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Title

First successful detection of oestrogen, progesterone and testosterone in multiple human hard tissues, and their use as potential biomarkers of pregnancy

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Abstract

The sex steroid hormones oestrogen, progesterone, and testosterone have never been detected in modern or archaeological human skeletal tissues using enzyme-linked immunosorbent assay (ELISA) analysis, and there are no standard protocols for their extraction. As progesterone is a biomarker of pregnancy in living individuals, its detection in skeletal remains would substantially improve the visibility of pregnant individuals in the archaeological record and furnish a novel means of exploring female life histories in the past. The present study demonstrates that oestrogen, progesterone and testosterone can be detected in the hard tissues of ten individuals of known sex dating from the 1st to 19th centuries CE and evaluates their potential as biomarkers of pregnancy. The cohort comprised seven females of varied parity status and three males. A novel ELISA methodology was developed for hormone extraction from prepared tissue samples of bone, dentine, enamel, root from second and third permanent molars, and dental calculus (n=74). Oestrogen, progesterone and testosterone concentrations were measurable in bone, dentine, enamel, and root samples, whereas only progesterone and testosterone were detected in dental calculus. Elevated progesterone concentrations were detected in the bone and tooth structures of one pregnant female, all individuals with *in utero* pregnancies had undetectable testosterone, and those associated with fetal remains presented elevated progesterone levels in dental calculus. Our findings demonstrate the feasibility and significant potential of the ELISA method for the detection of sex hormones in human skeletal remains to examine the reproductive histories of past populations.

Keywords

ELISA, hormones, fertility, pregnancy, skeletal remains

Abbreviations

Ancient Deoxyribonucleic Acid (aDNA)
Enzyme Immunoassay (EIA)
Enzyme-Linked Immunosorbent Assay (ELISA)
Percentage Coefficient of Variability (%CV)
Permanent second molar (M2)
Permanent third molar (M3)
4th Order Polynomial Curves (4PLC)

1. Introduction

Research into women's experiences in the past has moved to the forefront of archaeological agendas in the 21st century (Gilchrist, 2007; Gowland, 2018, 2020; Gowland and Halcrow, 2019; Le Roy and Murphy, 2020a), yet the methodological limitations of detecting pregnancy in skeletal remains have conspired to obscure evidence of women's reproductive lives. The aim of this study was to develop and apply a novel method to examine the reproductive histories of women in the past using archaeological human remains. We achieve this by demonstrating the first successful detection of sex steroid hormones oestrogen, progesterone, and testosterone in either modern or ancient human skeletal tissues using enzyme-linked immunosorbent assay (ELISA). This establishes their potential to substantially improve the visibility of pregnant individuals in the archaeological record and illuminate the reproductive histories of past populations.

The presence of *in utero* fetal remains in a burial context demonstrates pregnancy at the time of death and is the only direct bioarchaeological identifier of pregnancy (Le Roy and Murphy, 2020: 214). For fetal remains to be considered *in utero*, they must accompany a mature skeleton in an anatomical position in keeping with an unborn fetus, namely within the abdominal or pelvic cavity or partially within the pelvic inlet, the latter suggesting death in childbirth (Le Roy and Murphy, 2020a). The placement of remains is vital to differentiate an *in utero* fetus from those of a post-natal individual buried within the same grave or coffin as an adolescent or adult who may not be their mother (Lewis, 2022). This important distinction is not always possible in the archaeological record due to taphonomic degradation, grave disturbance, the misidentification of fetal bones (Roberts and Cox, 2003; Willis and Oxenham, 2013), and the surgical removal of the fetus post-mortem (Blumenfeld-Kosinski, 2019; Woodward, 1836). Consequently, examples of reliably *in utero* remains are rare (Halcrow, Tayles and Elliott, 2017: 88). In a review of 8000 graves from 70 cemeteries dating from the 11th - 17th centuries CE in England, Scotland and Wales, only one example was identified (Gilchrist and Sloane, 2005) and Craig-Atkins and Fissell (2024: 72) identified only seven examples from the 18th-19th centuries CE in England.

Indirect evidence of past pregnancies has been sought through skeletal markers of parity. Parturition scars – morphological features on the pelvic bones considered to be an indicator of childbirth – have recently received renewed attention, but their utility and reliability remain questionable (Pany-Kucera et al., 2022; Pany-Kucera and Rebay-Salisbury, 2022; Ubelaker and De La Paz, 2012). A modern study of parturition scars using CT imaging in a 21st-century population with known parity, demonstrated that average parity for a population could be estimated via a regression analysis model using a mean dorsal pitting scoring system, but individual parity status could not be predicted (Waltenberger et al., 2022). Despite these promising population-level results, no reliable methods exist to establish parity at the individual scale in osteological remains.

New methods that can accurately identify pregnancy from skeletal remains are needed if we are to accurately reconstruct past women's reproductive histories, interpret their experiences within social and cultural contexts, and make effective comparisons between modern and archaeological populations.

1.1. Sex steroid hormone biomarkers

The sex steroid hormones oestrogen, progesterone and testosterone are present in humans regardless of chromosomal genotype and are vital to sexual development and reproduction. Their levels can be measured in blood serum, hair, saliva, and urine and are used in clinical medicine to explore puberty, fertility, and pathology (Balen, 2007; Li et al., 2024; Lucaccioni et al., 2021; Marceau et al., 2021; Zeberg et al., 2020). To date, only oestrogen and testosterone have been successfully detected in ancient remains - in hair samples of mummified individuals dating to 50-450 CE (Tisdale et al., 2019) and bone samples of human remains dating to between 2007 CE - 4850 BCE (Mark et al., 2011). None of the three sex steroid hormones have previously been detected in tooth structures or calculus in ancient or

modern samples. However, cortisol, a known hormonal biomarker for stress, has recently been detected in archaeological hair (Kaufman, 2024; Tisdale et al., 2019; Webb et al., 2010), bone (Kaufman, 2024), tooth dentine, and enamel (Nejad and Jeong, 2016; Quade et al., 2023, 2021). These studies used ELISA to identify the presence of cortisol and quantify the hormone concentration in each sample type. The ELISA method also has potential to detect and quantify sex steroid hormones in human hard tissues, which survive considerably better and are recovered more frequently than hair and soft tissues in the archaeological record.

