An Investigation of Enamel Hypoplasia and Weaning through Histomorphological Analysis and Bayesian Isotope Mixing Models

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ABSTRACT Enamel hypoplasia (EH) is a developmental defect, frequently used in bioarchaeological research to assess the nutrition and health in infants and children. Anthropological studies suggest that EH relates to disease and malnutrition especially during weaning, a hypothesis that up to now has not been examined empirically in ancient populations.

In the present study, we reconstructed the weaning process of 66 individuals from ancient Thessaloniki (4th c. BC-16th c. AD), a metropole in southeastern Europe, to explore the effect of breast milk consumption and infant diet on the development of EH. For this, we estimated the duration of weaning using stable isotope analysis on dentinal collagen of permanent first molars and breast milk proportions using Bayesian modeling. In parallel, we determined the exact formation age and duration of EH defects on the canines or the incisors of the same individuals using histomorphological analysis.

The combined results of our analyses show that individuals consuming less than 50% of breast milk during weaning, developed multiple EH defects (between 2.0-5.0 years), mostly formed close to the age of weaning or later. Our results are consistent with similar studies and provide new insights into the living conditions of children in pre-industrial and pre-vaccination contexts.

Enamel hypoplasia (EH) is a developmental defect caused by metabolic and physiological stress that affects the formation of tooth enamel (Goodman and Rose, 1990; Lewis, 2018) and is the most frequently observed pathological lesion in the dentition of ancient populations (Caufield, Li and Bromage, 2012; Hillson and Bond, 1997; Krenz-Niedbała and Kozłowski, 2013). It has been used as a standard index of health and nutritional status of infants and children in ancient societies for more than 80 years (Dąbrowski et al., 2020; Goodman, Armelagos, and Rose, 1984; Katzenberg and Herring, 1996; Sarnat & Schour, 1941).

EH is caused by the insufficient secretion of mineral and organic substances carried by ameloblasts that create the enamel layers. During tooth development, enamel is deposited daily, forming cross-striations. After 6-12 days of constant secretion, cross striations form distinct bands called Retzius lines (or perikymata) (Antoine and Hillson, 2015; Smith, 2020). Any homeostatic disruption that occurs in parallel to this process can result in insufficient secretion of enamel layers and low mineral intensity, affecting the morphology of Retzius lines that appear accentuated and malformed (known as Wilson bands) (FitzGerald, Saunders, Bondioli, and Macchiarelli, 2006; Smith, 2020).

EH is classified in four types: a) furrow, b) linear, c) pits and d) plane (Goodman and Rose, 1990;

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Internationale, Fédération Dentaire, 1982). The age-at-formation of enamel defects can be estimated macroscopically based on dental growth-rate charts (Massler, Schour, and Poncher, 1941; Reid and Dean, 2000). However, the macroscopical examination of EH may lead to discrepancies in determining precisely the age-at-formation, since dental growth is affected by environmental and dietary factors (Goodman and Rose, 1990; Seow, 2017) as well as intra- and inter-population variability (Hillson and Bond, 1997; Krenz-Niedbała and Kozłowski, 2013). A far more age-specific approach that considers individual and population specific standards, is the histological examination of enamel defects (Reid and Dean, 2000). This approach enables the calculation of the enamel appositional rate and thus the precise determination of the age-at-formation and duration of each defect (Antoine, FitzGerald, and Rose, 2018; Dąbrowski et al., 2021; Martin, Guatelli-Steinberg, Sciulli, and Walker, 2008; Reid, Beynon, and Ramirez Rozzi, 1998; Reid and Dean, 2000; Risnes, 1986; Skinner and Anderson, 1991; Tagiguchi, 1966).

Despite the wide use of EH by biological anthropologists for the investigation of growth disruptions in the past, its etiology remains elusive. It has been associated with infectious diseases (e.g., yellow fever), vitamin deficiencies (A, C, D) and malnutrition (Aldred, Talacko, and Steyn, 2016; Blakey, Leslie, and Reidy, 1994; Caufield et al., 2012; Dąbrowski et al., 2020; Goodman and Armelagos, 1988; Larsen, 1987; Miszkiewicz, 2015; Roberts and Manchester, 2010; Smith, 2020). Considering that enamel hypoplasia reflects physiological and metabolic stress during early life, many researchers have linked EH to breastfeeding and weaning practices (Corruccini, Handler, and Jacobi, 1985; Dittmann and Grupe, 2000; Garland, Reitsema, Larsen, and Thomas, 2018; Goodman et al., 1984; Moggi-Cecchi, Pacciani, and Pinto-Cisternas, 1994; Sandberg, Sponheimer, Lee-Thorp, and Van Ger-

Table 1. Published studies that examine the association between enamel hypoplasia and weaning. EH: Enamel Hypoplasia, EH formation ages and weaning ages are in years.

Site	Time (AD)	N	Examination of EH	EH formation ages	Reconstruction of weaning	Weaning ages	Reference
Newton Plantation, Barbados	1660-1820	100	macroscopic examination	3.0-4.0	historical sources	2.0-3.0	(Corruccini et al., 1985)
Florence	1800-1900	83	macroscopic examination	2.0-3.0	historical sources	1.0-1.5	(Moggi-Cecchi et al., 1994)
Wenigumstadt (Aschaffenburg, southern Germany)	500-700	44	macroscopic examination	3.0	stable nitrogen isotope analysis of bone collagen	1.0-3.0	(Dittmann & Grupe, 2000)
Kulubnarti, Sudan	550-800	5	macroscopic examination	4.0-7.0	incremental dentine analysis (δ13C, δ15N)	3.0-4.0	(Sandberg et al., 2014)
Mission Santa Catalina de Guale, St. Catherine's Island, Georgia USA	1605-1680	14	histological examination	2.5-4.5	incremental dentine analysis (δ13C, δ15N)	2.5-4.5	(Garland et al., 2018)

ven, 2014) (Table 1). Indeed, weaning entails many nutritional and infectious risks for infants and is considered as an intrinsic source of physiological stress in ancient populations (Grueger and Canadian Paediatric Society, Community Paediatrics Committee, 2013; Halcrow, Miller, Pechenkina, Dong, and Fan, 2021; Katzenberg and Herring, 1996; Kendall, Millard, and Beaumont, 2021).

The study of Goodman et al. (1984), was the first to suggest the relationship between EH and weaning. However, due to methodological limitations of their time, it was not possible to acquire empirical data on weaning. Recent studies that empirically reconstructed the weaning process corroborate that poor nutrition during and after weaning causes the formation of EH (Corruccini et al., 1985; Dittmann and Grupe, 2000; Garland et al., 2018; Moggi-Cecchi et al., 1994; Sandberg et al., 2014) (Table 1). Furthermore, it has been suggested that hypoplastic defects develop some time after the completion of weaning as the immunological and nutritional benefits of breast milk are no longer provided (Cucina, 2002; Fernández-Crespo et al., 2022; Tomczyk, Tomczyk-Gruca, and Zalewska, 2012). However, it remains unclear whether the depletion of breast milk or the quality of supplementary foods instigate the formation of EH.

The precise reconstruction of the weaning process in ancient individuals has become feasible with the analysis of stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in dentinal collagen (Beaumont and Montgomery, 2015; Eerkens, Berget, and Bartelink, 2011). This analysis permits the accurate investigation of growth and development in ancient populations as dentinal collagen encloses dietary information during initial apposition and is hardly influenced by environmental and dietary changes thereafter (Beaumont, 2020). Experimental studies from mother-infant pairs indicate that infants during exclusive breastfeeding have elevated $\delta^{15}N$ and $\delta^{13}C$ values compared to their mothers. This difference is more evident in nitrogen (between 2-3‰) than in carbon (approximately 1‰) (Fogel, Tuross, and Owsley, 1989; Herrscher, Goude, & Metz, 2017). At the onset of weaning isotopic ratios decrease and after the complete cessation of breast milk, they stabilize and are similar to those of the adult population (Halcrow et al., 2021). These measurable shifts in isotopic ratios can be detected with the segmentation of the tooth, from crown to root into small sections (Beaumont and Montgomery, 2015; Eerkens et al., 2011). Since dentine apposition can be estimated (Dean, 2017), this analysis provides the opportunity to outline dietary changes in time-specific periods of infancy and childhood. Over the years, this temporal resolution has been increased through the optimization of the dentine sampling protocols (Beaumont nd Montgomery, 2015; Curtis, Beaumont, Elamin, Wilson, and Koon, 2022; Czermak, Fernández-Crespo, Ditchfield, and Lee-Thorp, 2020; Eerkens et al., 2011). Furthermore, the development of Bayesian isotope mixing models (Fernandes, Grootes, Nadeau, and Nehlich, 2015; Stock et al., 2018) has enabled the estimation of the relative proportions of food sources in individual diets, including the amount of breast milk consumed by infants (Chinique de Armas et al., 2022, 2017).

Leveraging Bayesian modeling, histology, and stable isotope analysis we aim to estimate the exact chronological time of appearance and duration of the EH defects, the weaning process and the infant diet, and to determine whether the duration of weaning and the amount of breast milk consumption *has an impact* on the development of enamel hypoplastic defects. The encompassing hypothesis of our study is to examine whether breast milk mitigates the risk of developing severe or recurrent forms of physiological stress during weaning that result in EH formation.

Our sample population comprises individuals from the ancient city of Thessaloniki, the capital of the Provincia Macedonia and one of the largest metropoles of the Roman Empire (Adam-Veleni, 2003). Previous stable isotope studies in the site (Ganiatsou et al., 2023; Ganiatsou, Vika, Georgiadou, Protopsalti, & Papageorgopoulou, 2022) have revealed that almost 10% of the examined individuals show evidence of physiological stress. We aim to delve deeper into this observation by examining the dentition of these individuals for hypoplastic defects. In parallel, we aim to use the Bayesian model MixSIAR on the stable nitrogen and carbon ratios (Stock et al., 2018) to estimate the relative proportion of breast milk during weaning. To the best of our knowledge, no other study has documented the breast milk proportions in relation to the formation of hypoplastic defects in archaeological populations. The present study provides novel insights into this hypothesis and utilizes advanced statistical methods to answer complex research questions about the living conditions of infants and children in ancient societies.

