The periodicity of enamel laminations in human deciduous molars

Petrina Barnard[1,2], Gina McFarlane[1], Chris Deter[1,2], Carolina Loch[3], Patrick Mahoney[1,2],

[1]School of Anthropology and Conservation, University of Kent, Canterbury, UK

[2]School of Chemistry and Forensic Sciences, University of Kent, Canterbury, UK

[3]Sir John Walsh Research Institute, Faculty of Dentistry, University of Otago, Dunedin, New Zealand

Abstract

Objective

Enamel laminations are closely spaced incremental lines that run parallel to Retzius lines or the developing enamel surface. Here, the timing of enamel laminations is calculated for naturally exfoliated deciduous molars (n=111) from three modern-day populations (Aotearoa New Zealand, Britain and Canada).

Design

Teeth were sectioned using standard histological methods and examined using a high-powered microscope. Mean daily secretion rates (DSR) were calculated for the outer enamel of each molar in cuspal, lateral and cervical enamel regions. These DSRs were used to determine the periodicity of enamel growth across laminations in each region. Lamination periodicity was compared between populations and sexes, and within molars to assess the relationship between lamination periodicity and the angle between laminations and the outer surface.

Results

Laminations were present in 57% of all molars (*n*=63 out of *n*=111). Their presence did not vary between populations or by sex. A mean two-day periodicity was observed in cuspal and lateral outer enamel sampling regions. A mean one-day periodicity was observed in the cervical outer enamel. The angle of laminations relative to the outermost surface of the enamel was significantly related to the presence of laminations.

Conclusions

A two-day periodicity for laminations indicates that this incremental marking is not a reliable proxy for a circadian 24-hour rhythm in human deciduous molars. The orientation of laminations was similar to Retzius lines but differed to the orientation of cross-striations.

Key words

Laminations, incremental growth, deciduous dentition

1. Introduction

Human dental enamel retains evidence of incremental growth markings. Short period markings occur every 24 hours in the form of cross-striations along enamel rods (Lacruz & Bromage, 2006; Papakyrikos et al., 2020; Schour & Poncher, 1937; Zheng et al., 2013). Longer period markings, also known as Retzius lines, manifest with a periodicity of 4 to 12 days that varies between individuals (Beynon et al., 1991; Dean et al., 1993; McFarlane, Guatelli-Steinberg, et al., 2021). Cross-striations are used to reconstruct the daily rate of enamel secretion, while Retzius lines are often incorporated into calculations of tooth formation time (Mahoney, 2012; Nava et al., 2022; Reid & Dean, 2000; Reid & Ferrell, 2006).

The outermost enamel of primate teeth can preserve evidence of other incremental markings named enamel laminations (EL) (Ripa et al., 1966). Laminations often occur near the enamel dentine junction (EDJ) and the enamel surface, as well as in the outermost prismless enamel. These EL markings are oriented parallel to Retzius lines (Fig. 1) (e.g., Smith, 2006; Whittaker, 1982). It has been suggested that ELs form as ameloblasts slow down and the Tomes process disappears in the maturation stage of amelogenesis (Osborn, 1973; Whittaker, 1982). Prior research reports that ELs can have a daily periodicity and are therefore equivalent to cross-striations in prismless enamel (Gustafson, 1959; Ripa et al., 1966; Smith, 2006; Whittaker, 1982). It has been reported that ELs can have a spacing (width) that is comparable to the width of adjacent cross-striations (Smith, 2006; Smith et al., 2003, 2004). No study has examined ELs in modern human deciduous teeth.

The aim of this study is to calculate the periodicity of enamel laminations in deciduous molars (n=111) from three modern-day populations (Aotearoa New Zealand, Britain, Canada). Variation in the presence and timing of the laminations was investigated between populations, and between males and females. In addition to the main aim, several other aspects of EL morphology are explored. The angle of laminations relative to the outer enamel surface was calculated and compared between enamel regions. Variation in enamel thickness and EDJ length is compared to the periodicity of ELs. The angle of laminations relative to the outer enamel surface was compared to EDJ length. The rate of enamel secretion was compared to ELs to explore whether an alteration in enamel production results in EL formation.

1.1Background

Enamel formation (amelogenesis) commences as the cells of the inner epithelium differentiate into ameloblasts (Aoba, 1996; Bui et al., 2023; Pandya & Diekwisch, 2021). The differentiation

of ameloblasts begins at the tip of the dentine horn and progresses cervically towards the dentine cervix along the EDJ (Berkovitz et al., 2018; Hu et al., 2007). Once an ameloblast has differentiated, it begins secreting enamel matrix and this continues until the full thickness of the layer has been achieved (Berkovitz et al., 2018). During the subsequent maturation phase, the diffusion of calcium ions and removal of proteins from the matrix by ameloblasts increases the dimensions of hydroxyapatite crystallites (e.g. Berkovitz et al., 2018; Boyde, 1963). During amelogenesis, temporary changes in enamel secretion produces incremental features (Antoine et al., 2009; Mahoney, 2008, 2012; Whittaker, 1982). These incremental features are indicative of long and short periods of enamel growth, evidenced in human enamel as Retzius lines and daily cross striations (DCS), respectively (Berkovitz et al., 2018).

Cross-striations form along enamel prisms with a daily circadian rhythm (Bromage, 1991; Lacruz et al., 2012; Zheng et al., 2013, 2014). They form parallel to the secretory face of the ameloblast (Tomes' process) every 24 hours (e.g. Antoine et al., 1999, 2009; Bromage, 1991; Dean, 1998). These short period growth lines are orientated at 90 degrees relative to the long axis of the prism. Cross-striations typically have an interval between 3-6μm per day in human teeth (Berkovitz et al., 2018; Boyde, 1979; Bromage, 1991; Guatelli-Steinberg & Huffman, 2011; Modesto-Mata et al., 2020). The enamel increments between two cross-striations are used to calculate regional daily secretion rates (DSR) (Aris, Mahoney, & Deter, 2020; Aris, Mahoney, O'Hara, et al., 2020; McFarlane, Guatelli-Steinberg, et al., 2021; McFarlane, Loch, et al., 2021; Nava et al., 2017).

Retzius lines are long-period growth lines (Retzius, 1837). They are orientated obliquely to the long axis of enamel prisms and mark the progression of the enamel forming front (Retzius, 1837; Boyde, 1989). Retzius lines are on average 25-40μm apart in modern humans and represent successive positions of the enamel forming front (Fitzgerald & Rose, 2008; Mahoney et al., 2020; Modesto-Mata et al., 2020). The periodicity of Retzius lines range between 4-12 days across deciduous and permanent human teeth (e.g., McFarlane et al., 2021).

