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TECHNICAL NOTE

Sex estimation of teeth at different developmental stages using dimorphic enamel peptide analysis

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Abstract

Objectives: This study tests, for the first time, the applicability of a new method of sex estimation utilizing enamel peptides on a sample of deciduous and permanent teeth at different stages of mineralization, from nonadults of unknown sex, including perinates.

Materials and methods: A total of 43 teeth from 29 nonadult individuals aged from 40 gestational weeks to 19 years old were analyzed. The sample included pairs of fully mineralized and just developing teeth from the same individual. The individuals were from four archaeological sites in England: Piddington (1st–2nd centuries AD), Coach Lane, Victoria Gate, and Fewston (all 18th–19th centuries). A method that identifies sex chromosome-linked isoforms of the peptide amelogenin from human tooth enamel was applied. The method utilizes a minimally destructive acid etching procedure and subsequent nano liquid chromatography tandem mass spectrometry.

Results: It was possible to determine the sex of 28 of the nonadult individuals sampled (males = 20, females = 8, undetermined = 1). Only one sample failed (CL9), due to insufficient mineralization of the sampled tooth enamel. Data are available via ProteomeXchange with identifier PXD021683.

Discussion: Sufficient peptide material to determine sex can be recovered even from the crowns of developing perinatal teeth that are not fully mineralized. The minimally destructive and inexpensive (compared to ancient DNA) nature of this procedure has significant implications for bioarchaeological studies of infancy and childhood.

KEYWORDS
amelogenin, mass spectrometry, perinate, sex, tooth enamel

1 | INTRODUCTION

The estimation of sex from the skeleton is fundamental to the study of past human populations and for establishing human identity in forensic contexts. Traditional macroscopic sex estimation methods in bioarchaeology are reliant on the presence and analysis of sexually dimorphic skeletal elements, including the innominate bones and skull (Buikstra & Ubelaker, 1994). In nonadults, morphological and metric analyses of the mandible, dentition, and ilium are occasionally undertaken to determine sex (Lewis, 2007; Luv et al., 2017; Schutkowski, 1993; Vlak et al., 2008). Prior to the development of secondary sexual characteristics, however, there is limited sexual dimorphism in skeletal features, rendering macroscopic methods unreliable before puberty (Hoppa & Fitzgerald, 1999; Scheuer & Black, 2000; Lewis, 2007). While

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ancient DNA analysis provides a potential solution, it does not always preserve in archaeological contexts and is too destructive and expensive for bioarchaeologists to use routinely. The inability to reliably determine the sex of nonadults has placed constraints on studies of infancy and childhood in the past and has contributed to their marginalization in archaeology (Lewis, 2007).

This study examines the applicability of an innovative method for sex estimation using sexually dimorphic enamel peptides (Stewart et al., 2017) to deciduous and permanent teeth in different stages of development. The method identifies sex chromosome-linked isoforms of the peptide amelogenin from human tooth enamel using a minimally destructive acid etching procedure and subsequent nano liquid chromatography tandem mass spectrometry (nanoLC–MS/MS). This method was originally developed on permanent teeth and has already been successfully tested on adult skeletal remains whose sex was either estimated using sexually dimorphic skeletal features or was known from associated coffin plates (Stewart et al., 2016, 2017). The aim of this study is to ascertain whether sufficient amelogenin peptides for sex estimation can be retrieved from nonadult teeth, including developing deciduous and permanent teeth.

Tooth enamel is the hardest human tissue and is highly resistant to diagenesis in burial contexts (Budd et al., 2000); it is therefore ideal for the biochemical estimation of sex (Stewart et al., 2016). Tooth enamel consists of only a small number of proteins, and is primarily comprised of the heterogeneous, dimorphic amelogenins (AMELX and AMELY). Several studies in recent years have successfully demonstrated that proteins can be extracted from human tooth enamel and used as a method for identifying the sex of archaeological individuals (Castiblanco et al., 2015; Froment et al., 2020; Lugli et al., 2019; Parker et al., 2019; Porto, Laure, de Sousa, et al., 2011; Porto, Laure, Tykot, et al., 2011; Stewart et al., 2016, 2017; Wasinger et al., 2019), including nonadults (Parker et al., 2019). The method has worked successfully on samples that are poorly preserved, are of variable time-depth (Demarchi et al., 2016; Lugli et al., 2019; Parker et al., 2019; Stewart et al., 2017; Wasinger et al., 2019), and where no DNA is preserved (Cappellini et al., 2018; Welker et al., 2020). The peptide method is also more cost effective than aDNA analysis for estimating sex. Results of false positive females due to differing levels of expression between the X and Y isoforms of amelogenin is also not of concern with this method, as the degree of difference (up to 10x) that can be observed is within the dynamic range of observation (Fincham et al., 1991; Parker et al., 2019).

Despite the proven robustness of this method, to date, there have only been a handful of deciduous teeth sampled using this technique (Parker et al., 2019), all of which were completely mineralized. This study, therefore, examined a larger sample of completely (n = 14) and incompletely (n = 29) mineralized nonadult permanent and deciduous teeth, including those of perinates. A successful application will have significant implications for our understanding of childhood in the past. It also has important applications to adult skeletal remains from forensic or archaeological contexts that are too fragmentary to reliably determine sex using morphological analysis.