Materials and Methods

The archaeological site

Thessaloniki was one of the first ancient urban centers in South-eastern Europe (Figure 1) (Adam-Veleni, 2003, 2012; Karamberi, 2000, 2003; Nigdelis, 1997a; Nikakis, 2019; Vakalopoulos, 1983). Historical and archaeological evidence suggest that the foundation of Thessaloniki took place in 315/16 BC by King Cassander of Macedon who named the city after his wife, who was the sister of Alexander the Great (Adam-Veleni, 2003; Allamani-Souri, 2003; Nigdelis, 1997b). During its historical transitions from Hellenistic to Roman and then to the Byzantine period, the city grew into a multicultural, economic and political hub of the ancient world (Adam-Veleni, 2012). In 2011 during the construction of the city's Metropolitan subway, the two ancient cemeteries of the city with numerous burials were unearthed (Acheilara, 2007, 2008, 2009, 2010, 2011; Bakirtzis and Pazaras, 2006; Bakirtztis and Pazaras, 2006; Kanonidis, Lamprothanasi, and Protopsalti, 2016; Makri and Vasileiadou, 2011; Misailidou-Despotidou, 2012; Misailidou-Despotidou, Lamprothanasi, and Protopsalti, 2014; Paisidou, Vasileiadou, and Konstantinidou, 2009; Vasileiadou and Pazaras, 2010).

The sample population

The sample population dates mostly to the Roman period (1st c. BC - 4th c. AD) (Table 2). Sex and age estimations are reported in Table S1 and were performed using standard anthropological methods (Acsádi, Nemeskéri, and Balás, 1970; Brooks and Suchey, 1990; Brothwell, 1981; Buikstra and Ubelaker, 1994; Ferembach, Schwindezky, and Stoukal, 1980; Işcan, Loth, and Wright, 1984, 1985; Lovejoy, Meindl, Pryzbeck, and Mensforth, 1985; Miles, 1962; Phenice, 1969).

For the weaning reconstruction we selected 66 individuals (*n*=34 males, *n*=26 females, *n*=6 indeterminate), who had intact permanent molars without pathological conditions (attrition, carries). First permanent molars (M1) were selected as they develop between birth and ten years of age (AlQahtani, Hector, and Liversidge, 2010), framing a suitable time period for weaning reconstructions. Fifteen (15) molars were newly processed (see *Reconstruction of the weaning process with the Bayesian model MixSIAR*) whereas the remaining 51 have

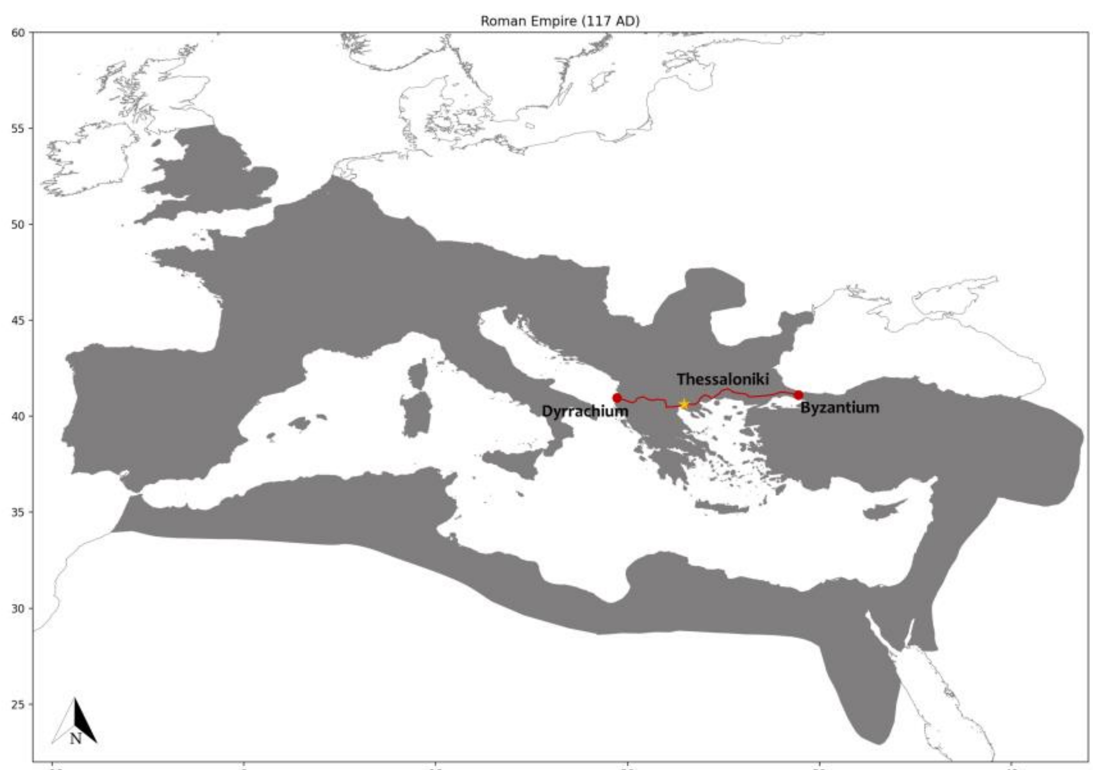

Figure 1. Extended map of the Roman Empire at 117 AD (colored in grey) showing Thessaloniki (4th c.BC- 16th c.AD). Via Egnatia (red line) connected the Adriatic (Dyrrachium) to the Black Sea (Byzantium). The map was generated in Python using the geopandas package and maps from http://awmc.unc.edu/awmc/map_data/shapefiles/political_shading/

been previously published (Ganiatsou et al., 2022; Ganiatsou et al., 2023).

Permanent canines (C) and incisors (I) from the 66 individuals were examined macroscopically for hypoplastic defects. Canines and incisors were selected as they are more susceptible to stress than premolars and molars due to their genetic canalization (Goodman and Rose, 1990; Krenz-Niedbała and Kozłowski, 2013). Furthermore, their crown forms between the first and the sixth year of life (Hillson 2014), which coincides with the period we reconstructed isotopically utilizing the molars. Twenty-seven (27) individuals with enamel hypoplasia (*n*=17 males, *n*=9 females, *n*=1 indeterminate) were identified but 26 were sampled and processed histologically, as one canine was not suitable for analysis (see *Histological examination of hypoplasia*).

Reconstruction of the weaning process with the Bayesian model MixSIAR

The weaning process was reconstructed using $\delta^{15}N$ and $\delta^{13}C$ measurements from incremental dentine collagen of first permanent molars corresponding to the life period from birth to the first six years of age. Sample preparation and collagen extraction was carried out following the Method 2 of Beaumont et al. (2015), and Brown et al. (1988), respectively and age-at-increment assignment is described in Ganiatsou et al., (2022). Weaning ages were estimated with the application WEAN, an automated tool that utilizes a computational approach to estimate the weaning duration (Ganiatsou, Souleles, and Papageorgopoulou, 2023).

The Bayesian model MixSIAR (Stock et al., 2018) was used to estimate the proportion of breast milk during weaning. This model has the advantage of allowing the incorporation of priors (e.g. fractionation factor) to account for uncertainty associated with any empirical or calculation errors (Galván, Sweeting, and Polunin, 2012; Moore & Semmens, 2008). MixSIAR necessitates three data files to provide estimates: 1) the isotopic values of consumers 2) the isotopic values of potential dietary sources and 3) the trophic discrimination factors (TDF) for each dietary source. As we are interested in the reconstruction of breastfeeding and weaning, we used the $\delta^{15}N$ and $\delta^{13}C$ values from birth until the completion of weaning. To discriminate between the weaning and the post-weaning period, we used the weaning age estimate from WEAN (Ganiatsou, Souleles, and Papageorgopoulou, 2023). In cases with no available weaning age estimate, we used the values corresponding to the first two years of life (mean weaning age estimate) (Ganiatsou, Souleles, and Papageorgopoulou, 2023). For the dietary sources' values, we used published data (breastmilk: Chinique de Armas et al., 2022, 2017, C3 and C4 plants, animal protein, freshwater fish: Dotsika et al., 2019). The trophic discrimination factors (TDFs) were taken from Ambrose (2002) and Chinique de Armas et al. (2022).

The MixSIAR model recommends that the number of dietary sources should be less or equal to the number of isotopic tracers +1 (Phillips, 2001; Schwarcz, 1991; Stock et al., 2018), i.e., in the present study $\delta^{13}C$ and $\delta^{15}N$. For this we had to reduce the number of sources to ensure the accuracy of the computational approach. To do this, we used the "isospace" function of MixSIAR to identify which dietary sources contribute to our distribution. This function plots the individual and dietary sources' isotopic values with the corrections based on the

Table 2. The number of male, female and indeterminate individuals analyzed in this study per chronological period (Hellenistic: 4th c. BC - 1st c. BC, Roman:1st c. BC - 4th c. AD, Byzantine and Post-Byzantine: 4th c. AD – 16th c. AD).

Chronological period	**Males**	**Females**	**Indeterminate**	**Total**
Hellenistic	1	4	-	5
Roman	14	15	2	31
Roman-Early Byzantine free burials*	6	1	3	10
Byzantine and post-Byzantine	13	6	1	20
Total	34	26	6	**66**

*free burials did not contain grave goods. They are dated to the Roman/ Early Byzantine period based on stratigraphy and archaeological documentation.

TDFs (Figure 2). In principle, the dietary sources should form a triangle, known as the mixing triangle (or polygon if more than three sources are included) and the consumer values should form a cluster within the mixing triangle. In both models, individual values cluster within breast milk, C3 plants and animal protein, whereas fish and C4 plants are far from the consumer values (see Figure 2). Therefore, in our analysis we selected to use as potential weaning food sources breast milk, C3 plants and animal protein and exclude the fish and C4 plants. It is important to highlight that the scope of this analysis is to examine the depletion of breast milk over time, and not to characterize diet per se. The reduction of dietary sources in our model does not exclude the possibility that some individuals had more diverse weaning diets (Ganiatsou et al., 2023; Ganiatsou et al., 2022).

After determining the number of dietary sources, two models with different breast milk values were compared to assess their validity (Chinique de Armas et al., 2022, 2017). Source data and TDFs were input as means and SDs (Table 3). Both models were run in the "short" version (chain Length=50,000, burn=25,000, thin=50, chains=3) with "Individual ID" and "age" as fixed and continuous factors respectively.

We assessed the predicted accuracy of the two models by computing the LOO (leave-one-out cross-validation) and WAIC (widely applicable information criterion), which are methods used in model selection and comparison in the field of statistics and machine learning (Risnes, 1986). LOO provides an estimate of how well the model is likely to perform on new, unseen data and WAIC quantifies the goodness of fit of a statistical model to the data, while taking into account the complexity of the model. Lower values in LOO and WAIC indicate which model has better performance (McElreath, 2020; Vehtari et al., 2017). Based on the results shown in Table 4, we selected the estimates of Model A since it has lower values than Model B, although the difference is not significant.

