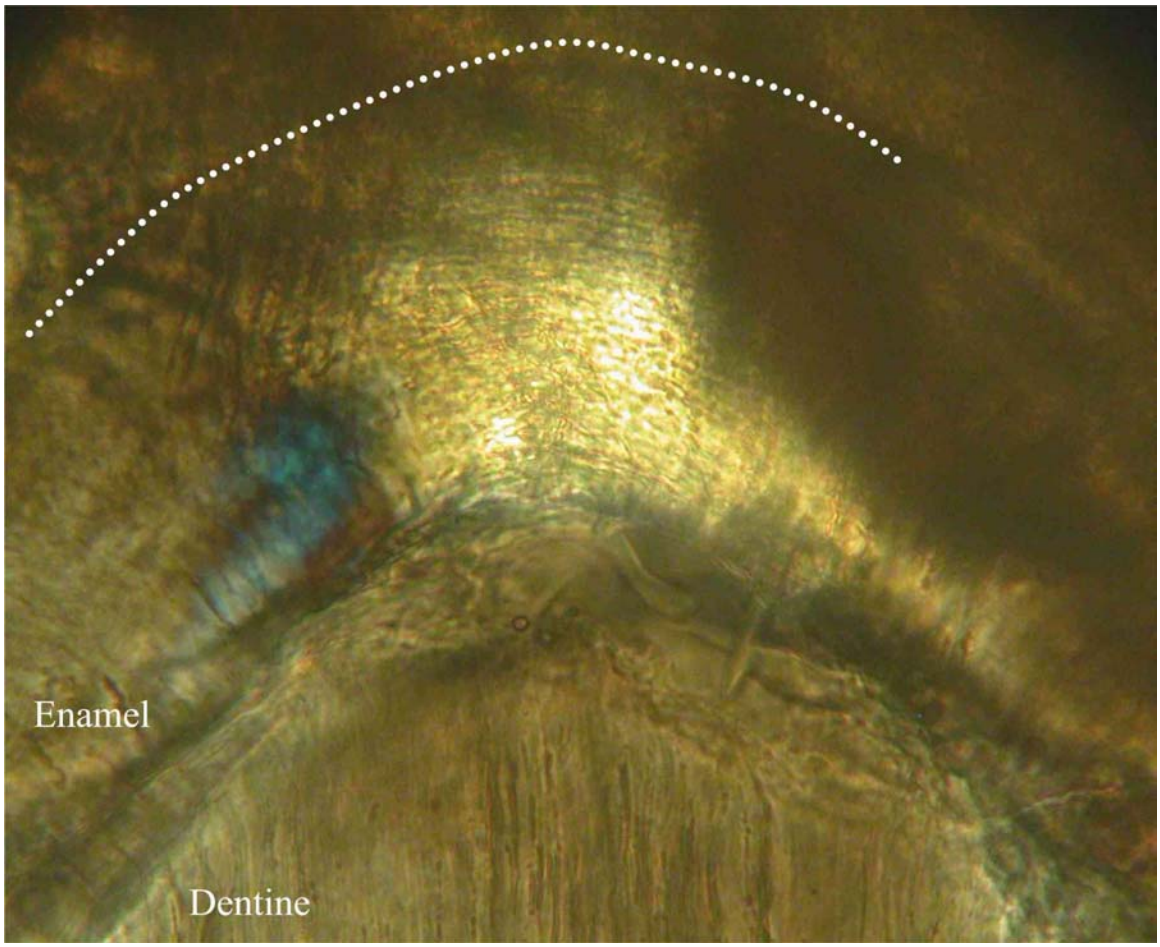

Figure 5.2 Prenatal enamel development in the protoconid of a chimpanzee lower first molar, shown under polarized light microscopy at high magnification (slide 88.89.46 m). In this image, the dentine horn is at the bottom, forming an arch under the enamel, which shows approximately 30 - 35 laminations paralleling the profile of the enamel dentine junction. The neonatal line in this tooth is partially indicated with a dotted white line (above), and the prenatal formation time was estimated to be 34 days.

Relationships between developmental variables

A correlation matrix showing the Pearson's product-moment correlation coefficient between ten developmental variables and two morphological variables is shown in Table 12. Several developmental variables were significantly positively correlated with one another, including periodicity, imbricational formation time, and total formation time. Cuspal enamel thickness was positively correlated with outer DSR. Several variables were also positively correlated with bi-cervical diameter, including periodicity, Retzius line number, and total crown formation time. Variables that were significantly positively correlated with EDJ length include Retzius line number, imbricational formation time, and total crown formation time.

The relationship between crown formation time and EDJ length was examined further. Crown formation time was regressed against cusp-specific EDJ length in 61 mixed cusps (mesial and distal cusps from maxillary and mandibular molars), which showed significant positive linear, logarithmic, and quadratic relationships (Table 13). It is suggested that the most appropriate theoretical model may be the quadratic equation, as EDJ length to the second power may serve as an approximation of the surface area of the EDJ. The relationship between crown formation time and EDJ length is shown in Figure 4, with the linear and quadratic best-fit lines indicated. The data were then separated by collections, and linear, logarithmic, and quadratic relationships were calculated (Table 14). The relationship between crown formation time and EDJ length for each of the four collections is shown in Figure 5, with the linear and quadratic best-fit lines indicated.

Table 5.12 Bivariate correlation matrix of developmental variables.

		Per	Ret	Imb	Thick	InnRate	MidRate	OutRate	AveRate	AveTime	CFT	BC diameter	EDJ
Per	Pearson	1	.088	.369(**)	.024	-.187	-.163	-.247	-.234	.160	.358(**)	.417(**)	.124
	Sig. (2-tailed)		.508	.003	.867	.198	.264	.088	.105	.217	.005	.001	.342
	N	61	59	61	52	49	49	49	49	61	61	56	61
Ret	Pearson	.088	1	.944(**)	.269	-.043	.021	.099	.031	.195	.919(**)	.381(**)	.425(**)
	Sig. (2-tailed)	.508		.000	.054	.769	.888	.501	.830	.139	.000	.004	.001
	N	59	59	59	52	49	49	49	49	59	59	54	59
Imb	Pearson	.369(**)	.944(**)	1	.255	-.075	-.014	.025	-.022	.221	.972(**)	.440(**)	.451(**)
	Sig. (2-tailed)	.003	.000		.068	.608	.923	.867	.878	.087	.000	.001	.000
	N	61	59	61	52	49	49	49	49	61	61	56	61
Thick	Pearson	.024	.269	.255	1	.081	.122	.312(**)	.224	.956(**)	.407(**)	.097	-.096
	Sig. (2-tailed)	.867	.054	.068		.510	.320	.009	.064	.000	.003	.512	.498
	N	52	52	52	72	69	69	69	69	72	52	48	52
InnRate	Pearson	-.187	-.043	-.075	.081	1	.442(**)	.337(**)	.735(**)	-.104	-.069	.118	.151
	Sig. (2-tailed)	.198	.769	.608	.510		.000	.005	.000	.397	.635	.440	.301
	N	49	49	49	69	69	69	69	69	69	49	45	49
MidRate	Pearson	-.163	.021	-.014	.122	.442(**)	1	.421(**)	.807(**)	-.099	-.022	.044	.081
	Sig. (2-tailed)	.264	.888	.923	.320	.000		.000	.000	.418	.879	.772	.580
	N	49	49	49	69	69	69	69	69	69	49	45	49
OutRate	Pearson	-.247	.099	.025	.312(**)	.337(**)	.421(**)	1	.774(**)	.118	.062	.037	-.003
	Sig. (2-tailed)	.088	.501	.867	.009	.005	.000		.000	.333	.670	.809	.982
	N	49	49	49	69	69	69	69	69	69	49	45	49
AveRate	Pearson	-.234	.031	-.022	.224	.735(**)	.807(**)	.774(**)	1	-.030	-.010	.107	.123
	Sig. (2-tailed)	.105	.830	.878	.064	.000	.000	.000		.805	.945	.484	.398
	N	49	49	49	69	69	69	69	69	69	49	45	49
AveTime	Pearson	.160	.195	.221	.956(**)	-.104	-.099	.118	-.030	1	.396(**)	-.017	-.136
	Sig. (2-tailed)	.217	.139	.087	.000	.397	.418	.333	.805		.002	.900	.298

	N	61	59	61	72	69	69	69	69	81	61	56	61
CFT	Pearson	.358(**)	.919(**)	.972(**)	.407(**)	-.069	-.022	.062	-.010	.396(**)	1	.400(**)	.376(**)
	Sig. (2-tailed)	.005	.000	.000	.003	.635	.879	.670	.945	.002		.002	.003
	N	61	59	61	52	49	49	49	49	61	61	56	61
BC diameter	Pearson	.417(**)	.381(**)	.440(**)	.097	.118	.044	.037	.107	-.017	.400(**)	1	.433(**)
	Sig. (2-tailed)	.001	.004	.001	.512	.440	.772	.809	.484	.900	.002		.001
	N	56	54	56	48	45	45	45	45	56	56	56	56
EDJ	Pearson	.124	.425(**)	.451(**)	-.096	.151	.081	-.003	.123	-.136	.376(**)	.433(**)	1
	Sig. (2-tailed)	.342	.001	.000	.498	.301	.580	.982	.398	.298	.003	.001	
	N	61	59	61	52	49	49	49	49	61	61	56	61

Developmental variables are: Per- Retzius line periodicity, Ret- Retzius line number, Imb- imbricational formation time in days, Thick- cuspal enamel thickness in μm, Inn Rate- inner cuspal enamel secretion rate in μm/day, Mid Rate- middle cuspal enamel secretion rate in μm/day, Out Rate- outer cuspal enamel secretion rate in μm/day, Ave Rate- overall average cuspal enamel secretion rate in μm/day, Ave Time- average of minimum and maximum estimates of cuspal enamel formation time in days, CFT- crown formation time (imbricational and cuspal formation) in days, BC diameter- the linear distance between buccal and lingual cervices (bi-cervical diameter) in μm, and EDJ- the length of the enamel dentine junction from the tip of the dentine horn to the tip of the cervix (within a cusp type) in μm.

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 5.13 Results of curvilinear estimation of the relationship between crown formation time and enamel dentine junction length.

Dependent	Mth	Rsq	df	F	Sig	Intercept	Slope	Quadratic
CFT	LIN	0.14	59	9.74	0.003	385.135	0.0812	
CFT	LOG	0.13	59	8.93	0.004	-2875.7	431.106	
CFT	QUA	0.16	58	5.47	0.007	1260.90	-.2359	2.8E-05

Dependent variable- CFT- crown formation time, Mth- method of regression: LIN-linear, LOG-logarithmic, QUA-quadratic, Rsq- r-squared value, df- degrees of freedom, F- F value, Sig- significance level. Y-intercept, slope, and quadratic terms may be used to construct a regression equation.

Table 5.14 Results of curvilinear estimation of the relationship between crown formation time and enamel dentine junction length divided into the four original collections.

Dependent	Mth	Rsq	df	F	Sig	Intercept	Slope	Quadratic
Harvard								
CFT	LIN	0.53	11	12.35	0.005	336.1	0.11	
CFT	LOG	0.54	11	12.86	0.004	-3694.8	539.26	
CFT	QUA	0.56	10	6.26	0.017	-959.03	0.66	-6.E-05
Martin								
CFT	LIN	0.89	1	8.40	0.211	-1833.4	0.48	
CFT	LOG	0.88	1	7.44	0.224	-25783	3087.52	
CFT	QUA	1.00	0	---	---	26829.7	-8.48	0.0007
Ashton								
CFT	LIN	0.23	16	4.75	0.045	257.117	0.08	
CFT	LOG	0.24	16	4.95	0.041	-3495.9	487.95	
CFT	QUA	0.26	15	2.58	0.109	-1372.9	0.64	-5.E-05
Newcastle								
CFT	LIN	0.24	25	7.77	0.010	271.223	0.10	
CFT	LOG	0.23	25	7.49	0.011	-4167.8	579.81	
CFT	QUA	0.24	24	3.90	0.034	1152.51	-0.20	2.5E-05

Dependent variable- CFT- crown formation time, Mth- method of regression: LIN-linear, LOG-logarithmic, QUA-quadratic, Rsq- r-squared value, df- degrees of freedom, F- F value, Sig- significance level. Y-intercept, slope, and quadratic terms may be used to construct a regression equation.

