Variation in Regional Enamel Growth Rates in Modern Humans Presenting Dental Evidence of Vitamin D Deficiency

Christopher Aris, [1,2*+] Katie A. Hemer, [1,3+] and Emma Street [4]

[1] Department of Archaeology, University of Sheffield, UK
[2] School of Chemical and Physical Sciences, Keele University, UK
[3] Institute of Archaeology, University College London, UK
[4] No affiliation

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ABSTRACT

Introduction: Enamel development (amelogenesis) research has been fundamental to our understanding of variation in human enamel physiology. However, research into internal enamel structures is often limited to exploring rates of enamel formation. This study addresses this gap by analysing enamel growth and the impact metabolic disease can have on that growth.

Materials and methods: Thin sections were produced for nine permanent teeth, five presenting zero or minimal evidence of vitamin D deficiency, and four presenting moderate-severe deficiency. Vitamin D deficiency was identified via interglobular dentine (IGD). Enamel development was analysed through daily secretion rates (DSRs). Statistical analysis investigated for variation in mean DSRs, and overall DSR distribution variance, across mid, inner, and outer lateral enamel regions between IGD-absent and IGD-present groups.

Results: Mean DSRs were significantly faster in the inner and mid regions in the IGD-present group. Distribution variance was significantly larger in all regions in the IGD-present groups.

Conclusions: These findings suggest that vitamin D deficiency impacts the formation of enamel concurrently with dentine. While more research into the correlation between IGD formation and changes in DSRs is needed, these findings allude to vitamin D deficiency regulating human enamel secretion and/or enamel undergoing catchup growth after vitamin D deficiency recovery comparable to bone.

Human enamel growth rates are frequently analysed through histological analysis within the fields of biological anthropology and bioarchaeology. Such analyses have primarily focused on the variation between cusps of the same tooth (e.g., Mahoney, 2008), within individual populations (e.g., Schwartz, Reid, and Dean, 2001), as well as comparison between different populations (e.g., Smith et al., 2007; Aris et al., 2020a, 2020b). Whilst the scope of research into enamel growth variation between groups has been wide, these projects have almost exclusively researched dental samples presenting no evidence of pathology or stress markers such as linear enamel hypoplasia. Select research has commented on how other human enamel growth features have varied between groups of individuals not suffering from physiological stress compared to those that were under stress, as identified from dental evidence; these studies have, however, typically focused on the influence of the methodologies used to calculate enamel growth across different tooth types (e.g., Lukacs and Guatelli-Steinberg, 1994; Guatelli-Steinberg and Lukacs, 1999; FitzGerald and Saunders, 2005). Indeed, relatively limited research has been published which directly considers the relationship between permanent enamel growth and stress. This includes studies such as those identifying slowing enamel formation rates after birth in deciduous teeth (Birch and Dean, 2009), and those whose data could be interpreted as showing drops in enamel formation rates potentially related to seasonal stressors (Macchiarelli et al., 2006). More recently, Aris and Street (2021) analysed growth rates of accessory enamel (defined by them as "growth of enamel outside of the features typically used to define and identify human tooth types"),

+ These authors contributed equally
*Correspondence to:
Christopher Aris
Keele University
Email: c.aris@keele.ac.uk

and found that the presence of accessory enamel growth correlated with a significant slowing of enamel growth within the normal enamel areas of the same tooth, and compared to 'normal' teeth from the same population. This evidence suggests that not only is there scope to further investigate the correlation between normal and accessory enamel growth in other populations, but there is also a need to consider the relationship between other dental defects and pathologies which could significantly influence the growth rates of human enamel. Interglobular dentine (IGD), often appearing as zones of black globular patches within the dentine, is an example of a defect observable in the same tooth cross sections used for collecting enamel growth data (e.g., Nanci and Smith, 2020; Snoddy et al., 2020). The presence of IGD has been linked to vitamin D deficiency at the time of dentinogenesis (e.g., Kagayama et al., 1997; Tsuchiya et al., 2002). By analysing teeth presenting IGD and those that do not from the same population, we can begin to examine whether dental enamel growth rates are influenced by vitamin D deficiency. It is further possible to use the specific location of IGD to identify whether any variation in tooth enamel growth between deficient and non-deficient individuals is regional within the tooth, since IGD has been shown to appear at different foci within the tooth crown and root (Jayawardena et al., 2009). This analysis will help inform our understanding of how tooth enamel growth rates vary in individuals presenting dental manifestations of pathology, and specifically the potential disruption to enamel growth caused by a metabolic condition, such as vitamin D deficiency.

Background

Amelogenesis and Daily Enamel Growth

Ameloblast cells secrete and mineralize protein matrix in a process known as amelogenesis (Boyde, 1989; Nanci and Smith, 1992; Smith and Nanci, 2003). During the amelogenesis stage in which the matrix is secreted, the behaviour of ameloblasts is altered according to a circadian rhythm, which produces short-period markers along the length of enamel prisms; these line markers are referred to as cross-striations (e.g., Boyde, 1963; 1990; Massler and Schour, 1946; Okada, 1943; Kajiyama, 1965; Dean et al., 1993; Dean, 1995; Antoine, 2001; Smith and Nanci, 2003; Antoine et al., 2009). Cross-striations possess a different refractive index to that of the rest of the volume of enamel prisms thus making them visible in dental thin sections using transmitted light microscopy (e.g., Berkovitz et al., 2002; Zheng et al., 2013).

By counting cross-striations, daily enamel secretion rates of enamel matrix (DSRs) can be calculated (Aris, 2022). Research on teeth without pathology or abnormal growth variations show DSRs to accelerate from inner to outer enamel regions, along the pathway of enamel prisms, from the enamel dentine junction (EDJ) towards the outer enamel surface (e.g., Beynon, Dean, and Reid, 1991; Beynon et al., 1998; Reid, Beynon and Ramirez Rozzi, 1998; Lacruz and Bromage, 2006; Mahoney, 2008; Aris et al., 2020a, 2020b; Aris and Street, 2021). Further variation in DSRs has been observed along the EDJ, with faster rates recorded with increased proximity to the dentine horn (Beynon, Dean, and Reid, 1991). As a result of DSRs varying within a tooth, both along and away from the EDJ, their analysis often involves calculating them for defined areas of the tooth crown: cuspal, lateral, and cervical enamel, which are then subdivided into inner, mid, and outer regions (e.g., Aris et al., 2020a, 2020b).

