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BRIEF COMMUNICATION

Desperately seeking stress: A pilot study of cortisol in archaeological tooth structures

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Abstract

Objectives: Cortisol is a glucocorticoid hormone produced through activation of the hypothalamic pituitary adrenal axis. It is known as the "stress hormone" for its primary role in the body's stress response and has been the focus of much modern clinical research. Within archaeology, only a few studies have analyzed cortisol in human remains and these have been restricted to hair (Webb et al., 2010; Webb, White, van Uum, & Longstaffe, 2015a; Webb, White, van Uum, & Longstaffe, 2015b). This study examines the utility of dentine and enamel, which survive well archaeologically, as possible reservoirs for detectable levels of cortisol.

Materials and methods: Then, 69 teeth from 65 individuals from five Roman and Post-Roman sites in France were tested via competitive enzyme-linked immunosorbent assay (ELISA) to assess and quantify the cortisol concentrations present within tooth dentine and enamel.

Results: In both tooth dentine and enamel, detectable concentrations of cortisol were identified in multiple teeth. However, concentrations were low and not all teeth yielded results that were measurable through cortisol ELISA. Differences in cortisol values between dentine and enamel could suggest different uptake mechanisms or timing.

Discussion: These results suggest that cortisol is incorporated within tooth structures and merits further investigation in both modern and archaeological contexts. Analysis of the results through liquid chromatographic–mass spectrometry would verify current results and might yield values that could be better integrated with published cortisol studies. Future studies of cortisol in tooth structures would greatly expand the research potential of cortisol in the past and could have implications for studies of human stress across deep time.

KEYWORDS

glucocorticoid hormones, bioarchaeology, dentine, enamel, ELISA

1 | INTRODUCTION

The detrimental impact of "stress" on the body has been an important focus of study within the clinical literature. In bioarchaeology, the subject of more generic "health stress" is also a primary concern, with a considerable body of research directed toward nonspecific skeletal indicators (see Klaus, 2014; Reitsema & McIlvaine, 2014; Temple & Goodman, 2014). However, studies addressing stress experiences within different societies and over time have been limited by methodological constraints. Cortisol has previously been identified and analyzed in human hair from archaeological contexts; however, hair preserves only in exceptional circumstances (Webb et al., 2010; Webb, White, van Uum, & Longstaffe, 2015a; Webb, White, van Uum, & Longstaffe, 2015b). To gain a broader understanding of "stress" in the past, this study aims to adapt and develop a new method for analyzing cortisol hormone concentrations (indicative of stress) from dental tissues (Nejad, Jeong, Shahsavarani, Sung, & Lee, 2016). Given the high survival of teeth in archaeological contexts, cortisol concentrations from teeth have the potential to provide an important new avenue for research on stress in the past.

1.1 | Cortisol

Studies assessing stress and irregularities in stress response in contemporary human and animal populations routinely test cortisol concentrations. Cortisol, often referred to as the "stress hormone," is the primary adrenal chemical messenger produced by the hypothalamic pituitary adrenal (HPA) axis in the presence of a stressor (Charmandari, Tsigos, & Chrousos, 2005). Cortisol prepares the body to respond to a threat, prioritizing processes serving immediate energetic needs (cardiac output, glucose, and red blood cell production), and diverting energy away from those biological processes that are secondary to immediate survival (growth, digestion, reproduction) (Panagiotakopoulos & Neigh, 2014; Sapolsky, Romero, & Munck, 2000). As such, activation of the HPA axis and heightened cortisol secretion are an adaptive and important part of the human stress response, promoting survival (Beehner & Bergman, 2017). However, when chronically activated by stressors, the HPA axis can become dysregulated, resulting in abnormal cortisol secretion and negative effects on the body. Cortisol inhibits inflammatory and allergic reactions, has immunosuppressive effects (decreases antibody production and the proliferation of immune B-cells, T-cells), can reduce growth in children (inhibits growth hormone, modulation of osteoclast, osteoblast, and osteocyte function), cause osteoporosis in adults (degradation of collagen, reduction of calcium), delay wound healing by reducing fibroblast proliferation, and also impacts metabolic processes through gluconeogenesis (releasing amino acids from proteins in skeletal muscle and bone), glycogenolysis, and lipolysis (Canalis, 2005; Panagiotakopoulos & Neigh, 2014; Sapolsky et al., 2000). Assessing cortisol concentrations in human tissues therefore can be informative about relative "stress experience" (stressors an individual or population encountered). This data can contribute toward a greater understanding of human responses to differing social and environmental stressors in the past.