Enamel laminations have been observed in prismless enamel along the outer surface (Table 1; Kierdorf et al., 2019; Smith, 2006; Tafforeau et al., 2007; Ripa et al. 1966). Ripa et al. (1966) examined the presence of prismless enamel (n=116) that consisted of erupted deciduous (n=28), erupted (n=88) and unerupted (n=40) human permanent teeth. Ripa et al. (1966) highlighted the presence of enamel laminations in 'prismless', or aprismatic, enamel and observed that the prisms bent when approaching the outermost prismless enamel. The prism

orientation deviated with respect to the prism axis, which is perpendicular to underlying dentine by 16º. Ripa et al. (1966) suggested that the crystallites in the outermost prismless layer lie perpendicular to the enamel surface, and that the laminations do not represent prism boundaries.

Whittaker (1982) observed an average width of 5µm in the ELs of human teeth (n=550, deciduous n=160, permanent n= 345). Laminations were orientated in the same direction as cross-striations in the outer 50µm of prismatic enamel, except in the outer 50µm towards the cervical margin. Laminations ran parallel to the outermost surface of the tooth. It was suggested that the occurrence of ELs were associated with the presence of the prismless enamel layer. Gustafson (1959) observed that prisms were compressed near the surface resulting in an alteration of the prism direction. Furthermore, Gustafson (1959) suggested the presence of prismless enamel and ELs could be related to accentuated incremental lines or compression of the prisms. An overall loss of continuity of the prism arrangement was also observed by Whittaker (1982). Whittaker (1982) further suggested the presence of laminations and the alteration in prism orientation was due to the reduction in the movement of the ameloblast and loss of the Tomes process. These results suggested laminations may be related to a diurnal (daily) rhythm. Whittaker (1982) further suggested that ELs are incremental features related to a different underlying biorhythm connected to the slowing of ameloblasts as they approach the external enamel surface.

Smith et al. (2003) identified laminations in second molars of *Afropithecus turkanensis* (n=2) at the outer enamel surface and along the EDJ. These laminations were present in cuspal and lateral enamel sampling regions. Smith et al. (2003) suggested that the development of laminations may be related to the production of prismless enamel, or specific patterns of enamel prism packing (as suggested by Gustafson, 1959). In Smith et al.'s (2003) study, ELs did not appear to have a daily periodicity, as four to five ELs were observed in an 8-day period.

Smith et al. (2004) examined laminations in *Graecopithecus freybergi* (=*Ouranopithecus macedoniensis*), a late Miocene hominoid (n=1). In this *G. freybergi* specimen, cross-striations and perikymata could not be observed in areas where ELs were present (Smith et al., 2004, pg. 558). Smith et al. (2004), further confirmed that in this sample laminations may be associated with pattern 1 enamel (Beynon & Dean, 1988; Smith et al., 2003). Pattern 1 enamel was first defined by Boyde (1964) as enamel with cylindrical prism boundaries with separate interprismatic regions, and is typical of enamel in many primates, including humans as well as

several other mammals. Smith et al. (2004) observed the periodicity of ELs was variable, so when used as a proxy for cross-striations, ELs may hinder the determination of Retzius periodicity (RP) (Smith et al., 2003, 2004).

Smith (2006) assessed the presence of ELs in deciduous maxillary and mandibular teeth (canines, premolars and molars) in pigtailed macaques (n=17). The laminations observed in this study demonstrated a daily periodicity. The results showed that laminations bisected the prisms obliquely. Smith (2006) confirmed previous findings (Smith et al. 2004) that the assessment of laminations can complicate calculation of Retzius periodicity, as one line can superimpose upon another. From these observations, Smith (2006) suggests ELs should not be utilised as a substitute for cross-striations when calculating DSR's in macaques. It was suggested that laminations are a result of interaction between short-period growth lines.

Enamel laminations have been identified in non-primate enamel. Tafforeau et al. (2007) examined ELs in different species of rhinoceros (*Gaindatherium*, *Rhinoceros sondaicus, Rhinoceros unicornis*) (n=3), and compared these to modern cows, horses, humans and a fossil Elephantidae. Laminations were suggested to be equivalent to cross-striations in their daily periodicity across all species. These results agree with those of Smith (2004, 2006) in that they suggest the daily periodicity of laminations in rhinoceros and other large herbivorous mammals can be used to determine crown formation time (CFT).

Kierdorf et al.'s (2019) examination of laminations in pig enamel (n=7) indicated a 1-day periodicity. They suggested that away from the enamel surface, without perikymata to identify them, long-period growth lines were indistinguishable from laminations. Kierdorf et al. (2019) emphasised the need to differentiate Retzius lines from laminations.

In summary, previous research into enamel laminations has highlighted the variable presentation of these features across primate and non-primate enamel. Enamel laminations have been observed both in the deeper enamel and in the prismless enamel towards the outer surface. Both forms of laminations can often confound the calculation of RP when assessed in both primate and non-primate enamel (Kierdorf et al., 2019; Smith, 2006). Ripa et al. (1966), Whittaker (1982) and Smith et al. (2004) associated the presence of ELs with prismless enamel and an alteration to prism orientation. Studies such as Whittaker (1982) and Smith (2006) have further suggested that laminations can be equivalent to daily cross-striations and as such demonstrate a daily periodicity. However, research into the presence and periodicity of laminations in modern human samples has consisted of mixed (permanent and deciduous)

samples. Our main objective is to understand the timing of laminations in modern human deciduous molars. To explore possible variation in DSR's and laminations, several populations were assessed across different geographic locations. An additional objective is to understand whether the presentation of surface ELs relates to the orientation of the outermost enamel surface.