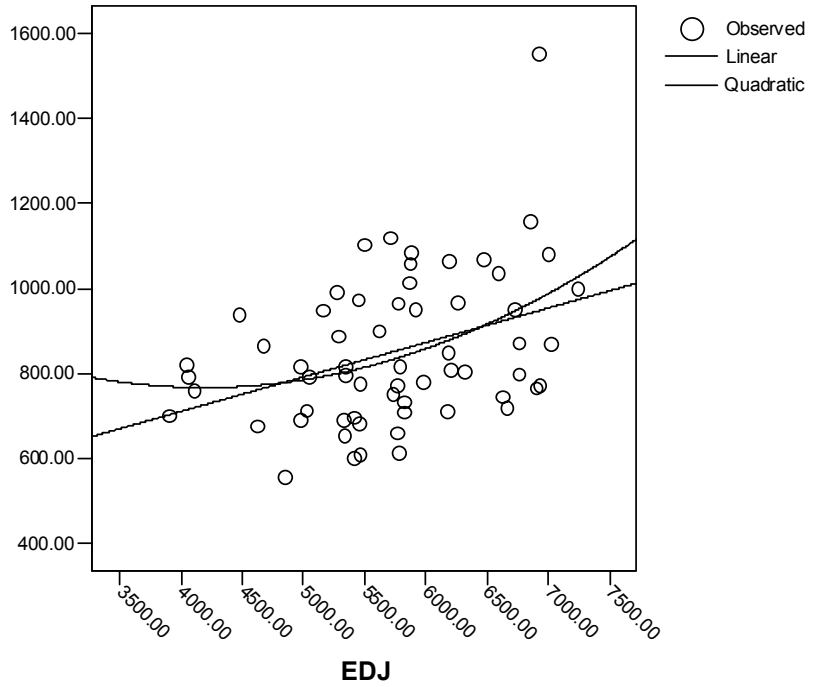

Figure 5.3 Relationship between crown formation time in days (y-axis) and enamel dentine junction length in μm (x-axis) showing the linear and quadratic best-fit lines for a mixed sample of cusps.

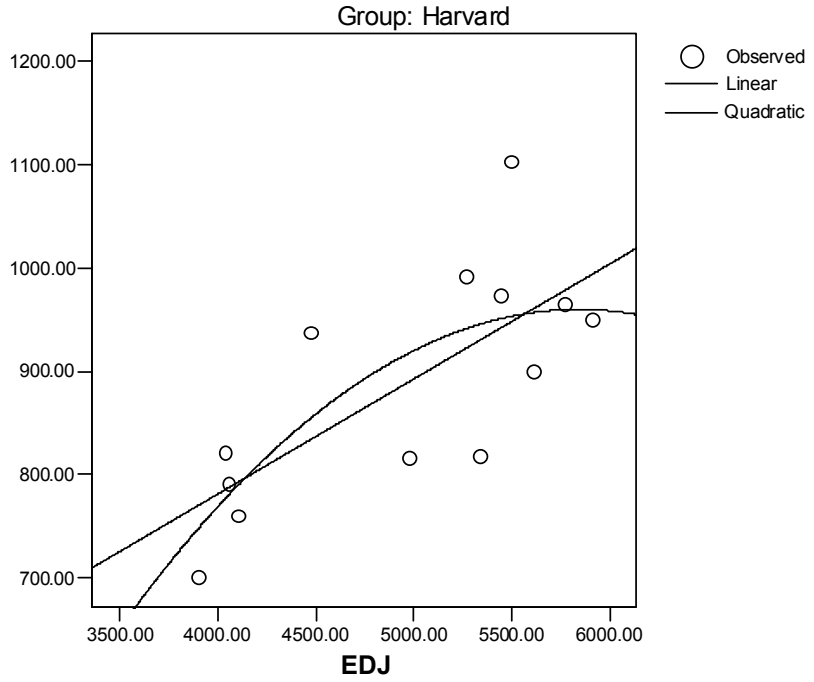

Figure 5.4 (a-d) Relationship between crown formation time in days (y-axis) and enamel dentine junction length in μm (x-axis) showing the linear and quadratic best-fit lines.
a) Harvard collection. (Figures b-d are below).

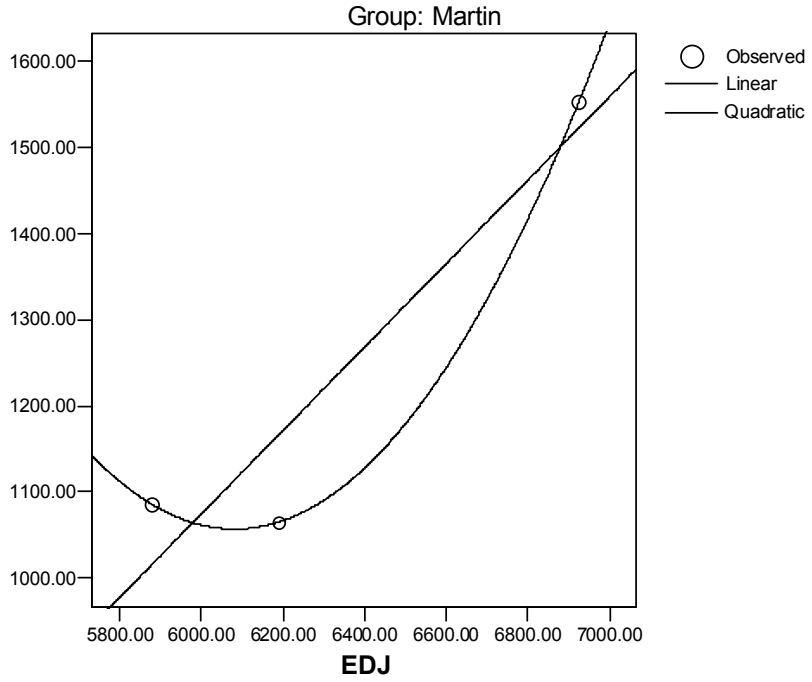

Figure 5.4 (b) Relationship between crown formation time in days (y-axis) and enamel dentine junction length in μm (x-axis) showing the linear and quadratic best-fit lines. b) Martin collection.

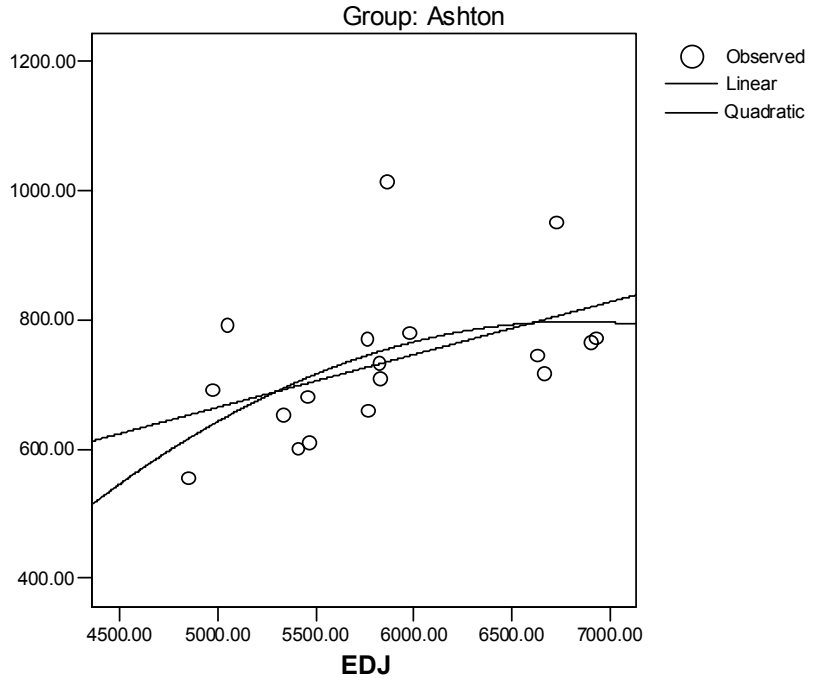

Figure 5.4 (c) Relationship between crown formation time in days (y-axis) and enamel dentine junction length in μm (x-axis) showing the linear and quadratic best-fit lines. c) Ashton collection.

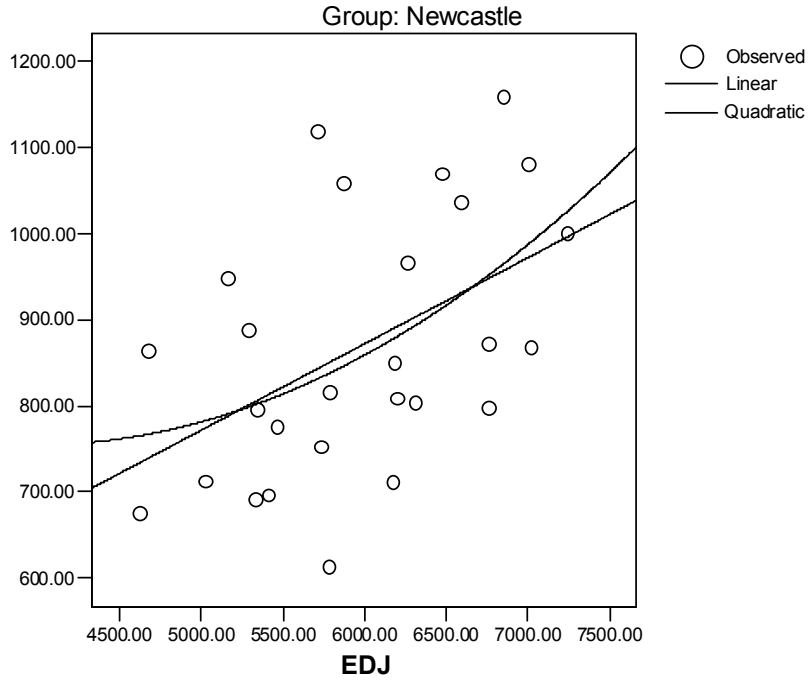

Figure 5.4 (d) Relationship between crown formation time (y-axis) and enamel dentine junction length (x-axis) showing the linear and quadratic best-fit lines.

d) Newcastle collection.

Discussion

Incremental Development

Daily secretion rate

DSR in chimpanzee cuspal enamel appeared to be fairly constant within zones among cusps and among molars. Average values for cuspal enamel ranged from approximately 3 - 5 μm/day, similar to published data (Table 15). Reid et al. (1998a) reported values for cuspal enamel zones in anterior and posterior teeth that also ranged from 3 - 5 μm/day, and the large majority of these values fall within the standard deviations reported in this study. The only tooth types that differ from the current study were upper and lower M3s, which showed slower rates than other teeth in each respective zone (but were not statistically evaluated). Examination of data from the current study by cusp and tooth type showed that most M3 cuspal secretion rates were similar to other molar teeth, save for relatively slower rates in distal lower molar cusps from single individuals. Given the limited sample sizes of these positions, statistical testing was not appropriate, and additional data are required to determine if third molars show slower rates than other molars. Schwartz et al. (2001a) reported averages for inner and outer cuspal enamel in chimpanzee canines, which fall within one standard deviation of the inner and outer values reported here. Thus, it appears that DSR within chimpanzees is consistent within analogous enamel zones among anterior and posterior teeth.[16]

Daily secretion rate increases from inner to outer cuspal enamel in all tooth types, which was also noted in numerous previous studies. However, the present study is the first to demonstrate a significant trend from inner to outer cuspal enamel zones.[17] Some variation was observed in the relationship between middle and outer enamel rates, as certain cusps showed an increase from inner to middle zones, and a decrease from middle to outer zones, while other cusps showed a linear increase from inner to outer zones.

[16] However, the table of DSR values reported in Reid et al. (1998a: Table 3, p. 436) does show a slight trend of decreasing cuspal enamel secretion from the anterior to posterior dentition. This should be examined further and tested within and among individual dentitions.

[17] Schwartz et al. (2001) demonstrated statistical differences between regions, but did not explicitly test for trends.

Table 5.15 Cuspal enamel daily secretion rate in chimpanzee molars.