In addition to the stress response, cortisol has important regulatory functions in cardiovascular, metabolic, and immunological systems (Charmandari et al., 2005). Without appropriate levels of cortisol, humans cannot survive (Dallman & Hellhammer, 2011). Outside of the stress response, the HPA axis has a natural circadian rhythm, secreting the highest levels of cortisol approximately 30–45 min after waking and then decreasing throughout the day, reaching the lowest levels during sleep (Dallman & Hellhammer, 2011). To synthesize cortisol, the HPA axis must be activated, initiating a hormone cascade beginning in the brain. The hypothalamus produces and transfers corticotropin-releasing hormone to the anterior part of the pituitary gland, stimulating the production of adrenocorticotropic hormone (ACTH). The ACTH is then transported throughout the body via the blood stream and detected by hormone-specific receptors in the adrenal gland, instigating the synthesis of glucocorticoid hormones, of which, cortisol is the most abundant. Cortisol travels through the bloodstream in both active (unbound) and inactive (bound to the protein transcortin or albumin) forms (Gow, Thomson, Rieder, van Uum, & Koren, 2010). From the blood stream, unbound cortisol passes through cell membranes to activate glucocorticoid receptors in the cytoplasm, where the complex is translocated into the cell nucleus, binding to glucocorticoid-response elements and modulating the transcription of target genes (Gow et al., 2010; Greaves, Jevalikar, Hewitt, & Zacharin, 2014). Although free or unbound cortisol makes up only a small proportion of total cortisol, usually 10% or lower, it is the free cortisol that acts upon and regulates systems within the body (Dallman & Hellhammer, 2011). The production of cortisol is tightly controlled through a negative feedback loop, where upon the arrival of cortisol back in the pituitary gland, the hormone cascade is suppressed (Dickerson & Kemeny, 2004; Sapolsky et al., 2000). The diurnal rhythm of the HPA axis and, therefore, cortisol secretion, is not uniform across the life course or between sexes, with the normal rhythm achieved at around 3 months of age (Gunnar & Donzella, 2002). Other major changes in cortisol production arise from reaching developmental thresholds such as adrenarche, puberty, pregnancy, and menopause (Greaves et al., 2014).

1.2 | Cortisol in bioarchaeology

Because stressors and the stress response have a significant impact on health and wellbeing, the study of stress is important in bioarchaeological analyses of past populations. Bioarchaeological methods are most often indirect and nonspecific measures of stress (growth disruption, dental enamel hypoplasia, cribra orbitalia) that require an unknown "threshold of stress" to be met for development of the lesions in the skeleton (Goodman & Rose, 1990). The nonspecificity of these skeletal and dental responses is problematic for interpretations of stress. Recent research has begun to explore cortisol and its role within the HPA axis in relation to skeletal stress markers and bone growth in archaeological populations

(Gowland, 2017; Klaus, 2014; Reitsema & McIlvaine, 2014; Rodney & Mulligan, 2014; Scott, Choi, Mookherjee, Hoppa, & Larcombe, 2016; Webb et al., 2010, 2015a, 2015b; Weston, 2011). Investigations of cortisol in archaeological hair have yielded successful results, demonstrating cortisol preservation over hundreds of years (Webb et al., 2010, 2015a, 2015b). The cortisol concentrations found in archaeological hair provide a measure of chronic "stress" that is direct, quantifiable and comparable with modern stress studies, circumventing some of the challenges in analyzing indirect stress indicators. Although progressive and exciting, analyses of cortisol from archaeological hair have several limitations. Some research has indicated that cortisol is removed from hair when washed (even just with water) (Davenport, Tiefenbacher, Lutz, Novak, & Meyer, 2006; Hamel et al., 2011; Meyer & Novak, 2012). More problematic is that very few archaeological individuals have hair preserved for analysis thereby severely limiting the application of this method. A recent study by Nejad et al. (2016) successfully detected cortisol from modern tooth dentine, suggesting that cortisol can be found in archaeological tooth structures. The ability to assess cortisol from teeth would have important implications for future research as teeth represent some of the best-preserved elements of the body in bioarchaeological contexts. Additionally, tooth structures have a much lower potential for contamination in the burial and post burial environment than hair or other skeletal elements (Turner-Walker, 2008), and are not subject to leaching in the same way as hair. Obtaining a more direct measure of this stress hormone in the past has the potential to provide a more nuanced understanding of the relationship between skeletal stress indicators and allows more direct comparisons with clinical measures in modern populations.