While the exploration of steroid hormones in archaeological human remains is in its infancy, there is a wealth of research on modern marine mammals. Cortisol, 17β-Estradiol (oestrogen), progesterone and testosterone have been detected in the dentinous tusks of narwhal (*Monodon monoceros*) and Atlantic walrus (*Odobenus rosmarus rosmarus*), and in the teeth of beluga (*Delphinapterus leucas*) and killer whales (*Orcinus orca*) (Hudson et al., 2021). Successful measurement of cortisol, 17β-Estradiol, progesterone and testosterone in the keratinous baleen of multiple whale species has also been demonstrated (Hunt et al., 2018, 2017, 2016, 2014; Lowe et al., 2021). These studies have highlighted the significance of the hormone progesterone as a marker of pregnancy. Incremental ELISA performed on the baleen plates of North Atlantic right whales (*Eubalaena glacialis*) (Hunt et al., 2016) and humpback whales (*Megaptera novaeangliae*) (Lowe et al., 2021) identified significantly raised progesterone concentrations in segments which corresponded with confirmed pregnancies, enabling reconstruction of parity history. Progesterone concentrations exceeded 100ng/g during known pregnancies in both studies, with sustained levels throughout pregnancy and the post-partum period providing further information on birth intervals (Hunt et al., 2016; Lowe et al., 2021). This research highlights these hormones' potential to investigate reproductive histories from human hard tissues. The incorporation of tissues other than hair will significantly increase the overall sample size, range of time periods and geographic regions that can be studied.

1.2. Progesterone as a biomarker of pregnancy in humans

During the first trimester in humans, progesterone rises significantly and is maintained throughout pregnancy (Lim et al., 2020; O'Leary et al., 1991; Schock et al., 2016; Van Der Molen, 1963). As such, it is implicated as a key biomarker for identifying pregnancy within archaeological skeletal remains. A serum progesterone level of ≥ 20-25 ng/mL is indicative of a viable pregnancy in 90% of women in the first trimester (Ghaedi et al., 2022). Serum progesterone levels rise from an average of 25.6 ng/mL in the first trimester to 130 ng/mL in the third trimester (Schock et al., 2016) and then drop following the delivery of the placenta, aiding in the transition of colostrum to milk in lactating mothers (Balen, 2007).

To investigate the hormone progesterone in archaeological tissues, we must consider its relationship with other sex hormones. Progesterone is produced by the hypothalamic-pituitary-gonadal axis, as are oestrogen and testosterone (Figure 1) (Balen, 2007). Furthermore, progesterone is metabolised to form testosterone and oestrogen, while testosterone can be metabolised into oestrogen (Figure 1). These hormones are also capable of stimulating each other's receptors. For example, the progesterone receptor found on osteoblasts, which stimulates osteoblast activity and the production of more osteoblast cells (Seifert-Klauss and Prior, 2010), can be bound to and activated by oestrogen (MacNamara et al., 1995; Mills et al., 2021). Oestrogen and testosterone levels also increase throughout pregnancy, albeit not to the same intensity as progesterone (O'Leary et al., 1991; Schock et al., 2016). Thus, a study which encompasses oestrogen, progesterone, and testosterone will

provide us with greater insight into the interaction between these hormones in skeletal tissue in comparison to a study of progesterone in isolation.

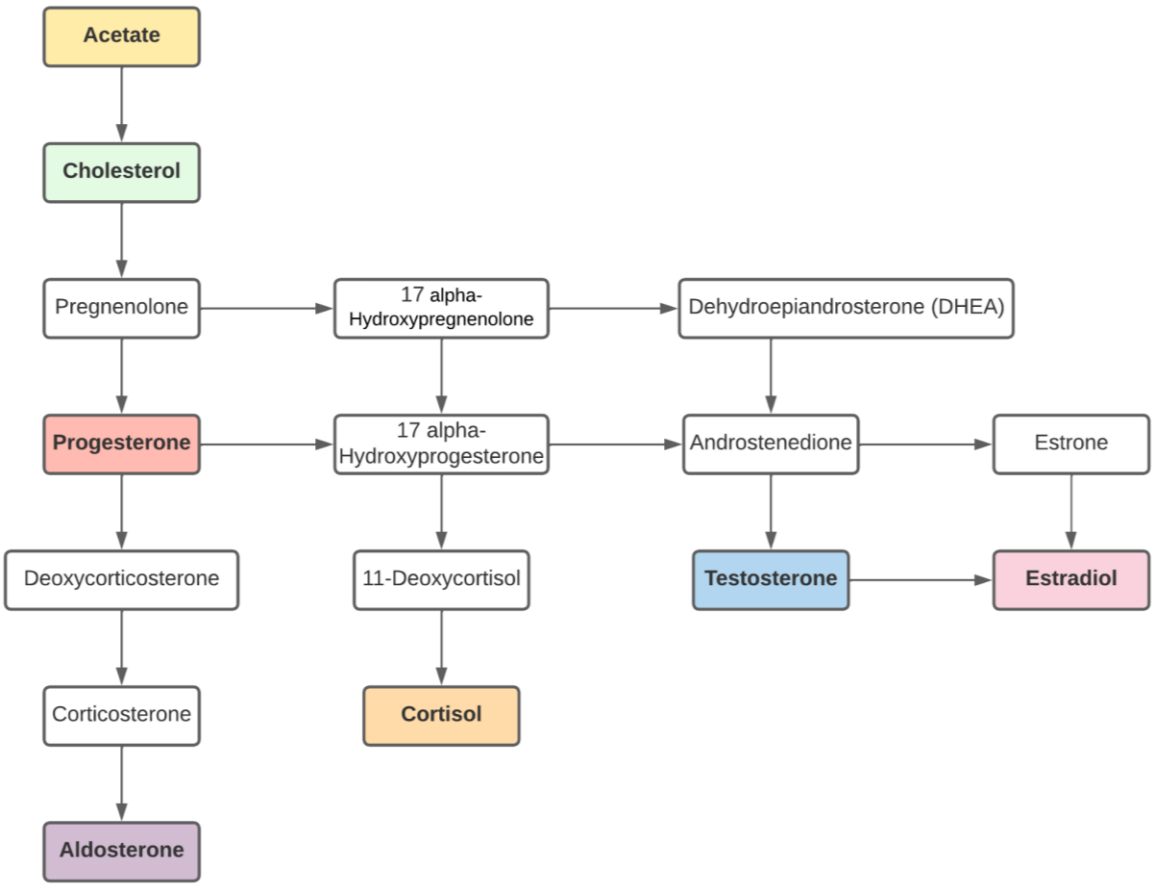

Figure 1: An overview of steroidogenesis. The metabolic pathway for the synthesis of steroid hormones. Aldosterone, estradiol (oestrogen), cortisol, progesterone and testosterone are metabolites of three molecules: acetate, cholesterol and pregnenolone.

It is unknown when hormones enter hard tissues, how they are incorporated, or if they are released following deposition in those tissues. There may be multiple routes of deposition and an accumulative effect across multiple pregnancies. Thus, understanding physiology is necessary to interpret the different concentrations within each tissue type. Once the mechanism by which sex steroids enter and act within skeletonised tissues is better understood, and the relationship between deposited concentrations and circulatory hormones is known, we may establish anticipated levels in incremental samples according to an individual's developmental stage and reproductive status, thereby offering insight into both past parity status and pregnancy at the time of death.