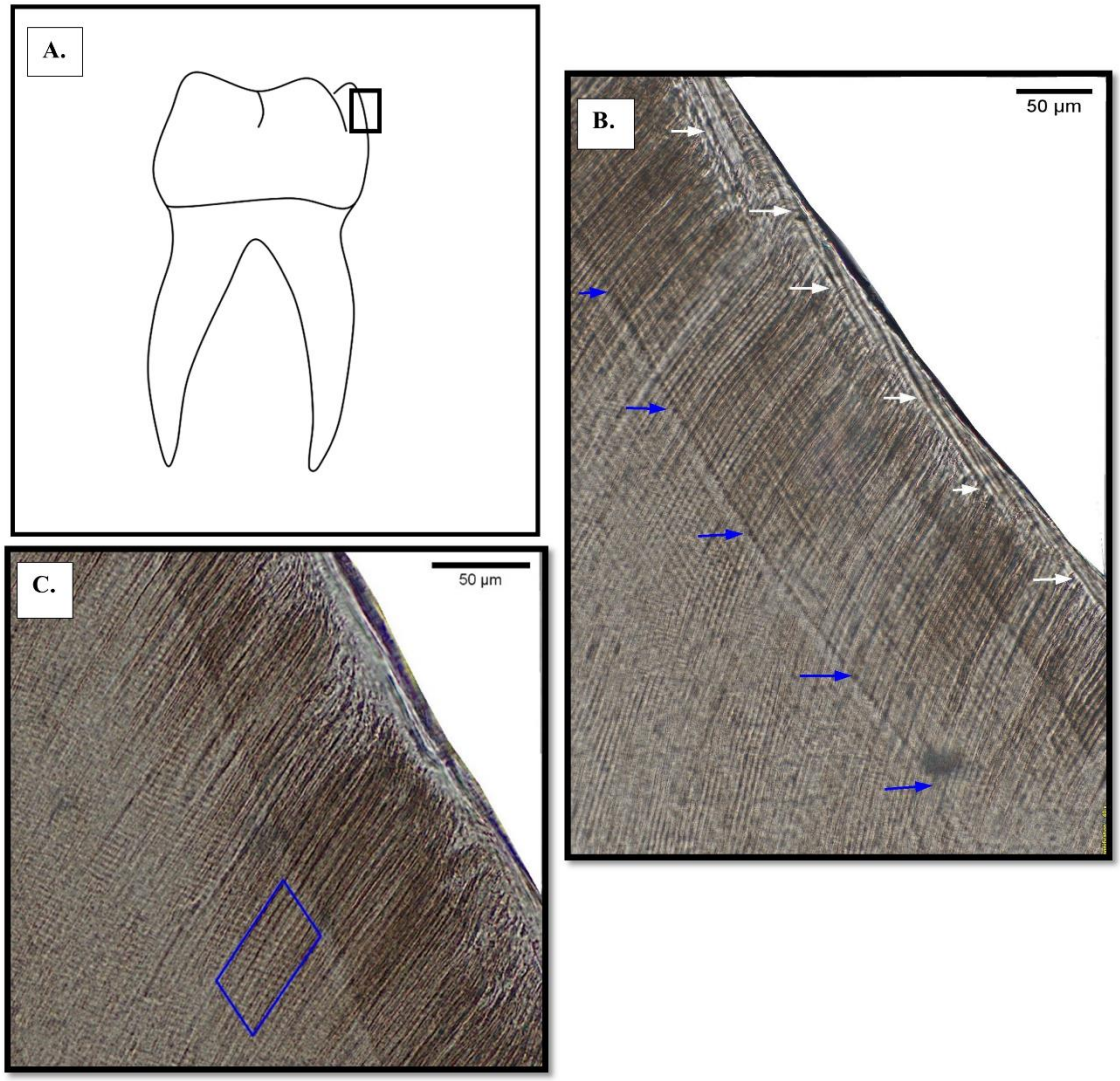

Fig. 1. Examination of deciduous molars for enamel laminations. (A) Deciduous second mandibular molar. (B) Light microscopy image of enamel laminations on the outermost edge of the enamel surface (white arrows) in contrast to accentuated lines (blue arrows). Deeper enamel laminations are present 200µm beneath the surface laminations (between white and blue arrows). These deeper ELs can affect RP and DSR calculations. (C) Outer enamel with cross-striations highlighted (blue rectangle).

Table 1.

Summary of previous research into enamel laminations

ecies	Periodicity	Observations	Source
man	Not specified	Laminations form in prismless enamel. Prisms bend as they approach the outermost layer of enamel.	(Ripa et al.,
man	Daily	Laminations occur concurrently with cross-striations. Prism orientation was altered in the presence of accentuated incremental lines. Compression of the prisms was observed.	(Whittaker,
opithecus kanensis	0.6 days (variable).	Laminations present at the outer enamel surface and along the EDJ. Formation of laminations attributed to prismless enamel and enamel packing patterns (pattern 1).	(Smith et al
necopithecus bergi	Daily but variable due to visibility in regions.	Laminations present in association with pattern 1 enamel. EL are variable so not a suitable substitute for cross-striations when determining RP.	(Smith et al
caque	Daily	Laminations bisect prisms obliquely but did not bend. Laminations can superimpose on Retzius lines (as they are parallel to each other) affecting RP calculations.	(Smith, 200
noceros *inindatherium ndaicus, cornis*)	Daily	Laminations and cross-striations are equivalent daily features. ELs shown to be three-dimensional alignment of cross-striations.	(Tafforeau e 2007)
	Daily	In contrast to the ELs observed in primates, Retzius lines and laminations were indistinguishable from each other in pig enamel. Intradian lines were observed in pig ELs. Retzius lines and ELs should be differentiated from each other prior to estimating RP and CFT.	(Kierdorf et 2019)

2. Materials and methods

2.1 Samples and selection criteria

Most of the samples were existing sections created for the Biorhythm of Childhood Growth project (Mahoney et al., 2020; 2022; McFarlane, Guatelli-Steinberg, et al., 2021; McFarlane, Loch et al., 2021). Samples originated from children from New Zealand, Britain and Canada (n=111). Thin sections were obtained from upper and lower first and second deciduous molars. Sex was known for n= 104 samples (Table 2). Five new thin sections were created for this project.

- The New Zealand samples consisted of existing sections of exfoliated deciduous molars collected from children (between the ages of 9-11) living in Dunedin, New Zealand, alongside additional samples from community oral health clinics in Whanganui and Hawkes Bay. Samples belong to children of New Zealand European, Māori or Pacific islander ethnicity. Ethical approval was obtained from the University of Otago Human Ethics Committee (approval number H19/030).

- New thin sections were created (n=5) for deciduous molars from the UCL/Kent collection, held in the Histology Lab at the University of Kent. These British samples were fully anonymised and were either from dental extractions or naturally exfoliated in routine dental treatments in the 1960's and 1970's. Ethical approval was granted by the UK National Health Service Ethics Committee (REC reference number: 19/EM/0126: 2019; project ID 203541.

- Existing thin sections were utilised from deciduous molars of Canadian children. These samples were collected in the 1970s at Simon Fraser University. These samples are held at the University of Kent's Histology lab. Ethical approval was granted by the UK National Health Service Ethics Committee (REC reference: 19/REM/0126, ID 261173).

The following criteria were adopted for inclusion in the project:

- The enamel surface should be intact along the lateral edge to allow analysis of the ELs.
- The section must have sharp enamel and dentine horn apices to ensure that oblique sections were not included.

Table 2.

Number of teeth by sex, tooth type and population.