This study seeks to develop and test a new method for assessing stress in archaeological human remains and has the following aims and objectives:

1. To determine if cortisol concentrations can be obtained from archaeological dentine or enamel through enzyme-linked immunosorbent assay (ELISA).
2. To investigate possible correlations between cortisol concentrations in dentine and enamel dental tissues.
3. To assess cortisol concentrations and variation based on biological sex.

2 | MATERIALS AND METHODS

To conduct the cortisol analysis, 69 teeth were selected from 65 individuals from five Roman and Post-Roman sites in France (Table 1). Of the 69 teeth, 29 were sampled twice, to test dentine and enamel concentrations within the same tooth, for a total of 96 samples. The skeletal collections are in the care of three laboratories: De la Préhistoire à l'Actuel: Culture, Environnement et Anthropologie (PACEA), Bordeaux; the Centre de recherches archéologiques et historiques anciennes et médiévales (CRAHAM), Caen; and the Centre de Conservation et d'Etudes de Lorraine (CCEL), Metz. Teeth from both male and female individuals were selected.

TABLE 1 Sites where teeth were collected

Site	Location	Dating	Individuals	Teeth	Dentine samples	Enamel samples	Total samples	Site source	Lab
Rue de Jacques Brel	Saintes	First–second c	10	11	11	10	21	Baigl, Farago-Szekeres, and Roger (1997)	PACEA
La Granède	Millau	Fourth–seventh c	4	4	2	4	6	Saint-Pierre (2010)	PACEA
Fontoy	Thionville	Fourth–seventh c	18	18	15	15	30	Seilly (1995)	CCEL
Rue du Tombois	Metz	Fourth c	9	9	5	9	14	de Filippo, Feller, Vidal, Genevieve, and Beck (2000)	CCEL
Saint Martin de Fontenay	Caen	Fourth–seventh c	24	27	27	0	27	Pilet, Alduc-Le Bagousse, and Buchet (1994)	CRAHAM
Total			65	69	60	38	98		

Abbreviations: CCEL, Centre de Conservation et d'Etudes de Lorraine; CRAHAM, Centre de recherches archéologiques et historiques anciennes et médiévales; PACEA, De la Préhistoire à l'Actuel: Culture, Environnement et Anthropologie.

Individual teeth were chosen based on several criteria, with a preference for permanent second molars wherever possible. The teeth selected had to be:

- Permanent teeth that began development after 4 months of age to exclude teeth that might have been affected by the period when the HPA axis is still developing and has yet to reach adult rhythms and values
- Free from pathological lesions or dental wear that might expose the dentine to the oral environment during life or the burial environment after death.

To test whether cortisol concentrations could be obtained from archaeological tooth structures, a method that identified cortisol in modern dentine (Nejad et al., 2016) was adapted and developed. Then, 65 individuals were tested for cortisol concentrations, with 38 enamel samples and 60 dentine samples deriving from a total of 69 teeth. For adolescents included in this analysis, age-at-death was determined by the stage of dental development (AlQahtani, Hector, & Liversidge, 2010). Adult age-at-death estimations were based on degenerative changes in the auricular surface and pubic symphyses (Brooks & Suchey, 1990; Lovejoy, Meindl, Pryzbeck, & Mensforth, 1985; Schmitt, Murail, Cunha, & Rougé, 2002) and dental wear (Brothwell, 1981). Sex estimates were based on sexually dimorphic features of the skull and pelvis (Brothwell, 1981; Bruzek, 2002; Phenice, 1969).