The relationship between enamel growth patterns, stress and pathology

While teeth presenting evidence of pathology or stress have been relatively absent from studies of enamel DSRs, different features of enamel growth have been investigated in dentition, especially in teeth showing physiological signs of stress (e.g., Lukacs et al., 1989; Lukacs, 1991, 1992, 1999; Lukacs and Joshi, 1992; Lukacs and Pal, 1993; Lukacs and Guatelli-Steinberg, 1994; Goodman and Song, 1999; Lukacs and Walimbe, 1998; Guatelli-Steinberg and Lukacs, 1999; Holt, Reid, and Guatelli-Steinberg, 2012; Birch and Dean, 2014). Much of this research has focused on the aetiology of external enamel growth defects via the impact of physiological stress on amelogenesis. For example, a series of papers have been published by Lukacs and colleagues on the pattern and expression of enamel defects in modern human populations (Lukacs et al., 1989; Lukacs, 1991, 1992, 1999; Lukacs and Joshi, 1992; Lukacs and Pal, 1993; Lukacs and Guatelli-Steinberg, 1994; Lukacs and Walimbe, 1998; Guatelli-Steinberg and Lukacs, 1999). Their results show that the expression of such defects, such as enamel hypoplasia, can vary between groups as a result of differing geographic location, climate, and diet. Of greatest significance to this study is that these articles also found evidence of increased crown formation times (CFTs) in individuals presenting stress-induced enamel defects. Further studies have subsequently been

published, and have all further stated that when physiological stress impacts enamel structures it significantly increases CFTs (e.g., Holt, Reid, and Guatelli-Steinberg, 2012; Birch and Dean, 2014; Primeau et al., 2015). Crown formation times, as a measure of enamel growth utilising cross striations, are directly related to other measures of enamel growth including DSRs (e.g., Massler and Schour, 1946). Therefore, if physiological stress can influence CFTs, it is reasonable to assume that physiological stress caused by nutritional deficiencies may also impact enamel matrix DSRs.

The potential to use enamel defects to predict the precise age at which a stressful event occurred has improved the way that we can investigate the impact of stress on enamel growth; this is possible due the regular, daily process by which cross-striations are formed (e.g., Antoine, 2001; FitzGerald and Saunders, 2005; Antoine et al., 2009). When cross-striations are altered, the ability to calculate the timing of these alterations can be correlated with when the individual is likely to have experienced the stressful event. Using a large sample of 274 teeth from 127 Roman subadults, FitzGerald and Saunders (2005) postulated that enamel formation is proportionally impacted in relation to the severity of the stressful event. They further concluded that there is no minimum level of stress required for enamel growth to be affected (FitzGerald and Saunders, 2005). It is therefore plausible that nutritional stress, such as that impacting on dentine formation, could equally impact on enamel growth.

Aris and Street (2021) expanded the research into DSRs by investigating the growth of accessory and non-accessory enamel presented in a modern-day incisor with a talon cusp. Their findings suggested that the presence of accessory enamel resulted in an overall slowing of enamel growth across the enamel cap. The exact aetiology of talon cusps is thought to be genetic, with predisposition to accessory cusp development further increased through stress and/or trauma during the development of the dental papilla (e.g., Mohan et al., 2013; Kalpana and Thubashini, 2015). This finding further shows how stress and/or genetically-determined pathological cases of dental manifestations have the potential to influence enamel growth. Furthermore, Aris and Street (2021) conclude that the lack of research on DSRs in association with different dental defects limits the overall understanding of how stress and pathology affects enamel growth, and whether that is always the case. Analysis of regional DSRs alongside the presence of IGD, as a marker of vitamin D deficiency, will work to address this.

Vitamin D

Vitamin D is essential for regulating calcium homeostasis within the human body; without the hormone, the body is unable to effectively absorb calcium and phosphate from the intestines and this results in the skeleton's inability to mineralize osteoid, that is, the precursor to bone (Holick, 2007; Brickley and Ives, 2008). Whilst dietary sources of vitamin D are available (e.g., oily fish, eggs), for most individuals, cutaneous synthesis is the main source of vitamin D. Vitamin D is synthesised following the exposure of the skin to ultraviolet B (UVB) radiation which creates the pre-hormone vitamin D3 (cholecalciferol). Vitamin D3 is inert, and therefore goes through a two-stage process to convert it into its biologically active form; this first step occurs in the kidneys, and then subsequently in the liver. It is the active form of vitamin D (1,25 (OH)2D) that is responsible for the absorption of calcium and phosphate from the blood (Nair and Maseeh, 2012).

The active form of vitamin D plays a significant role in most tissues within the body by binding to vitamin D receptors (VDR) in target cells (Holick, 2007). These vitamin D receptors (VDR) bind to specific regions of nuclear DNA known as vitamin D response elements (VDRE), and in doing so regulate the expression of more than 900 genes responsible for a variety of different physiological functions (Berdal et al., 1995; Bailleul-Forestier et al., 1996; Kongsback et al., 2013; Botelho et al., 2020). The VDR are therefore essential for regulating homeostatic processes, in particular by increasing the efficiency of calcium and phosphate absorption (Holick, 2007). This is especially true in the mineral-regulating organs such as the kidneys and intestines, as well as in bones and teeth where VDR are found in the bone-forming osteoblast cells (Dowd and MacDonald, 2013: 540; Keller & Wahli, 1997: 283).

A disruption to the synthesis of Vitamin D and inadequate calcium absorption can result in metabolic bone diseases due to its impact on bone osteoid. Osteoid is the precursor to bone formed by osteoblasts during growth and bone remodelling; during bone mineralisation, calcium phosphate nanocrystals populate a collagen-based organic matrix in order to create bone's dense structure (Brickley, Moffat, and Watamaniuk, 2014; Kuhn, 2001). Without adequate calcium phosphate, the osteoid remains unmineralized. As a consequence,

in the growing skeleton, defects in the bone at the sites of endochondral growth occur, including porosis, disorganised bone, and the splaying of the bone under mechanical force. The outcome includes the formation of bending deformities such as bowed limbs, deformation, and pseudofractures (Brickley and Mays, 2019).

