Biorhythms, deciduous enamel thickness, and primary bone growth in modern human children: a test of the Havers-Halberg Oscillation hypothesis

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Abstract

Across mammalian species, the periodicity with which enamel layers form (Retzius periodicity) in permanent teeth corresponds with average body mass and the pace of life history. According to the Havers-Halberg Oscillation hypothesis (HHO), Retzius periodicity (RP) is a manifestation of a biorhythm that is also expressed in lamellar bone. Potentially, these links provide a basis for investigating aspects of a species' biology from fossilized teeth. Here, we tested intra-specific predictions of this hypothesis on skeletal samples of human juveniles. We measured daily enamel growth increments to calculate RP in deciduous molars (n=25). Correlations were sought between RP, molar average enamel thickness (AET), and the average amount of primary bone growth (n=7) in humeri of age-matched juveniles.

Results show a previously un-described relationship between RP and enamel thickness. Reduced major axis regression reveals RP is significantly and positively correlated with AET and scales isometrically. The direction of the correlation was opposite to HHO predictions, as currently understood for human adults. Juveniles with higher RPs and thicker enamel had more primary bone formation, which suggests a coordinating biorhythm. However, the direction of the correspondence was again, opposite to predictions. Next, we compared RP from deciduous molars to new data for permanent molars, and to previously published values. The lowermost RP of four and five days in deciduous enamel extends below the lowermost periodicity of six days in permanent enamel. A lowered range of RP values in deciduous enamel, and data that contradicts the intra-specific HHO hypothesis for adult humans, implies that the underlying biorhythm might change with age.

KEY WORDS: Retzius lines, primary osteons, microstructure

Introduction

Evidence of growth rhythms are retained in primate enamel in the form of incremental markings (Boyde, 1979, 1989). One type of marking is a Retzius line (Retzius, 1837). Retzius periodicity (RP) is the number of days of enamel formation that occurs between adjacent Retzius lines (see Fig.1a). The RP of permanent enamel from some mammals matches the periodicity of growth increments in bone (lamellae), and also corresponds with rates of bone growth (Bromage et al., 2009). Correspondence between enamel RP and the periodicity of bone lamellae suggests an underlying mechanism that coordinates skeletal growth. It is hypothesised that the coordinating mechanism is a metabolic biorhythm termed the Havers-Halberg Oscillation (HHO) (Bromage et al., 2009). It is proposed that the HHO biorhythm has an origin in the hypothalamus (Bromage et al., 2012). Through hypothalamic signalling, which stimulates pituitary secretions that act upon endocrine glands, the HHO regulates cell proliferation, growth rates, and species-specific body mass (Bromage et al., 2012). Through its influence on body mass, the hypothesized HHO is further linked to the timing of life history events and related measures of mass, such as birth weight, age at sexual maturity, and life span (Bromage et al., 2012). Thus, RP reflects the hypothesised HHO, which is linked to life history through body mass.

When the HHO hypothesis is applied across mammals it predicts that those with smaller body sizes and accelerated life histories, such as mice, should have low mean RPs reflecting a shorter HHO rhythm, and faster rates of bone growth reflecting accelerated cell proliferation. Mammals with larger body sizes and protracted life histories, such as elephants, should have higher RPs and slower rates of bone growth. Evidence from inter-specific studies of RP of permanent teeth from some extant and fossil mammal species is generally consistent with this prediction. Smaller bodied species have lower RPs compared to those with a larger body (e.g., Lacruz et al., 2008), and the rate of bone growth, calculated from osteocyte lacunae density, was negatively correlated with body size in a selection of

mammals (Bromage et al., 2009). While there are exceptions to the HHO hypothesis amongst primates, these relate to species-specific life history adaptations (Schwartz et al., 2002, 2005; Hogg et al., 2015). Thus, the HHO holds potential for investigating aspects of a species' biology from fossilized teeth.