Tooth	Cusp	n	Inner	Middle	Outer	Source
UM1	mb	?	3.29±0.25	4.19±0.40	4.38±0.74	Reid et al. (1998a)
		2	3.35±0.37	4.11±0.64	3.78±0.08	**This study**
	ml	?	3.53±0.04	4.06±0.46	4.76±0.06	Reid et al. (1998a)
		1	2.82	4.55	4.60	**This study**
	db	?	3.45±0.35	4.36±0.06	4.79±0.95	Reid et al. (1998a)
		1	3.88	4.50	4.32	**This study**
	dl	?	3.16±0.63	4.29±0.01	4.76±0.35	Reid et al. (1998a)
		1	3.04	3.27	3.64	**This study**
UM2	mb	1	3.60	4.10	4.80	Reid et al. (1998a)
		2	4.04±0.5	3.99±0.01	4.48±0.26	**This study**
	ml	1	3.70	3.70	4.80	Reid et al. (1998a)
		1	4.16	4.05	5.77	**This study**
	db	1	3.65	4.77	4.67	**This study**
UM3	mb	4	3.35±0.30	4.28±0.45	4.63±0.27	**This study**
	ml	2	3.68±0.20	4.35±0.48	4.62±0.37	**This study**
	db	1	3.00	3.60	4.20	Reid et al. (1998a)
		6	3.32±0.36	3.84±0.50	4.32±0.38	**This study**
	dl	1	2.80	3.20	4.40	Reid et al. (1998a)
		2	3.72±0..57	4.19±0.45	4.68±0.36	**This study**
LM1	mb	?	4.06±0.19	4.71±0.49	4.88±0.51	Reid et al. (1998a)
		5	3.55±0.35	4.40±0.28	4.59±0.48	**This study**
	ml	?	3.67±0.23	4.61±0.66	4.66±0.81	Reid et al. (1998a)
		5	3.72±0.33	4.47±0.49	4.99±0.47	**This study**
	db	?	3.93±0.34	4.37±0.18	4.76±0.04	Reid et al. (1998a)

		4	4.15±0.46	4.70±0.36	4.48±0.45	**This study**
	dl	?	4.05±0.35	4.14±0.44	4.58±0.03	Reid et al. (1998a)
		4	4.07±0.38	4.64±0.76	4.62±0.45	**This study**
LM2	mb	?	3.82±0.38	4.42±0.22	4.66±0.19	Reid et al. (1998a)
		6	3.45±0.29	4.40±0.31	4.74±0.57	**This study**
	ml	?	3.68±0.17	4.39±0.16	4.85±0.44	Reid et al. (1998a)
		8	3.71±0.43	4.29±0.37	4.48±0.33	**This study**
	db	?	3.35±0.19	3.91±0.33	4.35±0.32	Reid et al. (1998a)
		4	4.15±0.46	4.20±0.71	5.12±0.58	**This study**
	dl	?	3.42±0.15	3.99±0.28	4.47±0.22	Reid et al. (1998a)
		2	3.77±0.54	4.26±0.98	5.08±0.69	**This study**
LM3	mb	?	3.00±0.27	3.77±0.37	4.16±0.74	Reid et al. (1998a)
		3	3.61±0.25	4.21±0.57	4.67±0.45	**This study**
	ml	?	3.06±0.38	3.61±0.54	4.03±0.59	Reid et al. (1998a)
		3	3.43±0.48	4.24±0.37	4.67±0.66	**This study**
	db	?	3.17±0.15	3.68±0.47	4.27±0.48	Reid et al. (1998a)
		1	3.09	4.12	4.11	**This study**
	dl	?	3.13±0.38	3.75±0.44	4.27±0.45	Reid et al. (1998a)
		1	3.03	3.58	4.48	**This study**
mixed	?	1-4	3.1±0.3	4.4	5.0±0.5	Beynon et al. (1991a)
?M1	?	?			3.97±0.2*	Beynon and Reid (1995)
?M2	?	1	~3.1	~3.7	~5.1#	Dean (1998a)

Cusps are given in Table 3, *n* is the number of cusps sampled, mean values are in μm/day with standard deviations (when given). Note that measurements by Reid et al. (1998a) were not made 100 μm from the enamel dentine junction (EDJ) or tooth surface.
* Data given for measurements made in a single area 100 μm below the surface.
# Data not given as inner, middle and outer zones; ten monthly zones from EDJ to surface were originally reported, which were averaged for inclusion in this table.

This variation has been observed in chimpanzees and in other hominoids (Reid et al., 1998a; Smith et al., 2003a, 2004). As shown above, cuspal thickness and outer secretion rate show a significant positive relationship. Thus, cusps with thicker enamel show a greater outer enamel secretion rate than cusps with thinner enamel. This may partially explain the variation in the patterning of cuspal secretion rate from middle to outer enamel zones, as well as the appearance of thin 'compressed' outer enamel in certain teeth with relatively slow outer secretion rates, and the rapidly formed and sometimes laminated appearance of thicker cusps (Figure 3). Additional examination of this phenomenon in other hominoids may provide more insight into this pattern.

Retzius line periodicity

The range of periodicity values in this sample is narrower than previously reported ranges for common chimpanzees (Table 16). Inter-observer error may affect comparisons of values from different studies. Schwartz et al. (2001a) reported that this was less than 3% among three observers in their study of hominoid canines. However, periodicity values from the material in Reid et al. (1998a) reported here are lower than the original values by 1- 2 days in two of four individuals. A preliminary assessment of inter-observer error was made in the present study using subsets of the Harvard and Newcastle collections, which showed error rates of about 10% and 40%, respectively.[18]

The periodicity range of 6 - 7 days is similar to published values on hominoids (see Smith et al., 2003a: Table 3, p. 293), although slightly less than several large-bodied hominoids (Dean and Schrenk, 2003; Smith et al., 2004). Smith et al. (2003b) demonstrated that periodicity is positively correlated with body mass among hominoids.[19] When considering extant great apes, it is not surprising that chimpanzees show the lowest mean periodicity. When compared to human values, which range from 6 - 12 days, this large sample of chimpanzees shows less variation. It is possible that periodicity in humans is more variable than in our closest living relatives.

[18] Re-evaluation of the Newcastle material with Donald Reid suggested that both intra- and inter-individual error may be responsible for high error rates and differences between values reported here and published values.

[19] Body mass, which is difficult to estimate in megadont fossil taxa, was calculated and analyzed using separate and averaged cranial and post-cranial estimates when possible, and the method used did not affect this result.

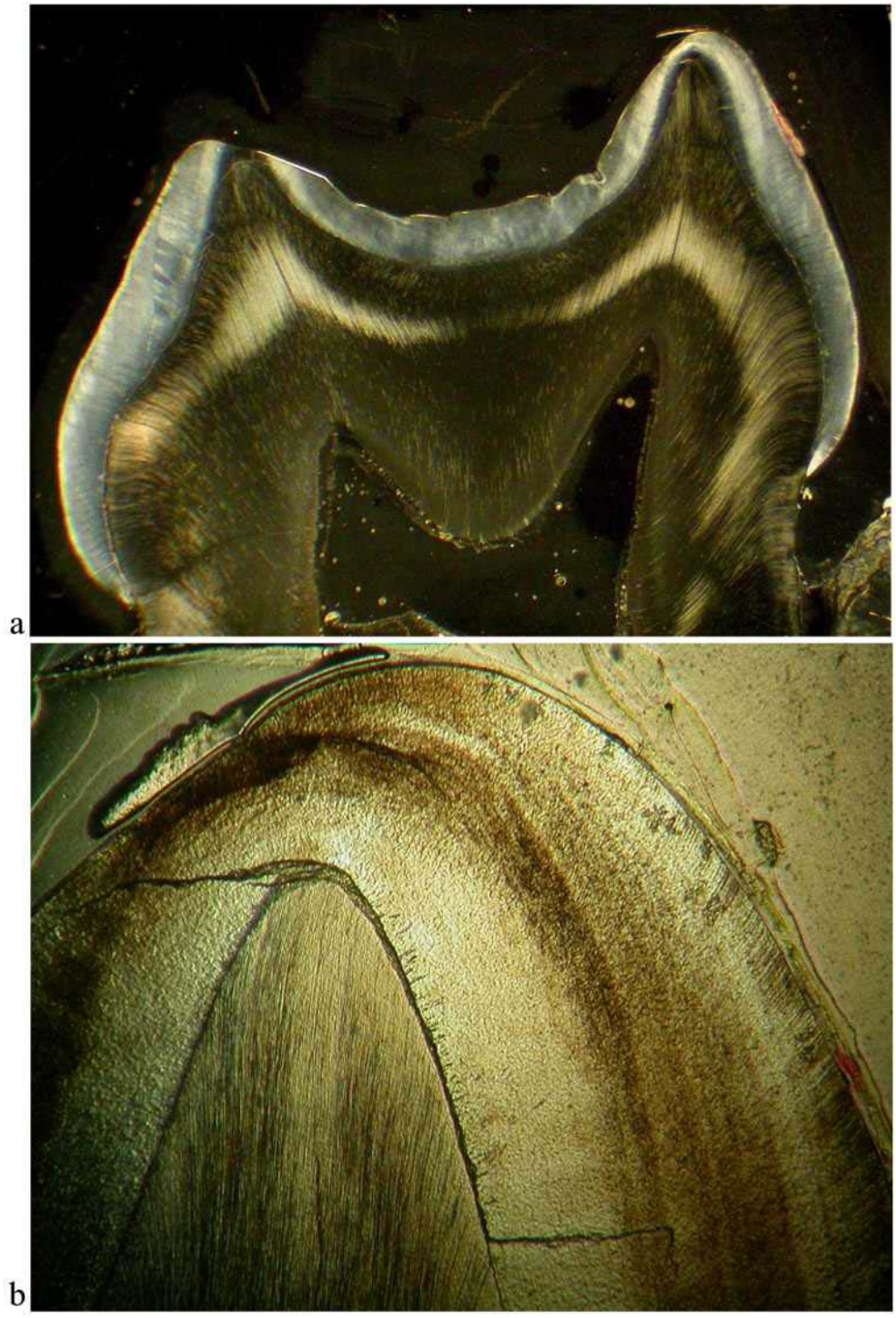

Figure 5.5 (a-d). An illustration of differences in the pattern of cuspal enamel formation in thin and thick cusps. a) Overview of the distal cusps of an upper M3, showing a lightly worn paracone (right side) with a 'compressed' enamel thickness condition. (slide 10.88.18 d) b) A higher magnification view of the paracone tip where the outer cuspal enamel has decreased in rate relative to enamel lateral to (and beneath) the cusp tip. The outer cuspal enamel in this tooth was estimated to form at a rate of 3.7 μm/day and cuspal crown formation time was estimated to take 55 - 64 days. (See below for images c and d.)

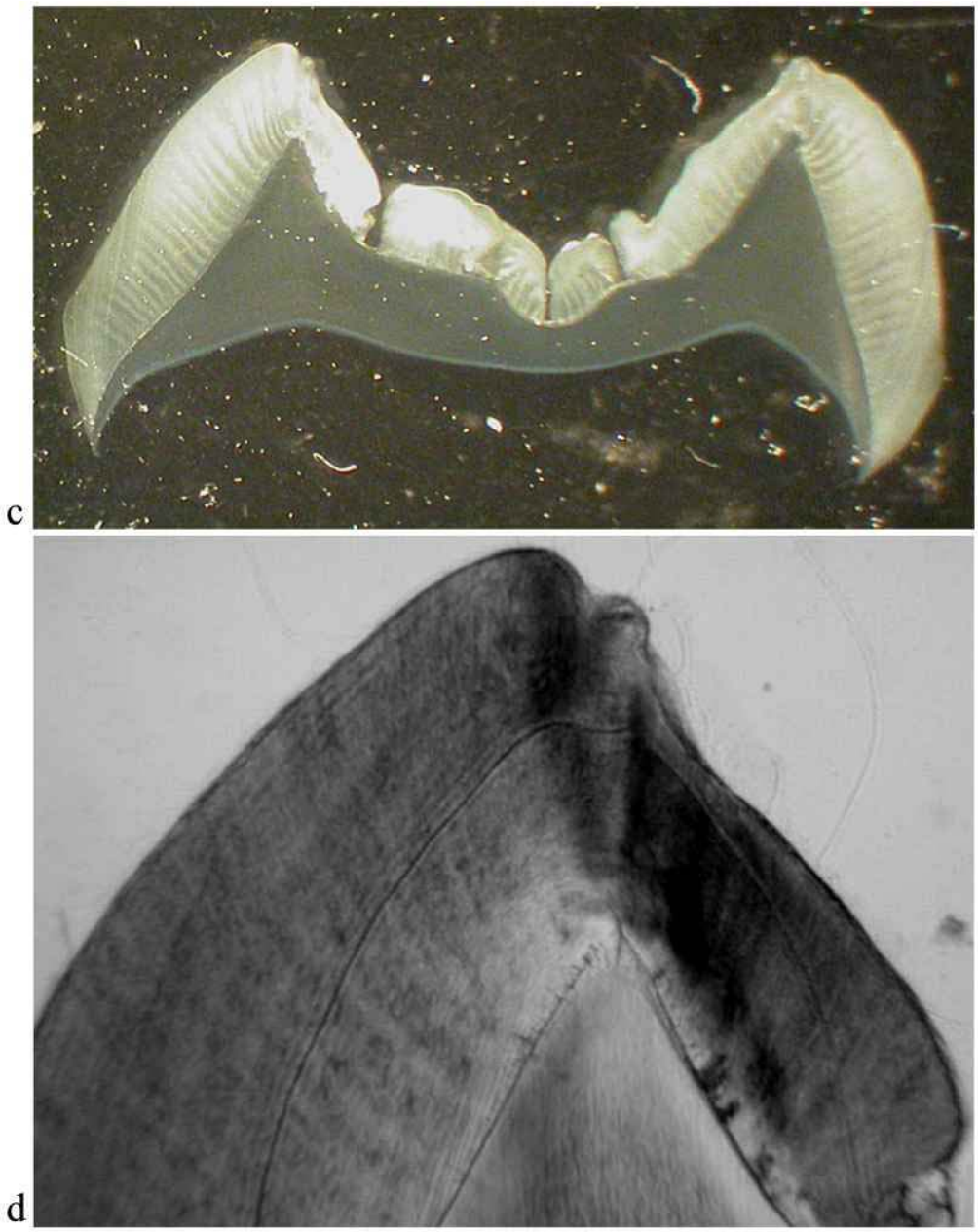

Figure 5.5 (c-d). c) Overview of a developing lower M3 representing the 'thick' cuspal condition, with the protoconid shown on the left (linear thickness 1055 μm). (slide 125-1) d) The protoconid shown under higher magnification, which shows a fairly uniform increase in daily secretion rate. The outer cuspal enamel secretion rate ranges from 5.0-5.7 μm/day, and the cuspal crown formation time was calculated to be 243 - 255 days.