2 | MATERIALS AND METHODS

Enamel peptide samples were collected from a total of 43 teeth from 29 nonadult individuals aged from 40 gestational weeks to 19 years old, excavated from four different sites from England (Table 1). The Coach Lane site was a Society of Friends (Quaker) burial ground located in North Shields, Tyneside, dating from the 18th to 19th centuries. North Shields during this period was a densely populated shipping, fishing, and coal-mining community, undergoing rapid expansion (Proctor et al., 2014). A total of 236 individuals were excavated from Coach Lane, including 81 individuals who were younger than 20 years of age (Gowland et al., 2018). The Victoria Gate site was part of the burial ground of Ebenezer Chapel, located in Leeds, West Yorkshire, dating predominantly to the 19th century. During this time, Leeds was a heavily industrialized city and the area around the chapel was associated with those of low socioeconomic status. A total of 21 articulated skeletons (and some disarticulated remains) were excavated from the site, including 12 nonadults (Caffell & Holst, 2014). Fewston is a small village in North Yorkshire and the churchyard of St Michael and St Lawrence was partially excavated in 2009. The skeletons predominantly date from the 18th and 19th centuries based on available coffin plates/grave monuments. A total of 154 individuals were excavated, including 50 nonadults (Gowland et al., 2018). The Piddington site, Northamptonshire, is a Roman Villa with an underlying Iron Age Settlement. A total of 17 infants were excavated from the site, dating from the first to second centuries AD (Hodson, 2017). The sample selected for this study was chosen to include permanent and deciduous teeth from individuals of varying ages and ontogenies.

All nonadults were assessed for age-at-death using dental development (AlQahtani et al., 2010), the appearance and fusion of the epiphyses (Scheuer & Black, 2000) and long bone length (Maresh, 1955; Scheuer et al., 1980). Age estimates for the perinates and infants are provided in gestational weeks of age (GWA). Evidence for pathological conditions, including infectious and metabolic disease, was also recorded following the diagnostic criteria outlined in Ortner (2003) and Lewis (2017). For 11 of the nonadults, more than one tooth from each, at different stages of development and mineralization, were sampled and analyzed (Figures 1 and 2). This was to test whether the intra-individual results were consistent regardless of the stage of tooth development.

The method used in this study is described in Stewart et al. (2016, 2017). In brief, the tooth surface was abraded to remove any obvious surface contaminants using a dental burr. The enamel was then washed with 3% H_2O_2 for 30 s before being rinsed with ultrapure water (Elga Purelab Ultra, 18.2 MΩ-cm). Approximately 60 μl of 5% (vol/vol) HCl was placed in the cap of a 0.2 ml Eppendorf tube, whereby an initial etch was performed by lowering the tooth onto the HCl and maintaining contact for 2 min; this first etch was discarded. The process was repeated for a second time and retained as the etch solution. A C18 resin loaded ZipTip (ZTC18S096; EMD Millipore) was conditioned three times with 100% acetonitrile using a 10 μl pipette, followed by three times with 0.1% (vol/vol) formic acid, before being discarded. The proteins were bound to the ZipTip by

TABLE 1 Result of dimorphic enamel peptide analysis of sampled nonadult teeth

Site	Period	Skeleton number	Sample no.	Age-at death	Burial type	Result	Retention time/peak area	Tooth sampled	Enamel appearance	Observed pathologies
Victoria Gate	18th century	2	VG2_1	5–6 years	Coffin	Male	X: 20.87 min/19552563 Y:17.21 min/9937685	ULdm2	White opaque, mineralized	Calculus, caries, DEH, ectocranial NBF
			VG2_2			Male	X: 22.35 min/269752 Y:18.40 min/748355	ULpM2	Gray/brown, not completely mineralized	
		3	VG3_1	10–16 months	Coffin	Male	X: 21.47 min/4891079 Y: 17.62 min/3028243	LLdM1	Brown/yellow not completely mineralized	DEH, rickets, possible scurvy, small for age
			VG3_2			Male	X: 22.76 min/205893 Y: 18.80 min/393285	LLpM1	Flakey, beige, pitting	
			VG3_3			Male	X: 21.85 min/4083743 Y: 17.87 min/1581289	LLdM2	Flakey, beige, pitting	
		4	VG4	8–10 years	Coffin	Female	X: 21.75 min/1947367 Y: Not present	LLpC	White opaque, mineralized	Caries, DEH, small for age, rickets, cervical rib and developmental anomaly of atlas
		5	VG5_1	8–10 years	Coffin	Male	X: 21.74 min/6958636 Y: 17.77 min/11095825	LRpM1	White opaque, mineralized	Calculus, caries, DEH, AMTL, sinusitis, porotic hyperostosis, rickets, unhealed fracture of mandible and rib
			VG5_2			Male	X: 21.58 min/20629960 Y: 17.68 min/12303224	URpC	White opaque, mineralized	
			VG5_3			Male	X: 21.46 min/14271819 Y: 17.60 min/9766371	ULpM2		
		6	VG6_1	7–8 years	Coffin	Male	X: 21.28 min/27778809 Y: 17.47 min/16839459	LRdM1	White opaque, mineralized	Calculus, caries, DEH, AMTL, small for age, endocranial NBF, rickets, border shift
			VG6_2			Male	X: 21.37 min/1445771 Y: 17.47 min/1386919	URP1	Brown/yellow not completely mineralized	
		12	VG12	16–19 years	Coffin	Female	X: 23.95 min/49884700 Y: Not present	LRpM1	White opaque, mineralized	Calculus, caries, DEH, abscess, woven NBF, endocranial NBF, sinusitis, bowing
		17	VG17	12–18 months	Inhumation	Male	X: 21.30 min/4001333 Y: 17.45 min/2733291	ULdC	Brown/yellow not completely mineralized	Endocranial NBF, woven bone
		19	VG19_1	6–12 months	Coffin	Male	X: 23.88 min/15636649 Y: 19.46 min/8222790	ULdI1	Brown/yellow not completely mineralized	
			VG19_2			Male	X: 27.18 min/95943	ULdM2		