Population	Total	Male	Female	Unknown	Lowdm1	Lowdm2	Updm1	Updm2
British	7	-	-	7	0	0	0	7
Canadian	10	3	2	5	0	1	7	2
New Zealand	94	31	63	0	27	28	19	20

2.2 Thin sections

Standard histological procedures were followed. Teeth were embedded in epoxy resin (Buehler Epoxy cure) to reduce the likelihood of splintering during sectioning (Silva et al., 2011). The teeth were then sectioned through the tip of the cusp and the dentine horn of the mesial molar cusp using a Buehler Isomet 1000 precision saw and diamond-wafering blade. Once cut, sections were mounted onto glass slides and ground using a series of grinding pads (p600 and p1200) and polished with 0.3µm aluminium oxide powder to produce 60-90 µm thick sections. Each section was then cleaned in an ultrasonic bath, dehydrated in 95% and 100% ethanol and cleared (with Histoclear ®) before being mounted with a cover slip and a xylene based mounting medium (DPX ®). Dental thin sections were examined using a high-powered microscope (Olympus ® BX53) with a mounted microscope camera (Olympus DP25). Images of the thin sections were examined using CellSens Entry software, calibrated to the magnification used.

2.3 EDJ length and average enamel thickness (AET)

The EDJ length was measured by following the junction path between the two distal-most points of the molar cervix (Mahoney, 2008; Martin, 1983; Smith et al., 2006). In some instances, portions of the EDJ were estimated when dental fillings were present. The average enamel thickness (AET) for each sample was calculated using CellSens software by measuring the area of the dental enamel cap and dividing this by the length of the EDJ.

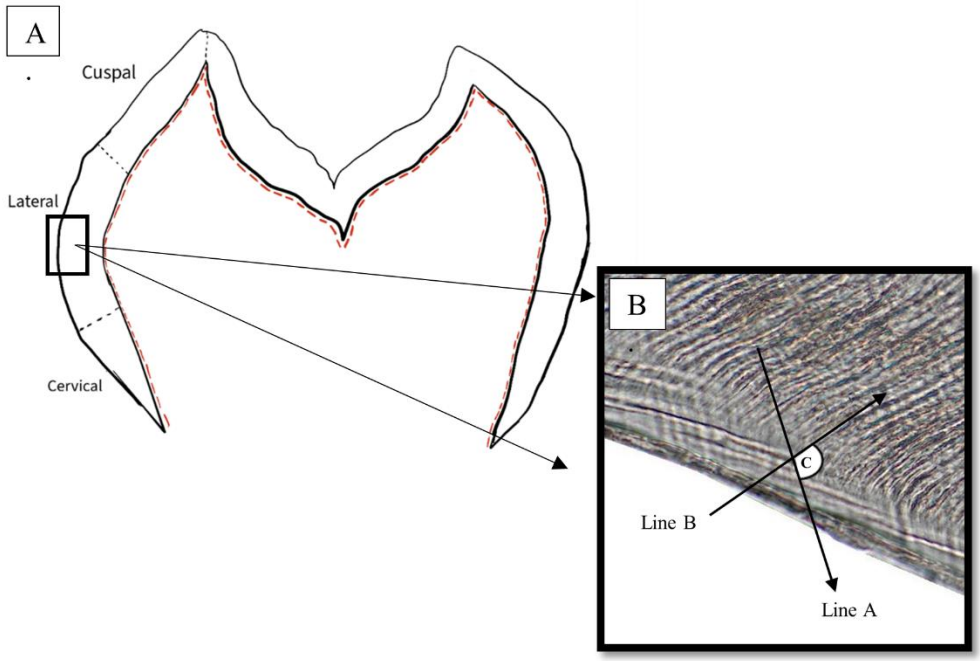

Fig. 2. A. Illustration of a deciduous molar with the cuspal, lateral and cervical sampling regions outlined and EDJ highlighted (red dashed lines) B. An example of the EL-surface angle (C) measurement. Line A takes into account the alteration in the outermost tooth surface and the bend in the prisms. Line B assesses changes in the horizontal plane of the tooth surface towards the EDJ.

2.4 DSR and Laminations

To ensure the DSR and laminations were recorded at equivalent intervals along each tooth as well as to assess variation along the tooth surface, enamel was first divided into three sampling regions: cuspal, lateral and cervical. This was achieved by dividing the EDJ into three equal lengths (Fig. 2). The cuspal region was defined as the area above the dentine horn apices.

Daily secretion rates were calculated using standard methods (e.g. Reid et al., 1998). Rates were measured along the long axis of an enamel prism within each region adjacent to the ELs (when present). Rates were calculated by measuring the length of prisms between 5 adjacent cross-striations to produce a mean value (Mahoney, 2008; McFarlane, Guatelli-Steinberg, et al., 2021). Multiple assessments of DSR were taken for each region and a final mean was calculated.

Lamination periodicity was recorded by measuring the distance between two adjacent laminations and divided by two. The process was repeated three times along the designated region and a final mean EL width was generated. The distance across one lamination was then divided by the local mean DSR to estimate periodicity. This process was repeated for the three sampling regions.

The angle of the EL relative to outer tooth surface (EL-surface angle) was recorded for the three sampling regions (Fig. 2). The EL-surface angle was measured using the 4-point measure tool in CellSens. To calculate the EL-surface angle (C), the degree of inclination of the outermost tooth edge was first established (Line A in Fig 2b). Line B was then established to accommodate for alteration in the enamel surface back towards the dentine and EDJ. The angle formed (C) between these intersections was used to measure the EL-surface angle. These measurements were repeated along the region and a mean EL-surface angle generated. The angle of the outer edge of the enamel surface was measured in teeth where no laminations were present, to compare the values for EL-surface angle.

2.5 Intra-observer reliability

Additional repeat measurements of all variables were conducted two weeks after the initial period of data collection. The repeated measurements were conducted blind to the original recordings.

2.6. Data analysis

All data analysis was conducted using IBM SPSS version 29. Statistical significance was set as $p<0.05$. Kendall Tau b analyses were conducted to ascertain the association between the presence of laminations and the metric variables (EDJ length, AET, DSR, EL-surface angle, and EL width at the three sampling regions) when accounting for sex, population and tooth type (Table 5). A Kendall Tau-b (τ_b) value of 0.3+ was indicative of a strong association between variables (Sharma, 2017). Sign tests were used to test for median differences between adjacent DCS and EL width measurements. Kruskal-Wallis tests were used to test for differences between enamel sampling regions (cuspal, lateral and cervical) and distance between adjacent laminations, as well as EL-surface angle and populations. Chi-square analyses were used to test for differences in lamination frequency between enamel sampling regions. Where multiple pairwise tests were carried out, Bonferroni adjustments were applied to the p-values utilising Dunn's (1964) procedure and the adjusted p-values reported therein.

Spearman's rank correlation coefficients were conducted to assess the relationship between mean DSR, EDJ length (µm), and EL-surface angle. A correlation coefficient value of 0.7 was considered strong (Akoglu, 2018).