Table 5.16 Retzius line periodicity in common and pygmy chimpanzee dentitions.

Species	*n*	Periodicity	Source
P. troglodytes	??	7	Beynon and Reid, 1995
P. troglodytes	2	7	Reid et al., 1998a
P. troglodytes	2	8*	Reid et al., 1998a
P. troglodytes	20	6-9 (ave 6.95)	Schwartz et al., 2001a
P. troglodytes	61	6-7 (ave 6.37)	**This study**
P. paniscus	1	6[2]	Reid, unpublished
P. paniscus	1	6	Smith and Martin, unpublished
P. paniscus	1	7#	Reid, unpublished

n- number of individuals, periodicity- number of cross-striations (days) between Retzius lines. * Reid (pers. com.) has recently re-examined this material and has concluded that the periodicity in these two individuals is less than 8 (new values- 6 and 7). # Reported in Smith et al. (2003a).

In this sample it was possible to examine the relationship between periodicity and Retzius line number. Previously, Reid et al. (2002) demonstrated a negative association between periodicity and Retzius line number in human canines, which suggested that these two developmental parameters may co-vary in a way that limits variation in anterior tooth crown formation times. As noted in Chapter 2, previous molar data suggested a variable relationship. Analysis in the present study did not show a significant relationship between periodicity and Retzius line number, which may be due to low variation in periodicity, or high variation in Retzius line number.[20] The lack of a relationship may result in variable imbricational and total crown formation times, which was found in this sample. Given that both periodicity and Retzius line number are significantly correlated with bi-cervical diameter (a linear surrogate for body mass), it may be worthwhile to investigate the relationships of these variables while controlling for the influence of body size. Ideally, this should be done using a sample of known-mass individuals. It is possible that the relationship between periodicity and Retzius line number is driven by crown formation limitations in longer-forming anterior teeth, and molar teeth are less

[20] This was analyzed for the lumped sample, which may have obscured a relationship due to differences in Retzius line number between cusp and molar types. However, it was not possible to assess this relationship within cusp and molar types, due to small sample sizes and low variance in periodicity.

constrained in the number of Retzius lines (and/or total crown formation time). Investigations of full dentitions may shed light on the relationships between developmental variables.

Retzius line numbers

The patterning of Retzius line numbers appeared to be similar to that of cuspal enamel thickness and crown formation time in lower M1, as mesiobuccal cusps showed a greater number of Retzius lines than mesiolingual and distolingual cusps, and the distobuccal cusp showed a greater number of Retzius lines than both mesiolingual and distolingual cusps. It is likely that this would be confirmed for other lower molar positions had sample sizes been larger. Similarly, the significant increase found in lower molar distobuccal cusp Retzius line number from M1 to M3 may also hold for other lower cusp types. Upper molars showed more variation in the patterning of Retzius line numbers, although sample sizes were generally smaller than for lower molars. Differences between upper and lower cuspal analogues were not significant except between mesiolingual cusps. This difference may indicate that these cusps do not represent developmental analogues, and that functional differences may be seen in developmental processes (Reid et al., 1998a). Of the three tooth positions, the highest number of Retzius lines was found in M2 for both upper and lower molars. Differences between cuspal positions and among molar types appear to contribute to the total variation observed in crown formation time within and among molar types.

Cuspal enamel thickness

Cuspal enamel thickness has been quantified in numerous studies, and is believed to show a functional signal in terms of patterning (Khera et al., 1990; Schwartz, 2000a,b; Grine, 2002). It is not surprising that significant differences were found between buccal and lingual cusps, given their different roles during occlusion. Had sample sizes been larger, it is likely that additional dyads would have revealed significant differences, particularly in lower molars. It is interesting to note that upper molars tended to show thinner enamel than their lower analogues, although this was not found to be significant. Additionally, cuspal thickness appeared to increase from M1 - M3, although this was

statistically significant in the lower distobuccal cusp only. Given the relatively uniform DSR in all molar cusps, these thickness differences may lead to differences in cuspal (and total) formation time among cusps and among molars. This was confirmed by the positive significant association between cuspal enamel thickness and cuspal (and total) crown formation time. When combined with data on Retzius line number, it appears that variation in cuspal enamel thickness and Retzius line number leads to larger differences between buccal and lingual cusps and among molars within the molar row.

Crown formation time

Crown formation time in chimpanzees, calculated as the sum of cuspal and imbricational enamel formation times, appears to show a high degree of variation within cusp types and molar types, as well as among cusp types and molar types in this sample. As noted above, differences in cuspal formation times reflect differences in cuspal enamel thickness, and differences in imbricational formation times reflect differences in Retzius line number. Additional imbricational formation times were reported to illustrate an even higher degree of variation in a large number of cusps that were excluded from the calculation of cusp-specific formation time. Average (minimum) crown formation times were estimated from these data by combining average imbricational times (from Table 9) with average cuspal times (from Table 8), and are shown in Table 17. These times markedly extend the range of average times for upper M1 and M2 mesiolingual cusps to over three years, suggesting that they may form over a longer period than their mesiobuccal analogues. These data also extend the time for the distobuccal cusp in upper M1 to more than two years, and extend the time for mesiobuccal and mesiolingual cusps in lower M2 and for the mesiobuccal cusp in lower M3 to more than three years. The impact of these data on the original estimates is not surprising given that the most poorly represented positions are most affected by the inclusion of additional average values, and underscore the need for additional data on these cusp types and tooth positions.

The additional data presented in Table 17 lend some support to the observed trend of increasing crown formation time from M1 to M2 and decreasing time from M2 to M3. This may be related to tooth size, as second molars generally show the largest cross-sectional (occlusal) area within the molar row in chimpanzees (e.g., Ashton &

Zuckerman, 1950; Schuman & Brace, 1954; Pilbeam, 1969; Swindler, 2002). This trend is supported in the current study by the significant positive relationship between crown formation time and bi-cervical diameter. If secretion rate is held constant, the other alternatives for generating a larger tooth are by increasing the number of cells secreting enamel, and/or by extending the period of developmental time (Grine and Martin, 1988). This is discussed further below in the section on correlates of crown formation time.

Table 5.17 Estimated mean crown formation times for an additional sample of cusps not used for statistical assessment of crown formation time.

Tooth	mb	ml	db	dl
UM1	818	1128	845	n/a
UM2	n/a	1176	n/a	n/a
UM3	998	991	815	877
LM1	862	699	874	663
LM2	1180	1073	719	853
LM3	1406	921	n/a	761

Cusps are as in Table 3. Average cuspal formation time was taken from Table 8 and combined with the average (minimum) imbricational formation time in Table 9 to yield an average cusp-specific crown formation time in days.

Data on crown formation time in chimpanzees are similar to published hominoid values (reviewed in Smith et al., 2003a: Table 7, p. 299), although it must be noted that several published values represent the period from crown initiation to completion (among cusps), which is longer than values from single cusps (and depends on the cusp under consideration).[21] Values reported in this study appear to be fairly similar to other extant great apes, and are frequently shorter than cusp-specific crown formation times for

[21] Smith et al. (2003a, 2004) added 0.1 years to the lower mesiobuccal cusp crown formation time estimate to compensate for the final (non-overlapping) period of distobuccal cusp formation. This was based on data from Reid et al. (1998a,b) on humans and chimpanzees.

humans, as reported by Reid et al. (1998b: Table 4, p. 471). These values are slightly greater on average than those from radiographic estimation (Table 2), as predicted by Beynon et al. (1998a). There does not appear to be such a large difference between radiographic and histological estimates of chimpanzee molar crown formation time as Reid et al. (1998a) suggested. The new estimates are more consistent (on average) with reports of age at molar eruption (Table 1), as the majority of M1 cusps are completed by approximately two years of age (crown formation time minus average neonatal time), which may allow for a year or more of root growth prior to eruption. Similarly, estimates of age at M2 and M3 completion may be reduced given shorter average crown formation times than reported by Reid et al. (1998a). Future work aimed at establishing the age at death and ages at crown completion will confirm this for M2 and M3.

As noted above, differences in calculated crown formation time between the present study and the study of Reid et al. (1998a) are largely due to differences in Retzius line periodicities. Calculations of DSR, counts of Retzius lines, and estimates of cuspal formation times were fairly similar. Differences of 1 - 2 days per Retzius line resulted in differences of a half a year or more for molars from two of the four individuals in common between the two studies. Results for several of the developing teeth or broken teeth presented in Reid et al. (1998a) were not included in this study, as the goal was to provide the most accurate dataset possible, which may be slightly more data-poor than in less conservative studies. Examination of material that was excluded from statistical analyses showed some results that were similar to those of Reid et al. (1998a), but were often lower due to differences in Retzius line periodicity.

This study also reports the largest histological dataset on prenatal crown formation time in a non-human hominoid. As noted above, small numbers of maxillary M1 appear to show variation in the order of cuspal initiation (n=2), unlike mandibular molars, which parallels the variation seen in cuspal enamel thickness, Retzius line numbers, and duration of crown formation (n=1 or 2 for upper M1 and M2). It is unclear whether this is an artifact of small sample sizes, or if there is more biological variation in the upper developmental field. Regardless of the reason, it appears that the conflicting sequences of maxillary cuspal initiation reviewed above may both be found in chimpanzee molars. Among lower M1, the protoconid is generally the first cusp to form,

and may begin as early as two months or more prior to birth. No evidence was found for prenatal formation in the hypocone or entoconid, which are only infrequently found at birth in several dissection based studies.

Correlates of crown formation time

The present study demonstrates that several developmental variables are correlated with one another and with two morphological variables: EDJ length and bi-cervical diameter. Several of the variables were expected to be correlated with crown formation time, such as periodicity, Retzius line number, and cuspal enamel thickness, as these are used for the calculation of crown formation time. It was surprising to note that DSR did not show a relationship with crown formation time, which may add further support to this as a relatively fixed developmental parameter among cusps and among molars. Crown formation time also showed a strong significant positive relationship with bi-cervical diameter and EDJ length, which suggests that body size and formation time are related. This was suggested to be true among primates by Macho (2001), but this study represents the first demonstration of an association within a primate species. Future work may incorporate animals of known body mass, permitting a more exact examination of this relationship.

It was suggested in Chapter 4 that the relationship between crown formation time and EDJ length could be used to derive a predictive equation. In the mixed-collection sample, significant linear, logarithmic, and quadratic relationships were found, but the correlation coefficient was low. One source of potential error was postulated to be the influence of variation in individuals from different subspecies or developmental conditions. Recent work has shown that wild chimpanzees show later ages of molar emergence than captive animals (Zihlman et al., 2004), which may be the result of differences in crown formation time. Although it was not possible to test this directly, the study sample was split into separate collections, which differed in composition. The Harvard sample was known to be the most homogenous sample, made up entirely *Pan troglodytes verus* upper M3 cusps. This sample showed the largest *F*-value and lowest *P*-value, with an r^2=0.56 for the quadratic regression. The graph of this (Figure 5.4a) shows a quadratic relationship that closely resembles the relationship between these two

variables in the fully formed (homogeneous) macaque sample (Figure 4.7). The Martin collection was too small to interpret statistically, but it is worth noting that this wild-shot collection of unknown provenience(s) showed a large degree of variation in a small number of individuals. The Ashton collection, which is believed to be a relatively homogenous captive sample, showed significant linear and logarithmic relationships between crown formation time and EDJ length in primarily first lower M1 cusps. The quadratic best-fit line in this sample was similar to that of the Harvard collection. Finally, the Newcastle collection, which is believed to be a heterogeneous collection of mainly captive animals, showed significant positive linear, logarithmic, and quadratic relationships between crown formation time and EDJ length. However, the correlation coefficients were lower than in most of the other groups and regression methods.

Thus, it appears that an association exists between crown formation time and EDJ length. This association may be most apparent when homogenous samples are considered, particularly when single cusp types (and molar types) are compared. Due to the limited samples in the present study, it was not possible to examine the relationship between crown formation time and EDJ length within individual cusp or molar types. Future studies of larger samples may allow this theory to be tested and refined, as it may represent a valuable means of assessing dental development through non-destructive techniques (e.g., micro-computed tomography).