Vitamin D deficiency and Interglobular dentine (IGD)

Whilst changes to the skeleton may be observed macroscopically, vitamin D deficiency also has an impact on the formation of dentine during tooth development. During the early stages of dentinogenesis, dentine - a proteinaceous calcified tissue - is formed by the action of odontoblast cells. It commences at the point where the tooth germ reaches the late bell stage; in first permanent molars, the formation of the cuspal dentine, the horns, begins in-utero at around 30 weeks gestation (Hillson, 1996: 122). Mantle dentine is formed first near the dentino-enamel junction, whilst circumpulpal dentine subsequently forms beneath the mantle (Kagaymama et al., 1997: 477-78). Dentine is laid down in an incremental fashion through the activity of the odontoblast cells which go through a process of cell differentiation, as well as the secretion of a collagen matrix, and the mineralisation of the matrix through the coalescence of spherical hydroxyapatite crystals (calcospherites) (Jayawardena et al., 2009; Opsahl Vital et al., 2012). In a vitamin D sufficient individual, who has adequate calcium and phosphate blood serum levels, the calcospherites fuse to form a homogenous mineralised dentinal matrix (Jayawardena et al., 2009; Opsahl Vital et al., 2012). If an individual is vitamin D deficient, however, the mineralisation of the dentine is disrupted and the calcospherites fail to grow and coalesce leaving behind bands of dark voids; these are areas of unmineralised dentine known as interglobular dentine (IGD) (Jayawardena et al., 2009; Opsahl Vital et al., 2012; D'Ortenzio et al., 2016: 152-153) (Figure 1). Through the histological analysis of teeth, it is possible to identify those individuals who experienced a single episode of vitamin D deficiency (represented by a single band of IGD), or multiple episodes of vitamin D deficiency (represented by more than one band of IGD), during tooth dentine formation. Bioarchaeological studies have linked the presence of IGD to skeletal changes indicative of vitamin D deficiency rickets in archaeological populations (e.g., D'Ortenzio et al., 2016; Veselka et al., 2019; Hemer and Verlinden, 2020), yet no studies have sought to identify a link between vitamin D deficiency and enamel growth rates, as proposed here.

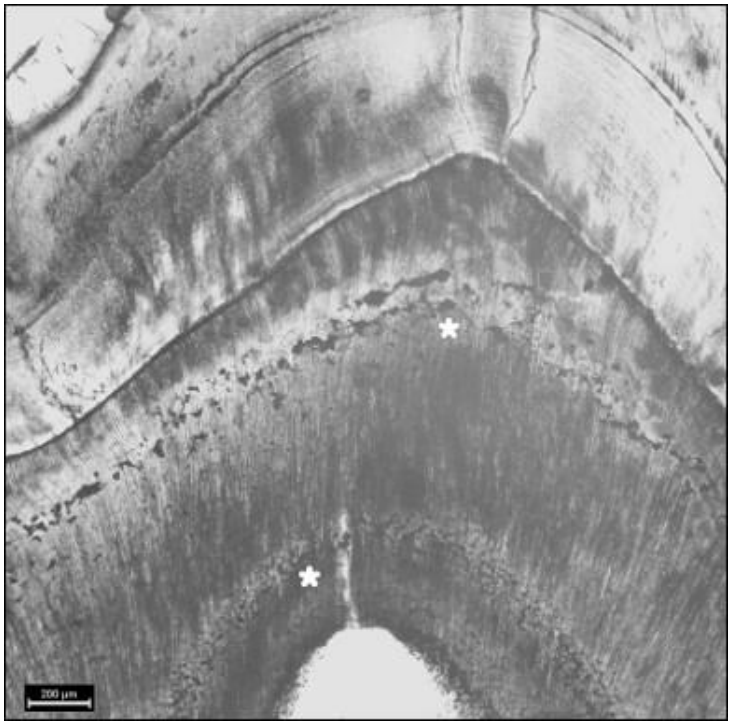

Figure 1. Thin section of the first permanent molar from Skeleton 278. Two bands of IGD are indicated by the white, star-shaped markers; these suggest this individual experienced two separate episodes of vitamin D deficiency within the first three years of life.

Materials and Methods

Dental sample

Teeth were sampled from an archaeological population from southwest Wales dating from the 8th-11th century AD. The sampled individuals form part of a wider bioarchaeological research project into the population led by KH. Preliminary analysis of a single juvenile skeleton from the site revealed skeletal and histological evidence for vitamin D deficiency rickets (Hemer and Verlinden, 2020); further investigation is ongoing to explore the impact of this metabolic condition on the wider population. Before destructive sampling of the teeth was undertaken, all individuals were subjected to a rigorous, macroscopic osteological assessment following the recommended guidelines of the British Association for Biological Anthropology and Osteology (Brickley and Mckinley, 2004) and the Chartered Institute for Archaeology (Mitchell and Brickley, 2017). Each skeleton was recorded including their degree of preservation, an estimation of age, sex, and stature (where possible), and any skeletal markers of physiological stress, disease, and trauma were also recorded.

Sample preparation

Histological thin sections were produced using standard procedures for dental sampling (e.g., Schwartz et al., 2005; Mahoney, 2008; Aris, 2020). Each tooth was embedded before cutting in a resin-hardener mixture (Buehler®) in order to reduce the chance of any enamel fracturing during the sectioning process. Embedded samples were then cut at a low speed using a diamond-edged wafering blade (Buehler® IsoMet 1000 Precision Cutter) at a longitudinal angle through the apex of the selected cusp (see below). The samples were then mounted on glass microscope slides and lapped using progressively finer grinding pads (Buehler®) until the dental material was around 100-120µm thick. Ground samples were polished using 0.3µm aluminium oxide powder to improve the clarity of the slides during microscopy. Polished samples were then placed within an ultrasonic bath for two minutes in order to remove any remaining debris before being dehydrated using 90% and 100% ethanol-based solutions (Fisher scientific®). All sections were examined using polarised light microscopy (Lecia DM2700 P system microscope). Analysis and image capture was conducted using micro imaging software (Leica microsystems LAS v4) (see below for detail).

Daily secretion rates

Daily secretion rates (DSRs) were calculated for the inner, mid, and outer areas of the lateral enamel region of each tooth using standard methods (e.g., Beynon, Dean, and Reid, 1991; Schwartz et al., 2001; Mahoney, 2008; Aris et al., 2020a, 2020b). Each section of the three areas was determined by dividing the length of the lateral enamel region into three equidistant portions, following the longitudinal axis of local enamel prisms (Figure 2). The lateral enamel region itself was determined within the section of imbricational enamel equidistant between the dental cervix and dentine horn. For molars, DSRs were collected from the lateral regions of buccal cusps, and for canines from the labial enamel. This approach was selected due to its prevalence in human enamel DSR studies, and for the fact that it accounts for any inter-prism pathway variation occurring within regional areas of the enamel cap.