3 Results

3.1 Sampling location and periodicity

When all molars were combined, mean EL periodicity was two days in cuspal and lateral sampling regions and one day in cervical regions (Table 3). The average width between laminations significantly differed between the enamel regions ($\chi 2(2) = 42.075$, $p =< 0.001$) which were wider in cuspal and lateral enamel compared to the cervical sampling region ($p < 0.001$, $p < 0.001$, respectively). On average the distance between laminations was highest in the cuspal (mean= 7.03 µm), and lateral (mean= 6.32µm) regions compared to the cervical region (mean = 4.41µm), Fig. 3. The presence of laminations significantly differed between regions ($p<0.001$), with ELs observed at a higher frequency in cuspal (n=53 (47.5%)) and lateral (n=56 (50.5%)) sampling regions, compared to the cervical region (n=23 (20.7%)) ($p<0.001$ and $p<0.001$ respectively).

ELs were wider than the local DCS measurements in all three enamel regions. In the cuspal region, there was a significant median increase in width (2.95µm) in ELs compared to local DCS (z=-6.87, p=<0.001). EL width was also significantly increased compared to the DCS in the lateral (median difference= 2.32µm, z= -7.35, p=<0.001) and cervical regions (median difference= 0.69 µm, z= -3.75, p=<0.001).

On average, the EL-surface angle was highest at the lateral (mean =109.35º) and cuspal (mean=109.29º) sampling regions compared to the cervical (mean = 104.37º). There was a positive correlation between the EL-surface angle in the cuspal region and EDJ length in µm (r=0.356, p=0.001), indicating that greater EDJ lengths corresponded to greater cuspal EL-surface angle. A slightly weaker positive correlation was identified between mean lateral EL-surface angle and EDJ length (mm) (r=0.279 p=0.002). There was no significant correlation between EL-surface angle at the cervical sampling region and EDJ length (mm).

There was a weak positive correlation between DSR and lateral (r_s =0.222, p=0.019) and cervical (r_s=0.207, p=0.029) EL-surface angle values. There was no significant relationship between DSR and EL-surface angle at the cuspal sampling region.

Table 3

Descriptive statistics for all lamination data, sd = standard deviation.

	Mean (sd)	**Min**	**Max**	***n***
Cuspal				
Lamination width (µm)	7.30 (1.54)	2.71	11.19	53
Lamination periodicity (days)	1.86 (0.42)	0.93	3.00	53
Angle at cuspal surface (when EL present)	118.12 (6.18)	105.92	135.12	53
Angle at cuspal surface (when EL absent)	101.22 (4.88)	88.53	109.99	58
DSR	3.68 (0.23)	2.92	4.58	111
Lateral				
Lamination width (µm)	6.32 (1.52)	4.07	12.78	56
Lamination periodicity (days)	1.71 (0.40)	1.13	3.49	56
Angle at lateral surface (when EL present)	116.51 (6.07)	105.29	129.28	56
Ange at lateral surface (when EL absent)	102.05 (6.91)	83.70	114.87	55
DSR	3.68 (0.23)	3.01	4.60	111
Cervical				
Lamination width (µm)	4.41 (0.93)	2.89	7.28	23
Lamination periodicity (days)	1.20 (0.23)	0.85	1.99	23
Angle at cervical surface (when EL present)	112.92 (8.55)	97.09	130.63	23
Angle at cervical surface (when EL absent)	102.14 (6.43)	87.18	125.99	88
DSR	3.67 (0.23)	2.85	4.58	111

Table 4

Mean (sd) values for enamel area, AET and EDJ length across populations and sex.

	Population			Sex	
	British	Canadian	New Zealand	Female	Male
Enamel area (μm^2)	14002685.64 (1226260.63)	10053311.64 (3368865.14)	10335022.78 (3034913.76)	10742604.47 (31363934.18)	9443402.66 (2500810.31)
AET (mm)	0.75 (0.07)	0.57 (0.15)	0.62 (0.16)	0.64 (0.17)	0.58 (0.11)
EDJ length (μm)	18823.47 (782.98)	17392.23 (1995.39)	17764.48 (14316.95)	18585.13 (17140.31)	16144.72 (2132.79)

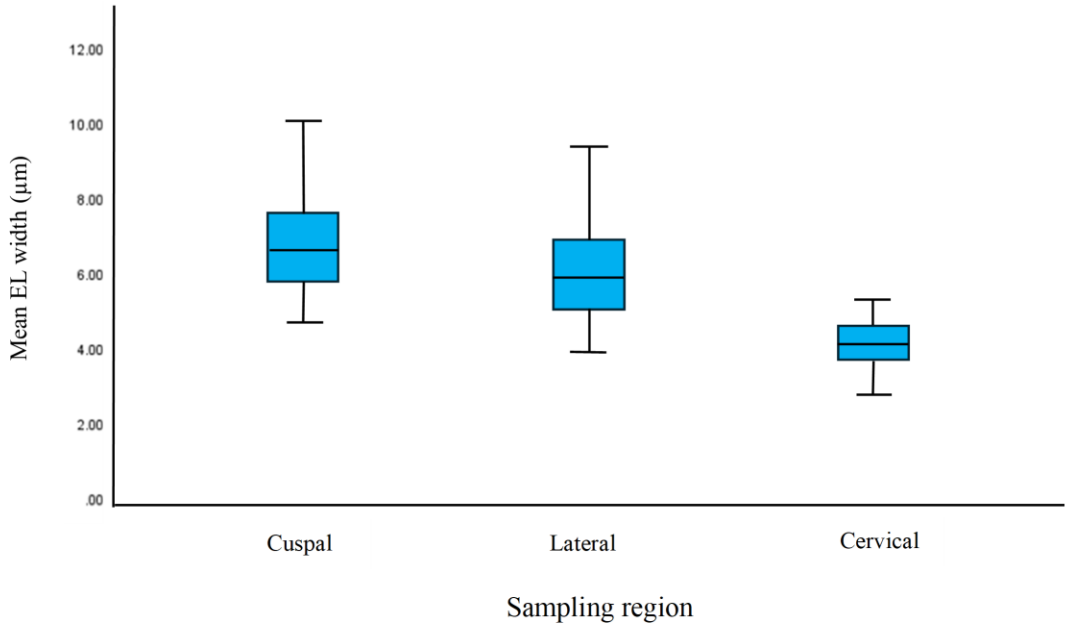

Fig. 3. Mean Lamination width (μm) observed across the three enamel sampling locations.

3.2. Association of laminations and biological variables

Table 4 displays the Kendall tau b analysis on the biological grouping variables and the EL metric values.