Variation in Dental Development

Eruption and crown formation

Variation in dental development, particularly in the sequence of eruption, is well documented within hominoids. Numerous studies of chimpanzees have described several distinct eruption sequences of the entire dentition (e.g., Krogman, 1930; Schultz, 1940; Clements and Zuckerman, 1953; Kuykendall et al., 1992; Kuykendall and Conroy, 1996). Kuykendall and Conroy (1996) noted that different sequences occur in both calcification (assessed via radiographs) and in the order of eruption, and that relationships among teeth fluctuate during development. It is has been noted in humans that, although substantial ranges exist in terms of age at eruption, and by inference eruption sequence, this variation

is not as great as that seen in osseous development (Chapter 1; see also Garn et al., 1959a; Lewis and Garn, 1960; Bailit, 1976).

Part of the apparent variation in age at eruption illustrated in Table 1 is likely due to methodological differences, as the method of age determination may differ (first appearance vs. average of interval), as well as the exam interval (especially for posterior molars) and radiographic technique (Bailit, 1976; Beynon et al., 1998a; Kuykendall, 2002). However, even with the documented underestimation of formation time in radiographic studies, the range seen within studies suggests that the age at eruption for individual teeth may vary by a year or more, especially in the case of third molars (but see Nissen and Riesen, 1964). This was the conclusion reached by Kuykendall et al. (1992) and Anemone et al. (1996) for large samples of chimpanzees, and Garn et al. (1959) for a large sample of human molars. Data on crown formation time in Table 2 do not show a range as large as age at eruption, although sample sizes are smaller. Kuykendall (2002) noted that neither radiographic nor histological studies of crown formation have yielded properly calculated population estimates for crown formation time. Data reported in the present study show a level of variation similar to published age at eruption, but are too limited to be considered appropriate population estimates.

To date, no one has analyzed variation in eruption sequence and age at eruption as they relate to variation in calcification sequence and crown formation time. It is likely that these factors are correlated, although data on root development are needed to evaluate these relationships. Data on molar eruption and crown formation (assessed histologically) in chimpanzees have yet to be reported in the same study for the same individuals, which leaves a number of important issues unresolved. As noted above, if the ages at eruption reported from large samples of captive animals are compared to crown formation times from published histological studies, it appears that there is very little time available for root formation prior to eruption. Given the range of reported variation in age at eruption and duration of crown formation time, it would be interesting to investigate whether individuals with short crown formation times erupt their first molars earlier than individuals with longer crown formation times. The alternative is that age at initiation and/or rates of dentine extension vary among individuals. Dean and Wood (1981) suggested that the latter scenario explains the differences between ages of molar

emergence in chimpanzees and humans, which have relatively similar periods of crown formation time. Without additional data on variation in crown initiation, crown formation time, and dentine extension in a larger sample, it will not be possible to establish conclusively the cause(s) of variation in age at emergence.

Sex differences

Kuykendall and Conroy (1996) suggested that, based on their examination of chimpanzee eruption sequence polymorphism, sex differences are an important component of intraspecific variation in dental development. However, a number of previous studies have shown that evidence for sex differences in calcification, crown formation, and age of eruption has been equivocal (Nissen and Riesen, 1945, 1964; Winkler, 1995; Marzke et al., 1996), including several by these authors (Conroy and Mahoney, 1991; Kuykendall et al., 1992; Kuykendall, 1996). The consensus is that female chimpanzees erupt their dentition slightly earlier than males (although the difference is not significant). It is not clear whether females also begin calcification earlier, produce teeth at faster rates, or produce roots at faster rates. As noted above, the most marked differences are found in canine DSR, crown completion time and eruption, which are faster and earlier in females (Kuykendall, 1996; Schwartz and Dean, 2000; Schwartz et al., 2001a).

Studies of human dental development have shown that female dental development is also advanced relative to males. Clements and Zuckerman (1953) presented data on a large sample of British children that showed that females consistently erupt their teeth before males. Garn et al. (1959) noted the same trend in calcification time, crown completion, and apical closure in American children. Demirjian and Levesque (1980) also reported a similar pattern in a large sample of French-Canadian children, but they noted that differences are not apparent until after five to six years of age. It is unfortunate that the gender of the majority of the individuals in the present study is unknown, as this may account for some of the variation in incremental development within cusps and molars. Future work is necessary to address the issue of sex differences in molar crown development.

Mandibular vs. maxillary analogues

Based on dissections of several chimpanzee dentitions, Siebert and Swindler (1991) suggested that mandibular first molars begin calcification slightly before maxillary analogues. However, Winkler (1995) reported that dissection of a single neonatal chimpanzee revealed M1 analogues at equivalent stages of development.[22] Reid et al. (1998a) presented histological data on molars from a single chimpanzee that showed a mixed pattern of analogues being first in initiation, longer in formation, and last in crown completion, which was confirmed in the present study. On average, mandibular cusps appear to initiate before maxillary cusps, but this may show some variation. In terms of eruption differences, Nissen and Riesen (1964) reported that their longitudinal data showed chimpanzee lower molars erupted earlier than upper molar analogues.

Radiographic or cross-sectional studies of homologous teeth are uncommon as radiographic imaging of maxillary dentitions is more difficult than mandibular dentitions (Winkler, 1995; Kuykendall, 1996). The results of several studies appear ambiguous, which may be due to methodological differences. Dean and Wood (1981) did not find developmental differences between mandibular and maxillary teeth in a cross-sectional hominoid sample. However, Conroy and Mahoney (1991) found that the mandibular M1 emerged significantly earlier than the maxillary M1 in a large longitudinal sample.[23] Clements and Zuckerman (1953) presented comparative data on humans, showing that mandibular permanent teeth tended to erupt before maxillary, but not always, and the pattern was reversed in males and female M1.

If a preliminary consensus may be drawn from some of these studies, it appears that mandibular molars generally begin calcification at the same time or prior to maxillary molars, and may erupt at or before the time maxillary molars erupt. Although it is tempting to assume that this has implications for the amount of root formed at eruption, or the rate of root growth, Dean and Wood (1981) noted that the original position of the tooth germ must also be considered, along with the speed of eruption and the length of the tooth. Additional work is required to examine variation in calcification, crown

[22] Winkler did note in a single orang utan that the mandibular M2 calcified before its maxillary analogue.

[23] Yet, significant differences were not reported when Kuykendall et al. (1992) reanalyzed these data.

formation time, and age at eruption, as well as differences in root growth between analogous teeth to provide further clarification.

Position in molar row

In their study of sequence polymorphisms, Kuykendall and Conroy (1996) noted that M1 appears to be more stable than other permanent teeth, as it exhibits little variation in eruption sequence between sexes (relative to deciduous and first-formed permanent teeth). Siebert and Swindler (1991) also noted in their direct examination of fetal and neonatal chimpanzee material that M1 appears to be less variable than deciduous molars. As noted above, few published data exist on the age at M1 crown initiation, but it appears to be less variable than second or third molars, which may also be true for the period of crown completion and eruption (see range data in Table 2). However, the current study demonstrates that crown formation times in all molar types appear to vary by one year or more, and M1 cusps did not appear to be less variable than other molar cusps (although sample sizes of cusp and tooth types were unequal). A study on a large sample of human children by Garn et al. (1959) also found relative molar variability to be equivalent. However, they did find that that the early developmental stages (calcification) of the earlier-forming molars were less variable than the later developmental stages (root completion) of the later-forming molars.

As reviewed above, Dean and Wood's (1981) radiographic study on great ape dental development assumed that there was no developmental overlap in crown formation between molars. However, both Anemone et al. (1991, 1996) and Kuykendall (1996) demonstrated molar overlap in their radiographic studies, and Reid et al. (1998a) confirmed this in a histological study. Based on bar charts published in these sources, it appears that M1 and M2 may overlap by approximately six months to one year, and M2 and M3 may overlap by approximately three months to one year and four months.[24] Anecdotal data in the present study also suggest that M1 and M2 may overlap by a half a year or more, and M2 and M3 may overlap by one year or more. Anemone et al. (1991)

[24] The lower ends of these ranges come from radiographic studies, and are probably too low as the initial period of calcification is not represented in radiographs, and the final period of crown formation is often underestimated (Beynon et al., 1998a).

first reported that crown formation time increases along the molar row from M1 – M3, which was also shown in their 1996 study. However, Kuykendall (1996) suggested that crown formation time increases from M1 - M2, and then decrease from M2 - M3. Histological work by Reid et al. (1998a) suggested a variable pattern, as the mandibular M3 required more time than the M2, but the opposite pattern was found for maxillary molars. Data from the present study appear to support Kuykendall's results, but this could not be demonstrated with significance for most cusps due to limited sample sizes.[25]

Effects of captivity

As reviewed in Chapter 1, it is suggested that non-human primates raised in captivity may show more rapid skeletal, dental, and sexual development than wild animals. Zihlman et al. (2004) demonstrated this recently for age at molar eruption in chimpanzees, and Phillips-Conroy and Jolly (1988) found the same results for wild and captive baboon populations. It is likely that both crown and root formation are advanced in captive populations, but this has yet to be examined directly in primates. Given the high probability that several of the individuals included in the present study were from captive collections[26], it is possible that some of the intra-tooth variation in crown formation time is due to sample heterogeneity. However, it was generally not possible to compare data from the known wild-shot samples with the potentially captive Ashton material, as data on lower first and second molars in known wild-shot individuals were not available. Future work should examine these differences explicitly, and current standards for the study of dental development in hominoids may need to be re-evaluated if environmental differences in crown formation time are confirmed.

[25] Although Dean and Wood's (1981) assumptions about a lack of molar developmental overlap have been challenged for *Pan*, *Pongo* does not appear to show overlap (Dean, pers. com.). Work in progress on the molar rows of several individuals of *Pongo* will examine this issue.

[26] It is tempting to suggest that the frequency of accentuated lines or enamel hypoplasias may provide insight into developmental environment. However, data on confirmed captive material are required to assess the potentially complex relationship between environment and dental disturbances or pathology.

Summary and Conclusions

This study represents the most comprehensive histological examination of molar development in non-human primates to date. Data were examined for differences within cusp types and among molar types, and the relationships among developmental variables were explored. Several conclusions may be drawn:

1) Daily secretion rate (DSR) appears to be consistent within analogous zones (inner, middle, and outer enamel) within cusp types, among cusp types, and among molar types. These rates appear similar to published data on anterior tooth formation, and suggest that DSR may be a fixed developmental variable within a primate species. Significant increasing trends were found from inner to outer cuspal enamel, ranging from approximately 3 - 5 μm/day, which is consistent with previous studies.

2) Retzius line periodicity minimally ranges from 6 - 7 days in 61 individuals, and may potentially range from as low as 5 days to as high as 8 days. No evidence was found to suggest that this value varied within individual dentitions. The range of 6 - 7 days is slightly lower than previous reports of chimpanzee periodicities, and is much less than the range of periodicity values reported for humans.

3) Retzius line number, cuspal enamel thickness, and crown formation time were found to show a similar trend of higher values in mandibular buccal cusps relative to lingual cusps, and a pattern of increase from M1 to M2. Some variation was seen from M2 to M3, and values in M3 were often less than in M2. Data on mandibular cusps showed more variation, but it appeared that mesiolingual cusps generally showed higher values than mesiobuccal cusps. This pattern between upper and lower molars suggests that functional similarity may influence cuspal development more than similarity in developmental fields. Thus, comparisons of upper and lower 'field analogues' used in the present study may be less appropriate than comparisons of functional analogues for conclusions regarding the order of cuspal initiation and crown formation duration. When upper lingual cusps are compared with lower buccal cusps, it appears that

maxillary teeth show longer periods of crown formation time. However, this should be examined within individual dentitions, as data on upper and lower molars were derived from different samples.

4) Cusp-specific molar crown formation in chimpanzees was most commonly found to range between two and three years. Individual cusps within molars ranged from 1.6 - 4.2 years. However, because crown formation generally begins in mesial cusps and finishes in distal cusps, crown formation times derived from different cusps should not be directly compared. Examination and registration of the first- and last-formed cusps are required to assess total crown formation time accurately. On-going studies of this sample will attempt to register cusps using accentuated lines in order to construct a chronology of cusp-specific development.

5) A correlation matrix revealed that several developmental variables were significantly positively associated with one another. Periodicity and Retzius line number were not correlated, which is in contrast to the relationship reported for anterior human teeth, and resulted in a greater range of imbricational (and crown) formation times. Several variables also showed a significant positive relationship with bi-cervical diameter, including periodicity, Retzius line number, and crown formation time. This suggests that molar size (or body size) may explain some of the developmental variation within this mixed sample. Trends in tooth size may parallel trends in crown formation time, but this would benefit from additional work.