It is not known if the HHO hypothesis holds intra-specifically. Evidence from a small sample of adult humans suggests it does, but the direction of the relationship between RP and growth patterns is opposite to the inter-specific patterns. Periodicity of permanent teeth was lower amongst adults with a greater height (n=6) (Bromage et al., 2009) and greater body weight (n=4) (Bromage et al., in press). Amongst five adult females, higher rates of secondary osteon bone growth calculated from osteocyte lacunae density were associated with greater body mass, though this measure of bone growth was not directly related to RP from the same person (Bromage et al., 2009). Instead, the time taken to form one secondary osteon lamella in one individual was found to lie within the range of human RPs (consistent with four other species), suggesting that the timing of incremental growth in enamel and bone are related within a species. Thus, the inter-specific link between higher periodicity and slower rates of bone growth in larger bodied mammals 'flips' to lower periodicity and higher rates of bone growth within larger bodied adult humans. This is not surprising, as relationships between the HHO and inter and intra-specific growth patterns are probably equally 'noisy', but the type of 'noise' in the data is likely different (e.g., effects of scale). The evidence from adult humans led Bromage and colleagues (2009) to predict that increases in body size *within* species are accomplished through increased cell proliferation, reflected by lowered RPs and an accelerated HHO. We refer to these ideas and predictions as the 'intra-specific HHO hypothesis'.

Here, we reconstruct RP in the largest sample (n=25) of human deciduous teeth to date. The RP values are used to test predictions of the intra-specific HHO hypothesis in a skeletal sample of modern human juveniles in order to address three research questions (see below).

Human enamel growth processes and Retzius periodicity

The secretory stage of deciduous enamel growth commences early in the second trimester and ends early in the second year after birth (e.g., Kraus and Jordan, 1965; Mahoney, 2015). Crown enamel increases in thickness as enamel forming cells, ameloblasts, travel outward and away from the enamel-dentin junction (EDJ) secreting structural proteins and increasing the length of hydroxyapatite crystallites (Nanci, 2013; Simmer and Hu, 2001). Crowns also increase in height as epithelium cells differentiate into pre-ameloblasts down along the EDJ (Simmer et al., 2010). The final thickness of the enamel coating upon a tooth crown is determined by the the number of active ameloblasts, the amount of enamel they secrete, and the length of time they remain active.

Evidence of growth rhythms are retained in enamel in the form of incremental markings (e.g., Boyde, 1979). One type of marking is a cross striation (see Fig 1.b), which corresponds with a circadian rhythm (Schour and Poncher, 1937; Bromage, 1991; Lacruz et al., 2012; Zheng, 2013). Another type of marking is a Retzius line (Retzius, 1837), which marks successive enamel layers. Retzius lines are formed from a discontinuity or altered activity of ameloblasts during the secretory stage of enamel growth (Risnes, 1998). The altered activity of ameloblasts can change the orientation of enamel rods (Weber, et al., 1974), decrease their dimension, or produce a ridge and underlying cleft (Risnes, 1990; Li and Risnes, 2004).

The RP of human permanent teeth ranges between six to 12 days (Schwartz et al., 2001; Reid and Dean, 2006; Reid and Ferrell, 2006; Smith et al., 2007; Mahoney, 2008), but remains constant in the permanent teeth of any one individual (FitzGerald, 1998; Reid et al., 1998). Retzius periodicity correlates with time taken to form the lateral enamel region of human permanent canines (Reid and Ferrell, 2006). The correlation between and RP and the length of time that secretory ameloblasts remain active in canines suggests underlying relationships between enamel growth processes and their periodicity.

By comparison with human permanent teeth, little is known about the periodicity of human deciduous teeth. A few values have been reported previously for a mix of deciduous tooth types and these ranged between four to eight days (*n*=3) (Huda and Bowman, 1994), and five to nine days (*n*=7) (Mahoney, 2011, 2012). The periodicity of four and five days is less than the RP of six days reported for human permanent teeth, though, Huda and Bowman (1994) note that the four day periodicity might reflect methodological limitations. No study has assessed RP against the amount of enamel secreted by ameloblasts in deciduous teeth, or the length of time that these cells remain active.