Sex

There was no significant difference in EL periodicity between males and females when cuspal, lateral and cervical sampling regions were compared. There was a moderate association

between sex and mean EL-surface angle at the cuspal and lateral sampling regions. Females were more likely to have higher mean EL-surface angle values at both the cuspal (τb=-0.249, p=0.003) and lateral (τb=-0.193, p=0.020) sampling regions compared to males. However, there was no significant association between EL width and sex.

Population

There was no significant association between population and the presence, width (μm), EL-surface angle and periodicity of laminations. Sample size varied considerably between populations, so care must be taken when interpreting these results. However, statistically significant differences were identified in EDJ length between Pacific Islander/Māori and British groups ($\chi 2(7)$ =-73.667, p=0.026). British samples had the largest mean EDJ values (18823.47μm). Further differences were detected between the enamel area values for Māori/NZ-European and British groups ($\chi 2(7)$ =-45.473, p=0.012). British samples had the largest mean enamel area values (14002685.64μm^2) (Table 4).

Tooth type:

The presence of laminations did not vary between first and second molars, or between maxillary and mandibular antimeres.

Presence of laminations:

There was a moderate association between presence of laminations and EDJ length, enamel area and AET (Table 5). This indicated that the presence of laminations is therefore more likely to occur in individuals with higher EDJ (μm), AET and enamel area values. Strong associations were identified between EL-surface angle and presence of laminations at both the cuspal and lateral sampling regions. However, no association was observed at the cervical region. The daily rate of enamel secretion did not correlate with the presence laminations.

3.3. Intra-observer reliability

The Cronbach's alpha value of 1 indicated a high level of internal consistency.

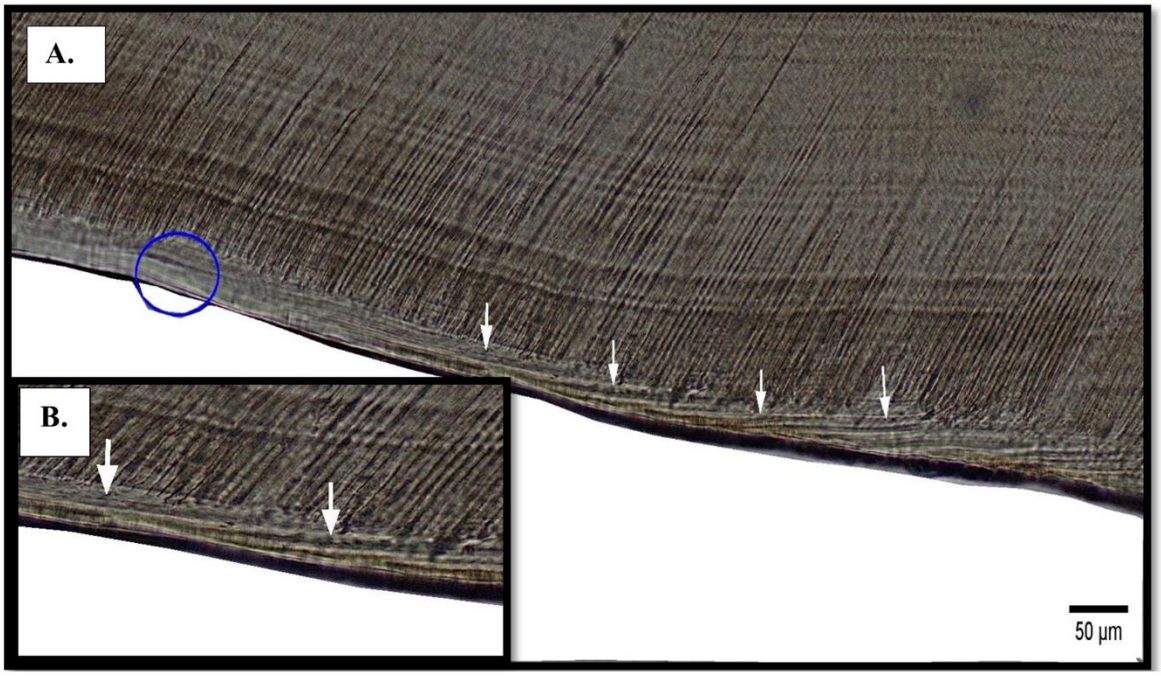

Fig. 4. Light microscope images of ELs from deciduous molars. (A) The presence of laminations (white arrows) on the outermost surface in contrast to the Retzius lines and the presence of an altered zone of prismless enamel (blue circle). (B) The 'stepped' appearance of laminations (white arrows) at the outermost tooth edge. The right side of both images is towards the cusp.

Table 5. Kendall Tau-B results from biological grouping and metric variables (statistically significant associations are shown in **bold**).

	Sex		Population		Skeletal collection		Tooth type		Presence of laminations	
	Correlation coefficient (τ)	Sig. (2-tailed)	Correlation coefficient (τ)	Sig. (2-tailed)	Correlation coefficient (τ)	Sig. (2-tailed)	Correlation coefficient (τ)	Sig. (2-tailed)	Correlation coefficient (τ)	Sig (2-tailed)
Enamel area (µm)	**-0.165**	**0.047**	0.097	0.178	**0.159**	**0.026**	0.087	0.222	**0.248**	**0.002**
EDJ (µm)	-0.66	0.426	**0.210**	**0.004**	**0.228**	**0.001**	-0.119	0.096	**0.213**	**0.006**
AET (mm)	**-0.196**	**0.018**	0.006	0.939	0.088	0.217	**0.168**	**0.019**	**0.229**	**0.003**
DSR	0.077	0.351	0.117	0.106	0.002	0.981	**-0.196**	**0.006**	0.069	0.375
Mean lamination width (cuspal) (µm)	-0.036	0.767	**-0.238**	**0.024**	-0.035	0.738	0.042	0.690	-	-
Mean lamination width (lateral)	0.142	0.232	-0.041	0.692	-0.002	0.982	**-0.199**	**0.050**	-	-
Mean lamination width (cervical) (µm)	0.114	0.552	-0.093	0.573	0.005	0.978	-0.063	0.700	-	-
Mean EL-surface angle at cuspal edge (µm)	**-0.249**	**0.003**	0.111	0.124	0.095	0.181	-0.010	0.887	**0.556**	**<0.001**
Mean EL-surface angle at lateral edge	**-0.193**	**0.020**	0.051	0.483	-0.012	0.865	-0.018	0.796	**0.627**	**<0.001**
Mean EL-surface angle at cervical edge	0.040	0.632	**0.166**	**0.021**	0.056	0.437	-0.128	0.074	0.079	0.319
Lamination periodicity in cuspal region	-0.092	0.455	**-0.240**	**0.023**	-0.026	0.805	0.146	0.165	-	-
Lamination periodicity in lateral region	0.127	0.288	-0.033	0.749	-0.005	0.958	-0.138	0.178	-	-
Lamination periodicity in cervical region	0.115	0.552	-0.098	0.554	-0.027	0.868	0.072	0.660	-	-

4. Discussion

4.1 The timing of laminations and sampling location

Here, we investigated laminations in modern human deciduous dentition. ELs were present in 57% of the sample across one or more sampling region. EL periodicity varied within a molar from two days to one day depending upon the location. The width of ELs decreased from the cuspal to cervical enamel, with a corresponding decrease in periodicity (from two days to one day). The presence of laminations was related to the EL-surface angle, but EL periodicity did not vary between the populations or between males and females.