In order to fully appreciate any difference in enamel formation rates across the enamel cap, and for each tooth type, the time periods between each isolated region were calculated. This was done using measured lateral enamel thickness (as per Aris, 2022) and the proportion of this separating each region (as above) and dividing them by regional DSRs (see below; e.g., Smith et al., 2003; Mahoney, 2008). This can be done with more precision by following single enamel prisms and counting the cross striations along the length of the space separating each region (e.g., Mahoney, 2011). Unfortunately, this approach was not possible for this study due to the level of diagenesis making counting cross striations over relatively long internal enamel cap distances impossible.

Within each enamel region a measurement was made of five consecutive cross striations along the length of an enamel prism. This measurement was subsequently divided by five, giving a mean daily rate of matrix secretion (µm/day). This process was repeated to produce six mean DSRs for each region. In previous studies, these six regional means have been averaged again to give a 'grand mean' (e.g., Beynon, Dean, and Reid, 1991**;** Beynon, Clayton, and Ramirez Rozzi, 1998; Reid, Beynon and Ramirez Rozzi, 1998; Lacruz and Bromage, 2006; Mahoney, 2008; Aris et al., 2020a, 2020b). This approach was, similarly to regional separation, due to its prevalence in human enamel DSR studies (e.g., Mahoney, 2008; Aris et al., 2020a, 2020b; Aris, 2022) and to help account for any local variation between different enamel prism pathways. In preliminary studies using smaller sample sizes, the six

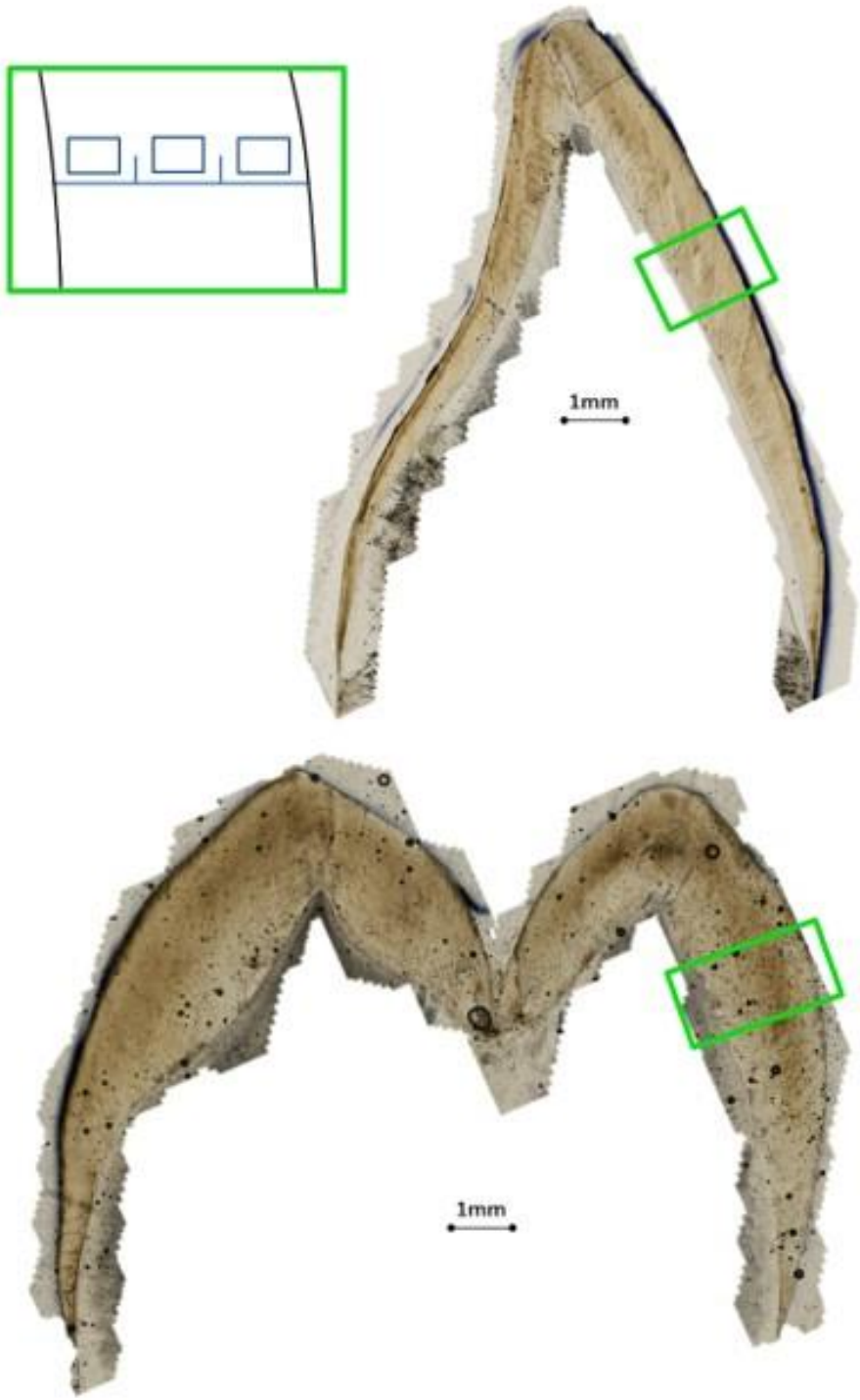

Figure 2. Digital images of a first molar and canine cross section displaying the locations from which lateral enamel regions were defined. The smaller green rectangles highlight the lateral areas from which DSRs were collected, and the larger green rectangle a representation of how inner, mid, and outer regions (moving left to right) were isolated.

mean DSRs for each region are, instead, used individually in analyses to better represent the variability of DSRs within enamel cap regions (Aris and Street, 2021). As a result, the six mean DSRs for each region of each tooth analysed here were kept separate and not used to form a 'grand mean'. All cross striation measurements were taken at 20x magnification (Figure 3).