2.1 | Tooth sample preparation

Before destructive sampling, each tooth was recorded and photographed on each of its five surfaces (occlusal, mesial, distal, lingual, buccal) in accordance with ethical guidelines for the destructive analysis of archaeological human skeletal remains (BABAO, 2019). To assess cortisol concentrations, the teeth were cleaned and prepared before subsequent ELISA analysis. These processes are a modified version of the methods proposed by Nejad et al. (2016). Each tooth was washed in isopropanol to remove any contaminants present on the tooth surface and left to dry. Once dry, the tooth was bisected from crown to root using a diamond-tipped saw to expose the dentine. Using a rosehead dental burr, 150 mg of dentine were drilled out of the tooth in a fine powder, avoiding circumpulpal dentine to prevent any possible pulp remnants. Enamel samples were abraded with a dental burr to produce a chip of core enamel of 300 mg. The enamel was then ground into a powder. To prevent contamination between the samples, all tools were washed in ultrapure water, placed in an ultrasonic bath for 5 min and then dried with acetone in between sampling each tooth. Aliquots of dentine (150 mg) and enamel (300 mg) were weighed into microcentrifuge tubes and 1 ml of methanol was added to the samples. The tubes were then left to incubate for 24 hr, with slow rotation at room temperature to elute the cortisol. After incubation, the samples were transferred to clean microtubes and dehydrated using a nitrogen stream. All samples were frozen until the day the ELISA was performed.

2.2 | Enzyme-linked immunosorbent assay

A competitive ELISA salivary cortisol kit by Salimetrics was used to assess and quantify the cortisol concentrations present in the tooth dentine and enamel. Currently, there is no kit developed specifically for the analysis of cortisol in any tooth or bone tissue; however, salivary kits have been successfully used to analyze both modern dentine (Nejad et al., 2016) and archaeological hair (Webb et al., 2010, 2015a, 2015b). This is a competitive ELISA immunoassay kit. On the day of analysis, samples were re-constituted with 30 μl of phosphate buffer. Cortisol conjugated to horseradish peroxidase for the antibody binding sites are coated on a microliter plate. After incubation, unbound components are washed away. Bound cortisol enzyme conjugate is measured by the reaction of the HRP enzyme to the substrate tetramethylbenzidine, producing a blue color. Stopping the reaction with an acidic solution yields a yellow product. The optical density is read on a standard plate reader at 405–450 nm. The amount of cortisol enzyme conjugate detected is inversely proportional to the amount of cortisol present in the sample. The ELISA kit was run according to all specifications made by the manufacturer. A standard curve was generated for each kit based on standards and controls, and fourth-order polynomial curve fit regressions were produced to define the cortisol concentrations within each sample. Student *t* tests and Mann–Whitney *U* tests were used to analyze differences between the sexes and tooth structures as appropriate.

3 | RESULTS

Teeth were selected from 25 females, 21 males, and 19 individuals of indeterminate sex. Age estimations followed standard age categories (18–25, 26–35, 36–45, and 45+ years) and are presented here by the mean of their respective age range (18–25 = 21.5 years) for visual clarity. Two individuals could only be aged as "adult" (18+ years) and are placed at the far right of the plot ("adult") (Figure 1).

Two ELISAs were performed, one for dentine and the other for enamel. Concentration sensitivity of the Salimetrics salivary cortisol kits were >0.007 μg/dl with an assay range of 0.012–3.000 μg/dl, as determined by the kit manufacturer. Of the 96 tooth samples tested for cortisol concentrations, 32 (16 dentine and 16 enamel samples) yielded results detectable through ELISA (Figure 1), the remaining 64 samples produced values below the 0.007 μg/dl concentration sensitivity threshold. In only 2 of the 29 teeth tested for both dentine and enamel cortisol, did both tissues yield detectable concentrations. In both cases, the dentine values were higher than enamel values (Figure 1). No other clear patterns were observed. Data variation was up to 10%.

Comparisons between valid dentine and enamel concentrations (32 samples) were performed using independent sample *t* test in SPSS with significant results ($p < .001$) (Table 2). Differences in cortisol concentrations between males, females, and indeterminate individuals were not significant in either dentine ($p = .808$) or enamel ($p = .407$) (Mann–Whitney) (Table 2).