1.2. Study objectives and hypotheses

This study had the following objectives:

1. Determine whether progesterone, oestrogen and testosterone can be detected in archaeological human bone, tooth, and calculus using ELISA analysis.
2. Assess whether progesterone levels vary between males, females and females who were pregnant at the time of death, thereby offering a marker for pregnancy at the time of death.

3. Offer new hypotheses and, where possible, explanations for patterns observed in hormone concentrations that address routes of absorption, survival and detection of hormones in ancient tissues, and align with current clinical and physiological understandings of hormone metabolism.
4. Provide a critical assessment of the efficacy and potential for evaluation of hormones in archaeological human remains, with targeted suggestions for future research.

With these objectives in mind, we have developed a protocol for the preparation of human hard tissues to enable the identification of multiple hormones using ELISA analysis. Hypotheses for the study were based upon clinical evidence:

1. Progesterone, oestrogen and testosterone are detectable in female and male bone, calculus, and second and third-molar tooth structures.
2. Progesterone concentrations will be higher in females and greatest in individuals who demonstrate *in utero* pregnancies, reflecting *in vivo* differences in serum concentrations.
3. The concentration of hormones detected in an individual tooth's enamel will be lower than in the dentine or root due to the higher proportion of inorganic compounds in tooth enamel.
4. Those with *in utero* pregnancies of the same gestation will demonstrate similar concentrations of hormones detected in hard tissues.
5. Oestrogen concentrations will be higher in females than males and highest amongst those with *in utero* fetal remains.
6. Testosterone concentrations will be higher in male individuals than in females.

2. Materials and Methods

2.1. Materials

Ten individuals were selected for analysis (Table 1). To investigate whether hormones could be used as a marker for pregnancy, the study population included five individuals whose parity status could be independently inferred: two females with *in utero* fetal remains (CS80.1 and Y-1302), two females associated with fetal remains (SCREM21 and SCREM42), suggesting mother and child burials, and one female (Y-2332) without associated fetal remains but identified as the mother to another individual (Y-2354) via ancient DNA (aDNA) analysis (Reich and Armit, 2024). Three males (BG493, BG499, BG567) and two females of unknown parity status (BG498, Y-2280) assigned sex via aDNA analysis and buried without fetal or infant remains were also included (Reich and Armit, 2024).

Skeletal Assemblage	Skeletal Individual	Biological Sex	Age-at-death estimation	Method of sex assessment
All Saints Church, Fishergate, York 1st -5th century and *11th - 14th century CE	Y-1302*	Female	15 - 25 years	Osteological analysis, *in utero* fetal remains
	Y-2280	Female	35 - 45 years	aDNA analysis
	Y-2332	Female	30 - 45 years	aDNA analysis, known historical pregnancy

Black Gate Cemetery, Newcastle-upon-Tyne 8th - 12th century CE	BG493	Male	17 - 25 years	aDNA analysis
	BG498	Female	15 - 25 years	aDNA analysis
	BG499	Male	25 - 35 years	aDNA analysis
	BG567	Male	15 - 25 years	aDNA analysis
Scremby Anglo-Saxon Cemetery, Lincolnshire 5th - 6th century CE	SCREM21	Female	16 - 20 years	Osteological analysis, recovered with perinatal remains
	SCREM42	Female	25 - 35 years	Osteological analysis, recovered with fetal remains
St Hilda's Cemetery, Coronation Street, South Shields 18th -19th century CE	CS80.1	Female	17 - 25 years	Osteological analysis *in utero* fetal remains

Table 1: Study assemblage. Genetic sex was determined via chromosomal genotyping (Reich and Armit, 2024). Each individual's age and biological sex were estimated via osteological analysis using standard methods (Brickley and McKinley, 2004; Mitchell and Brickley, 2017).

One permanent second molar (M2) and third molar (M3) were selected from each individual alongside a small rib fragment free from pathological bone changes. No rib fragments were present for one individual (Y-1302), therefore a miscellaneous cervical vertebral body fragment was sampled. Loose teeth were chosen for analysis where possible. If dental calculus was present on tooth surfaces, it was removed with a modern tartar scraper and retained for further analysis.

2.2. Methods

The protocol we present for the performance of ELISA on skeletal remains is derived from a series of preliminary studies conducted on porcine samples to refine novel preparation and steroid extraction techniques. Three distinct phases of the protocol are summarised in the workflow illustration of Figure 2: sample preparation, steroid extraction, and ELISA analysis. Reducing the risk of contamination was paramount throughout this study; measures implemented to minimise contamination are described in supplementary material (Supplementary material: sample preparation and data validation).

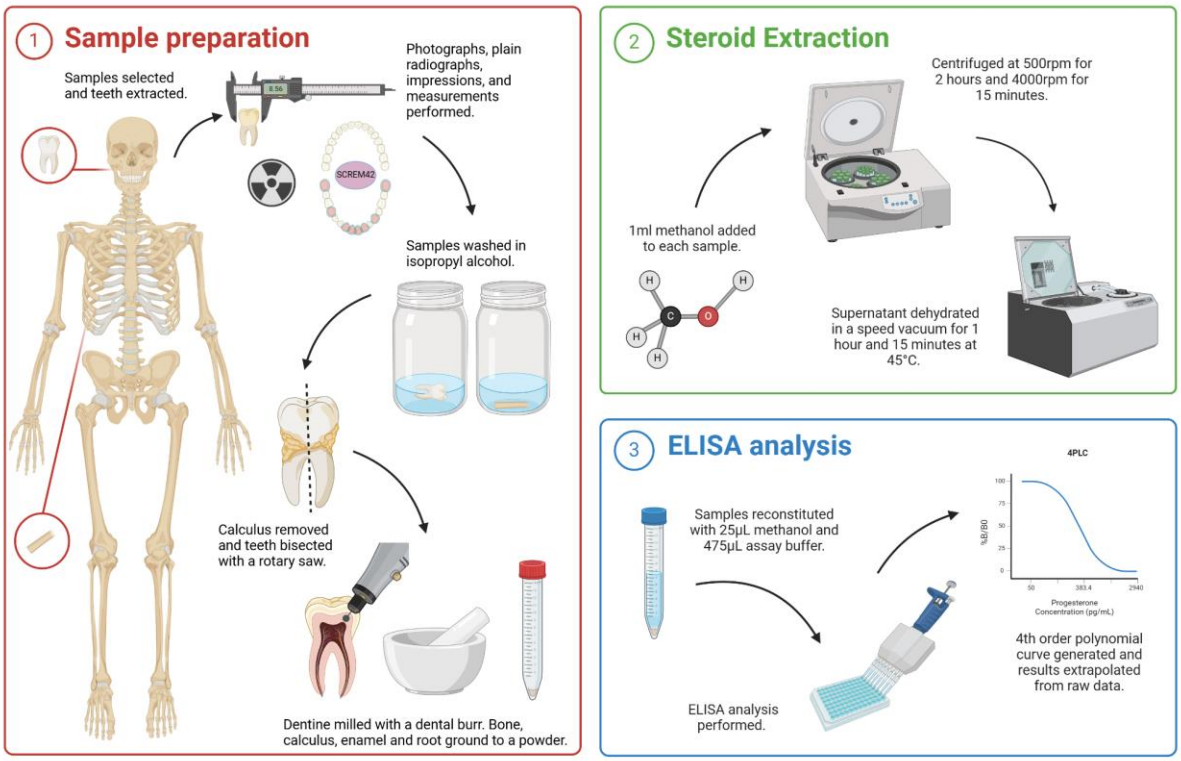

Figure 2: Methodology protocol. A summary of the key steps required to prepare skeletal material for the performance of ELISA. Prior to stage 1, photographs of bone fragments, maxilla and mandibles were taken. If the second and third molars required extraction, x-rays were first performed to determine the root structure and assist in removal. Following tooth extraction, dental impressions were cast, all five tooth surfaces were photographed, and measurements were taken of each tooth, including crown and cervical diameters (Hillson et al., 2005).