Histological preparation for EH age-at-formation estimation

Canines or incisors were examined histologically in order to estimate the precise age and duration of the EH defect. To achieve this, we determined the dental formation rate of appositional (cuspal) and imbricational enamel for each dental sample. This was a necessary step as there is no previous re-

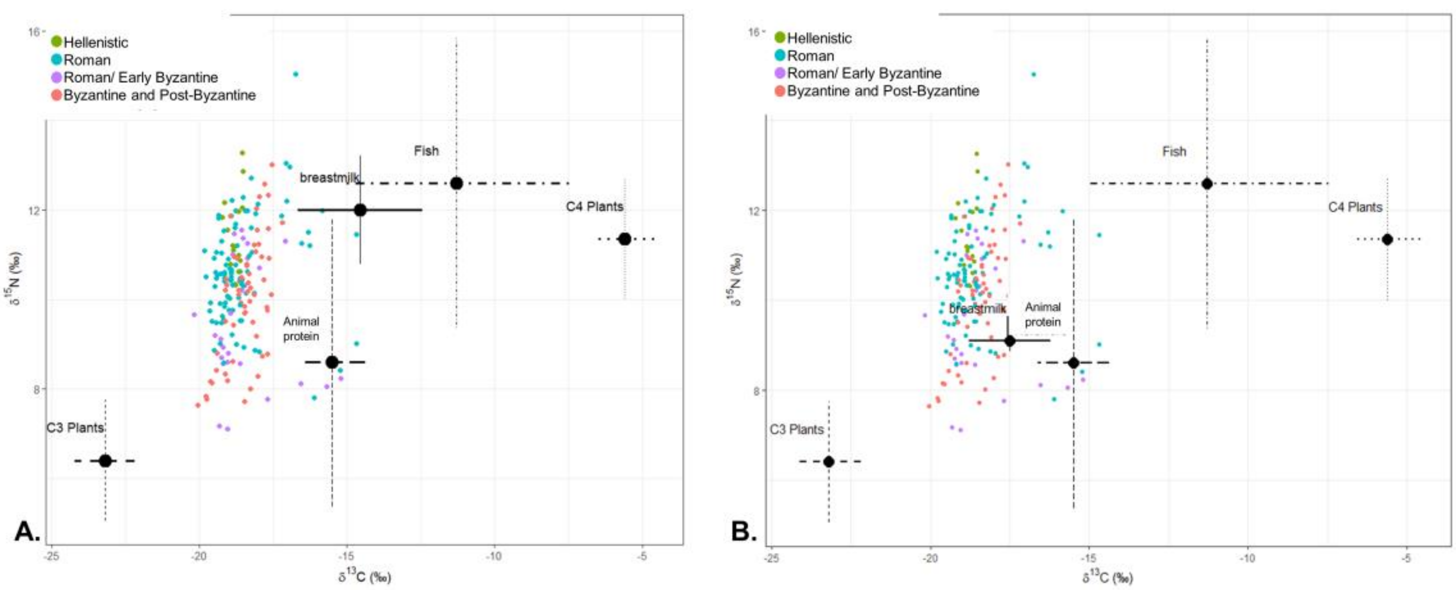

Figure 2. Isospace plots of Model A (A) and Model B (B) showing δ13C and δ15N values of individuals from ancient Thessaloniki (4th c. BC-16th c. AD) and potential dietary sources. Model A: breast milk: -19.55, 7.39, Chinique de Armas et al. 2017; C3 plants: -26.5,2.8, Dotsika et al., 2019; Animal protein: -20, 5.0, Dotsika et al., 2019). Model B: breast milk: -22.5, 5.1, Chinique de Armas et al. 2022; C3 plants: -26.5,2.8, Dotsika et al., 2019; Animal protein: -20, 5.0, Dotsika et al., 2019). Trophic discrimination factors (TDF) were taken from Chinique de Armas et al. (2022) and Ambrose (2002).

Table 3. Isotopic values and standard deviations (‰ AIR for nitrogen; VPDB for carbon) for probable dietary source components used in the MixSIAR computations for individuals from Thessaloniki (4th c. BC -16th c. AD). Source isotopic compositions and trophic discrimination factors (TDF) were taken from published literature (Ambrose, 2002; Chinique de Armas et al., 2022, 2017; Dotsika et al., 2019).

Model	Sources	Source isotopic compositions (‰) and SDs				TDF (‰) and SDs			
		$\delta^{13}C$	SD	$\delta^{15}N$	SD	$\delta^{13}C$	SD	$\delta^{15}N$	SD
A	Breastmilk	-19.55	1.1	7.39	1.19	5.5	0.7	4.4	0.1
	C3 Plants	-26.5	1.0	2.8	1.0	3.3	0.9	3.6	1.2
	Animal protein	-20	1.0	5.0	3.0	4.5	0.5	3.6	1.2
B	Breastmilk	-22.5	1.1	5.1	0.2	4.4	0.1	5.5	0.7
	C3 Plants	-26.5	1.0	2.8	1.0	3.3	0.9	3.6	1.2
	Animal protein	-20	1.0	5.0	3.0	4.5	0.5	3.6	1.2

Table 4. LOO and WAIC computations from the MixSIAR to assess the predicted accuracy of Model A and B. se_LOOic/ se_WAIC is the standard error of the two models, dLOOic/ dWAICic is the difference between each model. se_dLOOic/ se_dWAIC is the standard error of the difference between each model, weight, is an estimate of the model's probability to make the best predictions on new data, conditional on the set of models considered (McElreath, 2020; Stock et al., 2018).

Model	LOOic	se_LOOic	dLOOic	se_dLOOic	WAIC	se_WAIC	dWAIC	se_dWAIC	weight
A	168.7	32	0.0	NA	167.3	31.9	0.0	NA	0.512
B	168.8	32	0.1	0.4	167.4	31.9	0.1	0.4	0.488

search for dental formation rates in the Mediterranean populations and the acquisition of population specific data has been emphasized (Hillson and Bond, 1997; Krenz-Niedbała and Kozłowski, 2013; Ritzman, Baker, and Schwartz, 2008). Furthermore, histomorphological examination of enamel alterations caused by EH, specifically the morphological intensity of Wilson bands and the severity of enamel thinning, was conducted in order to investigate possible correlations of these micro-characteristics with the timing of the stress or sex. Prior to histological analysis, each tooth was scanned using micro-CT (SKYSCAN 1276 CMOS), to preserve the morphological characteristics for future anthropological studies.

Histological analysis of the teeth was carried out following the protocol of Hurnanen et al (2017) with minor modification. This includes: 1) rapid cleansing in accenting concentrations of ethanol (2 mins at 70% and 80% and 30 sec at 95% and 100%), 2) infiltration in xylene for 1h, 3) embedding in a two-parts epoxy resin (EpoFix, Struers). After the stabilization of the resin, the crown was separated from the root transversely, securing the root for future microscopical studies. Axial-buccolingual cross-sections of 200μm thickness were cut from the apex of the cusp to the cervix of the enamel, using a semi-automated Isomet Low Speed Shaw (Buehler) with diamond surface cutting disk (1.5mm thickness). The cross-sections were grinded, with the semi-automated grinder and polisher Labopol-20 (Struers), to 100μm, mounted on microscopic slides with mounting medium (BioMount DPX, Biognost CR) and covered with conventional coverslips. The histological analysis and observation of the enamel prisms were performed with an Axioscope A.1 (Zeiss) microscope with optical, transmitted and polarized light. The measurements of the cusp and counting of the prisms were regulated through microimages imported to Fiji software (Java 1.8.0) (Schindelin et al., 2012), captured with a digital microscope camera (AxioCam Icc3, Zeiss). In cases with minor attrition of the crown (*n*=6), the enamel was reconstructed in Adobe Illustrator software (CC 2015.3.1 (20.1)) and was then imported in Fiji software (Java 1.8.0_322).

To determine the formation time of cuspal enamel, we measured the enamel thickness from the dentine-enamel junction of the apex to the top point of the cusp (magnification X100) using the "Straight line" tool of Fiji software (Figure 3). The average cross-striation periodicity of enamel deposition was calculated by counting the cross-striations at 10 random locations of the cusp that were observable and countable (magnification X400). The measurement of the enamel thickness was then multiplied with the average cross-striation periodicity (Risnes, 1986) and divided by the total number of days in a year (365 days) as in formula (1).

$$Y = (X*a)/365 \quad (1)$$

Y=formation time of cuspal enamel, X=enamel thickness, a=average cross-striation periodicity

The duration of hypoplasia was determined by multiplying the sum of Retzius lines that were confined between the first and the last Wilson band, with the average cross-striation periodicity and divided by the average number of days in a month (30 days) (Figure 4). The chronological time when each hypoplastic defect began was determined by the formation time of the enamel from the cusp

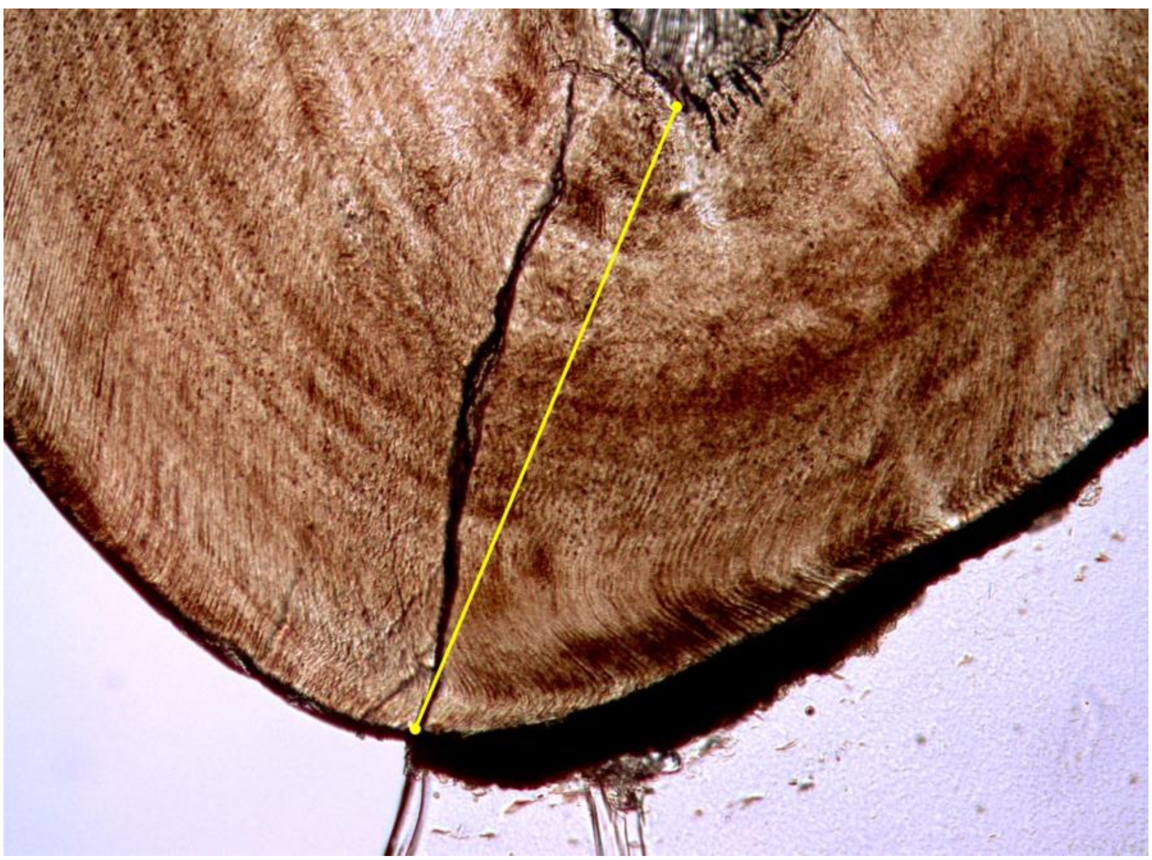

Figure 3. Microphotograph of lower canine (METi_466) of a subadult individual (4 years old) from Thessaloniki (2nd c. AD). Micro-image captured by Axiocam ICC3 (Zeiss) with magnification X100, under Axioscope A.1 (Zeiss), imported to Fiji software with the measurement of the cuspal thickness with the "Straight line" tool (yellow line).