6) The relationship between crown formation time and enamel dentine junction length was investigated, and a significant positive relationship was found with linear, logarithmic, and quadratic regression. When the sample was divided by collection, the association increased in the known homogenous sample, as well as a sample suspected to be relatively homogenous. The quadratic relationship within chimpanzees is similar to the relationship found in macaques (Chapter 4). Future work may examine this relationship within cusp types, which may show a stronger correlation than the mixed-cusp samples examined in this study.

7) Data on crown formation time in this study are more similar to reported values from radiographic studies than to a previous histological study. Differences between the current and the former histological study are largely due to differences in periodicity assessment, although the present study also includes more than three times as much data on molar formation than was previously available. Estimates of crown formation time in the present study are also more congruent with previously reported developmental data on age at first molar eruption.

8) A number of factors should be considered during future studies of dental development in chimpanzees. It appears that sex differences, analogous tooth positions, position in the molar row, and the developmental environment may all affect the timing, duration and variation of molar crown and/or development.

Chapter 6: Summary and Concluding Remarks on Primate Dental Development

Chapter 1 Summary and Conclusions

Several hundred years of investigation and several generations of innovative advances in microscopy have facilitated an understanding of the process of dental development from the level of the crystallite to the cusp. Knowledge of the embryology of dental development continues to benefit from experimental work on the enamel knot, as well as the genetic and biochemical basis of cellular interactions. Additional work on the interaction between enamel matrix proteins and the formation of enamel crystallites would be welcome, as would clarification of the process of organic replacement by inorganic components during mineralization. It is still not entirely clear how the most highly mineralized tissue in the body reaches its final and permanent composition.

Studies of the prismatic level of tooth organization will forever owe a debt of gratitude to the decades of studies conducted by Alan Boyde. His models of prism formation and prism packing pattern have shaped the last 40 years of investigations of both developing and fully mature enamel. Additional studies of developing enamel surfaces and the relationships among ameloblasts, Tomes' processes, and crystallite organization in the resulting prisms may serve to refine the seminal models Boyde introduced in 1964, 1979, and 1989. Additional work may also serve to integrate developmental models of prism packing patterns, prism decussation, and incremental features, an approach initiated by Lawrence Martin in 1983. Studies of the relationship between aprismatic enamel and incremental features are also needed, as well as a more rigorous examination of the distribution of aprismatic enamel among primates.

Recent debate over trends in prism width has underscored the need for a controlled study throughout the crown within and among primate taxa. This is particularly of interest for understanding enamel crown volume and increased surface area from the enamel dentine junction (EDJ) to the crown surface. As noted in Chapter 1, several models have been proposed to account for this change, yet empirical data are lacking. This would be of interest for studies of incremental features, particularly because it has been suggested that the cuspal and imbricational crown components may increase to a different degree from the EDJ to the surface. Ideally, given information on daily secretion rate (DSR) and prism width, it should be possible to calculate the volume of

enamel formed per day, which may be combined with information on the surface area of the EDJ and the duration of enamel secretion to predict the crown volume.

Conventional descriptions of incremental features in enamel have long recognized cross-striations and Retzius lines, short- and long-period features that may be observed with various forms of microscopy. Cross-striations in particular have been the subject of intense debate regarding their structure, formation, and periodic nature. Two schools of thought exist in regard to the relationship between cross-striations and prism varicosities/constrictions. Although many suggest that they either are, or are not, equivalent, it is suggested here that these structures may have a variable relationship. It is possible that in certain types of prism packing patterns, or at certain rates of enamel secretion, these structures are equivalent, while in other circumstances, prism varicosities and constrictions are not present or do not correspond to cross-striations in a 1:1 relationship. One of the difficulties in testing this relates to the fact that sectioned teeth preserve prisms cut in varying orientations, which may or may not represent an accurate prism profile (of the head) distinct from the prism tail. It is suggested that examination of non-destructive preparations such as naturally fractured teeth, or the use of confocal microscopy, may be the most effective means of resolving this on-going debate.

Alan Boyde also deserves credit for being one of the first researchers to integrate information on physiological rhythms and changes in blood chemistry with the production of cross-striations.[1] Experimental work by Okada, Mimura, and more recently by Shinoda and Ohtsuka-Isoya, has brought us closer to understanding the physiological basis of daily line production. Despite numerous experimental studies by Schour and colleagues and Okada and colleagues, the structural and periodic nature of cross-striations was not universally accepted, spawning decades of debate in the oral biology and anthropological communities. This was considered in Chapter 3, along with several studies that looked at daily line production in other mammals, including hibernating animals.

[1] Okada (1943) reviewed work that related biological rhythms, nutrient cycling, and hormone cycling to dental development prior to Boyde, but this research is believed to have been unknown until recently due to its publication solely in a Chinese journal.

In the present study, the subject of intradian lines was reintroduced, and a series of incidental references or images documenting them over the past 45 years was reviewed. Despite this, few recent studies have reported on these features, nor proposed how to distinguish them from cross-striations. Recent work by Smith and colleagues has conclusively refuted the traditional arguments used to dismiss them as artifacts of microscopy or preparation, but issue of their periodic nature remained unresolved. Similarly, incremental structures known as laminations have been documented for over 35 years, and they have received even less description and study than intradian lines. As noted in Chapter 1, these structures may show a relationship with aprismatic or pattern 1 enamel. Examination of a large sample of macaque and chimpanzee teeth has demonstrated that they are common in the first-formed enamel over the dentine horn, in the later-formed cuspal enamel that may become aprismatic below the tooth surface, in association with Retzius lines at the EDJ throughout the lateral and cervical enamel, and also in association with Retzius lines in the middle and outer lateral and cervical enamel. It does not appear that aprismatic enamel is necessary for the production of laminations. It is still unclear how these features may be formed throughout these various locations within the crown, and how they relate to the formation of cross-striations and Retzius lines. Additional examination of developing enamel surfaces from multiple planes of section may provide more clarity. The possibility that laminations may be associated with a lack of prism decussation may also represent an interesting area of future inquiry.

Studies of long-period Retzius lines have been appreciably advanced by the experimental work and theoretical modeling of Steiner Risnes. Risnes has demonstrated direct correspondence between Retzius lines and perikymata, including the three-dimensional nature of Retzius lines and the circumferential nature of perikymata. In addition, his models of Retzius line formation represent the most reasonable explanations to date for the appearance of prisms before and after a Retzius line. However, additional work is necessary to explain the variable appearance of Retzius lines, which are often poorly defined beneath the subsurface or outer enamel. Classic staircase Retzius lines modeled by Risnes are not ubiquitous, nor is it clear why other accentuated features may mimic the appearance of Retzius lines. It is suggested that developing teeth may provide more insight into this issue, particularly given the excellent definition of Retzius lines in

teeth that have not completed mineralization. Future work on this issue should also consider the influence of the three-dimensional nature of Retzius lines on their appearance in histological sections, as this may represent a confounding factor in light microscope investigations.

Additionally, the appearance and periodicity of 'regular' Retzius lines in the cuspal enamel is suggested to be an open question, due in part to the lack of published images of cuspal Retzius lines with a regular, periodic nature. Examination of chimpanzee material in this study revealed several developing teeth with what appeared to be regularly-spaced Retzius lines intersecting the EDJ and running into the cuspal enamel. However, when these lines were identified over the dentine horn, and cross-striations were counted within intervals, counts were generally greater than the periodicities recorded from imbricational Retzius lines in the same tooth (which met the tooth surface and formed perikymata).[2] It is possible that, given the necessary curvature of Retzius lines within the cuspal enamel, regularly spaced Retzius lines do exist in this region, but their appearance is obscured by section obliquity, prism path curvature, or superimposition from layers that are unlikely to be in phase with the layer in focus (due to the curvature of developing enamel front over the dentine horn). As noted above, the existence of cuspal Retzius lines is further supported by analogous long-period lines in dentine during the period corresponding to (the end of) cuspal formation.

A significant unresolved area in the study of incremental features concerns the physiological cause of Retzius lines. In contrast to cross-striations, there are fewer known biological rhythms with similar cycle lengths. A well-cited suggestion by Newman and Poole (1974) postulated that Retzius lines are the result of multiple short-period rhythms that interact and form a long-period beats frequency. However, given the seemingly tight control of circadian rhythms in biological systems, it is difficult to imagine how a second short-period rhythm could be maintained within an individual, yet vary among individuals or among hominoid species in a manner that explained the known diversity of Retzius line periodicities. A lack of understanding also applies to the etiology of accentuated lines, which may be formed in a manner similar to the neonatal line, but may

[2] A similar phenomenon was noted in between 'regular' Retzius lines deep to the cingulum in *Graecopithecus freybergi* and also in a few of the chimpanzees examined in this study.

not represent a clearly identified external stimulus that causes a change in enamel formation. Several recent studies have used accentuated lines as measures of health and survivorship in archaeological populations, along with enamel hypoplasias, although these types of studies would benefit from controlled experiments, which would most likely yield better resolution of the ultimate and proximate causes of accentuated lines.

Several decades of study on genetic anomalies have contributed to our understanding of the genetic basis of dental development, most notably sex-linked aspects. Advances in population genetic modeling have been applied to examine the heritibility of specific traits, which is of particular value for investigations of evolutionary relationships. A recent example is the work by Hlusko and colleagues that has suggested that variation in tooth size may be associated with the genetic control of body size, which is particularly interesting given the significant positive association found in the present study between crown formation time and bi-cervical width (a surrogate of body size) within a large sample of chimpanzee crowns.

Additional work on the endocrine component of dental development has benefited from a series of experimental studies on mammals, as well as numerous case studies of humans with endocrine disorders. Several hormones appear to affect dental development, including growth hormone, thyroid hormone, parathyroid hormone, and sex hormones. A review of the literature demonstrates several conflicting conclusions about the effects of these hormones on enamel and dentine growth, suggesting the need for additional experimentation. Yonaga's work with rodents is of particular interest, as he examined the specific effects of multiple hormones on extension and apposition, showing that parathyroid and thyroid hormones may influence different developmental processes in enamel- and dentine-forming cells in different ways.

Finally, the environmental basis of dental development is reviewed, particularly the influence of diet and environmental conditions (i.e., captive vs. wild populations). Demographic studies on several species of captive, provisioned, and wild populations of macaques show that captive and provisioned colonies reach specific life history stages (e.g., age at first reproduction) earlier than wild populations (Cleveland, unpublished).[3]

[3] Data on age at first molar eruption were not considered in this review.

Data on baboons and chimpanzees reviewed above suggested that captive populations show more advanced molar eruption than wild populations. This is particularly relevant for the interpretation of variation in incremental development and crown formation time in mixed samples of extant primate dentitions. It is likely that captive animals that are adequately cared for may benefit from better nutrition, allowing for earlier initiation of crown formation and/or more rapid dental development. This suggestion is consistent with published findings on skeletal development, and would benefit from additional work on dental development in known captive and wild populations.

Chapter 2 Summary and Conclusions

This chapter presented a comprehensive review of the methodology involved in assessment of incremental development in primate dental enamel. Methods were reviewed to quantify DSR, Retzius line periodicity and number, and the extension rate of crown formation. Each method was explained and critiqued, and several sources of potential refinement were suggested for future studies. The determination of Retzius line periodicity was highlighted as one of the most difficult aspects of studying incremental development, which was found to be particularly true during the analysis of a large number of chimpanzee molars, many of which showed few cross-striations in association with Retzius lines. Compared to limited examination of other living and fossil hominoid molar material, the chimpanzee sample examined in Chapter 5 generally showed poor/fair quality incremental features, particularly with respect to cross-striations.[4] Laminations were found to be very common between Retzius lines, which prohibited determination of periodicity in those areas. It was also suggested that prism or Retzius line curvature in the subsurface enamel may represent an additional source of error in periodicity estimation.

A model for the quantification of enamel extension proposed by Shellis was reviewed and was tested in the subsequent chapters. Several aspects of the model were suggested to be difficult to determine with precision, leading to potential error in the

[4] However, teeth that had yet to complete crown formation showed incremental features that were much more clear than those in crown complete molars.

predicted extension rate or time of formation. In particular, measurements of the angle of intersection between Retzius lines and the EDJ were suggested to be susceptible to error due to the subjectivity of defining an angle between two curvilinear lines that are converging. Data on this angle may be informative in a comparative context, provided that DSR at the EDJ is comparable between areas, as low angles may represent fast extension rates, but high angles may represent either low extension rates or high DSR. Several additional methods for crown formation time reconstruction were also presented, and two of these were tested in Chapter 4. Each method was reviewed and critiqued, including a number of methods for cuspal enamel formation. Cuspal enamel formation has proven to be a relatively complicated aspect of crown formation time determination, and a minimum of seven different approaches have been utilized. Although several of these methods have been shown to give consistent results with one another, the accuracy of most of these methods has yet to be tested.