2.2.1. Sampling

Osteological inventories were generated for each individual in this study, including visual and detailed dental inventories. This occurred before any sample collection in line with ethical guidelines for the destructive sampling of archaeological human remains (APABE, 2023; BABAO, 2019; Brickley and McKinley, 2004). Powdered samples of bone, dentine, enamel and root were prepared using the protocol described in supplementary material (Supplementary material: sample preparation and data validation). All bone, dentine, enamel, and root samples were weighed to 150mg. Calculus samples greater than 20mg were also included; however, not all individuals had dental calculus: BG499 (42mg), Y-2280 (28mg), SCREM21 (46mg) and SCREM42 (48mg) were tested in this study. All ground samples were stored in polypropylene centrifuge tubes at room temperature.

2.2.2. Steroid extraction

One ml of methanol was added to each sample and centrifuged at 500rpm for two hours, followed by 4000rpm for 15 minutes. The supernatant was pipetted off and transferred to Protein LoBind Eppendorf tubes. Calculus samples were allowed to incubate for 2 hours at room temperature following the initial 2-hour 500rpm centrifuge spin to maximise steroid extraction. Supernatant was dehydrated using an Eppendorf Concentrator Plus speed vacuum

for 1 hour and 15 minutes at 45℃. Dehydrated samples were stored at -20℃ until reconstitution and performance of ELISA analysis.

2.2.3. Enzyme-linked immunosorbent assay analysis

ELISA analysis was performed using Arbor Assays multispecies DetectX® 17β-Estradiol (oestrogen) (K030-H1), progesterone (K025-H1) and testosterone (K032-H1) enzyme immunoassay (EIA) kits. While these kits have not been directly validated for human bone, tooth or calculus sample testing, marine mammal studies have successfully used Arbor Assays multispecies EIA kits on dental structures (Hudson et al., 2021; Hunt et al., 2018, 2017, 2016, 2014; Lowe et al., 2021).

2.2.3.1. Reconstitution of samples

All samples were thawed to room temperature before adding 25µL 99.9% methanol and vortexed for a minimum of 30 seconds to reconstitute the samples. The generic Arbor Assays EIA kit concentrated assay buffer (X065) was diluted by one part concentrate to four parts deionised water. 475µL of diluted EIA assay buffer was added to the reconstituted samples and vortexed for a further 30 seconds to make up the total sample volume to 500µL. The buffer quantity was calculated to accommodate the performance of three EIA test kits, with leeway for pipetting losses and duplication. Samples were frozen at -20℃. On the day of testing, all samples were defrosted for 45 minutes and vortexed once thawed for 30 seconds.

2.2.3.2. Assay analysis

Assay protocols were followed per manufacturer instructions for the preparation of reagents and hormone standards, as well as the performance of assay analysis (Arbor Assays, 2023a, 2023b, 2022). To ensure accuracy and minimise error, an additional step was added to the manufacturer's instructions; 80µL of reconstituted samples were pipetted into a low-binding 96-well plate. Once all samples and standards were prepared, 50µL of each were pipetted into the 96-well microtiter-coated plates using a multichannel pipette and covered with a plate sealer to prevent evaporation. To conclude the analysis, the optical density of each well was read immediately in a Tecan Infinite M Nano+ plate reader reading at a measurement wavelength of 450nm and a reference wavelength of 540nm.

The raw data was inputted into the commercial free-to-use MyAssays (2023) software platform to generate 4th order polynomial curves (4PLC) for hormone standards and calculate individual sample readings. Any readings above the minimum detection threshold but outside of the range of standard measurements or generated curve on 4PLC were interpreted as negative results (Supplementary material: sample preparation and data validation). SPSS Statistics was used to perform further statistical analysis. Independent two-sample t-tests and dependent paired t-tests were used and are presented below only where sample size was adequate. Thus limited statistical analyses were performed due to the frequency of positive results within our small cohort.

3. Results

Results are presented by tissue type: bone (Table 2), enamel, dentine and root (Table 3), and dental calculus (Table 4). The positive measurements represent detectable levels of hormones. Their concentrations and corresponding percentage coefficient of variability (%CV) are provided. Samples with a concentration within the range of the 4PLC and standards, but below the Limit of Detection and/or sensitivity of the relevant EIA kit, are indicated. Unreported measurements were omitted because they were interpreted as negative (either absence of a hormone or presence below the minimum threshold for detection of the EIA kit, which cannot be differentiated experimentally), or as false positives (see Supplementary material: sample preparation and data validation).

1

Skeletal Individual	**Biological sex**	**Age-at-death estimation**	**Fetal remains**	Progesterone, **Mean Progesterone** pg/mL (%CV)	Estradiol, **Mean Estradiol** pg/mL (%CV)	Testosterone, **Mean Testosterone** pg/mL (%CV)
BG493	Male	17 - 25 years	-	81.79, <LOD **81.79** (-)	-	-
BG498	Female	15 - 25 years	none	73.52, 95.10 **84.31** (18.10)	-	52.49, 120.40 **86.46** (55.60)
BG499	Male	25 - 35 years	-	59.68, 56.52 **58.10** (3.85)	-	61.23 **61.23*** (-)
BG567	Male	15 - 25 years	-	61.53, 55.31 **58.42** (7.53)	<LOD, 47.67 **47.67** (-)	
CS80.1	Female	17 - 25 years	*In utero*	<LOD, 80.47 **80.47** (-)	-	-
SCREM21	Female	16 - 20 years	yes	-	-	-
SCREM42	Female	25 - 35 years	yes	<LOD, 73.82 **73.82** (-)	-	-
Y-1302**	Female	15 - 25 years	*In utero*	178.70, 183.80 **181.20** (2.00)	49.06, 51.24 **50.15** (3.07)	-
Y-2280	Female	35 - 45 years	none	73.16, 72.08 **72.62** (1.05)	-	60.79, 73.53 **67.16** (13.40)

Y-2332	Female	30 - 45 years	none		-	-	54.69, 32.07 **43.38** (36.90)

Table 2: Bone ELISA results. No bone sample was tested for testosterone for BG567. Extrapolated readings below the Limit of Detection (LOD) and/or sensitivity (S) are indicated. *Testosterone was tested in singlet for BG499. **A cervical body fragment was used for analysis for individual Y-1302.