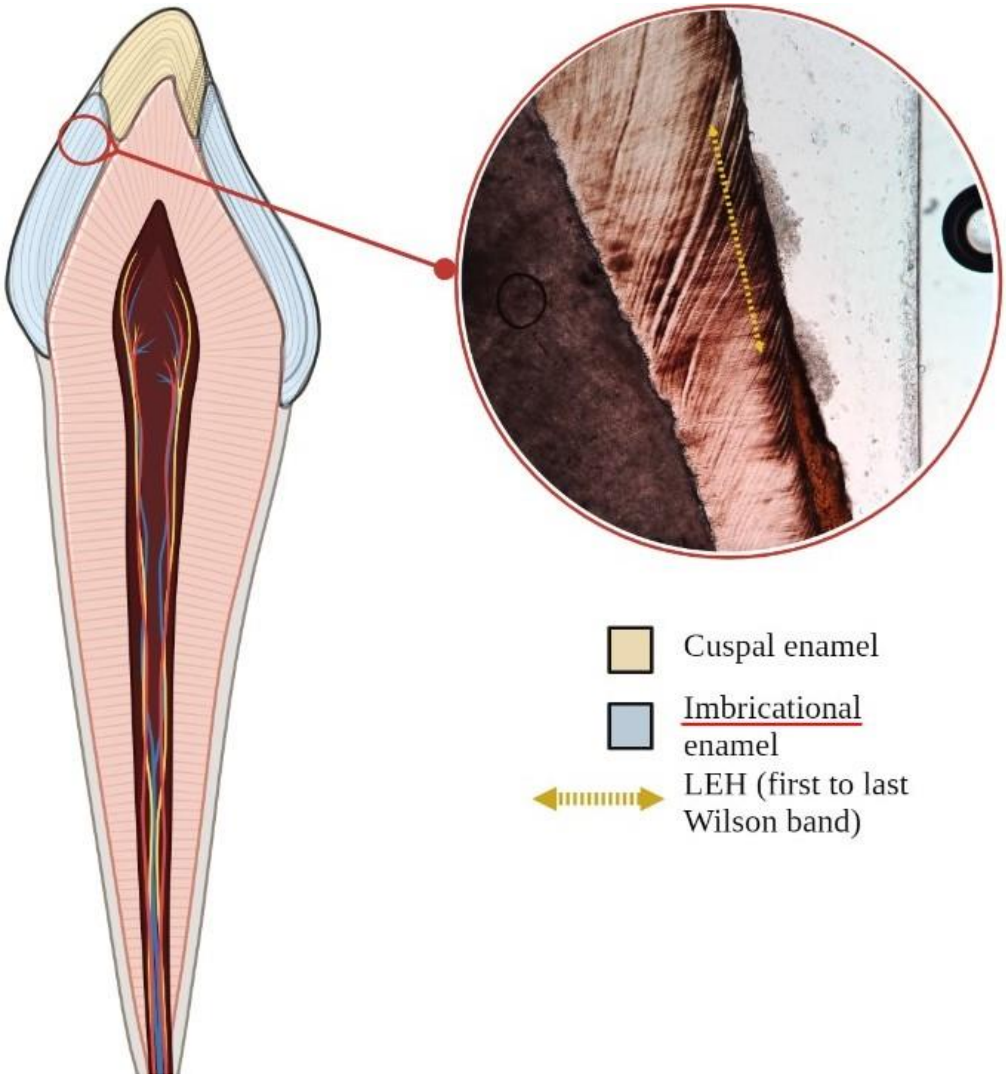

Figure 4. Canine cross-section and microimage of a LEH defect. Wilson bands are developed and demarcate the beginning and end of the LEH defect (yellow dashed arrow marking all the Wilson bands of a microphotograph of lower canine (METi_195) of a male individual (40-55 years old) from Thessaloniki (2nd c. AD Roman period)).

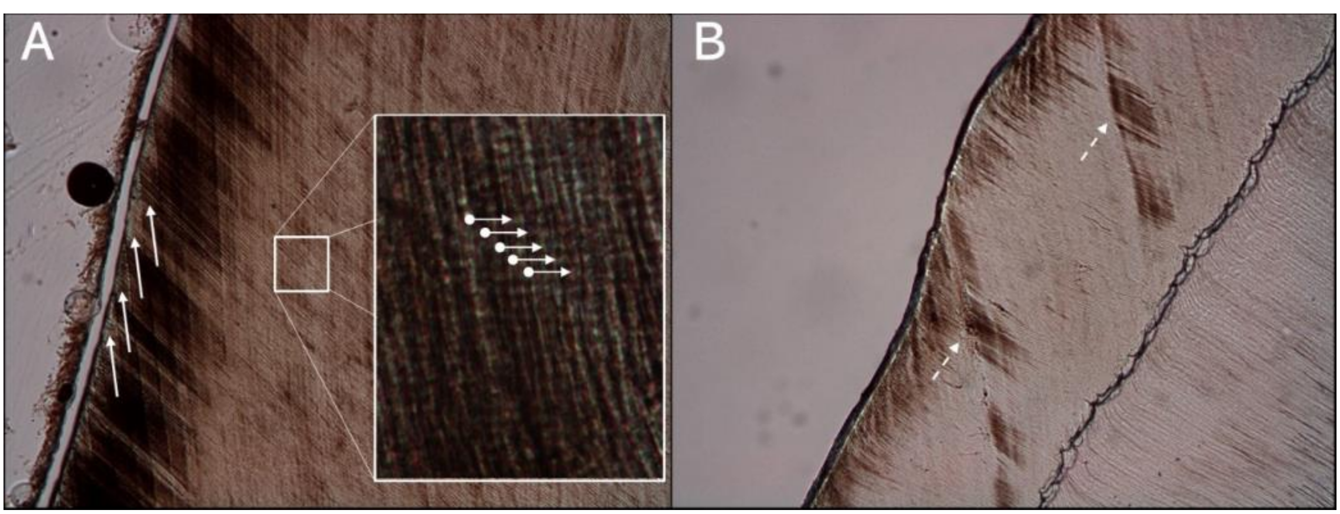

Figure 5. A. Microphotograph of lower canine (METi_199) of a young male (16-18 years) from Thessaloniki (16th c. AD). Retzius lines pointed by white arrows and cross-striations with bullet arrows on the pop-up window. B. Microphotograph of upper canine (METi_185) of a young adult male (25-30 years) from Thessaloniki (Post-Byzantine, 15-16th c AD). Wilson bands indicated with white dashed arrows. All microphotographs were captured by Axiocam ICC3 (Zeiss) with magnification X100 and X200, under Axioscope A.1 (Zeiss) optical microscope.

until the appearance of the first Wilson band (Figure 5).

In order to examine the EH episodes according to the timing of appearance we conducted descriptive analysis of the EH episodes and the chronological age of appearance. Kernel density plots were used for visualizing the concentrations and distribution of chronological ages of EH within the population and each sex group. Two-way ANOVA was performed to examine the correlation of EH defects timing between sexes after assessing the normality of the distribution with Shapiro-Wilk test.

The depth of the hypoplastic defects were examined by measuring the curvature (κ) of enamel. To calculate the curve of the EH, micro-images (magnification X100) were imported in Fiji software and run the plugin, Kappa. The plugin uses cubic B-spline curves formed by multiple 3rd degree Bézier curves. Thus, complex curvatures can be shaped with the application of a few points. Kappa plugin uses a minimization algorithm that fits the B-spline curve to the underlying data and scales them (Mary and Brouhard, 2019). Running the plugin we created open B-spline curves through point-clicking on the borders of the enamel before and through the hypoplastic defect. Calculus remnants were avoided as they do not follow the enamel shape (Figure 6). The retrieved data included the x and y coordinates of the curve and the control points (point curvature) that indicate the location and shape of the curve.

To statistically examine the curves according to the EH episodes and sex group we used area charts for visualization of the data. Considering the large size of the curve data, Kolmogorov-Smirnov test was used for normality testing instead of Shapiro-Wilk, as it is less affected by the sample size. ANOVA has three different types (Type I, Type II and Type III) to test split variations. Type III is used for data *that are unbalanced and not sequential*. Two-way ANOVA (Type III) performed for the correlation of the depth of hypoplasia through the curve data with the timing of EH episodes and sex.