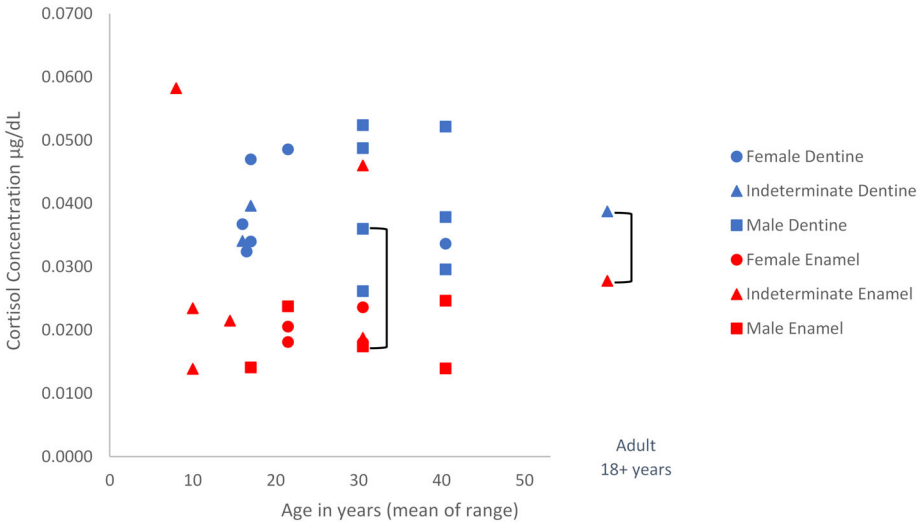

FIGURE 1 Tooth cortisol concentration (µg/dl) per 300 mg tissue sample, plotted against age-at-death mean of range. Red symbols denote enamel and blue symbols are dentine values. Circles are female, triangles are indeterminate sex, and squares are male individuals. Brackets indicate representative cortisol concentrations from the same tooth

TABLE 2 Detectable cortisol concentrations (mean) (µg/dl)

	Female	Indeterminate	Male	*p*-Value	Total
Dentine	0.0388	0.0375	0.0404	.808	0.0393
Enamel	0.0201	0.0300	0.0188	.407	0.0240

4 | DISCUSSION

Results of this study suggest that cortisol can be detected in archaeological tooth material. Although this finding is encouraging, the results are less straightforward than those obtained from either modern tooth dentine (Nejad et al., 2016) or archaeological hair (Webb et al., 2010, 2015a, 2015b). In common with other studies (Nejad et al., 2016; Webb et al., 2010, 2015a, 2015b), these results have not yet been validated with gas or liquid chromatographic mass spectrometry (GC/LC/MS), but this is an important next step to identify any cross reactivity within the assay between cortisol and other steroids or metabolites (Fraser et al., 2010; Wheeler & Barnard, 2010). In the absence of the possibility to perform this additional analysis, the results are cautiously interpreted in terms of their potential for future analyses. Although the observed cortisol concentrations were low, some patterns in the data do emerge. Values were broadly consistent, with only two individuals showing any marked difference from group averages. This result indicates that despite the low concentrations detected, differences between individuals and groups may be observed, rendering the method useful in assessing stress, especially with further development.