Skeletal Individual	Biological sex	Age-at-death estimation	Fetal remains	Tooth	Progesterone, **Mean Progesterone** pg/mL (%CV)			Estradiol, **Mean Estradiol** pg/mL (%CV)			Testosterone, **Mean Testosterone** pg/mL (%CV)		
					Enamel	**Dentine**	**Root**	**Enamel**	**Dentine**	**Root**	**Enamel**	**Dentine**	**Root**
BG493	Male	17 - 25 years	-	M2	-	-	<LOD, <LOD **-** (-)	-	-	-	-	-	-
				M3	-	71.37, <LOD **71.37** (-)	-	-		-	-	-	-
BG498	Female	15 - 25 years	none	M2	-	-	<LOD, <LOD **-** (-)	-	-	44.00, 102.20 **73.10** (56.30)	-	49.84, 51.38 **50.61** (2.15)	54.57, 86.66 **70.61** (32.10)

				M3	-	-	-	-	-	-	55.31, 81.95 **68.63** (27.40)	67.67, 66.25 **66.96** (1.50)	-
BG499	Male	25 - 35 years	-	M2	-	-	-	-	-	-	55.91, 134.20 **95.08** (58.30)	43.39, 136.60 **90.10** (73.30)	58.70, 113.60 **86.16** (45.10)
				M3	-	-	-	-	<LOD, <S **-** (-)	-	99.08, 157.50 **128.30** (32.20)	71.66, 150.30 **111.00** (50.10)	48.34, 138.30 **93.34** (68.20)
BG567	Male	15 - 25 years	-	M2	-	-	68.05, 69.87 **68.96** (1.87)	-	-	-	40.33, 42.31 **41.32** (3.39)	41.38, 47.08 **44.23** (9.12)	52.27, 43.33 **47.80** (13.20)
				M3	-	-	-	<S, <S **-**(-)	-	-	84.87, 39.04 **61.95** (52.30)	53.13, 52.98 **53.05** (0.20)	89.33, 43.09 **66.21** (49.40)
Y-1302	Female	15 - 25 years	*In utero*	M2	<LOD, 53.74 **53.74** (-)	56.47, <LOD **56.47** (-)	104.00, 92.96 **98.34** (8.12)	-	-	-	-	-	-

				M3	-	-	-	-	-	-	-	-	-
Y-2280	Female	35 - 45 years	none	M2	-	-	-	-	-	-	44.89, 41.81 **43.35** (5.03)	74.12, 47.10 **60.61** (31.50)	33.70, 80.15 **56.92** (57.70)
				M3	-	-	-	40.69, 44.67 **42.68** (6.61)	-	-	90.45, 50.97 **70.71** (39.50)	66.48, 38.17 **52.32** (38.30)	74.12, 46.29 **60.02** (32.70)
Y-2332	Female	30 - 45 years	none	M2	-	-	-	<S, <S **-**(-)	-	-	41.08, 44.03 **42.56** (4.91)	52.51, 50.02 **51.26** (3.44)	-
				M3	-	-	-	-	-	-	68.64, 58.74 **63.69** (11.00)	43.10, 78.36 **60.73** (41.00)	46.49, 61.72 **54.11** (19.90)

Table 3: Dental Structures ELISA results. No hormones were detected in the dental structures for CS80.1, SCREM21 and SCREM42. Extrapolated readings below the Limit of Detection (LOD) and/or sensitivity (S) are indicated.

Skeletal Individual	**Biological sex**	**Age-at-death estimation**	**Fetal remains**	**Calculus weight (mg)**	Progesterone, **Mean Progesterone** pg/mL (%CV)	Estradiol, **Mean Estradiol** pg/mL (%CV)	Testosterone, **Mean Testosterone** pg/mL (%CV)
BG499	Male	25 - 35 years	-	42	67.84 **67.84*** (-)	-	59.35, 104.70 **82.03** (39.10)
SCREM21	Female	16 - 20 years	yes	46	92.59, 128.20 **110.40** (22.80)	-	-
SCREM42	Female	25 - 35 years	yes	48	88.63, 203.80 **146.20** (55.70)	-	51.96, 31.90 **41.93** (33.80)
Y-2280	Female	35 - 45 years	none	28	-	-	42.27, 59.61 **50.94** (24.10)

Table 4: Dental Calculus ELISA results. *The calculus sample for BG499 was tested in singlet for progesterone.

3.1. Percentage coefficient of variability values

Samples were tested in duplicate to determine intra-assay reproducibility via %CV. Pipetting losses and evaporation of reconstituted samples were greater than anticipated. Consequently, 8 of 221 reconstituted samples tested were singlet (4/74 oestrogen, 2/74 progesterone, and 2/73 testosterone). For individual BG567, no bone sample was tested for testosterone due to the limited remaining reconstituted sample. Mean %CV was calculated for each hormone and tissue type, the results of which are displayed in Table 5.

Tissue	**Progesterone mean %CV** (n)	**Oestrogen mean %CV** (n)	**Testosterone mean %CV** (n)	**Mean %CV** (n)
Bone	**6.51** (5)	**3.07** (1)	**35.30** (3)	**15.72** (9)
Enamel	n/a (0)	**6.61** (1)	**20.00** (9)	**24.06** (10)
Dentine	n/a (0)	n/a (0)	**25.06** (10)	**25.06** (10)
Root	**9.99** (2)	**56.30** (1)	**39.78** (8)	**34.96** (11)
Calculus	**39.25** (2)	n/a (0)	**32.33** (3)	**35.10** (5)
Mean %CV	**13.45** (9)	**21.99** (3)	**30.48** (33)	-

Table 5: Mean percentage coefficient of variability (%CV) by tissue and hormone. Results are rounded to two decimal places, and the total number of %CV values provided (n).

Bone demonstrated the greatest intra-assay reproducibility, while calculus provided the least intra-assay reproducibility, with mean %CV of 15.72 and 35.10, respectively. Progesterone provided the best intra-assay reproducibility in the study with a mean %CV of 13.45. The greatest variability was found in oestrogen and testosterone root samples with a mean %CV of 56.30 and 39.78, respectively.