From the 57% of molars that had laminations, ELs were more likely to arise in the outermost 100µm of enamel of the cuspal and lateral regions. This finding is similar to previous research that identified laminations in the outermost region of enamel in macaques (e.g. Smith, 2006; Smith et al., 2003, 2004). However, laminations were not present along the EDJ or adjacent to the dentine horn (Smith, 2006). The laminations observed in this study agree with the descriptions provided by Whittaker (1982) and Smith (2006) as closely spaced bands in prismless enamel. In this study, ELs fluctuated in width and proximity to each other (Fig 1a and 4). We observed that ELs did not occur concurrently with cross-striations in human deciduous molars (Figures 1 and 4), which differs to that described in studies of fossil non-human primates and macaques (e.g. Smith, 2006; Smith et al., 2003, 2004). The ELs of the deciduous molars had the same orientation as Retzius lines (see Fig. 4).

Ripa et al. (1966) and Whittaker (1982) observed the orientation of ELs changed as they approached the outermost layer of enamel. This bend in the orientation of ELs was also observed in our study. Table 1 demonstrates the common association between prismless enamel and presence of ELs in previous research (Ripa et al., 1966; Smith et al., 2003; Whittaker, 1982).

The mean width of laminations recorded here, between 4.41 and 7.03 µm (cervical and cuspal regions, respectively), lie within the range of values (2-7 µm) reported in previous research of ELs in humans and non-human primates (e.g. Ripa et al., 1966; Shellis, 1998; Shellis & Poole, 1977; Smith, 2006; Smith et al., 2003, 2004; Whittaker, 1982). An increased frequency of laminations in the cuspal and lateral enamel regions was observed in this study. This may be due to the decreased visibility of enamel features in the cervical region of deciduous molars (McFarlane, Guatelli-Steinberg, et al., 2021). It has been suggested that in cervical enamel,

Retzius lines, perikymata and other incremental features are more clearly observed in deciduous second molars (Berkovitz et al., 2018). However, tooth type did not influence enamel lamination frequency in this study.

The presence of laminations related to the EL-surface angle when comparisons were undertaken in the same region. The EL-surface angle values were larger in regions where laminations were present (maximum difference of +16º). The mean EL-surface angle when laminations were present was 118º, compared to a mean angle of 101º when ELs were absent in the cuspal sampling region. This result indicated that the presence of ELs relates to the orientation of the outermost enamel surface. Whittaker (1982), Risnes (1998) and Ripa et al. (1966) reported a similar deviation in orientation of the enamel surface when examining laminations in the outer 50µm. They attributed this shift in enamel surface orientation to alteration in the crystallite alignment due to the loss of the Tomes' process when ameloblasts transition from the secretory to the maturation stage of amelogenesis. Whittaker (1982) and Ripa et al (1966) hypothesised that the alteration in crystallite orientation (and by association the outermost enamel surface) is reflective of the increased production of prismless enamel which is associated with the disappearance of the Tomes' process. This alteration in the angle of the outermost enamel surface has also been suggested to coincide with the fluctuations in the enamel matrix prior to mineralisation (Osborn, 1973; Whittaker, 1982). The association between presence of laminations and alteration in outermost surface angle suggests a connection between surface laminations and crown morphology. The curvature of the crown shape may influence the degree of bend observed at the outermost surface when ELs are forming via the interconnected sheets of ameloblasts (Beniash et al., 2019; Sabel, 2012). However, further research is needed to assess the likelihood of an association between cusp morphology and EL formation.

Moderate positive associations were identified between EDJ length, AET, enamel area and mean EL-surface angle at the cuspal and lateral sampling sites. These associations indicate that as EDJ length, AET or enamel area increases, there is a reciprocal increase in the EL-surface angle values. Thus, as the overall size of the tooth (EDJ, enamel area) increases, then the EL-surface angle increases. The presence of laminations was more likely to occur in individuals with higher EDJ (µm), AET and enamel area values.

4.2 Implications for the use of laminations

The results presented here indicate that enamel laminations in human deciduous molars are not a suitable substitute for daily cross-striations when calculating DSR's. This finding is consistent with previous research (e.g. Smith, 2006; Smith et al., 2003, 2004; Whittaker, 1982). Previous research has highlighted that ELs are a variable incremental feature that are often difficult to assess or differentiate from cross-striations or Retzius lines (Kierdorf et al., 2019; Smith, 2006; Smith et al., 2003, 2004; Whittaker, 1982). This difficulty is reflected in the frequent observation that ELs should not be utilised in RP or CFT calculations. The findings of this current study reinforce that care should be taken when attempting to utilise ELs as an indicator of a 24-hour circadian rhythm. If laminations were a reliable substitution for cross-striations, then we would expect their incremental intervals to be similar in width. However, the mean width of the enamel laminations observed in our study (6.27µm) did not have a similar spacing compared to the mean width between daily cross-striations (3.68µm). Furthermore, ELs did not superimpose upon cross-striations (Fig. 4a). This finding suggests the biological cycle underlying daily cross-striations may not be the same as that underlying ELs in human deciduous molars. The ELs observed in this study had the same alignment as Retzius lines. However, the orientation of ELs differs to the orientation of cross-striations (Fig. 1 and Fig. 4). Sometimes ELs and Retzius lines coincided, as both features marked the progression of the enamel forming front.

The difference between mean lamination widths and mean daily enamel increments varies along the tooth surface, indicating a shift in the EL periodicity from two days to one when transitioning from cuspal and lateral enamel regions to cervical. The variation in EL width from cuspal to cervical enamel was markedly larger (a difference of -3.37µm) than the 0.16µm deviation DSR observed across these regions in previous studies of human deciduous teeth (e.g. Mahoney, 2012; McFarlane et al., 2021). Whether the change in the periodicity of ELs relates to slower ameloblast secretion in the cervical region, or a coordination between the EL biorhythm and cross-striation biorhythm in that region, has not been determined in this study. The mean DSR for the samples utilised in our study are slightly lower, but comparable with those in McFarlane et al. (2021), which are 3.52µm and 3.88µm per day, respectively. This difference may result from the smaller sample and reduced number of populations in this study.