Human long bone growth processes and primary osteons

The humerus grows through a process termed endochondrial ossification (e.g., Long, 2012). During this process, a hyaline cartilage template is replaced by mineralized tissue, first through rapidly formed woven bone, and then more slowly, during modeling and remodeling (Allen and Burr, 2014). Signs of bone modeling are visible by week eight of the first trimester (Gray and Gardner, 1969), when mineralized tissue is deposited by primary osteons (Martin and Burr, 1989). Primary osteons are bone-forming units, which have a centralized vascular canal that produces lamellar bone though centripetal osteogenesis (Martin and Burr, 1989). During centripetal osteogenesis the vascular canal (see Fig 1b) is 'filled in' by osteoblasts, the cells responsible for bone matrix protein secretion and mineralization (Stout and Crowder, 2011; Jähn and Bonewald, 2012). Centripetal osteogenesis has been demonstrated experimentally in bird taxa (Castanet et al., 2000; de Margerie et al., 2004), guinea pigs and monitor lizards (Cubo et al., 2008), and rats (Kohara et al., 2015). Primary osteons are gradually replaced by secondary osteons through remodeling (e.g., Pitfield, 2015). Remodeling commences in the second trimester (Scheur and Black, 2007) and continues into adulthood.

Research questions and predictions

Here, we test predictions of the intra-specific HHO hypothesis to address three research questions. These questions are as follows:

Does RP in deciduous molars correspond with primary bone growth?

When the intra-specific HHO is applied to human juveniles it would predict that those with a lower RP will have increased developmental rates. Or at least in terms of the underlying biorhythm, those with a lower RP should be on a growth trajectory that leads to a greater body size, relative to other children of the same age. Thus, we predict that amongst age-matched children, those with a greater average amount of primary bone formation will have a lower periodicity. To test this prediction, we calculate RP of deciduous molars in our skeletal sample of human juveniles. Next, we determine age-at-death using standard osteological methods, and then separate out seven juveniles aged two years. The RP values of these age-matched juveniles are compared to the average amount of primary bone growth in their humeri. Bone growth is measured from the area of primary osteon vascular canals (Pr.On.Ca.Ar). We infer centripetal osteogenesis from canal size, whereby a smaller canal size reflects an increased amount of (preceding) primary bone deposition.

Does RP in deciduous molars correspond with enamel thickness?

If the reasoning underlying the intra-specific HHO hypothesis – that RP is related to adult height (Bromage et al., 2009) - is extended to RP and the amount of enamel secreted by ameloblasts, then it would seem likely that the two will *not* be related. This is because larger deciduous maxillary second molar crowns (dm^2) will have thicker enamel than smaller crowns, and will be succeeded by larger permanent first molars (M1) (Moorrees et al., 1957; Brown et al., 1980; Mahoney, 2013), but M1 size is only weakly associated with adult height (Garn et al., 1968; Henderson and Corruccini, 1976). So, a human child with a lower periodicity on a trajectory towards a greater adult stature should not necessarily have larger

dm^2s with thicker enamel (and larger M1s), compared to a child that is on a trajectory towards a shorter adult height. Alternatively, if tooth size and stature are (by chance) related in our sample, a human child with a lower periodicity on a trajectory towards a greater adult stature should have larger dm^2s with thicker enamel, relative to molars with thinner enamel. To test these two opposing predictions, we calculate RP for dm^2s from 14 juveniles, and compare these values to measures of enamel thickness (Martin, 1983, 1985) from the same molars.

Is the timing of RP in human deciduous teeth the same as RP in permanent teeth?

The lowered RP in deciduous relative to permanent teeth (see above) raises the possibility that either the timing of this growth rhythm is less than previously reported, or that RP and by implication the HHO, might change with age. Here, we compare the mode and range of RP values from our entire sample of 25 deciduous molars to RP values from adult permanent M1's (*n*=15) from the same population as the children, as well as to previously published values for adults.

Materials and methods

Samples

Retzius periodicity was calculated from histological thin sections of 25 deciduous maxillary molars (dm^2 n=17; dm^1 n=8). One tooth represented one individual (see Table 1 for sample breakdown). We re-sampled the humeri of seven of these juveniles (age matched, with humeri of similar length, and without skeletal indicators of pathology) to calculate primary bone deposition. We also calculated RP for 15 permanent first maxillary and mandibular molars (M1). All human samples were from skeletons excavated from one archaeological site that dated to the recent medieval period (11^{th} – 15^{th} Century AD) in England (Hicks and Hicks, 2000). The skeletal collection is curated in the Human Osteology Lab at the University of Kent, UK. All sectioning adhered to the British Association of Biological Anthropology and Osteoarchaeology code of practice (2014). No permits were required for the described study as these are archaeological samples from before the 19^{th} Century AD.