3.2. Progesterone

Sixteen out of 74 samples (21.6%) demonstrated detectable concentrations of progesterone (Tables 2-4). The most frequently positive tissue type was bone (Table 2), with 8 out of 10 samples testing positive. All males studied had detectable progesterone in bone samples, with relatively consistent mean concentrations ranging between 58.10 - 81.79 pg/mL. The mean bone progesterone concentration for females was much more varied, spanning both the lowest and highest concentrations detected (0 - 181.20 pg/mL) and, overall, there was no significant difference in bone progesterone concentration between males and females (Independent samples t-test, t = 1.793, 2-sided significance P-value 0.296). However, both females with *in utero* remains had progesterone detected in bone samples, with the highest mean concentration of all samples (181.20 pg/mL) found in the vertebral fragment of Y-1302. Meanwhile, the mean bone progesterone concentration for the other female with *in utero* remains, CS80.1, was 44% lower at 80.47 pg/mL.

Progesterone was detected in both male and female tooth samples in the enamel, dentine and root of M2, but only in the M3 dentine of one sample, a male individual (BG493) (Table 3). Progesterone was detected in all M2 samples of one individual with an *in utero*

pregnancy (Y-1302), but no progesterone was detected in any of their M3 samples. Y-1302 was the only individual to demonstrate detectable progesterone in M2 enamel and dentine. Furthermore, the concentration was consistent between enamel and dentine, at 53.74 pg/mL and 56.47 pg/mL, respectively. Y-1302 provided the highest concentration of progesterone within all tooth structures, with a mean concentration of 98.34pg/mL detected in the root of M2. CS80.1, the other female with an *in utero* pregnancy, had no detectable progesterone levels in any tooth structure.

Progesterone was detected in three out of four calculus samples (Table 4). Mean progesterone levels in the calculus of females associated with fetal remains (SCREM21 and SCREM42) were 47% greater than the male calculus sample (BG499). No progesterone was detected in the sample from the individual without any associated fetal remains (Y-2280).

3.3. Oestrogen

Four of 74 samples (5.4%) were positive for oestrogen. No oestrogen was detected in dental calculus. Only two bone samples demonstrated detectable levels for one male (BG567) and one female with *in utero* remains (Y-1302) (Table 2). Oestrogen was detectable in samples from female M2 root (BG498) and M3 enamel (Y-2280) (Table 3).

3.4. Testosterone

Testosterone was the most frequently detected of the three hormones investigated, with 34 out of 73 (46.6%) samples testing positive (Table 2-4). The majority of positive results were from dental structures. Only seven samples from four individuals provided positive testosterone levels in bone samples (BG498, BG499, Y-2280 and Y-2332) with the highest, 86.46 pg/mL, in a female without associated fetal remains (BG498). Three of the four calculus samples had detectable testosterone levels, with the highest measurement (82.03 pg/mL) found in the male sample (BG499).

Females with *in utero* fetal remains did not demonstrate detectable testosterone levels in any tissue type. Similarly, individuals associated with fetal remains did not have detectable levels of testosterone in M2, M3, or bone samples, but one individual (SCREM42) had detectable testosterone in dental calculus (Table 4). All females without associated fetal remains had detectable testosterone in M2 and M3 dentine, M3 enamel, and bone samples.

Testosterone was detected in all tissue types for two male individuals (BG499, BG567). The third male (BG493) did not have any samples with detectable levels of testosterone. The highest testosterone measurements in each tooth structure were found in samples from BG499, including the highest mean testosterone level in the study, 128.30 pg/mL from M3 enamel. In contrast, BG567 demonstrated the lowest levels of testosterone in M2 and M3 enamel, as well as M2 dentine and root.

On comparison between paired samples, there was no significant difference (dependent paired t-test, t = 0.101, 2-sided significance P-values, 8 degrees of freedom) between the mean testosterone concentrations of enamel and dentine (P-value 0.922), dentine and root (P-value 0.623), or enamel and root samples (P-value 0.267).

4. Discussion

4.1. Detection of sex steroid hormones in archaeological skeletal tissues.

As there is evidence that hormones impact growth and dimorphism in bones and teeth (Lam et al., 2016; Madalena et al., 2023; Miller, 1994; Ribeiro et al., 2013), it was deduced that hormones enter these structures and hypothesised that they would consequently be detectable (Hypothesis 1). We have demonstrated that oestrogen, progesterone and testosterone are detectable in samples from archaeological remains dating from the 1st - 19th centuries CE.

All individuals demonstrated at least one positive result from the trio of analyses. It is not yet possible to determine what concentrations of hormones are to be expected in hard tissues. Undetectable levels could represent concentrations below the ELISA kit's threshold for detection, incomplete steroid extraction, or destruction of hormones through funerary practices, degradation, and taphonomy. Diagenesis of archaeological proteins is also of particular concern to proteomic research. Issues arise when diagenic modification occurs during incorporation into tissues, tissue decomposition, and through the introduction of protein extraction reagents (Warinner et al., 2022). It is possible that diagenesis of the archaeological samples renders the presence of hormones either undetectable, or detectable but unquantifiable using ELISA. Analysis of archaeological bone, calculus, concretions, and tooth roots has demonstrated that human ancient peptide preservation is variable across sample types (Bonucci et al., 2025). However, tooth enamel is thought to preserve ancient proteins well (Hendy, 2021), and that enamel proteins are the most resistant to diagenetic change in vertebrates (Warinner et al., 2022). Indeed, recent research on medieval human teeth has demonstrated that not only are antibodies detectable within archaeological teeth, but are, in fact, present with intact disulphide linkages, and retain immune reactivity (Shaw et al., 2023). Nonetheless, future research should consider the impact of diagenesis on hormones within hard tissues to enable confident interpretation of concentrations.

While testosterone and progesterone were detected in 46.6% and 21.6% of samples, respectively, in contrast, few samples had measurable oestrogen levels, with no detection in calculus, despite the limit for detection (26.50 pg/mL) being the lowest of all three EIA kits (Arbor Assays, 2023b, 2023a, 2022). The most likely explanation is that the serum concentrations of oestrogen are significantly lower than circulatory progesterone and testosterone levels. For example, in premenopausal women, oestrogen levels range between 72 - 1309 pmol/L, whereas testosterone concentrations are between 0.4 - 2.1 nmol/L and progesterone levels fluctuate between 0 - 89.1 nmol/L during the menstrual cycle (Barnsley and Rotherham Integrated Laboratory Services, 2022). Furthermore, the half-life of 17β-Estradiol (oestrogen) is only 20-30 minutes (Kuhl, 2005), limiting its opportunity to be incorporated into tissues. Further consideration must be given to the metabolism of these hormones. Multidisciplinary research on modern humans into the impact of steroid hormone binding to other proteins, such as albumin, sex hormone-binding globulin and corticosteroid-binding protein, would be particularly informative and guide the development of reliable reference ranges.

4.1.1. Bone progesterone concentration may not reflect serum levels.

There is a large difference between the serum concentrations of progesterone in males and females in their luteal phase (Filicori et al., 1984; Oettel and Mukhopadhyay, 2004; Zumoff et al., 1990); therefore it was hypothesised that progesterone concentrations would be highest in samples from female individuals (Hypothesis 2). Where sample size permitted statistical comparisons, no significant differences in progesterone concentration were observed in bone from males and females. Therefore, our results suggest that progesterone uptake into bone does not reflect this difference.