The severity of the EH was examined through the color intensity of Wilson bands. "Segment line" tool of Fiji software creates a Region of Interest (ROI) of the line area that includes all the underlying data. In every EH of all teeth we *drew* segment lines on Wilson bands and retrieved data of the gray-scale color intensity and length (μm) through profile histograms of the software (Figure 7). We

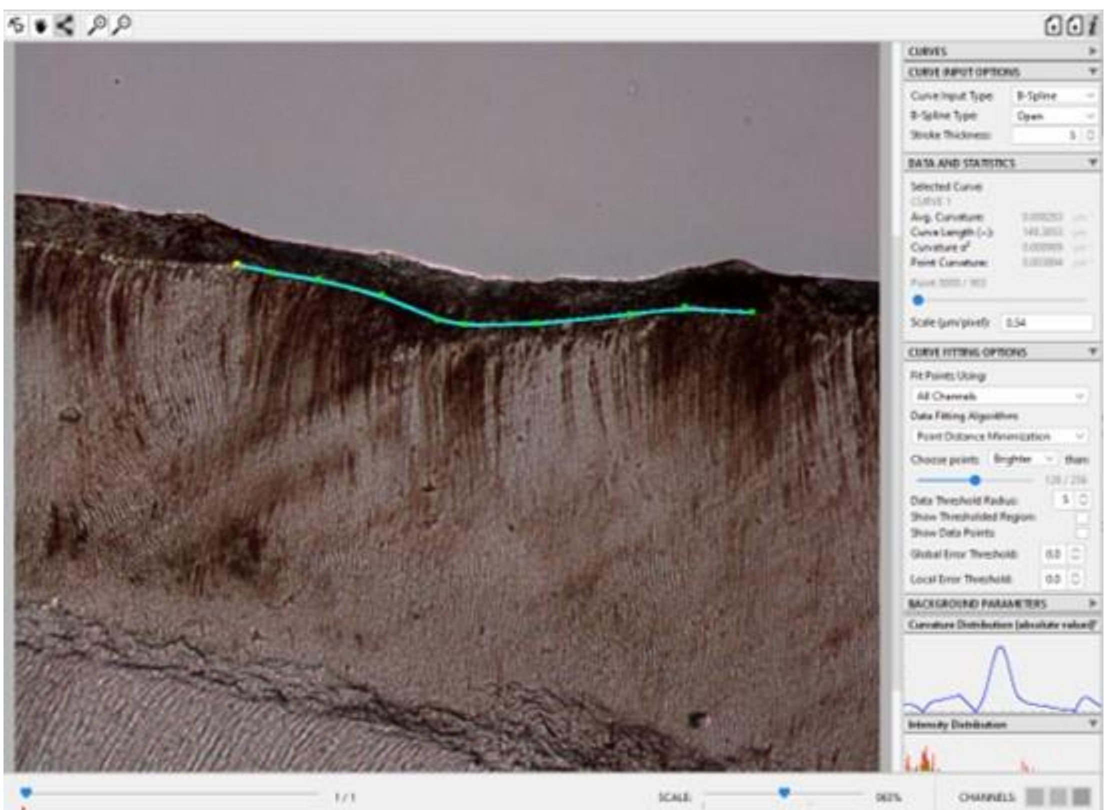

Figure 6. Micro-image of individual (METi_197) (magnification X100) of B-spline created in Fiji software using Kappa plug in.

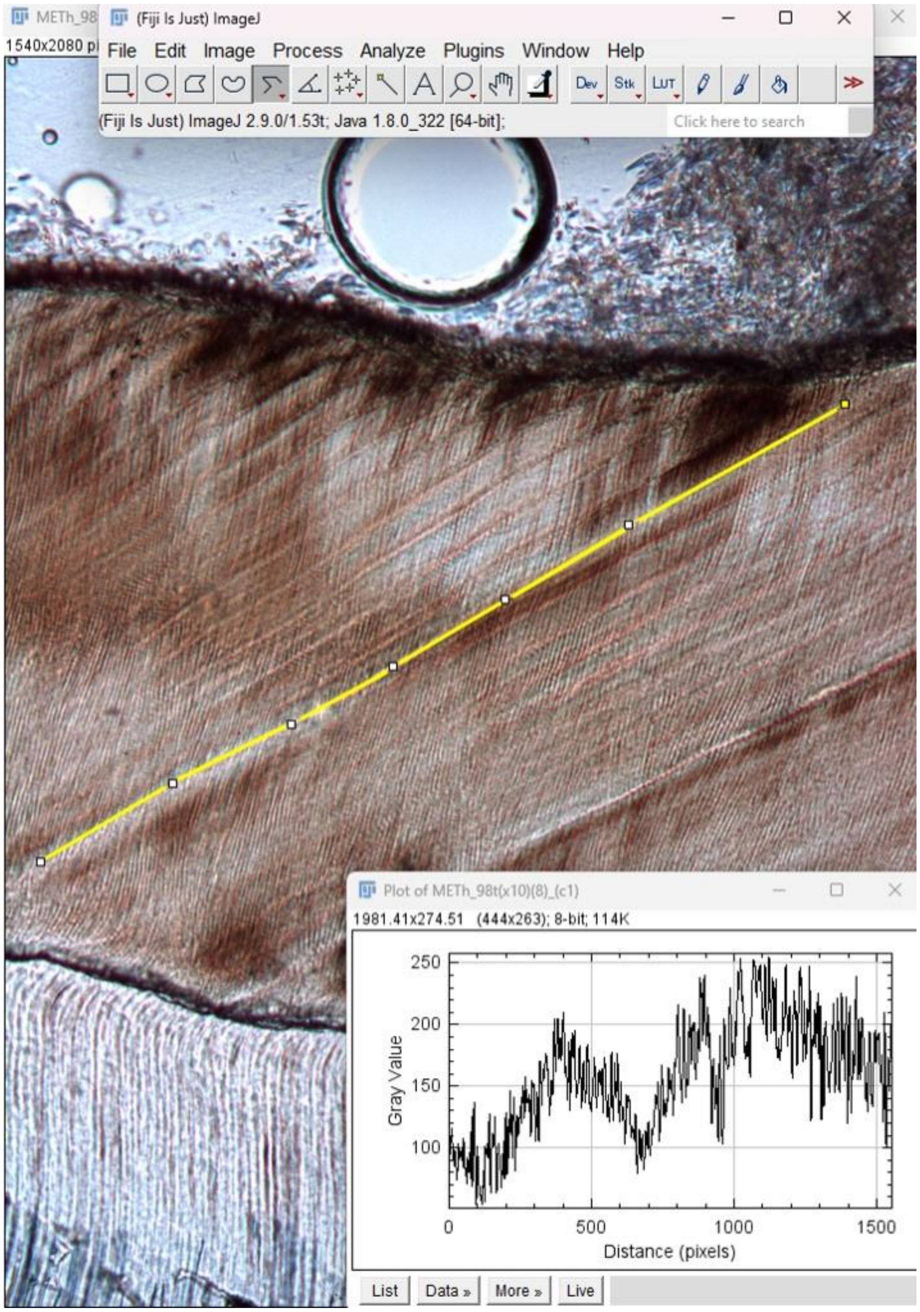

Figure 7. Micro-image of individual (METi_228) in Fiji software. Segment line tool (yellow line) marking a Wilson band, with the profile histogram showing the length of the line and the values of gray scale.

statistically examined the gray-scale color values of the Wilson bands with the EH episodes. Shapiro-Wilk test was performed for normality inspection of Wilson bands gray-scale values. Two-way ANOVA (Type III) test performed for the correlation of the gray scale intensity of Wilson bands with EH episodes.

Statistical evaluation of breast milk estimates between individuals with and without hypoplasias

The association between EH and breast milk proportion was determined by a Fisher's exact test, which is similar to a Chi-square test but more efficient in small sample sizes. Individuals were categorized into two groups based on the presence or absence of EH. In the absence of universally accepted breast milk proportions during weaning, it is not feasible to score them as "low", "medium", or "high" regarding its consumption. Therefore, to categorize breast milk proportion estimates, we used the percentiles of the distribution obtained from MixSIAR. The relationship between the breast milk proportion and the weaning duration was assessed by computing the Spearman's correlation.

Descriptive, inferential statistics and data visualization were performed in R (version 4.2.2).

Results

Reconstruction of the weaning process

A total of 225 increments were generated from the 15 newly reported individuals. Two individuals (METi_105, 109) date to the Hellenistic period, three (METi_107, 113, 466) date to the Roman period, ten (METi_133, 135, 149, 155, 173, 175, 209, 215, 231, 233,) date to the late Roman-early Byzantine period and one (METi_121) dates to the post-Byzantine period.

Out of this total, 163 were measured as we aimed to reconstruct weaning and diet during the first six years of age. Isotopic values of the 163 samples ($\delta^{15}N$ and $\delta^{13}C$) and elemental indicators (%C, %N, C: N) for collagen quality control of each sample are reported in Table S2. Overall, $\delta^{15}N$ values range between 7.06‰ and 13.68‰ (mean: 9.58‰) and of $\delta^{13}C$ range from -14.68‰ to -20.75‰ (mean: -18.43). All analyzed collagen samples (n=163) fall within the acceptable range for collagen integrity (Ambrose, 1990; DeNiro, 1985; van Klinken, 1999) (%C: 13-47%, %N: 5-17, C/ N: 2.9-3.6). The WEAN estimates of weaning completion are reported in Table S3.

The MixSIAR model estimates for potential die-

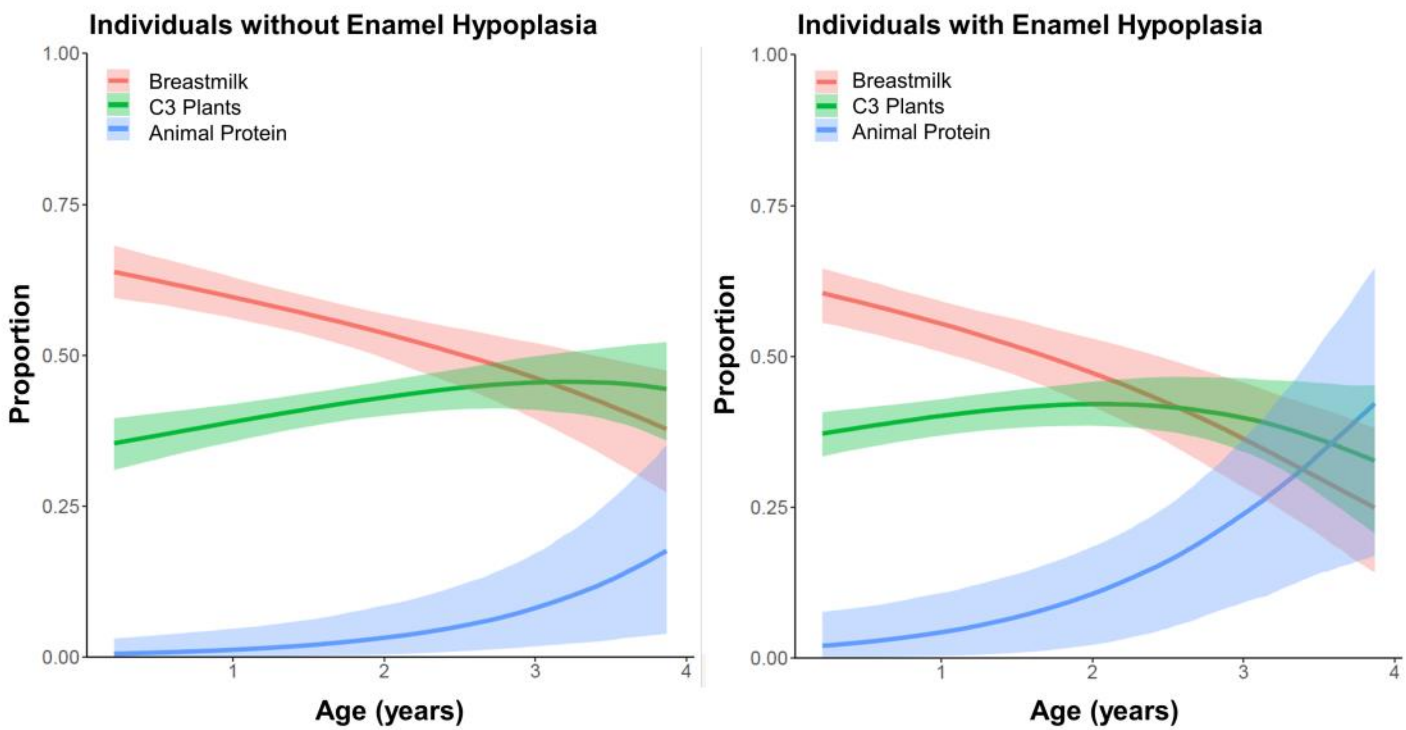

Figure 8. The proportion of breast milk, C3 plants and animal protein during the first four years of age for the individuals with and without enamel hypoplasia (EH) from ancient Thessaloniki (4th c. BC-16th c. AD) obtained from MixSIAR.