Sample preparation

We used standard histological techniques (e.g., Bancroft and Gamble, 2008; Mahoney, 2008; Miszkiewicz, 2015). Each tooth was embedded in polyester resin to reduce the risk of splintering while sectioning. Using a diamond-wafering blade (Buehler® IsoMet 4000 precision saw) sections were taken through the outermost molar enamel cusp tip, the tip of the dentin horn, and the most cervical extension of the enamel. Buccal-lingual sections captured the paracone and protocone of the maxillary deciduous molars, and the protoconid and metaconid of the mandibular permanent molars. Each section was mounted on a microscope slide, which was lapped using a graded series of grinding pads (Buehler® Eco-Met 300) to reveal incremental lines, polished with a 0.3 mm aluminum oxide powder (Buehler® Micro-Polish II 0.3μm) placed in an ultrasonic bath to remove surface debris,

dehydrated through a series of alcohol baths, cleared (Histoclear®), and mounted with a coverslip using a xylene-based mounting medium (DPX®).

Two parallel transverse cuts approximately 0.5cm apart were made into the anterior mid-shaft region of the humerus using an electronic drill (Dremel Rotary®) resulting in a "C" shaped section. The bone section was embedded in polyester resin and each specimen was cut (Buehler® IsoMet 4000 precision saw) in half from the medial towards the lateral end reducing the longitudinal cortical thickness to approximately 0.25cm. Sections were mounted onto microscope slides, cut to 200-100μm, ground and polished (see above) to reveal primary osteons.

Enamel histology

RP values for deciduous molars were calculated from protocone lateral enamel (see Fig. 2). The first Retzius line that emerges at the outer lateral enamel surface marks the division between cuspal and lateral enamel. Retzius lines were not examined in the most cervical enamel region as it can be difficult to accurately reconstruct RP in this region because of the 'packing' effect of Retzius lines towards the end of human deciduous enamel growth. We counted the number of cross-striations along a rod between two adjacent Retzius lines in the lateral enamel of four humans. For all other sections RP was calculated by measuring the distance between several adjacent Retzius lines. The measurement was divided by local daily enamel secretion rates (DSRs) (Mahoney, 2012 for a methodology).

All sections were examined at magnification (20-60x) using a high-resolution microscope (Olympus® BX51). Images were captured with a microscope digital camera (Olympus® DP25) and analyzed in CELL® Live Biology imaging software. Retzius periodicity values for deciduous molars were produced over a five-year period. Each slide was recorded three times. If values were not the same from one recording to the next then the

slide was not included in this study. An inter-observer examination of thin sections of five human molars selected for this study produced the same RP value as the original observer.

Bone Histology

Primary bone deposition was recorded in five regions of interest, which were created to standardize recording between thin sections. Two ROIs (region 1 and 5 in Fig.1b) were selected in the medial and lateral ends of the section. One ROI (3 in Fig.1b) was selected below the anterior surface, which bisected the section. All ROIs were from sub-periosteal bone (bone adjacent to the periosteum). This minimized the variation in canal size that can occur through a diaphyseal cross-section (Starck and Chinsamy, 2002; Villa and Lynnerup, 2010; Kohara et al., 2015). Each ROI was subdivided into quarters and one image was captured within each quarter at a magnification of 20x, approximately 0.56mm^2. Slight deviations from the canal wall will result in very little error at that magnification. We measured the area in μm^2 of primary osteon vascular canals within each quarter. We measured approximately 50 primary osteons for each section (it is recommended 25-50 osteons should be examined in a thin section: Stout and Crowder, 2011). Thus, we calculated one mean value, for each individual, using the combined data from the five ROIs, for that individual.