Given the vascularity of bone, we theorise that circulatory hormones primarily enter bone through blood serum via smaller blood vessels, capillaries and bone marrow. These

hormones are then transported into the bone matrix. The hormones detected may be bound to the receptors on the cell membranes of osteocytes and osteoblasts or be the internalised hormone within these cells following receptor activation rather than representing unbound hormones within the bone's vascular system. A study on the relationship between serum and intraskeletal oestrogen and testosterone in rats supports the theory that bone may act as a hormonal reservoir (Yarrow et al., 2010). Yarrow et al. (2010) found that intraskeletal concentrations of androgens, such as testosterone and oestrogen, were strongly correlated with serum concentrations. Yet when the primary source of serum testosterone and oestrogen had been removed, via the performance of a bilateral orchiectomy, intraskeletal testosterone and oestrogen levels remained the same 29 days post-procedure (Yarrow et al., 2010). This provides a dilemma, as on one hand our results support the notion of a hormone reservoir, but we are unable to determine if they are truly correlated with serum concentrations in skeletonised remains.

4.1.2. Hormonal concentration within enamel and dentine appears balanced.

We hypothesised that hormone concentrations in enamel would be less than in dentine, as their inorganic compositions are 96% and 70%, respectively (Hypothesis 3, Lee et al., 2017). On examination of testosterone levels in paired dentine and enamel samples, there is no significant difference between the concentrations. In the only individual for which progesterone was detected in the enamel (Y-1302), the difference in concentration between the enamel and dentine of the M2 was negligible. Furthermore, in the one case (Y-2280) where oestrogen was detected in tooth enamel, it was not identified in any other tooth structures from this individual.

An exploration of the mechanisms of hormonal deposition in tooth structures offers various explanations. There is evidence that oestrogen, progesterone, and testosterone receptors are present in the vascular dental pulp (Inaba et al., 2013; Jukić et al., 2003; Whitaker et al., 1999). Furthermore, odontoblasts possess progesterone receptors (Whitaker et al., 1999), so we can infer that the circulating hormone enters the dentine through the pulp. It is possible that the hormones detected in this study resided within the tubules that lead from the dental pulp into the dentine rather than adjacent dentine. Some serum-derived proteins, such as albumin, can also be incorporated into the developing dentine and enamel (Mazzoni et al., 2009; Orsini et al., 2009). Steroid hormones bind to albumin (Hammond, 2016), thus also making it plausible for bound hormones to be transported into the developing tooth.

Although dentine and enamel are not subject to remodelling, they can be remineralised. Thus, it is difficult to ascertain whether hormone levels in tooth structures remain constant, are altered during remineralisation, or can change following mineralisation. Another mechanism for hormonal deposition to consider is through contact between unerupted enamel with the overlying oral mucosa. Furthermore, dental wear from attrition, abrasion, or erosion can remove the surface enamel, exposing the underlying porous subsurface enamel and potentially allowing the absorption of hormones via saliva. The absorption of hormones into enamel from saliva could be higher than we anticipated if, once absorbed from the saliva into the enamel, they are transported into dentine between the boundaries of enamel prisms where the enamel is less mineralised.

Alternatively, it is possible that the balance between concentrations in enamel and dentine reflects the degree of impact of post-mortem diagenesis on hormones in their respective tissues. There is evidence that the preservation of proteins, such as collagen and osteocalcin, benefits from mineral stabilisation within hydroxyapatite (Collins et al., 2002). As enamel contains the greatest proportion of hydroxyapatite of any tissue, if hormones incorporated into enamel are subject to less diagenesis than those in dentine, this may result

in a mismatch between the endogenous incorporated hormone and the concentration of hormone detectable in different tooth structures.

4.2. Biomarkers of pregnancy in hard tissues

4.2.1. Progesterone as a marker of pregnancy

We hypothesised that progesterone levels would be highest in individuals with *in utero* fetal remains, thereby providing a biomarker for pregnancy at the time of death in archaeological remains (Hypothesis 2). Among positive calculus samples, progesterone concentrations were 47% higher among females with in-situ fetal remains than in the male control sample. No progesterone was detected in the calculus sample of the female without any associated fetal remains (Y-2280). Thus, positive progesterone calculus samples in female individuals, with concentrations significantly higher than the male or female controls, may indicate a recent pregnancy in skeletal remains.

The two individuals with *in utero* remains in this study were young adults in their third trimester of pregnancy, with CS80.1's fetus was estimated to be between 32 and 40 weeks gestation and Y-1302's fetus was estimated to be 40 weeks gestation. We hypothesised that these individuals would demonstrate a high circulating progesterone level in keeping with their third-trimester pregnancies and, thus, the same concentration of progesterone in bone (Hypothesis 4). The concentration of progesterone detected in the vertebral body fragment from Y-1302 was the highest of all tissue types at 181.20 pg/mL. Meanwhile, CS80.1's progesterone level in a rib sample was 80.47 pg/mL, 44% lower than Y-1302 and comparable to the upper range of values from males.

Y-1302 is from a chronologically earlier population than CS80.1, so the degradation of hormones over time does not appear to be a significant factor in this case. Instead, the difference in results between these individuals might be due to the different bone types, the vascularity of bone or the distance of the bone from the pelvic organs. While the venous system of the pelvic organs and the vertebrae are distinct, a small proportion of blood from the pelvic organs may pass through the anterior sacral foramina into the vertebral venous plexus (Gosling et al., 2017), thus offering hormones a route to the vertebrae. Other variables and mechanisms of deposition should be considered, such as the involvement of the lymphatic system, cerebral spinal fluid or blood pooling in a supine body after death. Conversely, as serum progesterone ranges between 48.40 ng/mL to 422.51 ng/mL (Barnsley and Rotherham Integrated Laboratory Services, 2022) in the third trimester, these results may in fact illustrate the variation within a normal range of progesterone in pregnancy.

CS80.1 had no progesterone detected in any tooth structures, while Y-1302 was the only individual with progesterone detectable in M2 enamel and dentine. Furthermore, progesterone detected in the M2 root of Y-1302 was 30% greater than the male for whom progesterone was detected. Yet no progesterone was detected in the M3 of Y-1302. With the distribution of progesterone throughout the M2, it suggests that progesterone was incorporated into the tooth during pregnancy, and not necessarily while the tooth was developing. It remains unclear why deposition occurred in M2 and not M3, but if this finding is replicated in further studies, it could be another valuable marker of pregnancy at the time of death.