The lamination widths presented in this study range from 4.41µm in cervical enamel to 7.03 µm in cuspal (overall mean = 6.27µm) and are larger than the DSR established for this sample, with cervical mean = 3.67µm and cuspal mean =3.68µm (overall mean= 3.68 µm). From a combined sample of deciduous and permanent teeth, Whittaker (1982) suggested that a lamination distance less than 4µm is indicative of the ameloblast slowing down as it reaches the outermost surface of the enamel. Investigation of DCS and EL width in the current study has further shown that laminations are on average wider than cross-striations across all enamel regions. The greatest width difference (median=2.95µm) was observed at the cuspal region with minor fluctuation in median differences observed across lateral and cervical enamel regions (2.32µm and 0.69µm respectively). The reduction in median difference between EL and DCS width down the tooth surface may reflect how crown formation slows along the crown surface and is not suggested to be as a result of deviation in EL formation. The shift in EL width observed in this study supports Whittaker's (1982) findings and suggests that ameloblasts are speeding up as they approach the outermost surface. However, there remains a level of uncertainty whether this is the mechanism behind the production of laminations in deciduous dentition and further research is required. Furthermore, it should be noted that DSR's in permanent teeth are faster towards the outer surface compared to inner enamel regions, relative to deciduous teeth, so the threshold utilised by Whittaker (1982) may not be relevant for a sample based exclusively on deciduous molars.

Ameloblast speed of enamel deposition is not the only factor to be considered when assessing the width and formation of ELs. In prismatic enamel, wider daily cross-striations reflect faster ameloblast movement per day, therefore if ELs are thinner than local DCS, it could suggest the ameloblasts have slowed relative to their pace prior to the ELs (Whittaker, 1982). However, the quantity of matrix being secreted by the ameloblast, the subsequent mineralisation process and the morphology of prismless enamel also needs to be considered. The outer prismless enamel is more mineralised than prismatic enamel and subsequently the apatite crystals grow larger with increased mineralisation (Beniash et al., 2019; Song et al., 2021). The loss of the Tomes' process also results in an altered alignment to the apatite crystals. Therefore, it may be that the alteration in EL width and the associated change in formation timing observed in this study reflects the larger crystal sizes (post mineralization) and, potentially, their different organisation (Beniash et al., 2019). An additional consideration is that the ameloblasts may slow as they reach the outer enamel surface but still secrete a similar amount of matrix within a 24-hour period, which then mineralises into a thicker increment (Fig 1 and 4). Further

research is required on a larger sample to determine whether increased mineralisation in prismless enamel impacts the rate of enamel secretion and width of ELs.

The results from this study support the interpretation that EL's may reflect underlying biorhythm that differs to that which produces cross striations. A consistent periodicity in the ELs of two days was observed across cuspal and lateral enamel as well as one day in cervical. Although the pace of DSRs can increase towards the outer enamel (McFarlane et al. 2021; Nava et al., 2024), our findings point to an abrupt slowing at the very final stage of enamel secretion when EL are formed. The lack of interaction with cross-striations supports the separate biorhythm origin hypothesis (Whittaker, 1982). The variation in width values for ELs compared to cross-striations within the same local region in this study, alongside the lack of visual synchronicity, indicates a separate biological cycle may underpin these incremental features.

Further research is needed to explore the nature of laminations across multiple samples as well as across deciduous canines and incisors. This would allow for the examination of intraspecific tooth variation (Mahoney, 2008; Whittaker, 1982), and the opportunity to further explore relationships of prismless enamel thickness and the periodicity of laminations, as suggested by Whittaker (1982). Further, it is important to note that this study explored surface enamel laminations and not those present in deeper in the enamel surface (Fig.1). Surface ELs in deciduous teeth may have a different aetiology than laminations present deeper in the enamel.

4.3. Influence of sex, population and tooth type on ELs

There was no evidence for sex-differences in enamel lamination width or the presence of laminations. An association was observed between the mean EL-surface angle and sex at the cuspal (p=0.003) and lateral sampling regions (p=0.020). Females were more likely to have a higher EL-surface angle enamel at these sampling sites than males. This result indicates that mean EL-surface angle may be useful as an indicator of sex that can be assessed in future studies.

Laminations were not population specific. Furthermore, tooth type was not associated with the presence of laminations, distance values or mean EL-surface angles overall.

5. Conclusion

Enamel laminations varied in width and frequency when cuspal and lateral regions of human deciduous molars were compared to cervical enamel. ELs were not temporally synchronised with daily cross-striations in this modern human sample. Cuspal and lateral sampling regions had a 2-day periodicity and a 1-day periodicity in cervical enamel. These findings demonstrate that ELs were not a reliable indicator of a consistent 24- hour circadian rhythm. The width and orientation of ELs differed to the width and orientation of adjacent cross striations. These findings suggest the biological cycle underlying daily cross-striations may not be the same as that underlying surface ELs in human deciduous molars. We further noted EL-surface angle values were greater in regions where laminations were present.

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Figure legends:

Fig. 1. Examination of deciduous molars for enamel laminations. (A) Deciduous second mandibular molar. (B) Light microscopy image of enamel laminations on the outermost edge of the enamel surface (white arrows) in contrast to accentuated lines (blue arrows). Deeper enamel laminations are present 200µm beneath the surface laminations (between white and blue arrows). These deeper ELs can affect RP and DSR calculations. (C) Outer enamel with cross-striations highlighted (blue rectangle).

Fig. 2. A. Illustration of a deciduous molar with the cuspal, lateral and cervical sampling regions outlined and EDJ highlighted (red dashed lines) B. An example of the EL-surface angle (C) measurement. Line A takes into account the alteration in the outermost tooth surface and the bend in the prisms. Line B assesses changes in the horizontal plane of the tooth surface towards the EDJ.

Fig. 3. Mean Lamination width (µm) observed across the three enamel sampling locations.

Fig. 4. Light microscope images of ELs from deciduous molars. (A) The presence of laminations (white arrows) on the outermost surface in contrast to the Retzius lines and the presence of an altered zone of prismless enamel (blue circle). (B) The 'stepped' appearance of laminations (white arrows) at the outermost tooth edge. The right side of both images is towards the cusp.

Table legends:

Table 1. Summary of previous research into enamel laminations

Table 2. Number of teeth by sex, tooth type and population.

Table 3. Descriptive statistics for all lamination data, sd = standard deviation.

Table 4. Mean (sd) values for enamel area, AET and EDJ length across populations and sex.

Table 5. Kendall Tau-B results from biological grouping and metric variables (statistically significant associations are shown in **bold**).