Enamel thickness measurements

Average enamel thickness in mm^2 was calculated for dm^2, the largest sample size for one tooth type. The AET measurement was calculated by dividing the area of the enamel cap by the length of the dentin-enamel junction (DEJ), which provides the average distance between DEJ and the outer enamel surface (Martin, 1983, 1985). We selected AET to address our second research question, as this measurement does not control for tooth size. In addition to average enamel thickness, we also calculated dm^2 relative enamel thickness (RET; Martin,

1983), a unit-less measure that scales AET for tooth size. The RET measurement is calculated by dividing AET by the square root of the dentin area, and the resulting figure is multiplied by 100. RET will allow us to assess the relationship between enamel thickness and RP after the effect of tooth size is removed.

Age-at-death

Age-at-death was reconstructed from tooth formation times (Moorrees et al., 1963a,b), the timing of dental eruption (Schour and Massler, 1941; Al-Qahtani et al., 2010), long bone length (Hoppa, 1992), and fusion of cervical vertebra (Scheuer and Black, 2000).

Analyses

First, the strength of the relationship between RP and AET was measured with correlation and regression statistics (linear, log, quadratic, reduced major axis). Following this, allometric scaling relationships between log-transformed RP, and log-transformed AET and RET, were examined through reduced major axis regression (RMA). In linear regression analysis, r-squared values indicate the proportion of variance in the dependant variable that is explained by the independent variable. A residual value is the error not explained by the regression equation. In RMA regression the slope captures the growth ratio between variables such that isometry is reflected by a slope equal to one. Significantly positive (greater than 1) and negative (less than 1) allometry is identified using the 95% confidence intervals (CI's). Statistical analyses were conducted in SPSS, and in PAST (Hammer et al., 2001).

Results

Does RP in deciduous molars correspond with primary bone growth?

Retzius periodicity data for deciduous molars is in Table 1. Primary osteon data is in Table 2. Children with a seven and eight-day periodicity had, on average, a smaller osteon canal area compared to those with a six-day periodicity.

Does RP in deciduous molars correspond with enamel thickness?

Enamel thickness data is in Table 3. The RMA regression is illustrated in Figure 3a-b. RP in dm^2 was tested as a linear, log, and quadratic function of AET. Whilst there was a significant (n= 14, p<0.005) and positive correlation between RP and AET (mean= 0.69mm^2, sd= 0.18) in each model, the quadratic function was the best fit (y= 0.667x +5.658x^2 +3.599), with the highest r^2 value of 0.636 and lowest residual of 36%. An RMA regression revealed that log-AET (n= 14) and log-RP were significantly correlated and scaled isometrically (r^2= 0.591; slope=1.131; p=0.001; 95% CI=0.744 to 1.415; intercept =1.019). When the RMA regression was repeated using dm^2 relative enamel thickness, log-RET and log-RP were significantly correlated and scaled isometrically (r^2= 0.564; slope=1.424; p=0.001; 95% CI=0.815 to 1.791; intercept = -0.677).

Is the timing of RP in deciduous teeth the same as RP in permanent teeth?

RP ranged between four to 11 days in dm^2 and four to eight days in dm^1 (Table 1). The modal RP for human deciduous teeth is six days. The range of human deciduous RP's extends below the six to 12 day periodicity of permanent molars from the same population as the children (Table 1). The mode of six days and lowermost value of four days in deciduous teeth is also less than the mode of eight or nine days (Reid and Dean, 2006) and lowermost RP of six days previously reported for permanent teeth (Smith et al., 2007).

Discussion

RP and primary bone growth

Correspondence between RP and mean primary osteon canal area is consistent with the idea that aspects of enamel and bone growth are centrally coordinated (Bromage et al., 2009). However, we observed that higher RP values corresponded with a greater average amount of primary bone deposition. The correspondence in our small sample of human children is opposite to that predicted by the intra-specific HHO hypothesis, as currently formulated for a small sample of adults. Therefore, if primary bone growth is linked to the HHO, then a slower oscillation over a greater number of days (reflected by a higher RP) corresponds with increased bone formation in humeri of age-matched human children.