4.2.2. Oestrogen and testosterone as markers of pregnancy

The results of this study suggest that both oestrogen and testosterone should also be considered as potential markers for pregnancy in archaeological tissues, emphasising the necessity to investigate these hormones alongside progesterone. Interestingly, neither individual with *in utero* remains had oestrogen detected in any tooth structure. Although, Y-

1302 was the only female with a detectable oestrogen level in a bone sample. As oestrogen levels in pregnancy increase on average by 876% (Schock et al., 2016), the detection in the bone sample of Y-1302 is not entirely unexpected. However, its absence in the tooth structures of both females with *in utero* remains was surprising and does not support our original hypothesis (Hypothesis 5).

As hypothesised (Hypothesis 6), male and female individuals had measurable testosterone in all tissue types, with the highest concentration of testosterone found in the tooth and calculus samples from a male individual (BG499). Females with *in utero* remains did not have detectable testosterone in any tissue type, whereas the females without associated fetal remains had testosterone detected in dentine, bone and M3 enamel. Individuals associated with fetal remains did not have detectable levels of testosterone in M2, M3, or bone samples. While this finding does not align with the elevated testosterone concentrations that are known to occur in pregnancy (Schock et al., 2016; Svensson et al., 2019; Van De Beek et al., 2004), an absence of testosterone from skeletal tissues could be an indicator of an *in utero* pregnancy. However, the detection of testosterone in samples from individual Y-2332, the mother of a 10- to 12-year-old individual (Y-2354) (Reich and Armit, 2024), suggests that the absence of testosterone cannot be used as an indicator of historical pregnancy. Furthermore, the detection of testosterone from the calculus of an individual with associated fetal remains (SCREM42) may reflect raised salivary testosterone concentrations during pregnancy and the post-partum period (Svensson et al., 2019; Voegtline et al., 2017).

4.3. Study limitations and suggestions for future research

Sample size was restricted by ethical requirements associated with destructive sampling (APABE, 2023), which dictate the application of an untested technique on a limited sample until its feasibility can be demonstrated. The requirement for genetic sex and maternity status constrained the sample further. We acknowledge that this investigative technique is in its infancy, and we welcome further studies to enable the refinement of this methodology. Specifically, future analysis of modern individuals with known medical and life histories will be required to generate tested reference ranges for hormonal concentrations in bone, teeth, and calculus. Moreover, the comparison between the detection and quantification of hormones via ELISA and high-resolution mass spectrometry would enable a discussion on the accuracy, accessibility, and affordability of such investigations in skeletal remains of variable macro- and microscopic preservation.

The variability in %CV values has highlighted some challenges within processing archaeological hard tissue samples and the performance of ELISA. As the R^2 values for each polynomial curve were very good, ranging from 0.9963 to 0.9999, the poorer %CV values may result from sample preparation and storage rather than pipetting issues and plate washing. Further investigation into the impact of storage techniques on intra-assay reliability and the automation of the ELISA process is needed to increase accuracy in future studies. Furthermore, given the quantity of values below the Limit of Detection and sensitivity of the EIA kits, research involving a larger sample size would benefit from the calculation of extraction efficiency via a spiked control, the Lower Limit of Quantification and independent Limit of Detection in order to improve precision and accuracy.

4.3.1. Dental calculus as a focus of further research

This research has demonstrated that salivary progesterone and testosterone can be incorporated into dental calculus either at the time of deposition or through absorption from

saliva implicating dental calculus as an alternative resource to explore the endocrinological profiles of individuals. We propose that hormones become incorporated into the dental calculus via saliva and from contact with the gingival crevicular fluid. Hormonal deposition in dental calculus could occur during the mineralisation of the calculus. Moreover, with the evolving nature of calculus, hormones would be absorbed into the structure as it enlarges, resulting in a cumulative effect whereby more extensive calculus deposits absorb, and therefore record, hormones over a longer period. Indeed, an exponential incorporation of trace elements during the initial 9 months of maturation has previously been observed (Abraham et al., 2005). If the same process of deposition occurs for hormones, any concentration measured will not represent a proportional average of salivary hormones over the lifespan of the calculus. Until the method of deposition is better understood, calculus hormonal concentrations must be interpreted cautiously. However, there is no doubt that this tissue type is a worthwhile focus for further hormonal ELISA analysis.

4.3.2. Applications beyond pregnancy

The successful detection of sex hormones in archaeological remains opens up a range of potential future applications, including alternative methods to assign age at death, estimate biological sex, and identify other stages of male and female reproductive life histories such as puberty and menopause. Further consideration should be given to the theory that bone acts as a hormonal reservoir, releasing hormones locally when they are required, such as during skeletal remodelling.

The study of sex hormones can provide insights into aspects of an individual's lived experience that are distinctive but complementary to the evidence that aDNA offers in genetics. While oestrogen, progesterone and testosterone are primarily associated with regulating sexual reproduction, they also serve important roles in other biological systems. For example, sex hormones and their metabolites influence an individual's appearance and behaviour beyond sexual dimorphism (Cai et al., 2018; Danby, 2015; Kilpi et al., 2020; Pearson Murphy et al., 2001; Shu and Maibach, 2011; Stefaniak et al., 2023). Hormones also affect an individual's sensory experience and how we communicate with others (Cassiraga et al., 2012; He and Ren, 2018; Horner, 2009; Jost et al., 2018). In addition, steroid hormones have the potential to explore malignancy and morbidity (Alshakhouri et al., 2024; Ghoumari et al., 2020; Klossner et al., 2021; Morfoisse et al., 2021) in archaeological populations. For instance, modern studies have demonstrated that low testosterone is associated with a lower risk of prostate cancer (Watts et al., 2018), whilst allopregnanolone (a metabolite of progesterone) is involved in ovarian tumour development (Pelegrina et al., 2020).

5. Conclusions

The purpose of this study was to determine whether progesterone, oestrogen and testosterone have the potential to be identifiers of pregnancy in human skeletal remains and thus provide a greater understanding of fertility in the past. This paper has presented a novel method for preparing archaeological skeletal tissues and extracting steroid hormones to perform ELISA. We have successfully detected oestrogen, progesterone, and testosterone in bone and tooth, and progesterone and testosterone in calculus samples. This study has revealed a series of consistencies and inconsistencies between the archaeological data and clinical and physiological expectations. High progesterone levels in dental structures and calculus, the presence of oestrogen in bone, and a lack of testosterone in hard tissues was consistent with

pregnancy at the time of death. This raises a series of new questions about sex steroid hormone metabolism and demonstrated the ability of this method to detect pregnancy in archaeological individuals; this has the potential to revolutionise the way we study reproductive histories of past populations.

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Declaration of Competing Interest

Aimée Barlow received a research grant for this study from the British Association for Biological Anthropology and Osteoarchaeology. All other authors have no competing interests.

Data Availability Statement

The data that support the findings of this study are openly available in the University of Sheffield data repository ORDA at https://doi.org/10.15131/shef.data.29069777.v1

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Supplementary material: sample preparation and data validation

Caption: *Sample preparation and data validation. Protocol followed for the preparation of bone, tooth and calculus samples for the performance of steroid hormone extraction and ELISA analysis.*

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