(Continues)

TABLE 1 (Continued)

Site	Period	Skeleton number	Sample no.	Age-at death	Burial type	Result	Retention time/peak area	Tooth sampled	Enamel appearance	Observed pathologies
							Y: 23.08 min/239983		Brown/black not completely mineralized	
Coach Lane	18th century	9	CL9	0–1 months		N/A	Undetected	LRdM1	Brown/black not mineralized	Possible scurvy
		14	CL14_1	2–3 years		Female	X: 0.22.71 min/ 10822157 Y: Not present	LRdM2	white/ yellow, pitting	Rickets, ectocranial porosity, caries, DEH
			CL14_2			Female	X: 23.27 min/511891 Y: Not present	ULpC	Brown/black not mineralized	
		57	CL57_1	7–9 years		Male	X: 21.27 min/1495084 Y: 17.44 min/3270339	ULpM1	White/yellow opaque, mineralized	DEH, CO, ectocranial porosity,
			CL57_2			Male	X: 21.47 min/841020 Y: 17.52 min/ 2683184	ULpM2	White/yellow opaque, opaque, mineralized	Phossy jaw, DEH. CO
		104	CL104	1.5–2.5 years		Male	X: 21.31 min/11827792 Y: 17.48 min/ 5880374	LLdM2	White opaque, mineralized	rickets, ectocranial porosity, caries, DEH
		107	CL107	16–17 years		Male	X: 21.37 min/ 9318414 Y: 17.53 min/ 7537087	URpI1	White opaque, mineralized	Rickets, DEH
		215	CL215_1	1–2 years		Male	X: 23.09 min/2184843 Y: 18.94 min/ 2152678	ULdM2	Flakey, beige, pitting	CO, scurvy, DEH
			CL215_2			Male	X: 22.26 min/5210208 Y: 18.22 min/5540212	URdC	White/yellow not completely mineralized?	
			CL215_3			Male	X: 26.78 min/1331549 Y: 22.79 min/1726177	LRdI1	Gray/brown, not completely mineralized	
		227	CL227	2–4 years		Female	X: 23.59 min/4646683 Y: Not present	LLdM21	White opaque, mineralized	CO, possible rickets, DEH, fractured left radius and ulna
		231	CL231_1	5–8 months		Male	X: 23.45 min/1376538 Y: 19.01 min/1541955	URdI1	Brown/yellow not completely mineralized	Possible scurvy, DEH
			CL231_2			Male	X: 30.96 min/ 307,449 Y: 27.12 min/426519	ULdM2	Brown/black not mineralized	
Fewston	18th century	331	F331	12–14 years	Coffin	Male	X: 25.36 min/ 21964899 Y: 20.90 min/ 31860603	LLpP2	White opaque, mineralized	Rickets, possible scurvy, DEH, caries, arrested growth
		334	F334	11–12 years	Coffin	Male	X: 25.02 min/6995810	LLpP2	White opaque, mineralized	

TABLE 1 (Continued)

Site	Period	Skeleton number	Sample no.	Age-at death	Burial type	Result	Retention time/peak area	Tooth sampled	Enamel appearance	Observed pathologies
							Y: 20.51 min/10824322			Rickets, arrested growth, rib lesions, DEH, caries
		423	F423	13–14 years		Female	X: 23.60 min/34812067 Y: Not present	LLpP2	White opaque, mineralized	Rickets, scurvy, CO, sinusitis,
Piddington	1st /2nd century	4	PID4	43GWA	Inhumation	Male	X: 26.06 min/7739105 Y: 22.57 min/7110317	LRdI2	Flakey, beige, not mineralized	NBF, possible rickets
		6	PID6	40GWA	Inhumation	Female	X: 24.20 min/8739528 Y: Not present	LRdM1	Beige, possibly partly mineralized?	NBF, possible rickets, metaphyseal expansion
		17	PID17	46GWA	Inhumation	Male	X: 27.90 min/3226795 Y: 23.51 min/4089298	URdM1	Brown/beige, possibly mineralized?	NBF, possible rickets
		18	PID18	46GWA	Inhumation	Male	X: 25.19 min/4570182 Y: 20.76 min/5633058	ULdI1	Brown, beige and opaque—possibly mineralizing?	NBF
		19	PID19_1	58GWA	Inhumation	Male	X: 23.29 min/4761025 Y: 18.81 min/17232397	LRdI2	Brown, beige, possibly mineralized?	NBF
			PID19_2			Male	X: 24.29 min/4500661 Y: 19.85 min/13712910	LRdM1	Brown, beige, possibly mineralized?	
		24	PID24	46GWA	Inhumation	Female	X: 23.50 min/17327684 Y: Not present	LLdI1	Beige, possibly partly mineralized?	NBF, possible rickets, metaphyseal expansion
		27	PID27	46GWA	Inhumation	Male	X: 24.19 min/12074232 Y: 19.72 min/11293643	ULdI2	Brown, beige, flakey	NBF, vertebral cleft
		29	PID29	46GWA	Inhumation	Male	X: 26.20 min/3576447 Y: 21.84 min/10165385	URdM1	Brown/beige	NBF
		31B	PID31_1	46GWA	Inhumation	Female	X: 30.32 min/467397 Y: Not present	ULdI1	Brown, possibly partly mineralized	NBF
			PID31_2			Female	X: 28.45 min/3953057 Y: Not present	URdC	Beige, flakey	

Abbreviation: GWA, gestational weeks of age.