Interglobular dentine

The ten histological thin sections were observed microscopically, and IGD was recorded as present or absent. In those cases where IGD was present, the scoring system of D'Ortenzio et al. (2016: 157) was employed in order to score the degree of severity according to their classification system of Grades 0 – 3, with Grade 0 representing normal dentine without IGD present, and Grade 3 representing the most severe manifestation of IGD including many large, interglobular spaces with a distinctive scalloped appearance covering >75% of the area of interest. Consideration was also given to the location of the IGD and the method of D'Ortenzio et al. (2016) was used to estimate the age/ages – represented by multiple bands of IGD – at which the individual experienced a deficiency in vitamin D and disruption of calcospherite growth occurred.

Statistical analysis

Mann-Whitney tests were run in order to identify any differences between the DSRs of equivalent regions from the IGD-present and IGD-absent groups. Subsequent F-tests were also conducted for each equivalent region in order to identify whether there was any significant difference between the variance in the distribution of DSRs between the two groups. Boxplots and descriptives were also produced to investigate any variation occurring between the tooth types analysed, in case this may have influenced the identification of any differences between the pooled IGD-present and IGD-absent groups. All statistical analyses were performed using SPSS 26.0.

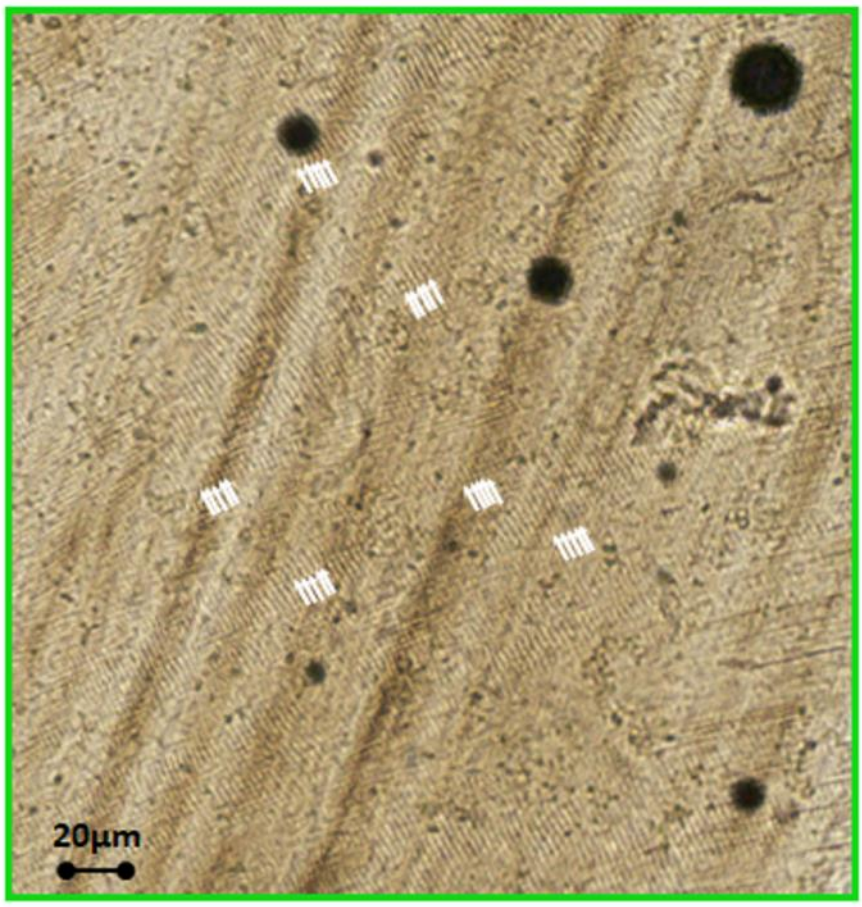

Figure 3. Digital image of an isolated enamel region displaying cross striations, captured at 40x magnification. Clusters of white arrows display how groups of adjacent cross striations were used for DSR calculations.

Results

Interglobular dentine

Of the ten teeth subjected to a microscopic assessment, six presented evidence of IGD (Table 1). There was variability in the severity of the IGD present, with most teeth exhibiting Grade 1 or Grade 2 severity, whilst only one case exhibited severe IGD which impacted >75% of the area relative to the amount of normal dentine. Whilst most teeth have a single band of IGD, representing a single influential episode of vitamin D deficiency, two teeth (belonging to STP 278 and STP 245) both exhibited two distinct bands of IGD occurring on two separate occasions during dentine formation. In skeleton STP 278, the first episode of disruption to the dentine formation occurred between 6 and 18 months of age, whilst the second band of IGD suggests another episode of disruption to dentine formation between 2 and 2.5 years of age (Hemer and Verlinden, 2020: 10). In skeleton STP 245, two bands of IGD were present; the first band / episode occurred within the first 6 months of life, whilst the second band / episode occurred between 6 and 18 months of age. Overall, the sample represents a high proportion of individuals whose dentinogenesis was disrupted by inadequate vitamin D synthesis.

Daily secretion rates

Table 2 shows the results of the Mann-Whitney U and F-tests for differences between group mean regional DSRs and group DSR distribution respectively. The Mann-Whitney U tests identified significantly faster DSRs in the IGD-present group for the inner (p=0.03) and mid (p=0.05) enamel region. While the difference was not significant between the group outer regions, mean DSRs were still faster in the IGD-present group by 0.17µm compared to the IGD-absent group - a mean difference equal to that observed in the inner and mid enamel region data (Figure 4). In contrast the F-tests identified significant differences for all enamel regions, with significantly larger variance in DSRs observed across the whole enamel cap in the IGD-present group compared to the IGD-absent group (inner: p<0.01; mid: p=0.05; outer: p<0.01).

Table 3 shows (with Figures 5 and 6 visualising) the DSR distribution for un-pooled tooth type samples for both the IGD-present and IGD-absent groups (respectively). While the deviation between the canines and molars in the IGD-present group appears notable, this is likely due to the disproportionate sample sizes (see Table 3), and in fact the mean values are relatively consistent - varying consistently by <0.5µm in all enamel regions. Even less

Table 1. Samples analysed including the presence/absence and severity of IGD recorded for each tooth.