Clear differences were observed between dentine and enamel concentration values ($p < .001$), indicating a potential divergence in uptake mechanism or timing. As with hair, the mechanisms for incorporating cortisol in tooth structures are not yet understood, but are based on studies that have used human hair and/or teeth to assess exposure to drugs, toxins, and pollutants (Altshuller, Halak, Landing, & Kehoe, 1962; Andra, Austin, & Arora, 2015; Chiesa et al., 2017). It is hypothesized that free cortisol circulating in the bloodstream is taken up into the structures during periods of growth or development (Haustein, Thiele, & Stangel, 1994; Klima, Altenburger, Kempf, Auwärter, & Neukamm, 2016; Klima, Huppertz, Altenburger, Auwärter, & Neukamm, 2015; Meyer & Novak, 2012; Nejad et al., 2016; Spinner et al., 2014; van Uum et al., 2008). As such, it is suggested that cortisol concentrations in dentine and enamel would reflect cortisol in the blood during development of the tooth structures. Dentine and enamel have different properties that would affect the mechanisms and timing of cortisol incorporation in the different tissues (Cippitelli et al., 2018; Klima et al., 2016), which could explain observed differences in this study. Enamel is composed of 96% inorganic materials and once mineralized is isolated from the blood supply and does not remodel (Lee, Seo, Park, Bae, & Cha, 2017). As a result, any chemical or analyte retrieved from the enamel is likely to derive from the period of childhood when that part of the tooth was mineralized (Rubin, 2018). Tooth specific patterns of enamel formation have been correlated with biological age (AlQahtani et al., 2010; Moorrees, Fanning, & Hunt Jr, 1963a; Moorrees, Fanning, & Hunt Jr, 1963b), localizing the period during which exposure could have taken place, but also revealing limitations in the amount of time chemical signals could be incorporated into enamel (Rubin, 2018). Concentrations of other biochemicals obtained from enamel have been low in comparison with dentine and other types of tissue potentially because of its high mineral content and limited window of exposure (Klima et al., 2016). Dentine is avascular with no blood supply and is composed of approximately 70% inorganic, 20% organic materials (including proteins and proteoglycans) and 10% water (Lee et al., 2017; Orsini et al., 2009; Turner-Walker, 2008). Once formed (approximately 3–4 days), dentine undergoes very little remodeling (Beaumont & Montgomery, 2016) and like enamel, should capture exposure to biochemical concentrations during a discreet period of development

(Andra et al., 2015). Studies have found drugs in higher concentrations in tooth dentine than in enamel, likely because of the less mineralized nature of dentine (accepts molecules more easily) (Klima et al., 2015; Klima et al., 2016; Nässtrom, 1996). This explanation does fit with the data in the present study.

Alternatively, it is possible for exogenous substances to become incorporated in tooth structures after mineralization. Some studies have been able to detect drugs in dental hard tissues, when drug usage was known to have occurred close to the time of testing/death (Houari, Loiodice, Jedeon, Berdal, & Babajko, 2016; Klima et al., 2016). Unless the highly mineralized structure of enamel has been degraded in some way (ex. carious lesions), drug incorporation via oral fluids has been reportedly quite low (although detectable) in drug users (Klima et al., 2015, 2016). Dentine is a more complicated tissue to consider. Despite its avascularity, dentine has a relationship with dental pulp (and thereby the blood supply) through dentinal tubules. Dentinal tubules could transmit chemicals or substances in the still living, enervated pulp to the dentine (Ghazali, 2003; Spinner et al., 2014) and the inverse from dentine to dental pulp and blood supply (Pashley, 1979). Higher concentrations of drugs in tooth dentine (compared with enamel) are purportedly the result of its relationship with dental pulp through the dentinal tubules (Ghazali, 2003; Klima et al., 2015; Klima et al., 2016; Nässtrom, 1996). That enamel cortisol levels identified in this study were significantly lower would seem to suggest that as with hair, free cortisol circulating in the blood gets trapped in enamel during formation, and is largely unaffected thereafter (especially as damaged or degraded teeth were excluded from analysis). However, it is not entirely clear whether dentine cortisol levels reflect circumstances during the development of the tooth or an amalgam of exposure across a lifetime. Based on the results of this analysis, tooth dentine and enamel generate different concentrations of cortisol, even from within the same tooth. However, because only two teeth sampled for both dentine and enamel generated detectable concentrations within the assay, future research should explore this relationship in more detail. It is worth noting that in both cases, the dentine values were higher than enamel values.

4.1 | Sex-based differences

No differences in cortisol concentrations between the sexes were observed in either of the dental tissues. This could be a result of the small sample size or the overall low values obtained in this analysis. Sex-based differences in HPA axis and cortisol concentrations are thought to develop at puberty, but reported results are not consistent (Kirschbaum, Wüst, & Hellhammer, 1992; Raven & Taylor, 1996). Nejad et al. (2016) also did not find differences between male and female cortisol concentrations in their analysis of modern tooth dentine. If cortisol is being deposited within the dental tissues during mineralization, the results are reflecting cortisol exposure before puberty and might explain the lack of difference between male and female cortisol concentrations in both this analysis and in modern dentine.