Table 5. MixSIAR model estimates depicting the probability of each dietary source during weaning for the individuals from ancient Thessaloniki (4th c. BC-16th c. AD) combining carbon and nitrogen stable isotope ratios of dentinal collagen. The percentiles serve as confidence intervals (CI) measuring this probability.

	Dietary source	5%	25%	50%	75%	95%
Individuals without hypoplasia	Breastmilk	0.48	0.52	0.54	0.57	0.60
	C3 plants	0.32	0.35	0.38	0.40	0.44
	Animal protein	0.06	0.07	0.08	0.09	0.11
Individuals with hypoplasia	Breastmilk	0.43	0.47	0.49	0.51	0.55
	C3 plants	0.31	0.34	0.36	0.38	0.42
	Animal protein	0.11	0.13	0.15	0.17	0.19

tary sources for every individual (15 newly and 51 previously analyzed) are reported in Table S4. Table 5 presents the results of the MixSIAR modelling. The provided percentiles serve as confidence intervals, showing the likelihood associated with the contribution of each dietary source into the diet. For instance, considering the breastmilk of individuals without hypoplasia, the 5th and 95th percentiles are reported as 0.48 and 0.60, respectively. To provide a more robust representation of the probability, we highlight the median at 0.54, accompanied by a 95% confidence interval ranging from 0.48 to 0.60. Individuals without EH consumed more breast milk until the cessation of weaning (54%) compared to individuals with EH (49%) (Figure 8). Individuals without EH had a diet richer in animal proteins than the ones with EH. There was no difference between the two groups on the C3 plants consumption (Table 5).

Histomorphological examination and age estimation of enamel hypoplastic defects

From the 66 individuals, 26 individuals (*n*=16 males, *n*=10 females, *n*=1 indetermined) were processed histologically. Three individuals (METi_139,201,221) date to the Hellenistic period, eleven (METi_157, 163, 189, 195, 197, 203, 223, 239, 257, 466, 71) date to the Roman period, four (METi_173, 175, 209, 231) date to the late Roman-early Byzantine period and eight (METi_121, 131, 185, 193, 199, 217, 228, 267) date to the post-Byzantine period.

One individual exhibited pitting enamel hypoplasia (PEH) (METi_231), one exhibited plane hypoplasia (METi_163), and the remaining 24 exhibited linear enamel hypoplasia (LEH). Histologically, plane hypoplasia is characterized by extensive destruction of the prismatic structure of the enamel and bending of the Wilson bands. Linear and pitting hypoplasia appear with localized thinning of the enamel and extensive number of Wilson bands (Hillson and Bond, 1997; Lukasik and Krenz-Niedbała, 2014).

Histomorphological examination of EH proved to be advantageous because it enabled the detection of defects that were not visible during the macroscopical examination. As a result, the number of hypoplastic episodes increased in 13 individuals i.e. in three individuals increased from two to three, in eight individuals from one to two and in two individuals from one to three. Furthermore, defects in two individuals (METi_199 and METi_217) that were recorded as EH by macroscopic examination were proven to be taphonomic weathering after the histological examination.

In Table 6 we present the developmental rates of cuspal and imbricational enamel. We could not estimate the cuspal enamel formation time in three individuals (METi_193, 221, 71), as the cross-striations that result in the average periodicity of enamel deposition were not observable due to taphonomic degradation. For these three individuals we used the average formation time of cuspal enamel that were generated from the same tooth type of the other individuals (Table S6). The average cross-striation periodicity emerging from the total sample of canines (upper and lower) for the population of Thessaloniki is 7.07 days. For details of the histological results see Table S6.

One individual exhibited five defects of enamel hypoplasia, six individuals three defects and 25 individuals two defects (Table S5). A young (15-21 years old) male individual (METi_163) exhibited one hypoplastic defect that was incessant for 3.9 years, appearing at the age of 2.9 years old until the age of 6.8 years old. The individual with the five defects was a subadult (6-10 years of age) (METi_466) (Table S5) who suffered five consecutive events of stress at the ages of 1.9, 2.1, 2.11, 3.7 and 4.07 years old.

For statistical analysis we grouped the episodes

Table 6. Average cross-striation periodicity of cuspal (n=23) and imbricational (n=26) enamel from the individuals of ancient Thessaloniki (4th c. BC-16th c. AD) according to tooth type (UI= Upper Incisor, UC= Upper Canine, LC= Lower Canine).

Number of teeth per tooth type	Cuspal average cross-striation periodicity (in days)	Imbricational average cross-striation periodicity (in days)
UI = 1	9.63	8.2
UC = 9	8.34	7.4
LC = 16	7.05	6.79

Table 7. Summary results of the 26 individuals from ancient Thessaloniki (4th c. BC -16th c. AD) that exhibited hypoplastic defects. Descriptive statistics of the timing of EH for each group independently.

	Values of ages		
Descriptive Statistics	**First EH**	**Second EH**	**Third EH**
Minimum	1.660	2.080	2.510
Maximum	4.500	6.160	4.670
1st Quartile	2.330	3.160	2.953
Median	2.500	3.510	3.715
Mean	2.591	3.524	3.645
3rd Quartile	2.757	3.750	4.350

of enamel hypoplasia according to the order of appearance: first, second and third EH. The fourth and fifth appearance of enamel defects were not tested as they were present only in one individual. Shapiro-Wilk test showed normal distribution ($p < 0.05$) of the EHs values. The statistical analysis revealed two peaks of stress, at the age of 2.4 years and 3.6 years (Figure 9). The first hypoplasia occurred at the mean age of 2.5 years, and the second hypoplasia occurred at the mean age of 3.5 years. The third episode of hypoplasia does not result in a significant peak as the range is wide and the sample size (n=6) is small (Table 7). Males exhibit a peak of the first EH at the age of 2.5 years with a wide range (1.6 - 3.3 years) whereas females at the age of 2.5 years with a smaller peak at the age of 4.5 years. The differences were statistically significant (two-way ANOVA) ($p < 0.05$). For the second hypoplastic defect, the difference between males and females was not statistically significant ($p > 0.05$) (Figure 10). Figure S1 shows the distribution of EH episodes according sex through the historical periods.

The statistical examination of the enamel morphological alterations, namely the intensity of the Wilson bands and the thinning of the enamel created by EHs, showed different correlation patterns. Kolmogorov-Smirnov test showed normal distribution of the control point curvature values ($p < 0.05$). The area plots show that the first EH defects have more intense curves, with an increased thinning of the enamel whereas the hypoplastic defects that follow are progressively shallower (Figure 11). Differentiation between sexes is also apparent with the males exhibiting deeper defects than females in all first and second EH episodes (Figure 12). The other three EH episodes cannot be compared as only one male individual exhibited a third EH and only one subadult exhibited a fourth and a fifth EH defect. Two-way ANOVA showed correlation between the depth of the defects and the sex with the EHs ($p < 0.05$).

The intensity of Wilson bands however, examined through the gray-scale intensity, resulted no correlation with each EH defect (i.e. first, second etc). Shapiro-Wilk test showed that the data of the gray scale of Wilson bands are not normally distributed. The two-way ANOVA (Type III) showed no correlation with EH ($p > 0.05$).

Statistical evaluation of breast milk estimates between individuals with and without hypoplasias

The MixSIAR model estimates for the breast milk proportion did not show significant differences for the four quartiles (Table 4). Therefore, we used only the 2nd quartile (50%) and created two

Table 8. Crosstab table showing the number of individuals with and without EH who consumed less or more than 50% of breast milk during the weaning process used for Fisher's exact test (p-value: 0.02, significance level set at 0.05).

	less than 50%	**more than 50%**
Individuals with hypoplasias	15	12
Individuals without hypoplasias	6	20

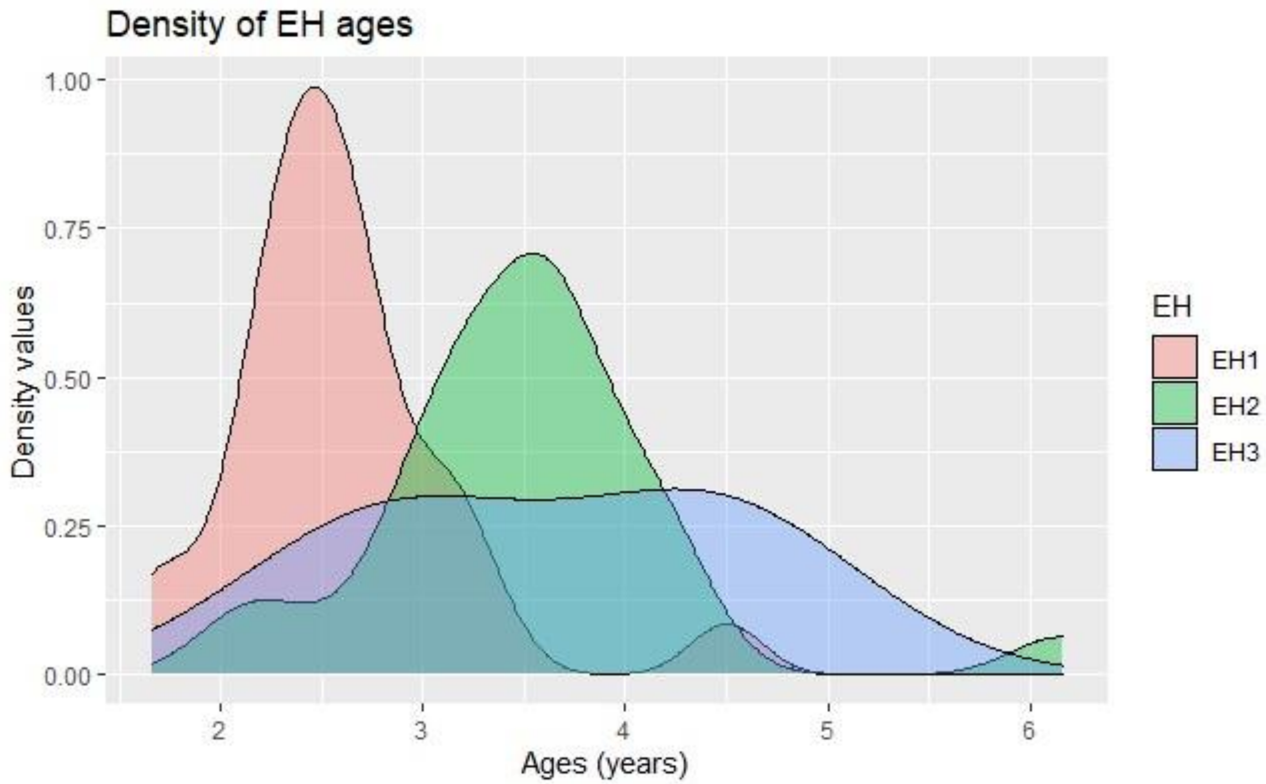

Figure 9. Kernel density plot showing the density and distribution and peaks of timing of appearance of each EH group incidences from the individuals of Thessaloniki (4th c. BC-16th c.AD). EH1 = first enamel hypoplasia, EH2= second enamel hypoplasia, EH3= third enamel hypoplasia.