Sampled Skeleton	Tooth analysed	IGD Present / Absent	# IGD episodes	Severity of IGD
STP 262	L. Max. Canine	Absent	0	N/A
STP 240	L. Max. Canine	Absent	0	N/A
STP 206	R. Max Canine	Absent	0	N/A
STP 242	L. Max M1	Absent	0	N/A
STP 216	L. Max M1	Present	1	Grade 1
STP 261	R. Man. Canine	Present	1	Grade 1
STP 245	L. Max M1	Present	2	First IGD band - Grade 2 Second IGD band - Grade 1
STP 278	L. Man M1	Present	2	Both IGD bands - Grade 2
STP 218	R. Max M1	Present	1	Grade 2
STP 257	L. Max M1	Present	1	Grade 3

Table 2. *Results of the Mann-Whitney and F-tests for variations in regional mean DSRs (µm/day) between the IGD-present and IGD-absent groups. Significant results are marked in bold, p < 0.01.*

Region	IGD group	N	Mean	SD	Min	Max	F	Mann-Whitney U test Sig.	F-test Sig.
Inner	Present	24	1.9	0.34	1.38	2.6	4.63	**0.03**	**0.00***
	Absent	30	1.73	0.2	1.39	2.23			
Mid	Present	24	2.13	0.28	2.13	2.64	3.93	**0.05**	**0.05**
	Absent	30	1.96	0.33	1.57	2.82			
Outer	Present	24	2.32	0.55	1.53	3.7	1.84	0.18	**0.00***
	Absent	30	2.15	0.31	1.6	2.79			

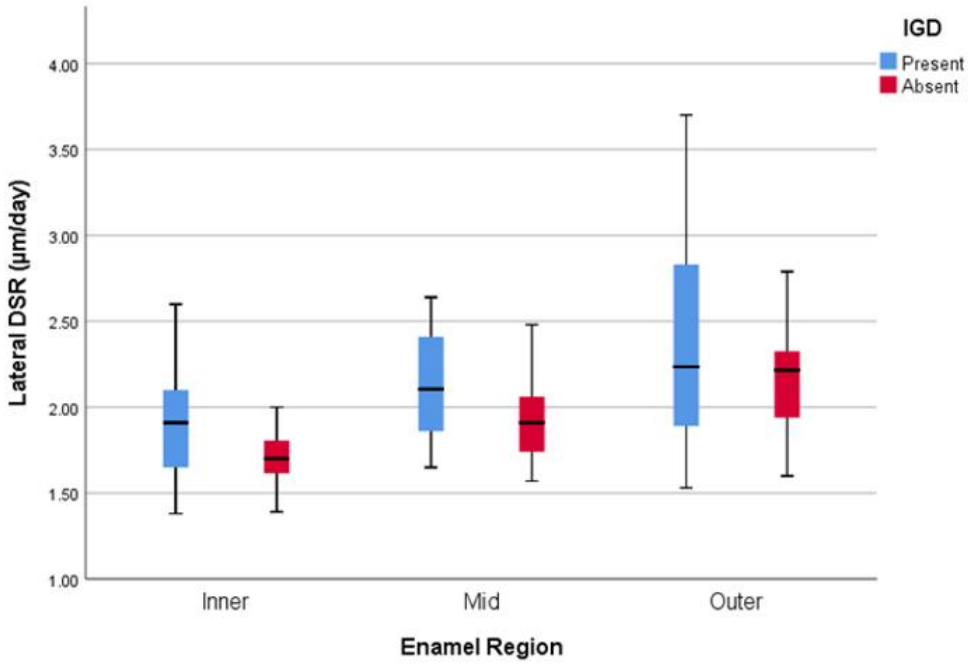

Figure 4. Plot of DSR data distribution of each sample group and enamel region. The central line displays the mean DSR value for the associated group and region.

Table 3. Descriptive statistics for regional mean DSRs (µm/day) for canine and molar split for the IGD-present and IGD-absent groups.

Region	IGD group	N	Mean	SD	Min	Max
			Molars			
Inner	Present	24	2.07	0.29	1.56	2.60
Mid	Present	24	2.20	0.27	1.65	2.64
Outer	Present	24	2.43	0.57	1.53	3.70
			Canines			
Inner	Present	6	1.49	0.10	1.38	1.66
Mid	Present	6	1.83	0.01	1.81	1.86
Outer	Present	6	1.91	0.04	1.86	1.97
			Molars			
Inner	Absent	12	1.83	0.23	1.39	2.23
Mid	Absent	12	2.07	0.40	1.60	2.82
Outer	Absent	12	2.19	0.41	1.60	2.79
			Canines			
Inner	Absent	12	1.63	0.10	1.41	1.79
Mid	Absent	12	1.85	0.20	1.57	2.26
Outer	Absent	12	2.11	0.14	1.84	2.29

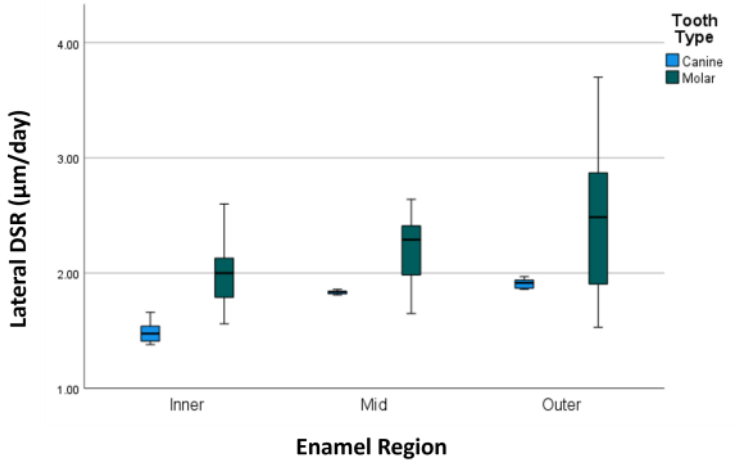

Figure 5. Plot of DSR data distribution for the IGD-present groups of the canines and molars. The central line displays the mean DSR value for the associated group and region.