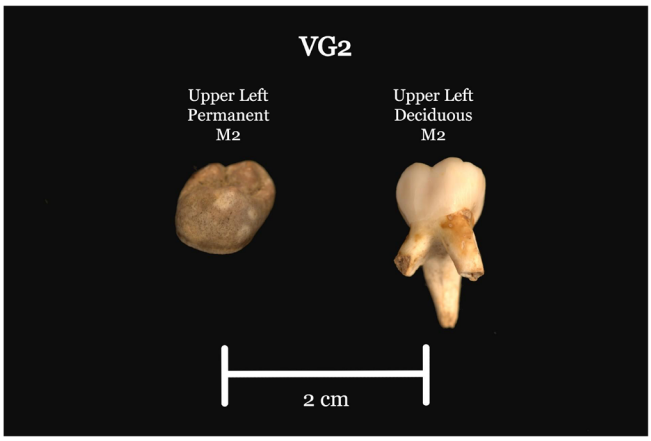

FIGURE 1 The permanent and deciduous teeth sampled from individual VG2

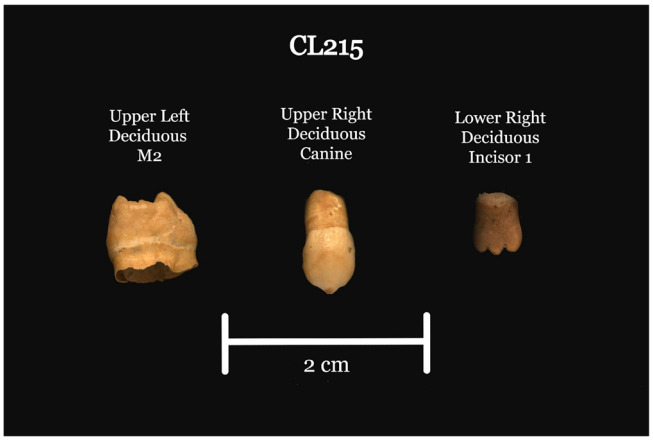

FIGURE 2 Three deciduous teeth sampled form individual CL215

drawing the etch solution through the ZipTip 10 times, discarding the last draw. The ZipTip was then washed six times with 0.1% (vol/vol) formic acid; each wash was discarded. The resin-bound peptides were eluted by drawing a 4-µl 60% acetonitrile/0.1% formic acid elution buffer through the ZipTip 10 times and the eluted peptides subsequently lyophilized. Samples were then dissolved in 12 µl of 0.1% trifluoroacetic acid in water, centrifuged for 5 min on a desktop centrifuge, and 10 µl transferred to glass autosampler vials.

A sample of 5 µl was injected for analysis by reversed-phase nanoLC–MS (UltiMate 3000 RSLCnano; Thermo Fisher Scientific) coupled to a hybrid quadrupole Orbitrap mass spectrometer (Q Exactive Orbitrap; Thermo Fisher Scientific) equipped with a nanospray ion source (Nanospray Flex, Thermo Fisher Scientific). Peptides were first loaded onto a C18 trapping cartridge (Pepmap100 C18; Thermo Fisher Scientific; 0.3 × 5 mm i.d.; 5 µm particle size) for 4 min at a flow rate of 20 µl/min using mobile phase A (0.1% [vol/vol] formic acid in hypergrade water, Merck KGaA). Separation was achieved at a flow rate of 200 nl/min on an analytical column (PepMap100 C18; 15 cm × 75 µm; 2 µm particle size) using a gradient of mobile phase B (0.1% [vol/vol] formic acid in acetonitrile, LiChrosolv, Merck KGaA) from 1 to 28% B (Curve 4) over 42 min, 28 to 99% B (Curve 6) over 8 min, 99% B for 5 min and back to 1% B over 1 min to equilibrate for 9 min, with a total chromatographic run time of 65 min. A stainless-steel emitter (40 mm, 1/32″ OD) was used post column. Blank injections were used between runs to reduce possible carryover. The mass spectrometer was operated in the positive ion mode with a spray voltage 1.6 kV and a capillary temperature of 250°C. MS data were acquired in a data-dependent manner, with full scan MS spectra (300–1650 m/z, (R = 140,000 at m/z 200) followed by the fragmentation of the top 10 most abundant precursor ions. A lock mass of 445.1200 m/z, corresponding to polysiloxane ($[M + H]^+$, $(C_2H_6SiO)_6$), was used. Dynamic exclusion was set to 45 s, with charge exclusion set for unassigned and singly charged species. Automatic gain control (AGC) target was set to 1×10^6 with a maximum injection time of 20 ms for full scan. Fragmentation of precursor ions was performed by higher-energy collisional dissociation with a normalized stepped collision energy of 20, 25, and 30, with a default charge state of 2. MS/MS scans (R = 17,500 at m/z 200) were performed with an AGC target value of 1×10^5 and a maximum injection time of 120 ms using an isolation window of 2.2 m/z.