The remainder of this chapter was devoted to a review of previously published data on incremental development in hominoids. A survey of these data from the last two decades made clear the need for additional data on a larger sample of non-human primates. For example, data on DSR has shown that discrete ranges may characterize or distinguish certain hominoids from one another. It was suggested that these studies would first benefit from the application of a consistent methodology, and secondarily from an examination of rate variation of within a species. Presumably mixed subspecies data on chimpanzees presented here from specific zones within cuspal enamel suggested that rates are statistically indistinguishable within cusps and within the molar row, and published data suggest that this is true in the anterior dentition as well.[5] These results lend additional support for the taxonomic value of comparisons of DSR within analogous regions among hominoids.

Reports characterizing crown formation times have been limited by a paucity of comparative data, particularly on posterior tooth development. A recent example is the study of two *Afropithecus turkanensis* second molars by Smith et al. (2003a), which found some variation in DSR and periodicity between the two lower molars, and an

[5] These data also suggest a high degree of consistency between studies, which is reassuring given the complications associated with rate determination.

approximate six month difference in crown formation times. These differences are within the range of variation observed within a cusp type in the sample of chimpanzees from the present study. Published data on crown formation time in most fossil and living apes and humans also fall within the range of the cusp-specific chimpanzee data (see Smith et al., 2003a: Table 7, p. 299), save for *Proconsul heseloni* at the low end, and *Gigantopithecus blacki* at the high end (Dean and Schrenk, 2003). However, the proportions of crown formation components may show some distinction within hominoids (Smith et al., 2003a, 2004). Chimpanzees are characterized by relatively thin cuspal enamel that forms in under a year, and relatively longer imbricational formation consisting of more Retzius lines, while some thicker-enameled hominoids show more than a year of cuspal enamel formation, and tend to show relatively fewer Retzius lines (and often higher periodicities).

In closing, Chapter 2 reviews a number of criticisms that have made of analyses of incremental development. Several issues that have been addressed by recent studies are outlined and are considered further in the following chapters. These include several methodological points, such as the effects of section obliquity and the accuracy of incremental analyses. Additional theoretical issues are raised, including the nature of variation of incremental features and the relationships of developmental parameters. These questions frame the experimental and investigative work conducted with a large sample of experimentally labeled macaque dentitions in Chapters 3 and 4, and the large sample of chimpanzee molars examined in Chapter 5.

Chapter 3 Summary and Conclusions

This chapter was devoted to a study of the time-dependent nature of incremental features in macaque (*Macaca nemestrina*) material that had been experimentally labeled during the course of a previous study. The sample included mandibular dentitions from 16 individuals that ranged in age from neonatal to slightly older than one year, which included primarily deciduous teeth as well as several first molars. The three fluorescent labels used were clearly identified and distinguished from one another in the dentine, and

related to accentuated lines in the enamel (when the tissues were forming simultaneously at the time of injection). The periodicities of four incremental structures were examined: cross-striations, intradian lines, Retzius lines, and laminations. It was demonstrated that both cross-striations and laminations show a daily periodicity. Twelve-hour subdivisions known as intradian lines were identified between the two daily features. This represented the first empirical demonstration of the periodicity of both laminations and intradian lines. Retzius lines in six individuals were shown to have a 4 day periodicity, which was shown to be consistent over a series of lines in the cervical enamel of a single individual. Local extension rates were calculated from Shellis' formula and from the known period of EDJ formation, and formation time was compared between calculated and known values. It was shown that the former method tended to yield significantly greater overestimations of formation time, particularly in fast-forming teeth.

Experimental studies of labeled dental material have been conducted since the 1930's. The present study represents the first attempt to examine the periodicity of all known incremental features in a single sample. Information on the daily nature of cross-striations refutes earlier criticisms of these features. Images from tandem scanning reflected light microscopy are presented, and confirm that short-period features are not an artifact of conventional light microscopy. However, the developmental basis of several of incremental features is still unclear. Given information from the present study, it is hoped that future work will refine and integrate models of short- and long-period feature development. An understanding of incremental development may also be advanced by research conducted in the field of biological clocks. When considering the essentially universal existence of circadian rhythms in plants and animals, it appears that dental tissues are one of many hard tissues that preserve a record of their growth. Experimental work on the development and control of biological rhythms, particularly in relation to fluctuating hormone levels and the role of the suprachiasmatic nucleus, should lead to a better understanding of the proximate and ultimate factors that direct enamel- and dentine-forming cells to secrete in a consistent incremental fashion rather than in a random or uniform manner.

Chapter 4 Summary and Conclusions

This chapter was intended to investigate the accuracy of histological assessments of both age at death and crown formation time. Five lower first molars were selected from five individuals described in the previous chapter, and the age at death, duration of crown formation, and duration of root formation were determined and compared to the known ages at death. It was found that age at death, and by inference crown formation time, may be determined with a high degree of accuracy (>90%), particularly in material that preserves clear incremental features and is sectioned appropriately. Additionally, Shellis' method for crown formation time estimation was tested against the adjusted crown formation time, and was found to significantly overestimate crown formation time.

The relationship between crown formation time and EDJ length was examined, and it was found that significant positive linear and quadratic relationships existed between log-transformed variables. A model was then proposed that illustrated the expected change in extension rate along the EDJ from the dentine horn to the cervix within a cusp. This model may also explain the relationship of crown formation time and EDJ length among cusps, provided that extension proceeds along a similar continuum among cusps. Given the similarities in DSR within chimpanzee molars (shown in Chapter 5), it may also be the case that extension rates do not vary within analogous regions among molars either. Additional data, including crown formation time, extension rates, and EDJ length in larger samples, are needed to investigate this issue.

Chapter 5 Summary and Conclusions

Having established the periodicity of incremental structures and the relatively high accuracy of histological analyses of dental development, this chapter reported on incremental development in the largest sample of non-human primate molars known to date. Several aspects of incremental development were quantified, including DSR, periodicity, Retzius line number, cuspal enamel thickness, and crown formation time. Differences in these variables were examined among cusp types and among molar types,

as well as between buccal and lingual analogues and mandibular and maxillary analogues. Data on upper molars generally did not show significant differences, likely due to small sample sizes and higher level of variation than mandibular molars. Daily secretion rates were indistinguishable within zones among cusps, and within cusps among molars, suggesting that this variable may be under relatively tight developmental control. A significant increasing trend in rate was demonstrated from inner to outer cuspal enamel in the combined sample, which has been noted in a number of previous studies.

Differences were found in Retzius line numbers, cuspal enamel thickness, and crown formation time. In mandibular molars, buccal cusps were generally greater than lingual cusps, and these variables tended to increase from M1 to M2. Results for M3 were mixed. Data on maxillary molars were more limited (and more variable), but some evidence suggested that lingual cusps show greater Retzius line number, cuspal enamel thickness, and crown formation time than buccal cusps. If this is confirmed, it appears that functional similarities, rather than similarity in developmental position, may account for the patterning of these variables among upper and lower analogues. Cusp-specific crown formation time, the product of cuspal and imbricational enamel formation, was shown to vary by a year or more within each tooth type, and by more than two years among tooth types. This implies that previous reports of crown formation time which did not specify the cusp type used may not be comparable. Unfortunately, comparisons of cusp and tooth positions among study collections were limited, which may have provided more precise ranges of variation within a cusp type. On average, individual cusps within first molars formed in two years, cusps in second molars formed in just under three years, and cusps in third molars formed in about two and a half years. This trend seems to parallel changes in molar tooth size. However, data on cusp-specific initiation and completion, accomplished among cusps via registry of accentuated lines, will give more precise estimates of crown formation.

A noteworthy finding in this chapter is the significant positive relationship between crown formation time and both EDJ length and bi-cervical width within a species. If it is the case that crown formation time is related to other life history traits among primates, as Macho (2001) suggested, a pattern of size-related scaling may also exist in crown formation time within primate species. Kelley and Smith (2003) originally

critiqued Macho's (2001) theory, noting differences in life history traits between chimpanzees and humans, but similarities between published crown formation times. However, given new data on a larger sample of chimpanzees, it appears that mean crown formation times are lower than in humans, which would be in keeping with a more rapid developmental schedule. This area would clearly benefit from additional examination, including the analysis of more recent data on life history variables and more extensive and reliable data on primate crown formation time in known mass individuals (Kelley and Smith, 2003).[6]

Finally, the relationship between EDJ length and crown formation time was investigated within a lumped sample of 61 cusps, as well as among the different collections used in this study. In the lumped sample, a significant positive relationship was found with both linear and quadratic regression, although a high degree of variation in both time and length was noted. When this relationship was examined within collections, a higher correlation was found in a homogenous sample of upper M3s, and also in a sample of mainly lower M1s that may be from a homogenous population (relative to the two other collections in this study). The quadratic best-fit lines of these two groups also resembled the relationship of extension rate and formation time within macaque M1s presented in Chapter 4. Additional data from large sample of primate molars are needed to examine this relationship further, as well as the potential application of this relationship for the prediction of crown formation time from EDJ length.

Final Concluding Remarks

The intention of this dissertation was to identify a number of ways that future work may complement and refine our current appreciation of the complexity of tooth formation. Some of the most critical research needed includes additional experimental studies on Retzius line and accentuated line formation, particularly as reports of accentuated lines and enamel hypoplasias have been the subject of a number of recent

[6] As noted in Chapter 2, Macho (2001) used primate crown formation time data from Shellis (1998), which has been shown in Chapters 3 and 4 to be questionable due to the use of calculated extension rates to infer crown formation time.

studies of living and fossil primates. Other aspects of prismatic and incremental development may benefit from additional work on developing enamel. Differences in the quality and appearance of incremental features in histological sections of developing enamel are striking (relative to mature enamel), and it would worthwhile to establish what causes this as it may provide insight into enamel formation. Continuing experimental work on the genetic, hormonal, and physiological basis of dental development is of particular significance for the integration of information from the level of the cell to the whole organism. Recent studies on the control and expression of biological rhythms in dental development by Ohtsuka-Isoya and colleagues represent one of the most exciting areas of current research. Their experimental manipulations of the suprachiasmatic nucleus showed the first direct association between neuroanatomy and the expression of daily lines in dentine. Future work will hopefully also address the role of melatonin and the pineal gland in the production of rhythms during dental development.

We are fast approaching the day when we may be able to model not only the path of a group of prisms, as Radlanski et al. (2001) have done with confocal microscopy and three dimensional reconstruction software, but we may also be able to model the daily growth of a tooth from initial calcification to crown completion. Advances in microscopy and image analysis have provided a means to facilitate a deeper understanding of the three dimensional paths of ameloblasts and their secretory products, while careful histological studies have recently detailed the two dimensional nature of daily apposition and extension. Micro-computed tomography may also prove to be an invaluable addition to our investigative toolbox, particularly with advances in resolution and slice thickness, which may provide a nondestructive means of exploring incremental development. The integration of these data may hold the key to producing a holistic three-dimensional model of crown formation.

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Appendix 1. Histological sections of macaque (*Macaca nemestrina*) material.

Prenatal			Postnatal		
Indiv	**Slide**	**Tooth**	**Indiv**	**Slide**	**Tooth**
285	A3	dc	317	A5	dc
	B5	dp3 mes		B1	dp3 mes
	B9	dp3 dis		B3	dp3 mid
	B13	dp4 mes		B6	dp3 dis
	B18	dp4 dis		B8	dp4 mes
	T5	transverse		B11	dp4 dis
296	A2	dc		B18	M1 mes
	B5	dp4 mes		T5	transverse
	B8	dp4 dis	319	A2	dc
	B14	M1 mes		B3	dp3 mes
	T5	transverse		B6	dp3 mid
300	B2	dp3 mes		B8	dp3 dis
	B4	dp3 dis		B13	dp4 mes
	B11	dp4 mes		B18	dp4 dis
	T4	transverse		T5	transverse
	T5	transverse	324	B6	dc
302	A3	dc		B14	dp3 mes
	B3	dp3 mes		B17	dp3 dis
	B10	dp4 mes		B21	dp4 mes
	T6	transverse		B25	dp4 dis
303	B1	dc		B29	M1 mes
	B7	dp3 mes		B33	M1 dis
	B10	dp3 dis		T5	transverse
	B15	dp4 mes	325	A4	dc
	B19	dp4 dis		B5	dp4 mes
320	A3	dc		B8	dp4 dis
	B4	dp3 mes		B17	M1 mes
	B6	dp3 dis		T3	transverse
	B12	dp4 mes	326	A5	dc
	B17	dp4 dis		B5	dp3 mes
327	A4	dc		B9	dp3 dis
	B3	dp3 mes		B17	dp4 mes
	B12	dp4 mes		B21	dp4 dis
	B14	dp4 dis		B26	M1 mes
	T4	transverse		B32	M1 dis
	T6	transverse		T5	transverse
333	B8	dp3 dis	330	A7	dc
	B15	dp4 mes		A12	dc dis
	B18	dp4 dis		B6	dp4 mes
	T5	transverse		B10	dp4 dis
				B19	M1 mid
			336	B5	dp3 mid/ dev p3
				B12	dp4 mid/ dev p4
				B24	M1 mid

	SB 1	dp4 mes
	SB 2	dp4 dis
	SB 3	dp4 dis
	SB 4	M1 mes
	SB 5	M1 mes
	SB 6	M1 mes
337	A3	dc
	B4	dp3 mid/ dev p3
	B13	dp4 mid/ dev p4
	B20	M1 mid
6898	M1	M1 mes
	M2	M1 mes
	D1	M1 dis
	D2	M1 dis

Indiv- individual animal numbers assigned during the original studies. Slide- slide number. Slides labeled A and B are coronal right mandibular sections, slides labeled T are transverse left mandibular sections, SB sections are left upper sections, and M/D slides are from a lower left M1. Tooth- tooth type as indicated, unless the plane of section cut across several teeth, indicated by transverse.