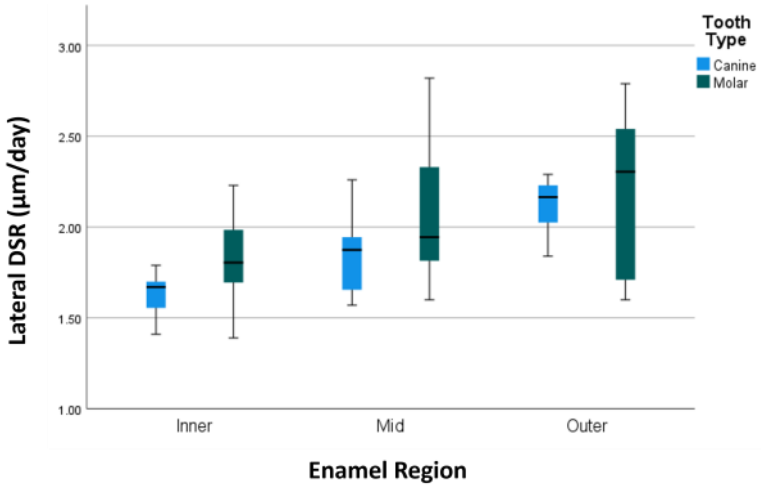

Figure 6. Plot of DSR data distribution for the IGD-absent groups of the canines and molars. The central line displays the mean DSR value for the associated group and region.

variation can be observed between the IGD-absent canine and molar groups, with consistently ≤0.3µm difference between regional mean DSRs. Even less variation was observed in the deviation in this group also, however this was likely due to the sample sizes being equal between tooth types when split in this way.

Inter-enamel region formation time

Inter-enamel region formation times were calculated by dividing proportional quantities (see Figure 2) of the total lateral thickness (LT) of the corresponding enamel region by the mean regional growth rates. The mean LT of the canines was 1.12mm, and the mean LT of the molars was 1.48mm - both comparable to LT values of multiple analysed human populations (Aris, 2022). Moreover, LT values did not vary by any notable measure between the IGD-present and IGD-absent groups, suggesting no impact of total enamel thickness as a result of vitamin D deficiency. Lateral enamel formation periods all overlapped with IGD formation periods (6-18 months; see Section 3.1) although some variation between sample groups was noted (see below).

For IGD-present molars inner, mid, and outer regions represented 236, 222, and 201 days of growth respectively - suggesting just under two years total lateral enamel formation time. For IGD-present canines inner, mid, and outer represented 248, 202, and 193 days respectively - suggesting again a formation time of just under two years.

For IGD-absent molars inner, mid, and outer regions represented 267, 236, and 223 days of enamel secretion respectively - suggesting roughly two years of total lateral enamel formation time. For IGD-absent canines the inner, mid, and outer regions represented 226, 200, 175 - suggesting around 18 months of total lateral enamel formation.

Discussion

With the exception of outer lateral enamel matrix DSRs, enamel growth measures were found to vary significantly across multiple factors between the IGD-present and IGD-absent samples. While the initial analysis indicates that the presence of IGD correlates with faster enamel growth and potential 'catch-up growth' in teeth similar to bone (e.g., Mays et al., 2009; Rajah et al., 2008), additional analysis instead suggests that the significant variations are a result of a drastically larger distribution of growth rates across the lateral enamel of IGD-present teeth. While it is plausible that some variation may be the result of multiple tooth types being analysed, the comparative analysis here suggests this is minimal (although future research should consider analysing this with larger sample sizes). Moreover, lateral enamel formation times for the IGD-present samples was near-identical between the tooth types, suggesting reliable combination of DSRs for these groups - although there was more notable difference in formation times between IGD-absent groups (discussed more below). Overall, this all alludes to a potential interruption of standard enamel growth patterns which is likely caused by the same interference arising from vitamin D deficiency and the incomplete formation of dentine.

Inter-group equivalent region analysis

The mean lateral DSRs were significantly faster in the IGD-present teeth for the inner and mid regions alluding to potential catch-up growth later in the development of the dentition. Such variations between the growth of equivalent enamel regions are not uncommon, and those seen here are comparable to similar differences which have been observed both within and between groups from British populations (Aris et al., 2020a, 2020b). However, within the context of analysing dentition showing evidence of pathology/nutritional deficit, this finding is unexpected yet is supported by the lateral enamel formation time analysis, which found notably shorter formation times in the IGD-present molar group compared to the IGD-absent molar groups - again indicating faster enamel growth in a number of the IGD-present samples. The research which has been conducted in the past has found links between physiological stress and the slowing trajectory of enamel cap formation (e.g., Reid and Dean, 2006; Holt, Reid, and Guatelli-Steinberg, 2012; Birch and Dean, 2014; Primeau et al., 2015), and thus it would be reasonable to expect to see slower DSRs in IGD-present groups.

Moreover, recent research on accessory enamel growth has found evidence of enamel defects being more influential on the trajectory and pattern of enamel DSRs, rather than simply reducing the speed of enamel development (Aris and Street, 2021). It is therefore possible that the variations observed here could suggest that DSRs and CFTs can vary independently according to different external factors. Similar suggestions have been made to this effect in the past, with the additional discussion of enamel thickness (Aris et al., 2020b). What remains potentially unexplained by the analysis of mean DSRs however, is why the outer region dis-

played no significant difference between the IGD-present and IGD-absent groups, while the difference in mean was the same for all three equivalent region comparisons (0.17µm/day), and graphically appearing as the most variable region between groups.

One cause of this may have been the reverse pattern in lateral enamel formation time occurring between the molar and canine groups, where instead the IGD-present canines had formation times roughly 6 months slower than the IGD-absent canines. This suggests that the IGD-present canines formed faster overall with the potential for inter-tooth variation being more notable than that which can be observed from DSRs alone. Another possibility is that the outer regions of lateral enamel for all tooth types and IGD groups were forming after the 6-18 month period where all IGD observed here had formed. This could suggest that any difference in the inter-group variation of the outer DSRs was the result of levelling vitamin D levels and thereby a return to unaffected enamel formation patterns.

DSR distribution between and within groups

While the comparisons of the equivalent enamel region mean DSRs between the two groups was unclear in places, a review of the variation within each group and region by way of the distribution of growth rates may further illuminate the potential impact of vitamin D deficiency on concurring enamel growth. For all lateral enamel regions, DSRs were found to vary in their distribution significantly more in the IGD-present group than in the IGD-absent group. Similar, if not statistically tested variations, have been observed in past research. For instance, in a recent case where all published human DSR data (at time of publishing) was collated for regions of lateral and cuspal enamel, SD variation was observed to typically lie around <0.30µm/day, with outliers normally <0.40µm/day (see Table 5 in Aris et al., 2020b). Moreover, this DSR data and that which has been subsequently published, appears to show the most common regions to present high SD are the inner and outer regions, but with minimal fluctuations within individual populations (Aris et al., 2020a, 2020b). In regards to inner and outer regions of enamel, our findings follow this trend, with the most marked differences in DSR distribution occurring in these regions between the IGD-present and IGD-absent groups (Table 1). What is new, however, is that these identified variations come from within a single population suggesting that the presence of IGD, and therefore vitamin D deficiency, correlates with a wider distribution and less consistent pattern of enamel growth.