The data were searched against the Human FASTA file (Swiss-Prot [21/09/2020] canonical 20,375 protein count) using MaxQuant (v1.6.3.4), with an "unspecific" digestion mode and default parameters. Sex was estimated by visualizing the reconstructed ion chromatogram of 440.2233 m/z and 540.2796 m/z at 1 PPM mass accuracy; corresponding to Ser-Met(oxidized)-Ile-Arg-Pro-Pro-Tyr (from AMELY) and Ser-Ile-Arg-Pro-Pro-Tyr-Pro-Ser-Tyr (from AMELX), respectively. Estimation was further supported with correct relative retention times, a correct charge state of 2 in the full MS and, if present, an accompanying MS/MS spectrum matching predicted fragment ions. The data were also searched against the Human FASTA file (Swiss-Prot [10/09/2020], canonical and isoforms, 214,628 valid protein count) using PEAKS Studio 10.5 build 20200219. Digest mode was set to "unspecific", parent mass error tolerance was 10.0 ppm, fragment mass error tolerance was 0.05 Da with the variable modifications; deamidation of N and Q and oxidation of M, fragmentation mode was high energy CID (y and b ions), MS and MS/MS scan modes were FT-ICR/Orbitrap. Default settings were used for all other parameters. The datasets generated during and/or analyzed during the current study are available in the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the PRIDE [1] partner repository with the dataset identifier PXD021683.

3 | RESULTS AND DISCUSSION

Sufficient amelogenin peptides were recoverable for 28 of the 29 nonadult individuals (male = 20, female = 8, undetermined = 1) for a qualitative estimation of sex. One sample (CL9, a perinate) failed to produce sufficient signal for the AMELX peptide and therefore sex could not be estimated.

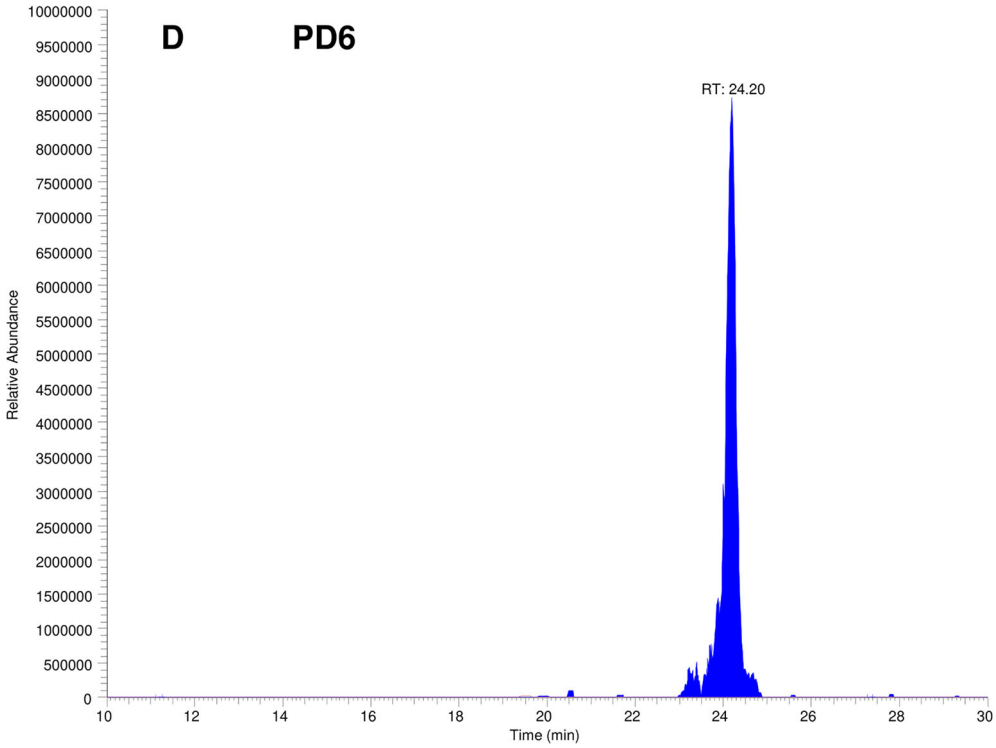

FIGURE 3 Results for tooth sampled from perinate (40 gestational weeks of age [GWA]) PID6 (female)

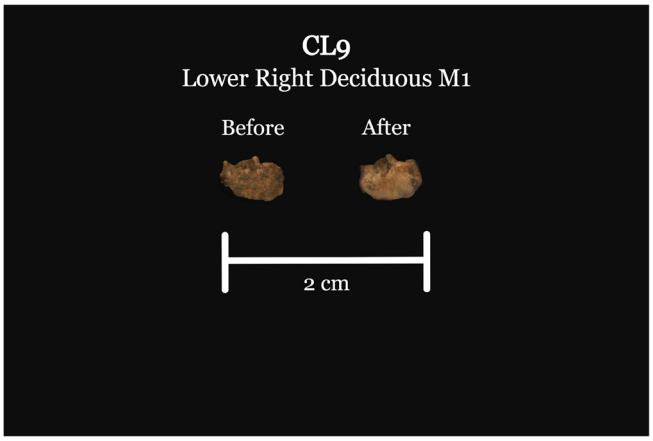

FIGURE 4 CL9 lower right deciduous M1 prior to and after sampling

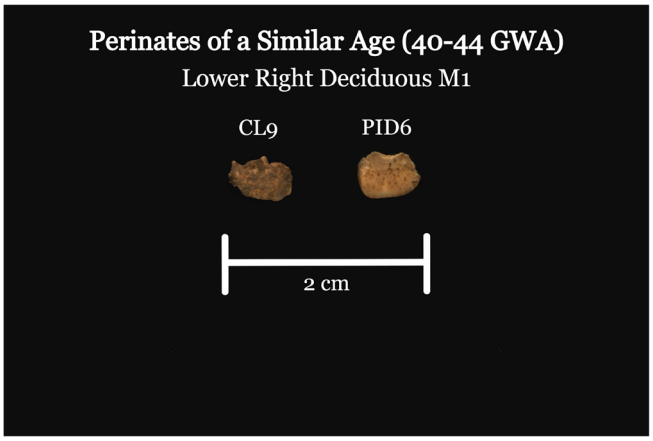

FIGURE 5 Perinates of a similar age showing different rates of enamel mineralization