Appendix 2. Histological sections of chimpanzee (*Pan troglodytes* sp.) material.

Collection	Individual	Slide	Tooth	Collection	Individual	Slide	Tooth
Harvard	6907	6907.2.1.2a	LUM1	Martin (cont.)	3385	002-1	RUM1
		6907.2.1.2b	" "			002-2	" "
		6907.2.2.1	LUM3			002-3	" "
						005	" "
	6908	6908.2.2.1	RUM1			029	RLM2
	6909	6909.2.1.3	RUM3		3387	135-1	RUM1
		6909.2.2.2	RUM1			135-2	" "
						003	RUM2
	6911	6911.2.1.2	LUM1			136-1	" "
		6911.2.2.2	LUM3			136-2	" "
						136-3	" "
	6912	6912.2.1.2	RUM3			023	RLM1
						022	" "
	6915	6915.2.2.1	RUM3			139-1	" "
		6915.2.2.3	" "			139-2	" "
						027	RLM2
	6916	6916.2.1.1	RUM1			028	" "
		6916.2.1.2	" "				
		6916.2.1.3	" "	Ashton	389A	114-1	RLM1
						114-2	" "
	6919	6919.2.1.2a	LUM1				
		6919.2.1.2b	" "		389B	111-1	RLM1
						111-2	" "
	6924	6924.2.2.3	RUM1			024-1	" "
						024-2	" "
	6926	6926.2.1.1	LLM1			024-3	" "
		6926.2.1.4a	" "				

	6926.2.1.4b	" "
	6926.2.1.4c	" "
	6926.2.1.4d	" "
6928	6928.2.1.1	LUM1
	6928.2.1.3	" "
6937	6937.2.1.1	RUM3
	6937.2.2.2	RUM1
6945	6945.2.1.1	LUM1
	6945.2.1.2	" "
	6945.2.2.1	LUM3
	6945.2.2.3	" "
6946	6946.2.1.2	LUM1
	6946.2.1.3	" "
	6946.2.2.1	LUM3
	6946.2.2.2	" "
	6946.2.2.3	" "
6949	6949.2.1.1	RUM1
	6949.2.1.3	" "
	6949.2.2.3	LUM3
6950	6950.2.1.3a	LUM3
	6950.2.1.3b	" "
	6950.2.2.2	LUM1
	6950.2.2.3	" "
6952	6952.2.1.1	RUM3
	6952.2.1.3	" "
	6952.2.2.1	LUM1
	6952.2.2.2	" "

389C	120-1	RLM1
	120-2	" "
	020	" "
	119-1	RLM2
	025	" "
	119-2	" "
	131-1	RLM3
	131-2	" "
	131-3	" "
389D	116-1	LLM1
	019-1	" "
	019-2	" "
	019-3	" "
	116-2	" "
	115-1	LLM2
	115-2	" "
	122-1	LLM3
	122-2	" "
389E	113-1	RLM1
	021	" "
	113-2	" "
	123-1	RLM2
	123-2	" "
389F	128-1	LLM1
	128-2	" "
	032	" "
	124-1	LLM2
	129	" "
	124-2	" "
	124-3	" "

				389G	112-1	LLM1
6953	6953.2.1.1	LUM3			016	" "
	6953.2.1.2	" "			112-2	" "
	6953.2.1.3a	" "				
	6953.2.1.3b	" "		389I	117-1	LLM1
					017-1	" "
6954	6954.2.1.1	LUM1			017-2	" "
	6954.2.1.2	" "			117-2	" "
	6954.2.2.2	LUM3				
				389J	118-1	LLM1
6956	6956.2.1.1	LUM1			018	" "
	6956.2.1.4	" "			118-2	" "
					126-1	LLM2
6957	6957.2.1.1	RLM1			126-2	" "
	6957.2.1.2	" "				
				389K	121-1	LLM1
6958	6958.2.1.1	RUM3			127	" "
	6958.2.1.4	" "			121-2	" "
					125-1	LLM2
					125-2	" "
6975	6975.2.2.1	RUM1			125-3	" "
6978	6978.2.2.1	LUM1	Newcastle	15.00	#1	RLM1
					#2	" "
6983	6983.2.1.3	LUM1			#3	" "
	6983.2.2.1	LUM3			#1	RLM2
	6983.2.2.3	" "			#2	" "
					#3	" "
6985	6985.2.1.3	RUM3			#1	RLM3
	6985.2.2.1	RUM1			#2	" "
					#3	" "
7014	7014.2.1.2	RUM3				
				28.90	46	RLM1
7036	7036.2.1.3	LUM1			46	" "

	7036.2.2.1	LUM3		47 #1	RLM2
				47 #2	" "
7038	7038.2.1.1a	RUM3		47 #1	" "
	7038.2.1.1b	" "		47 #2	" "
	7038.2.1.1c	" "			
			4.01	46	RLM1
7059	7059.2.2.3	RUM1		46	" "
				47	RLM2
7061	7061.2.1.1	LUM1		47	" "
	7061.2.2.1	LUM3		48	RLM3
				48	" "
7067	7067.2.1.1a	LUM1			
	7067.2.1.1b	" "	88.89	16	RUM1
				16	" "
7072	7072.2.2.3	LUM1		16	" "
				17	RUM2
7077	7077.2.1.1	LUM1		18	RUM3
	7077.2.2.2	LUM3		18 #1	" "
				18 #2	" "
7079	7079.2.1.1	LUM3		46 #1	RLM1
				46 #2	" "
7080	7080.2.1.3	LUM3		46	" "
	7080.2.2.2	RUM1		47	RLM2
				47	" "
7081	7081.2.1.2	LUM1		47	" "
	7081.2.2.3	LUM3		48	RLM3
				48	" "
7084	7084.2.2.1	LUM3		36	LLM1
7089	7089.2.1.3	LUM1	43.87	36 #1	LLM1
	7089.2.2.2	LUM3		36 #2	" "
				36 #1	" "
7091	7091.2.1.3	RUM1		36 #2	" "
	7091.2.2.3	LUM3		37	LLM2

				37	" "
7099	7099.2.1.1	LUM1		38	LLM3
	7099.2.2.1	LUM3		38	" "
			PTS-1	mes	LM1
7101	7101.2.1.2	RUM3		dis	" "
	7101.2.2.1	RUM1			
			59.89	36	LLM1
7105	7105.2.1.3	LUM1		36	" "
				37	LLM2
7106	7106.2.1.1	RUM3		37	" "
	7106.2.2.1	LUM1			
			89.89	26	LUM1
7117	7117.2.1.1	RUM3		26	" "
	7117.2.2.1	LUM1		26	" "
				37	LLM2
7118	7118.2.1.2	RUM3		37	" "
	7118.2.2.2	RUM1	10.02	36	LLM1
				36	" "
7119	7119.2.1.1	LUM1		37	LLM2
	7119.2.2.2a	LUM3		37	" "
	7119.2.2.2b	" "		38	LLM3
				38	" "
Martin 2a	038	RUM1			
	039	" "	6.88	?8	LM3
	004-2	RUM2			
	004-3	" "	7.88	46	RLM1
	004-1	" "			
	028-1	RLM2	10.88	18	RUM3
	028-2	" "			
	133	" "	11.88	38	LLM3
	034	LLM3		38	" "
	134	" "			

	033	" "
	035	" "
995	007	LUM2
	132	" "
	006-2	" "
	006-1	" "
	008	LUM3
3373	137	RLM1
	030	RLM2
	031	" "
	138	" "
	037	RLM3
	036-2	" "
	036-1	" "

Collection- source of material (see text). Individual- designation given for animals during the original studies. Slide- slide number.

Appendix 3. Sample data sheet for collection of chimpanzee incremental data.

Date: Section:
Overall Quality (1-5): Completeness (1-5):

Qualitative Observations (including pathology, presence of short-period lines, dentine):

Quantitative Observations:

1) Periodicity:

2) Retzius lines Cusp 1: Cusp 2:

Estimated missing:

3) Cuspal thickness Cusp 1: Cusp 2:

4) DSR quality:

Cuspal montage ?

Other notes:

Associated photos/files:

Appendix 4. Cross-striation spacing (daily secretion rate) data sheet.

Cross Striation Spacing Spreadsheet

Taxon:
Specimen:
Magnification:

Zone:	X Coord:	Y Coord:	Area:	Units:	XS:	Ave Unit:	Factor:	Ave Int:	

Appendix 5. The periodicity values of 61 chimpanzee molar dentitions.

Group	Individual	Periodicity
Harvard	6907	7
	6908	6
	6909	7
	6911	6
	6912	6
	6915	7
	6916	7
	6924	6
	6926	6
	6928	6
	6937	6
	6945	6
	6946	6
	6949	6
	6950	7
	6952	6
	6953	6
	6954	6
	6956	6
	6975	6
	6983	6
	6985	6
	7014	6
	7038	6
	7059	6
	7067	7
	7077	6
	7079	7
	7080	7
	7089	6
	7091	7
	7099	6
	7101	6
	7105	6
	7117	7
	7118	6
	7119	6
Martin	2a	7
	3373	6

	3385	7
	3387	7
Ashton	389A	6
	389B	6
	389C	6
	389D	6
	389E	6
	389F	6
	389G	6
	389I	6
	389J	6
	389K	7
Newcastle	15.00	7
	28.90	6
	4.01	7
	88.89	7
	43.87	7
	59.89	6
	PTS	6
	89.89	7
	10.02	7
	10.88	6

Appendix 6. Cusp-specific crown formation time in 62 cusps of chimpanzee molars.

Tooth	Cusp	Slide	Time
UM1	paracone	135-1	2.92
		89.89.26 m	2.06
	protocone	88.89.16 m	2.22
		89.89.26 m	2.39
	metacone	89.89.26 d	1.95
	hypocone	88.89.16 d	1.85
		89.89.26 d	1.90
LM1	metaconid	28.90.46 m	1.68
		59.89.36 m#1	2.24
		113-1	1.87
		032	1.81
		112-1	1.65
		118-1	1.67
	protoconid	114-1	2.12
		113-1	2.61
		112-1	2.04
		017-1	2.01
		59.89.36 m#1	2.96
	entoconid	114-2	1.52
		128-2	1.79
		121-2	1.90
		59.89.36 d#2	2.18
	hypoconid	28.90.46 d	2.38
		4.01.46 d	2.65
		114-2	1.94
		111-2	2.12
		116-2	2.14
		128-2	2.12
		112-2	1.97
UM2	paracone	88.89.17 m	2.90
	protocone	88.89.17 m	2.93
	metacone	136-2	4.26
LM2	metaconid	027	2.97
		88.89.47 m	2.33

		10.02.37 m	1.95
		115-1	2.17
	protoconid	28.90.47#2	2.84
		4.01.47 m	2.75
		115-1	2.78
	entoconid	28.90.47#2	2.43
	hypoconid	28.90.47#2	2.91+
		88.89.47 d	3.18
UM3	paracone	6909.213	2.72
		6911.222	2.29
		6946.223	2.51
		7038.211a	2.67
	protocone	6909.213	3.02
		6953.213b	2.65
	metacone	10.88.18 d	2.19
		6937.211	2.28
		6953.212	2.11
		7099.221	2.28
		7101.212	2.64
		7119.222b	2.19
	hypocone	7079.211	2.61
		7119.222a	2.11
LM3	metaconid	15.00.48.3	2.60
		10.02.38 m	1.91
	protoconid	4.01.48 m	2.74
	entoconid	4.01.48 d	2.13
		88.89.48 d	2.37
	hypoconid	88.89.48 d	3.07

Note: the lower second molar hypoconid of 28.90.47#2 was not included in the statistical analyses, as it had yet to complete crown formation.