We recorded canal area from approximately 50 primary osteons in sub-periosteal bone at one anatomical location using fixed regions of interest for each juvenile. Whilst this methodology standardised our results (e.g., Villa and Lynnerup, 2010), it was also limited to one axis (one thin section). Bone microstructure can vary through a diaphyseal cross section (Starck and Chinsamy, 2002). Whether the relationship we have reported transfers to deeper cortical bone remains to be determined. Furthermore, we could not assess other measures of bone histomorphometry against RP, such as osteon size, because the absence of a cement line made it difficult to delineate the boundary of primary osteons in thin sections. Further work using a larger data set is needed to explore relationships between primary bone histomorphometry and RP.

RP and deciduous enamel thickness

Children with the highest RP of 10 and 11 days had, on average, the thickest second molar enamel while those with the thinnest enamel had the lowest RP's of four and five days. When interpreted alongside the data for humeri, greater enamel deposition by ameloblasts, and greater bone deposition by osteoblasts of primary osteons, may be linked to a slower oscillation producing a higher periodicity. However, if RP and average enamel thickness are associated through a third variable, and that variable is body size, then the direction is again, opposite to that predicted by the intra-specific HHO as currently formulated for adults. Furthermore, if body size is the 'linking' variable, then it is unclear why periodicity and relative enamel thickness are still associated after the effect of body (tooth) size has been removed.

The length of time that secretory ameloblasts remain active in lateral enamel of permanent canines is related to RP (Reid and Ferrell, 2006). Permanent canines with longer lateral enamel formation times had lower periodicities. Further analysis of our sample of dm^2s also reveals a relationship between periodicity and formation time (see Fig 4, and legend for data). Cusp formation times[1] of nine dm^2 paracones were significantly correlated with RP values from the same teeth, and these variables scaled isometrically (RMA regression: r^2= 0.556; slope=1.359; p=0.025; 95% CI=0.668 to 1.781; intercept = -2.699). These correlations in deciduous molars and permanent canines suggest underlying relationships between periodicity and enamel growth processes. However, unlike permanent canine lateral enamel, ameloblasts in enamel layers of deciduous molars with a higher periodicity remain active for a longer period of time than those in molars with a lower periodicity (and see discussion below). When the data for deciduous molars is considered alongside our data for AET and RP, they suggest that a higher periodicity and longer formation time might exist because it leads to thicker enameled deciduous molars in human children. This proposal is consistent with our periodicity values for both deciduous and

permanent teeth (Table 1). Permanent teeth have a higher modal RP, and thicker enamel crowns that take longer to form, compared to their deciduous isomeres (e.g., Mahoney, 2010, 2011).

Five juveniles had an RP of six days but AET varied between them (Fig. 3a). Therefore neither average, nor relative enamel thickness explains all of the RP variation in our sample, and hence there was a residual of 36% in the quadratic function. One explanation for this might be the sex of the juveniles, which we could not estimate from skeletal remains. Modal RP's in permanent teeth can vary between males and females from some but not all adult populations (Schwartz et al., 2001; Smith et al., 2007). If the periodicity of deciduous molars varies between sexes, then this could have contributed to the residual in our sample.

The timing of RP in human deciduous teeth

Our sample of human deciduous molars had a lower range of RP values relative to human permanent teeth. Our data implies that periodicity is not the same for these two tooth types. Indeed, 26% of all values reported for deciduous teeth to date (Table 1) are less than those reported for permanent teeth. Even if we estimated the periodicity of some of these teeth incorrectly, it is unlikely that we miscalculated by more than one day. Thus, it is unlikely that a molar with a periodicity of six days could be mistaken for a periodicity of four days. We did not examine RP in deciduous and permanent teeth from the same jaw (see Mahoney 2012, who reported periodicity did not vary between a deciduous and permanent molar from one individual). Additional work is needed on RP values from deciduous and permanent teeth from the same jaw. Furthermore, even though we have reported RPs for the largest sample of deciduous molars to date, it is still a small sample (n=25). It may be that we missed lower or higher RPs, and that the full range of RP values for deciduous molars has yet to be determined.

[1]Formation time for five dm^2 paracones were taken from Mahoney (2015), as RPs were calculated from the same thin sections. Formation times for four dm^2s were calculated for this study. Data is in Figure 4 legend. See Mahoney (2011) for a methodology.