Two previously identified peptides of similar ion intensities (Stewart et al., 2016, 2017), Ser-Met(oxidized)-Ile-Arg-Pro-Pro-Tyr from AMELY and Ser-Ile-Arg-Pro-Pro-Tyr-Pro-Ser-Tyr from AMELX, were used to assess sex. Many AMELX peptides have greater ion intensities than AMELY peptides, presumably because the expression of the AMELY protein has the potential to be 10% that of the AMELX (Fincham et al., 1991; Parker et al., 2019). In order to account for potential differences in expression, this study targets an AMELX peptide that has similar intensity to an AMELY peptide; the intensity of the Ser-Ile-Arg-Pro-Pro-Tyr-Pro-Ser-Tyr AMELX peptide is consistently much lower than other AMELX peptides. For example, the ion intensity for the N-terminus peptide Met(oxidized)-Pro-Leu-Pro-Pro-His-Pro-Gly-His-Pro-Gly-Tyr-Ile-Asn-Phe is approximately two orders of magnitude higher.

The data were recorded in a data-dependent fashion, as previously described (Stewart et al., 2016, 2017) for a proteomic overview of these samples. The peptide features identified from these samples are presented in Supplementary Table 1. The reconstructed ion chromatogram for the two peptides mentioned above were used for sex estimation. A database search using MaxQuant did not return Ser-Met(oxidized)-Ile-Arg-Pro-Pro-Tyr from AMELY from any of the samples (data not shown). Due to the inherent limitations of database search algorithms, especially with respect to short, nontryptic peptides, the identifications of these are not guaranteed. The data were also searched using a different software package (PEAK Studio) and samples with AMELY peptides identified (males) corroborated with those identified using the two

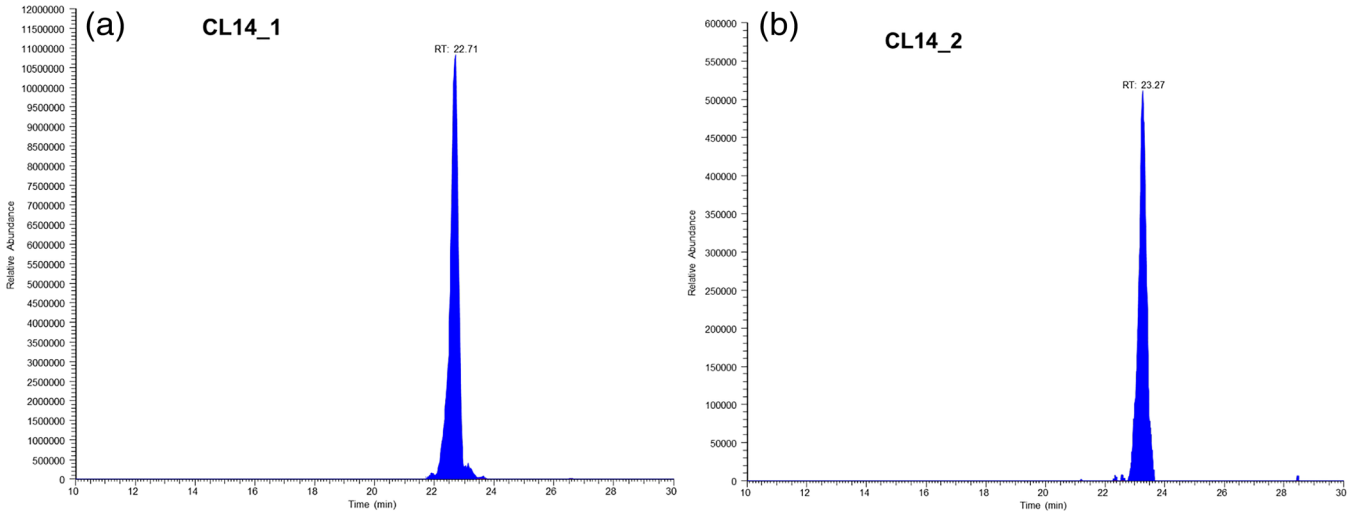

FIGURE 6 Results for two teeth sampled from CL14 that demonstrate internal consistency of sex (female)