To further contextualise the levels of distribution seen here, the SD levels of the IGD-present group, while particularly high by the standards outlined previously, are not unheard of. While examples of this level of variation are rare, they can be seen in analysis of British Roman teeth (Aris et al., 2020b) and modern South African teeth (Lacruz and Bromage, 2006). However, in both these cases the SD was high across all regions of the given population; whereas here, while the IGD-present group was significantly more variable than the IGD-absent group in all regions, the individual SD values for the mid and inner regions were within expected ranges for human populations (see Table 5 of Aris et al., 2020b). Our data are therefore unique with the IGD-present group showing SD on the expected scale for the inner and mid region, but exceptionally high in the outer region, while still significantly varying from the IGD-absent group of the same population.

These observations potentially explain the inconsistencies within the analysis of equivalent mean DSRs between the groups. This expanded analysis therefore suggests that vitamin D deficiency (identified via IGD) does not necessarily cause an increase in mean DSRs (as inner and mid test results indicate), but rather it results in an interruption in secretion of enamel matrix, subsequently causing inconsistent developmental rates across the enamel cap. Future research would benefit from replicating the analysis here on cervical and cuspal DSRs on a larger sample to further investigate this. This would also help explore the idea that the impact of vitamin D deficiency on enamel growth is localised (similar to how it is in enamel), influencing the variability in enamel growth and formation rates for regions forming at the same time as IGD. Future research is therefore also encouraged to analyse dental samples with more extensive evidence of multiple IGD formations.

Potential differences between tooth types

Typically, analyses such as those presented here investigate individual tooth types such as molars (e.g., Aris et al., 2020b; Beynon, Dean, and Reid, 1991; Lacruz and Bromage, 2006; Mahoney, 2008; Smith et al., 2007) or anterior teeth (canines and incisors; Aris et al., 2020a; Aris and Street, 2021; Birch and Dean, 2009; FitzGerald, 1998; FitzGerald and Hillson, 2009; Reid, Beynon, and Ramirez Rozzi, 1998a; Schwartz et al., 2001). In some cases teeth

have been pooled within these categories, but it is unusual to pool teeth from between these groups as has been done here. There is also only one case where DSRs are not compared between groups through a grand mean (Aris and Street, 2021). It is possible therefore that the DSR variations and high distribution between the IGD-present and IGD-absent groups could be the result of this pooling of tooth types, and/or taking six DSR measures for each region of each tooth. However, comparison of tooth-type specific groups show relatively small differences between the relative IGD groups (see Table 3), and all tooth types analysed here were relatively equal in their representation of each group analysed, and for each enamel region analysed. As a result, any impact of pooling growth data collected from different teeth would have been consistent in both groups. Therefore, we do not expect that pooling growth data for different teeth has any negative impact on the conclusions drawn here. However, the enamel formation calculations suggest some variation in that data between the canines and molars, and thus future research is recommended to analyse tooth types separately when factoring in enamel thickness to confirm the previous suggestion.

The impact of Vitamin D deficiency on daily secretion rates

In seeking to explore the potential relationship between the occurrence of IGD and the variable DSRs observed in the study sample, further consideration was given to vitamin D's role in cellular activity. As noted previously, vitamin D plays a significant role in gene expression through its relationship with vitamin D receptors (VDR) in the target cells. Two calcium-binding proteins whose expression is regulated by the presence of Vitamin D are Calbindin-D28k and Calbindin-D9k. They have been identified in numerous tissues including those of the kidney, placenta, and cartilage (Onisihi et al., 2008: 117). Of the two proteins, Calbindin-D9k is most closely regulated by Vitamin D and directly associated with vitamin-D dependent calcium homeostasis (Onishi et al., 2008: 122). Indeed, Calbindin-D9k is directly involved in the mineralisation of tissues; for example, in bone, it is present in both osteoblasts and osteoclasts. Moreover, Bailleul-Forestier et al. (1996) demonstrated that vitamin D plays a significant role in regulating odontogenesis from the very earliest stages of tooth formation through to mineralisation. The reason being that both Calbindin-D28k and Calbindin-D9k are present in teeth, where they serve different purposes. Onishi et al. (2008) found that Calbindin-D9K was localised in the maturation ameloblasts where the active transportation of calcium was required. In contrast, Calbindin-D28K was expressed by secretory ameloblasts, and was not involved in the vitamin-D dependent calcium transport that occurs during enamel maturation.

Through their investigation of IGD in rodent molars, Kagayama et al. (1997) found a strong correlation between IGD and the early stages of dentine formation. It was found that IGD formation was not associated with the secretory stage of amelogenesis but, rather, with the maturation stage of enamel formation. They suggest that the interaction between the epithelial-mesenchymal cells during the later stages of tooth formation is fundamental in determining whether or not interglobular dentine appears; Onishi et al. (2008) later showed that this process was associated with the vitamin-D regulated expression of Calbindin-D9K. If vitamin D deficiency has such an impact on Calbindin-D9K and the disruption of calcium homeostasis during the maturation stage of enamel formation then it seems possible that it could also disrupt the mineralisation of the tooth enamel to such an extent that we have seen variable lateral enamel DSRs in those individuals who exhibited IGD in our study sample.

Conclusions

The presence of IGD correlates with variable lateral enamel DSRs, particularly with an increase in distribution of regional growth rates between IGD-present and IGD-absent groups within the same population - with most notable variations occurring when enamel was forming at the same time as IGD. While it should be noted that DSRs here relate to the rate of more organic enamel matrix (as opposed to enamel mineralisation during the amelogenesis maturation stage), this evidence strongly suggests that the interruption of vitamin D deficiency on the development of dentine also impacts the development of enamel, potentially in a similarly time-period-localised manner. This highlights the value of conducting histological research on populations and individuals with identifiable pathologies and/or nutritional deficiencies in order to expand our knowledge on the plasticity of enamel growth in early life.

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