4.2 | Low concentrations detected

The concentration values obtained in this study were low in comparison with modern dentine data (Nejad et al., 2016), archaeological hair concentrations (Webb et al., 2015a), and modern standards from hair, saliva, blood, and urine (Aardal & Holm, 1995; Deutschbein et al., 2011; Gonzalez et al., 2019). Not all of the samples in this analysis met the minimum detection threshold of the ELISA cortisol kits. Although this finding is not exclusive to this study (Gow et al., 2010), it is not clear why the values are low. Accordingly, there are several possible explanations for these results with implications for future analyses. Cortisol may not be as readily deposited within tooth structures as in other tissue types. If metabolized too rapidly, biochemicals or compounds cannot be incorporated into the growing structure (Greff et al., 2019), and this includes hair as well as teeth. Circulating cortisol is thought to have a half-life of 80 min in the body, which may not be long enough for detectable volumes to become consistently incorporated into dental structures (Greff et al., 2019; Isaac et al., 2017). However, cortisol is constantly in circulation, relatively small in size (362 Da) and lipophilic, characteristics that predispose and increase the likelihood of deposition within tooth dentine and enamel (Greff et al., 2019; Haustein et al., 1994; Klima et al., 2015, 2016; Spinner et al., 2014). Because the mechanisms for incorporation are not yet understood, it may be that the time window for potential exposure during amelogenesis and odontogenesis is more limited than for hair or other tissues. Cortisol concentrations from modern dentine suggest that detectable quantities of cortisol can be present within the tissue, seemingly refuting hypotheses that cortisol is too rapidly metabolized to be incorporated into dental tissues (Nejad et al., 2016). Further, there is evidence that enamel and dentine can be influenced by hormones and exogenous chemicals (Ahlgren, 1968; Houari et al., 2016). Ameloblasts (enamel forming cells) have been found to contain glucocorticoid hormonal receptors depending on their developmental stage (Houari et al., 2016). It has even been suggested that amelogenesis is regulated by endogenous steroid hormones, affecting the quality, hardness and mineralization of the enamel (Houari et al., 2016; Pawlicki, Knychalska-Karwin, Stankiewicz, Jakób-Dolezal, & Karwan, 1992). Hydrocortisone (a synthetic variant) has been found to retard dentine formation under experimental conditions (Ahlgren, 1968). Studies of drug concentrations within dental hard tissues (Cattaneo, Gigli, Lodi, & Grandi, 2003) have been criticized for not adequately removing pulp tissues before processing, potentially confounding reported results (Rubin, 2018; Spinner et al., 2014). Although Nejad et al. (2016) reported multiple cleaning processes, they do not specify whether the pulp chambers of sampled teeth were cleaned. It is, therefore, possible that their reported values are in fact reflecting cortisol concentrations from dental pulp, containing a blood and nerve supply, in addition to tooth dentine.[1] This could explain why their reported values were higher than the values obtained purely from tooth dentine or enamel in this study. Further analyses would be necessary to explore this explanation of results.

Alternatively, tooth cortisol could have degraded during decomposition, in either the burial or post-burial environments. Obtained