If RP is a reflection of the HHO rhythm, then lowered RP in deciduous relative to permanent teeth suggests that the rhythm might change with age, between these tooth types. The timing of lateral enamel formation provides support for this proposal. On average, lateral enamel in dm^1 forms between birth and the middle of the eighth post-natal month, while in dm^2 it forms between three and a half months after birth to about 1.2 years of age (Fig. 2). By comparison, the earliest lateral enamel growth in permanent molars from Europeans occurs, on average, between 1.3 to 11.3 years after birth (Reid and Dean, 2006). Therefore, even though permanent first molar *cuspal* enamel growth overlaps with deciduous molar lateral enamel growth during the first year after birth, the region from which Retzius lines are calculated, *lateral* enamel, does not normally form at the same time in these two tooth types. Therefore, usually, periodicity values calculated from lateral enamel in deciduous and permanent molars represent different childhood chronological ages.

Two implications for the HHO hypothesis

Our findings might provide new evidence for the complexity of HHO evolution in modern humans. The intra-specific HHO predicted that lower RP would correlate with increased bone formation, but this study found the opposite in deciduous molars and primary bone. Similarly, there was a prediction of lower RP correlating with thicker enamel, assuming a link with body size, but this study found the opposite. Our data seems to contradict findings reported for adults (Bromage et al., 2009), but the link between the HHO and body size in human juveniles compared to adults might still hold if the oscillation changes during ontogeny. Perhaps humans follow the inter-specific pattern early in development, or an ancestral pattern, while in later childhood, growth patterns follows a derived version of the HHO, a secondary evolution on top of the mammalian pattern. The 'flip' in the relationship between formation time and RP in early forming deciduous molars (Fig. 4) compared to later forming permanent canines (Reid and Ferrell, 2006), is compatible with this idea.

Our data has an additional implication for the HHO, which future studies might explore further. The presence of an association between relative enamel thickness and RP in human children after the effect of tooth size (body size) is removed implies that additional variables might exert an influence on the oscillation, even if the oscillation changes with age. Others have already reported exceptions to the relationship between body size and RP in lemurs, whereby the HHO adjusts to ecological constraints (e.g., Hogg et al., 2015). Furthermore, at least one life history characteristic, oestrous length, links to RP independently of body size (Bromage et al., 2012). Future studies might explore relative enamel thickness and RP across primates.

Methodological implication

Our findings have important implications for the way that periodicity is used when calculating age-at-death and enamel formation times. Retzius periodicity is often incorporated into calculations of crown formation time and estimates of age-at-death (e.g, Mahoney, 2011, 2012). Lowered RP values in deciduous relative to permanent teeth suggest that the periodicity of this growth rhythm should not be transferred these two tooth types in the same individual when calculating age-at-death and formation time.

Conclusion

We calculated Retzius periodicity for the largest sample of human deciduous teeth to date, and sought correlations with enamel thickness and primary bone growth to test predictions of the intra-specific HHO hypothesis. Our data supports the idea that aspects of bone and enamel growth might be co-ordinated. However, we found that the direction of the correlation between RP, primary bone growth, and enamel thickness was opposite to predictions derived from the intra-specific HHO, as currently formulated for adults. Greater primary bone deposition and thicker enamel was linked to a slower oscillation, reflected by a higher Retzius periodicity. We propose that RP, and by implication the HHO, may change with development in modern humans, from deciduous to permanent teeth.

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Author contributions

Mahoney and Guatelli-Steinberg designed the project. Mahoney (dental), Miszkiewicz and Pitfield (bone), Deter (osteological) produced the data. Mahoney, Guatelli-Steinberg, Schlecht, and Deter drafted and revised the content. All approved the final version.