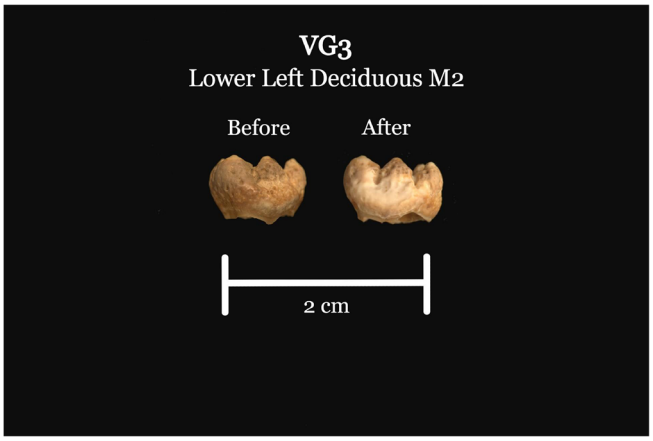

FIGURE 7 Before and after sampling condition of lower left deciduous M2 of VG3

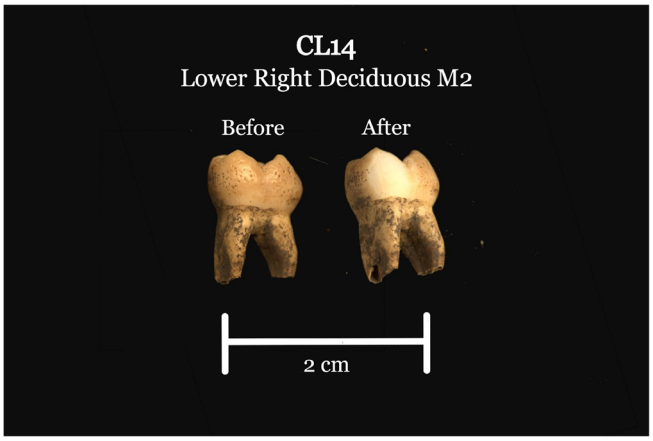

FIGURE 8 Before and after sampling condition of lower right deciduous M2 of CL14

peptides above. Not all male samples identified through this search method, however, possessed an identification for the AMELY peptide (Ser-Met[oxidized]-Ile-Arg-Pro-Pro-Tyr). Then, 19 of the 31 samples estimated to be male had this peptide identified (CL107, CL215_1, CL215_3, CL231_1, CL57_1, CL57_2, CL69, F331, F334, PID19_1, PID19_2, PID27, PID29, PID4, VG19_1, VG2_1, VG5_1, VG5_3, VG6_1, see Supplementary Table 2).

A drift in retention time was observed over the time course of the experimental analysis of these samples (Table 1). A polymer was clearly observable in some of the less abundant samples; potentially polyethylene glycol, which could account for the alteration in retention time. The polymer may originate from plasticware used during the ZipTip desalting step. Carry-over was kept at a minimum (<1%); however, the nature of these samples do affect the chromatography over time. This can be rectified by running a few blanks or standards with the chromatography returning to starting conditions. As these samples are simple acid etches, they are complex samples and will contain species other than peptides, such as proteins and lipids, which may interfere with the chromatography.

It was possible to retrieve sufficient peptide material even from incompletely developed perinatal teeth to estimate sex (Figure 3). The only sample for which this was not possible was CL9, although amelogenin peptides were identified. This tooth consisted only of the crown cusps, which were dark brown in color, very thin, and with little macroscopically observable enamel present (Figure 4). Tooth enamel is formed by secretion of the enamel protein matrix by ameloblasts and within this matrix very thin enamel crystallites grow from the enamel–dentine junction to the tooth surface (Fincham & Simmer, 1997; Robinson et al., 1981; Smith, 1998). This immature, organic-rich enamel is thus "formed," but it is only lightly mineralized: approximately 30% (Smith, 1998). As the enamel matures, this protein "scaffold" guides the nucleation and elongation of the enamel crystallites and is eventually resorbed during the transitional phase to permit the enamel crystallites to expand widthways during the maturation

phase and occlude the space previously occupied by the organic matrix (Fincham et al., 1999; Mann, 1997; Smith, 1998). As the organic matrix is resorbed, individual crystallites expand in a similar manner to the growth of tree rings, with the first enamel to form at the very center of each mature enamel crystal, leaving some residual protein trapped within the mature enamel (Boyde 1997, p. 18). It seems likely that CL9 was at an early stage of development when either no enamel had started to form at the enamel dentine junction, or any enamel that had formed was so poorly mineralized that it did not survive burial, due to either dissolution or mechanical abrasion.

There were other individuals in this study with the same age-at-death estimation (0–1 months/40GWA) as CL9 (e.g., PID6 and PID12) from which peptides were successfully recovered (Figure 5). For example, the teeth sampled from CL9 and PID6 were both lower right deciduous first molars and at a similar stage of cusp development, although the former was browner in color suggesting that it was less well mineralized. The discoloration of incompletely mineralized enamel during burial in soil is an indication that the trace element integrity is compromised (Montgomery, 2002, p. 331). For the most part, this does not appear to be the case for residual peptides in the enamel and the results indicate that peptides can be recovered from incompletely mineralized teeth at a very early stage of development if sufficient enamel is present. The CL9 and PID6 samples came from different sites (Coach Lane and Piddington) and therefore were subjected to different burial conditions (e.g., pH, soil, geology, hydrology, etc.), which may account for the failure to obtain a sufficient amount of peptides from CL9. However, all the other samples from Coach Lane were successful. It is more likely that there was no/insufficient enamel present on the dentine of this tooth and that this is the main constraint for the recovery of enamel peptides from archaeological infants.

Within the sample overall, incompletely mineralized teeth that were darker brown in color tended to yield fewer peptides, while those which were beige/light brown and gray in color (e.g., PID6) were more developmentally advanced and had greater quantity of peptides. The very early stage of development of the tooth from CL9 and lack of success in this instance demonstrates the lower qualitative limits of observable enamel mineralization needed from an individual tooth (deciduous or permanent) to successfully collect chromosomally linked peptides.