cortisol concentrations from modern tooth structures (7.92 ± 0.91 ng/mg) (Nejad et al., 2016) were markedly higher than results in the present analysis. Although cortisol is a stable natural steroid, long-term exposure to 37°C and above can lead to significant degradation, and this is worth considering in the future (i.e., historic climate factors, including temperature, humidity, and effects on biological markers) (Khonmee et al., 2020). It is possible that higher cortisol concentrations in modern analysis were a result of the inclusion of dental pulp and blood supply, and lower environmental temperature exposure. Again, further testing of cortisol degradation should clarify these findings. However, excellent preservation of cortisol in archaeological hair from comparable time periods (1–1,000 AD) has been reported (Webb et al., 2010, 2015a, 2015b). As hair is rarely preserved, it may be that burial conditions and post-burial treatment are better for individuals with hair than individuals decomposed to only skeletal material. For hair to be preserved archeologically, certain burial conditions must be present and because of their rarity, skeletons or mummies with preserved hair are more likely to be handled more delicately by fewer people than entirely skeletonized individuals. In clinical analyses, however, cortisol can withstand multiple heating and cooling cycles without appreciable decomposition until reaching a heat of 220°C (Hamel et al., 2011). It is highly unlikely that the individuals in this analysis, excavated and stored in laboratories, had ever experienced such high heats, leaving no other trace. It is thought that teeth undergo very little diagenetic change in post burial environments and can maintain chemicals substances, protecting them from alteration or degradation within the hard tooth structures (Cippitelli et al., 2018; Lee et al., 2017; Spinner et al., 2014). Although ideal for preservation over long periods of time, the structure of both dentine and enamel can make the extraction of organic chemicals challenging (Lee et al., 2017). Perhaps the low values in this study are attributable to challenges in separating out cortisol bound to hard dental tissues, as has been found in drug analysis of hair (Spinner et al., 2014). Again, future analyses should test for cortisol degradation and may be able to identify or overcome some of these challenges.

As with hair, cortisol within tooth structures reflects chronic stress levels, averaging out normal functioning cortisol and acute stress events (Russell, Koren, Rieder, & van Uum, 2012). Cortisol or its animal equivalent (corticosterone) is only produced by mammals, and although external animal or human corticosterone/cortisol infused fluids or fats could be present in burial environments, several cleaning steps were undertaken to remove possible external contaminants from the samples. Dentine and core enamel would only have been exposed to the burial environment if the tooth was damaged or degraded. Other related steroid hormones, and cortisone, a precursor and metabolite of cortisol, could display cross-reactivity with antibodies on the ELISA plate. GC/LC/MS techniques can identify these different hormones, which would clarify this issue further. However, the cortisol ELISA utilized in this study displays high sensitivity and selectivity for human cortisol, with no detectable cross-reactivity (<0.004%) to related steroids. Minor-cross reactivity is possible with some synthetic steroids. This is not expected to be an issue within this study, as the teeth date to Roman and Post-Roman periods and precautions were taken to avoid modern pharmaceutical contamination. Although this study has generated promising results, several steps need to be taken to further refine the method, for appreciable conclusions to be drawn. The first step is to validate the result of the assays using GC/LC/MS or other methods for controlling cross-reactivity (Fraser et al., 2010; Wheeler & Barnard, 2010). Once validated, further experimentation on potential uptake mechanisms and different methods of extraction have enormous potential to advance the method and perhaps permit a reduction in the minimum required sample (e.g., hair—10–15 mg; van Uum et al., 2008). This method allows the identification of stress in skeletal individuals in a different way, which has the potential to be more quantitative and direct than current macroscopic methodologies. Future studies will consider cortisol concentrations in relation to various qualitative pathological indicators, including growth disruption and dental enamel hypoplasia.

5 | CONCLUSION

In both tooth dentine and enamel, measurable concentrations of cortisol were identified in multiple teeth. However, concentrations were very low (0.02–0.04 μg/dl) and not all teeth yielded results that were detectable through cortisol ELISA methodologies. These results suggest that cortisol is deposited within tooth structures and merits further investigation in both modern and archaeological contexts. Future testing for cortisol degradation and analysis of the results through GC/LC–MS would verify results and may yield values that could be better integrated with published cortisol studies. This study has acted as a proof of concept, and these results expand the research potential of cortisol in the past and could have implications for studies of human stress across deep time.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Leslie Quade: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; writing-original draft; writing-review and editing. **Paul L. Chazot**: Formal analysis; methodology; resources; supervision; writing-review and editing. **Rebecca Gowland**: Conceptualization; funding acquisition; supervision; writing-review and editing.

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ENDNOTE

[1] It is also not clear in the Nejad et al. (2016) study if dentine was separated from enamel prior to analysis. From the wording of the text, it seems likely that after cleaning, the entire tooth was ground down to generate the 1 g of tooth powder.

DATA AVAILABILITY STATEMENT

The data generated in this study are available from the corresponding author upon request, with eventual plans to make the data available in an appropriate research data repository.

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