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TABLES

Table 1 Retzius periodicity in human deciduous teeth**.**

	Days							
Tooth	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Ldi2	-	1	-	-	-	-	-	-
Ldc	-	-	2	-	-	-	-	-
Ldm1	-	1	-	-	-	-	-	-
Ldm2	-	-	-	1	-	1	-	-
Udc	-	2	1	-	-	-	-	-
Udm1	1	1	2	3	1	-	-	-
Udm2	1	2	5	3	1	2	2	1
Frequency	2	7	10	7	2	3	2	1

Deciduous tooth types: L-lower jaw, U-upper, di2-lateral incisor, dc-canine, dm1-first molar, dm2-second molar. Values for Udm1 and Updm2 are from this study. Values for Ldi2, Ldc, Ldm1, Ldm2, Udc taken from Mahoney, 2011, 2012. Values for permanent M1 from the same skeletal collection as the children were: RP of 6 (n=2), 7 (n=3), 8 (n=4), 10 (n=3), 11 (n=2), 12 (n=1).

Table 2 Retzius periodicity and primary osteon canal area in μm^2.

Age	**Tooth**	**RP**	**Pr.On.Ca.Ar**
2 years	Udm2	6	1282.71
2 years	Udm2	6	1668.27
2 years	Udm2	6	1418.82
2 years	Udm2	7	981.49
2 years	Udm2	7	1166.23
2 years	Udm2	8	978.10
2 years	Udm1	8	1132.84

Deciduous tooth types: Udm1-upper first molar, Udm2-upper second molar.

718 **Table 3** Enamel thickness measurements and Retzius periodicty

Tooth	AET (in mm^2)	RET (unit-less)	RP
Udm2	0.4611	7.749	4
Udm2	0.4231	7.943	5
Udm2	0.690	11.626	5
Udm2	0.779	14.137	6
Udm2	0.786	12.368	6
Udm2	0.651	11.199	6
Udm2	0.631	11.088	6
Udm2	0.560	9.624	6
Udm2	0.621	11.199	7
Udm2	0.582	10.154	7
Udm2	0.657	11.692	9
Udm2	0.845	13.030	10
Udm2	1.049	15.470	10
Udm2	1.032	15.170	11

719 AET=Average enamel thickness. RET=Relative enamel thickness.
720 Deciduous tooth type: Udm2-upper second molar.
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Figure legends

Fig. 1 Deciduous enamel Retzius lines and primary osteons in a human humerus.
(A) Thin section through a human dm^2 at a magnification of 4x revealing Retzius lines indicated by black arrows (scale bar: 60µm). Cross striations revealed at a magnification of 20x, indicated by white arrows (scale bar: 20µm). White dashed line indicates direction of enamel rods. (**B**) Regions of interest (ROI) on the anterior mid-shaft sub-periosteal region of a humerus revealing a primary osteon (white arrow) at a magnification of 20x, with vascular canal (scale bar: 40µm).

Fig. 2 The timing of lateral enamel growth after birth.
Lateral enamel in dm^1 and dm^2 is highlighted in grey. On average, dm^1 cuspal enamel is formed through 121 days of prenatal growth, and is complete at birth. Following this, lateral enamel grows for about another 210 post-natal days. Enamel growth continues in the most cervical enamel (not used for calculating RP), for an additional 70 days. On average, dm^2, cuspal enamel formation commences 71 days before birth and is complete about 94 days after birth. Following this, lateral enamel continues to grow for another 290 days, finishing around 384 days after birth. Enamel growth continues into the most cervical enamel after that, for about another 73 days. Raw data taken from Mahoney (2015) and re-calculated. Inset show the location of lateral enamel in a thin section, taken through the dm^1 protocone. The lateral enamel region from which Retzius periodicity was calculated in thin sections is also highlighted in grey.

Fig. 3 Plot of log-transformed Retzius periodicity against log-transformed average (**A**) and relative **(B**) enamel thickness for deciduous second maxillary molars. A reduced major axis regression line is fitted to the data. Periodicity and enamel thickness are significantly (p<0.001) and positively correlated, and scale isometrically. See Results section for regression statistics. All data taken from Table 3.

Fig. 4 Plot of log-transformed Retzius periodicity against log-transformed dm^2 paracone formation times. A reduced major axis regression line is fitted to the data. Periodicity and cusp formation time are significantly (p<0.025) and positively correlated. See Discussion section for regression statistics. Dm^2 paracone formation times (RP value): 304days (5), 344days (6), 347days (7), 406days (9), 435days (6), 459 days (6), 464 days (8), 526days (11), 539 days (10).

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