The largest quantities of peptides were generally recovered from the white/opaque, completely mineralized teeth. This was true for both deciduous and permanent teeth for individuals of all ages. As part of the study design, multiple samples were taken from teeth at different developmental stages from the same individual (n = 11), including deciduous and permanent teeth (e.g., VG2, VG3, VG6, CL14). In some cases, the amount of peptides recovered were greater in a completely mineralized (white/opaque) deciduous tooth than from an enamel sample (gray/brown) from a still mineralizing permanent tooth from the same individual (e.g., VG2, VG6, CL14). This further demonstrated that tooth type and dentition was not an important factor. The crucial determinant was the stage of enamel mineralization at the time of death. Importantly, for those individuals for whom a developing and mineralized tooth were sampled, all were internally consistent in terms of sex (Figure 6).

There was no relationship between the quantities of peptides recovered and whether the nonadult was male or female, or the tooth permanent or deciduous. Some of the samples with the largest quantities of peptides were the perinates excavated from Piddington, which was the earliest dated site. Parker et al. (2019) also noted that some of their older samples returned strong signals. It is currently not clear whether this is coincidental, or linked to burial environment, changes to the tooth surface during burial, or time. Nevertheless, there does not appear to be a negative correlation between duration of burial and quantity of peptides recovered (Parker et al., 2019; Stewart et al., 2017). Several challenges arise when sampling partially developed deciduous tooth crowns using the method described above. Many of these are related to the structural integrity of the developing tooth crowns. Firstly, this surface can weaken when exposed to acid (although the tooth crowns did not fragment). If enamel fragments become present within the acid, this etch should be discarded to prevent sample contamination, and the enamel etch repeated. The poorly mineralized tooth crown can absorb acid during sampling, but despite this, adequate recovery of amelogenin peptides was achieved, providing not all the acid solution was absorbed. The delicate nature and size of partially developed teeth, particularly perinatal tooth crowns, means that they are often difficult to handle during the etching process. Metal forceps and/or tweezers can be used to handle the teeth, so that the crowns are not damaged. It is imperative not to touch the acid etch with these implements to avoid contamination. Overall, the acid etch is slightly more noticeable for developing teeth, as it penetrates more deeply into the tooth compared to completely mineralized teeth. Images of two of the teeth prior to and after the sampling procedure are shown in Figures 7 and 8.

The biochemistry of enamel formation is unique in that, from the protease activity of KLK4 and MMP20, it results in many different peptides produced predominantly from specific regions of the protein (Lugli et al., 2019; Parker et al., 2019; Stewart et al., 2016, 2017). Peptides of variable length are mainly identified from the N and C-terminus. These "variforms" are present in different amounts and are identified across the samples. A lack of identification does not necessarily represent a lack of presence, it may simply reflect the shortcomings of the search algorithm used. Previous studies determining sex using amelogenin by LC–MS have advised caution when interpreting the results of these types of analyses due to the potential for higher levels of expression (up to 10x in adults) for the X-isoform of the protein compared to the Y-isoform, which may lead to false positive females (Fincham et al., 1991; Parker et al., 2019). However, it is unlikely that the interpretation of the data presented here resulted in false positive females as the two peptides used to identify chromosomal sex (Ser-Met[oxidized]-Ile-Arg-Pro-Pro-Tyr and Ser-Ile-Arg-Pro-Pro-Tyr-Pro-Ser-Tyr) are equivalent in intensity (for males), much lower in intensity compared to other AMELX peptides, and within the dynamic range of the mass spectrometer. Moreover, there is no evidence suggesting that nonadult teeth are expressing different amounts of the X and Y isoforms of the protein compared to adult

teeth. If there had been uncertainty about any of the data, the results of those samples would have been identified as indeterminate.

This method and others (Lugli et al., 2019; Parker et al., 2019) are essentially qualitative, albeit different approaches are used to ascertain sex. Absolute quantities of the peptides are not known and further research is required to identify the limits of quantification and detection of AMELX and AMELY peptides, in order to more confidently distinguish presence from absence of these in a sample. A limitation of this study is that the method was used on archaeological individuals of unknown sex, and since they are subadults, other than aDNA, there is no corroborative method to support these results. Part of the advantage of the Stewart et al. (2017) method continues to be that the technique is minimally destructive (Figures 7 and 8) compared to other variations that utilize bulk sampling methods (Froment et al., 2020; Parker et al., 2019) making it more palatable from a conservation and ethical perspective.

4 | CONCLUSION

The successful application of this method for estimating sex in nonadults, particularly perinates, has the potential to revolutionize the way that bioarchaeologists study infancy and childhood, including studies related to growth, epidemiology, and demography in the past. Moreover, bioarchaeologists are now better equipped to explore questions related to sex-dependent cultural treatment of infants and juveniles, including questions related to identity, weaning, infanticide, childcare, and puberty. This method can also contribute to the reliable, minimally destructive, and cost-effective identification of nonadult human remains in forensic contexts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rebecca Gowland: Conceptualization; funding acquisition; methodology; project administration; writing-original draft. **Nicolas Stewart**: Conceptualization; data curation; formal analysis; investigation; methodology; writing-review and editing. **Kayla Crowder**: Data curation; formal analysis; investigation; writing-review and editing. **Claire Hodson**: Data curation; formal analysis; investigation; writing-review and editing. **Heidi Shaw**: Project administration; writing-original draft; writing-review and editing. **Kurt Gron**: Formal analysis; investigation; methodology; writing-review and editing. **Janet Montgomery**: Conceptualization; formal analysis; methodology; supervision; writing-review and editing.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available in the ProteomeXchange Consortium (http://www.proteomexchange.org/) via the PRIDE [1] partner repository with the dataset identifier PXD021683.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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