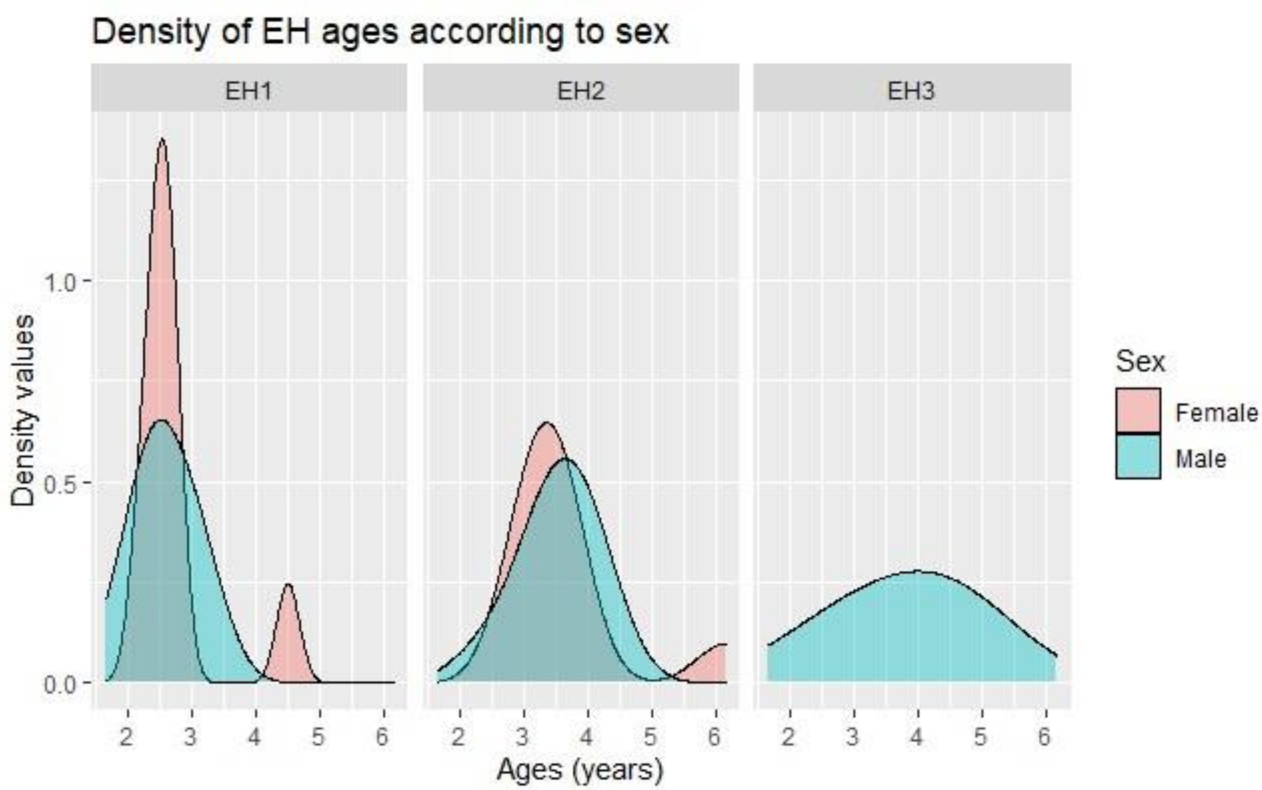

Figure 10. Kernel density plot showing the distribution and peaks of timing of appearance of each EH group incidences from the individuals of Thessaloniki (4th c. BC -16th c. AD) according to sex. Males exhibit a peak of the first EH at the age of 2.5 years with a wide age range (1.6 - 3.3 years) whereas females at the age of 2.5 years with a smaller peak at the age of 4.5 years.

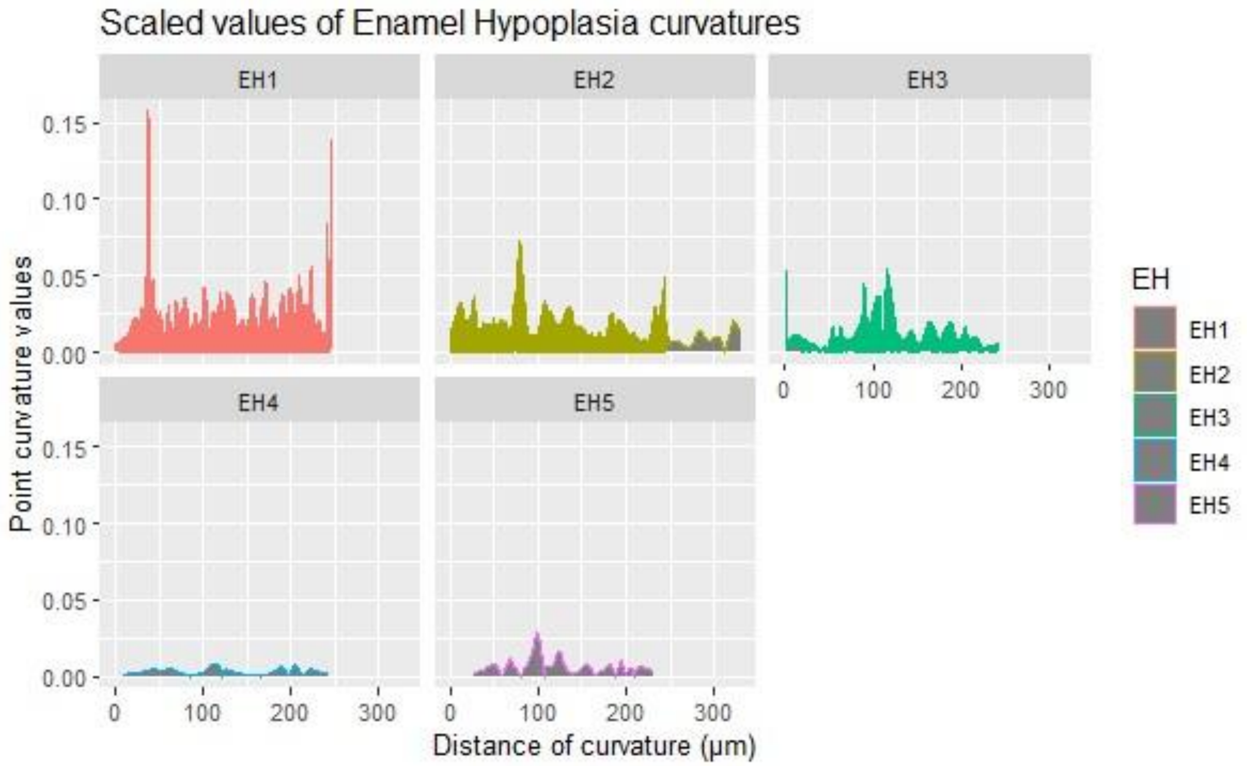

Figure 11. Area plots showing the curves of EHs from the individuals of Thessaloniki (4th c. BC -16th c. AD) according to the timing of EH. The first EH has increased points of curvature that indicate deeper defects. The second EH is less deep but are lengthier showing larger duration. The other EHs are progressively shallower.

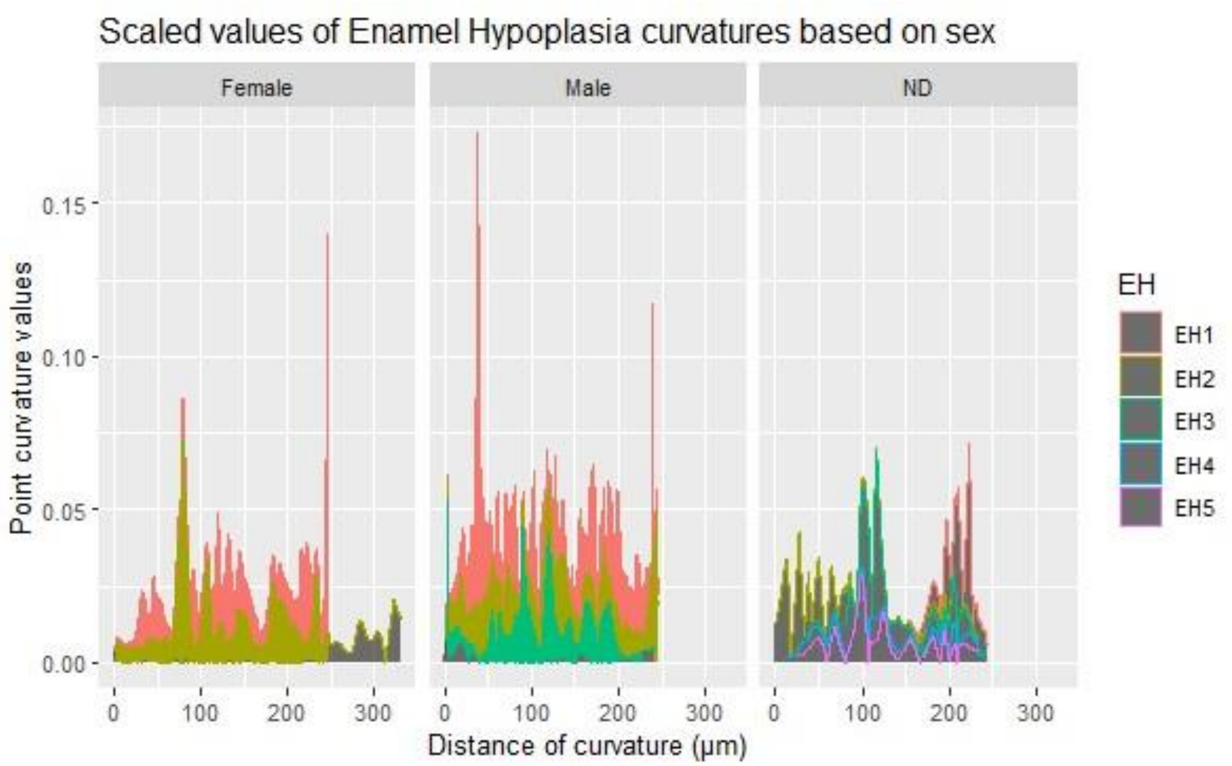

Figure 12. Area plots showing the curves of EHs from the individuals of Thessaloniki (4th c. BC -16th c. AD) according to sex. At the first and second EH males exhibited higher points of curvature than females, that indicate deeper defects.

groups: in the first, breast milk constitutes less than 50% of the total diet (“Less than 50%”), whereas in the second, breast milk constitutes more than 50% of the total diet ("More than 50%”) (Table 8). The results of Fisher's exact test showed that the difference between the two groups *is statistically significant* (p: 0.02, significance level set at 0.05). We have also found a weak positive but not statistically significant correlation between breast milk proportion and weaning age (rho = 0.040, p = 0.776). Figure S2 shows the distribution of breastmilk proportions and other food sources according to historical periods.

Discussion

The overarching question of this study was to test whether the proportion of breast milk during weaning *has an impact* on the *formation* of enamel hypoplasia. We have explored this hypothesis in 66 individuals from ancient Thessaloniki (4th c. BC-16th c. AD) by estimating: 1) the duration of weaning using incremental dentine analysis (163 newly reported increments), 2) the proportion of breast milk and other potential dietary sources during weaning using Bayesian modeling and 3) the formation ages and duration of enamel hypoplasia using histological analysis.

Enamel hypoplasia in ancient Thessaloniki

Multiple EH defects were identified in 27 out of 66 individuals (40%), formed between 2.0-5.0 years of age. All individuals developed at least two hypoplastic defects with a modal age of 2.4 years and of 3.6 years respectively, whereas six individuals exhibited a third one (Table S5). The high frequency of EH and more specifically of linear enamel hypoplasia (LEH) on Greek populations has been previously reported (Pitsios, 2012). In particular, in the study of Pitsios (2012), ancient skeletal material from five different ancient Greek cities (Leonidio, Arkadia; Tripoli, Arkadia; Markopoulo, Attica; Athens, Attica; Eretria, Euboea; Abdera, Thrace) were studied macroscopically. According to this study the frequency of LEH varied between the cities from 17% to 51% and the most affected tooth type was the upper canine. However, there is no information about differences in sex or age or more specific chronological distribution of the datasets.

In the present study, we did not identify significant differences in dental growth formation rates between males and females but found statistically significant differences in the frequency and occurrence of the first incidence of EH between the two sexes (Figure 10). In particular out of the 27 individuals that exhibited EH, the 61.5% (17/27) were males while 34.6% (9/27) were females. In females the first EH defect appears at the age of 2.2 years and does not exceed the age of 2.9 years (except for one individual), whereas males exhibit the first EH defect between the age of 1.8 and 3.4 years. Furthermore, the examination of the enamel thinning revealed that the first EH is more severe than the later and males develop more severe hypoplasias than females. These reveals a shorter and less acute stress period for females than males. This result may relate with the observation that male infants are biologically weaker than female, a phenomenon also known as the male disadvantage (Hossin, 2021). Medical studies suggest that male infants exhibit higher neonatal mortality and *more* severe morbidity compared to females (Eriksson, Kajantie, Osmond, Thornburg, and Barker, 2010; Hossin, 2021; Wong, Schreiber, Crawford, and Kumar, 2023). Alternatively, the sex difference in timing and severity of EH defects could be associated to different feeding practices as showed by previous studies (Ganiatsou et al., 2022) (Table S6). The underlying cause of this significant disparity between sexes remains unclear and is likely multifactorial. Overall, EH on a Roman-Byzantine population from Greece was a common condition indicating that in pre-vaccination and pre-industrial contexts, acute manifestations of physiological stress during childhood were frequently experienced.

Exclusive breastfeeding shapes the immune system of infants.

As infant feeding practices could be an important stress factor, we examined the breastfeeding and the weaning patterns in ancient Thessaloniki and found that the majority of EH defects (n=20/27) formed after the end of weaning and few (n=7/27) during the last phase of weaning. Similar patterns of EH formation and weaning age were found in 14 individuals from Mission Santa Catalina de Guale (1605-1680 AD) in St. Catherine's Island (Georgia, USA) and were attributed to malnutrition due to a maize-based weaning diet (Garland et al., 2018). Sandberg (2014) examined 5 individuals from the site of Kulubnarti (550-800 AD) in Sudan and suggested that morbid events resulted in the formation of EH between 4.0 and 7.0 years of age whereas, weaning was completed between 2.0-5.0 years of age according to incremental dentine analysis.

Therefore, based on the results of the present study, we suggest that breast milk consumption and EH could have a causative relationship. As we have shown, hypoplastic defects were developed in individuals consuming less than 50% of breast milk during weaning. Furthermore, in individuals with EH, the defects did not develop during the first year of life, when breast milk had constituted the major source of their nutrition (Table 8). This possibly highlights the immunological support provided by breast milk, at least during periods that is consumed in larger amounts. Breast milk is a nutritionally complete food source and is microbiologically safer compared to other foods, such as the non-pasteurized milk (Andreas, Kampmann, and Mehring Le-Doare, 2015). A number of studies have reviewed the immunological advantages of breast milk against specific pathogens (viruses, bacteria, and parasites) as well as separate clinical illnesses (e.g. necrotizing enterocolitis, bacteremia, meningitis, respiratory tract illness, diarrheal disease, and otitis media) (R. A. Lawrence, 1997; R. M. Lawrence & Lawrence, 2004). The significance of breast milk for the newborns' immune system was recognized since ancient times according to the treatises of Hippocrates, Soranus and Galen, who advised breastfeeding from the mother or a wet-nurse (Fulminante, 2015).

Furthermore, according to the results of MixSIAR, the individuals that developed EH defects consumed more animal protein, most likely in the form of milk (Garnsey, 1999), compared to those who did not (Table 8). It is possible that these individuals were not only lacking the immunological advantages of breastmilk consumption but were also exposed to non-sterilized feeding vessels, which, especially in settings with poor hygiene, can increase the risk of infections (Kendall et al., 2021).

It is also important to consider that children can benefit from the nutritional characteristics of breast milk for a limited period (approximately for the first six months of age) (Kendall et al., 2021; Pérez-Escamilla, Buccini, Segura-Pérez, and Piwoz, 2019). As infants grow their nutritional expectations change and breast milk alone is not sufficient (Kendall et al., 2021). Although it is significant that supplementary sources of nutrition must be introduced, there is no guarantee that the nutritional quality of these foods is adequate. A possible explanation for the individuals we examined is that their diet was not nutritionally sufficient, and these children were severely malnourished.

Overall, we found diverse weaning practices in the site, which adhere to a baby-led weaning pattern (Cichero, 2016). Specifically, according to this pattern, parents provide the supplementary foods but the infant decides what they will eat, how much and how quickly (Cichero, 2016). This weaning pattern may prove harmful for some infants, such as premature ones, who have higher iron needs than term babies (Baker, Greer, and Committee on Nutrition American Academy of Pediatrics, 2010) and also have difficulty managing lumpy solids even at 12 months of age (Hawdon, Beauregard, Slattery, and Kennedy, 2000). Furthermore, the early introduction of solids may lead to the development of bad feeding behaviors, such as picky eating or skipping meals (Chung, Lee, Spinazzola, Rosen, and Milanaik, 2014), which overall increase the risk of malnutrition.

Methodological insights: histology and stable isotope analysis in the assessment of physiological stress during weaning

The combined results of histological and isotopic analysis highlight that the individuals in ancient Thessaloniki exhibited high levels of physiological stress. This was also discussed in Ganiatsou et al., (2023) that used a machine learning approach to identify inconsistent changes in isotopic ratios during weaning, which are related to malnutrition (Beaumont & Montgomery, 2016; Craig-Atkins, Towers, & Beaumont, 2018; Garland et al., 2018). According to their results, isotopic patterns related to physiological stress were identified in 21 individuals out of the 51 examined in total. In the present study we examined 13 out of the 21 individuals as the remaining had no available canine or incisor to sample (Table S1) and found that all 13 showed hypoplastic defects. As EH is a robust sign of developmental disturbances the overlap of the two conditions i.e. the inconsistent changes between carbon and nitrogen isotopic ratios and the presence of EH during infancy indicates a "powerful duo" for detecting physiological stress in archaeological populations. However, due to the small sample size of the present study, *conclusions are drawn with caution against overinterpretation of these results*.

Finally, this study underscored the importance of histological analysis in the investigation of EH, which despite of its destructive nature, yields, so far, unprecedented precision. Furthermore, this analysis provided the means to examine for the first time the average short periodicity of enamel and average growth rates between cuspal and imbricational enamel in the individuals of ancient Thessaloniki that showed significant differences, compared to other studies (Table S7) (Dąbrowski et al., 2021; Goodman and Rose, 1990; Krenz-Niedbała and Kozłowski, 2013; Lukasik and Krenz-

Niedbała, 2014; Reid and Dean, 2000). This underlines the significance of dental growth rates estimation, since fluctuations due to stress, environmental, dietary factors (Goodman and Rose, 1990), and even social status (Nakayama, 2016) can lead to inaccurate age-at-formation estimation of LEH defects (Goodman and Rose, 1990; Witzel et al., 2008). Dental developmental charts and regression equations based on macroscopic measurements (Goodman and Rose, 1990; Massler et al., 1941), although they offer the advantage of non-destructive methodology, they do not take into account the fluctuations of growth rates among the different tooth types, different populations and the cuspal enamel growth, leading to incorrect age estimation of up to 6-12 months of age (Dąbrowski et al., 2021; Goodman and Rose, 1990; Krenz-Niedbała and Kozłowski, 2013; Lukasik and Krenz-Niedbała, 2014; Reid and Dean, 2000).

Conclusions

The present study examined EH in individuals from ancient Thessaloniki and found that this condition was more frequently developed in infants consuming less than 50% of breast milk during weaning. This highlights the significant immunological support that breast milk provided in ancient times and protected infants from experiencing severe episodes of physiological stress. Nevertheless, these episodes were frequent, recurrent and severe in the site considering that almost 50% of the dataset (27/66 individuals) developed multiple hypoplastic defects. This is evident by the results of the histological analysis showing that hypoplastic defects, mostly of the linear type, occurred between the ages of 2.0-5.0 years and usually after the completion of weaning, consistent with other studies. Overall, the study employs a novel methodological approach to examine EH, and sheds light on a previously unexplored aspect on this condition. Our results, obtained from high-precision assays and statistical techniques, provide empirical data that support the heatedly debated causation of EH in ancient populations.

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