Amino acid dating of Pleistocene mammalian enamel from the River Thames terrace sequence: a multi-taxon approach

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ABSTRACT

Amino acid geochronology can provide effective relative dating frameworks for the Pleistocene and has enabled correlation of terrestrial deposits to the global climatic fluctuations described by the marine oxygen isotope record. Using methods developed for the analysis of intra-crystalline amino acids in tooth enamel, we aimed to construct an enamel-based amino acid geochronology for the terrace deposits in the valley of the River Thames in southern Britain using different mammalian taxonomic groups: elephant, horse and bison. To achieve this, chiral amino acid analysis was applied to 58 elephantid, 21 horse and 15 bison teeth from 10 horizons in the Upper Thames Valley, three in the Lower Thames Valley and one from a Thames tributary in the Lea Valley. We evaluate differences in the rates of amino acid breakdown between the taxa and establish which species are similar enough to enable comparison for relative dating purposes. The relative dating of the river terrace deposits is in good agreement with the terrace stratigraphy, biostratigraphy, and other independent estimates of age for all three taxonomic groups. These frameworks demonstrate the potential of enamel-based amino acid geochronologies for relative dating of Middle–Late Pleistocene deposits in the UK, and establish an aminostratigraphic framework from which the dating of other tooth material can be refined. Enamel offers an opportunity to evaluate the age of sites where shell material is absent or poorly preserved. It can also, crucially, provide direct relative dating of mammalian fossils, which are often the focus of study in terms of their evolution, distributional changes or extinction. Direct dating negates the risk that the mammal fossils themselves might be reworked, or of different ages to shell, sediments or other dated material in the same deposits; it also enables archived samples with insecure provenance (e.g. from early 17th-19th century collections) to be directly dated.

1. Introduction

Dating palaeontological and archaeological sites is critical to the interpretation of paleoenvironmental records and for the reconstruction of past climates; yet, dating and correlation of many terrestrial Pleistocene sequences remain a substantial challenge. When suitable biominerals are present, amino acid geochronology is able to provide effective frameworks for relative dating, as well as correlation of deposits with the marine oxygen isotope record (e.g. Miller and Mangerud, 1985; Kaufman, 2003; Penkman et al., 2013; Ortiz et al., 2018; Tesakov et al., 2020). Past studies have primarily focused on calcium carbonate-based biominerals (e.g. mollusc shell), rather than calcium phosphate-based biominerals, such as those present in mammalian

skeletal material. This is partly due to the preparative and analytical challenges with calcium phosphate-based biominerals (Griffin, 2006), as well as concerns about their reliability (Bada, 1985; Marshall, 1990). However, enamel is an extremely dense mineral, leading to a high potential for organic preservation in the mineral structure. This protected environment for amino acids, combined with relative abundance of teeth in the fossil record, makes tooth enamel a favourable substrate for building amino acid geochronologies. Furthermore, enamel offers an opportunity to evaluate the age of sites where shell material is absent or poorly preserved. It can also, crucially, provide direct relative dating of mammalian fossils, which are often the focus of study in terms of their evolution, distributional changes or extinction. Direct dating overcomes the risk that the mammal fossils themselves might be reworked, or of

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different ages to the sediments or other dated material in the same deposits.

Recent developments have provided improved preparative methods for the analysis of calcium phosphate-based biominerals (Dickinson et al., 2019). By isolating amino acids which exhibit closed-system behaviour using the intra-crystalline protein decomposition (IcPD) approach (Sykes et al., 1995; Penkman et al., 2008), it has been shown that several amino acids in elephantid tooth enamel break down reliably and predictably, making them an ideal target for amino acid geochronology (Dickinson et al., 2019). Therefore, there is now the potential to expand the application of amino acid geochronological dating to other mammalian taxa.

In this paper we apply amino acid geochronology to Pleistocene enamel material from the terrace sequence of the River Thames in the UK, a securely dated sequence of deposits, to test the reliability of this approach as a relative dating technique. As peptide sequence differences between taxa may lead to possible taxonomic effects on the rates of amino acid breakdown, we tested chronologically constrained tooth enamel from four commonly occurring mammalian taxa within these deposits: mammoth, straight-tusked elephant, horse and bison. Using these results, we create relative aminostratigraphic frameworks, from which material of unknown age in the region can be correlated and therefore dated. This expands the applicability of IcPD amino acid geochronology to not only another biomineral, but one that can provide direct dates on mammalian fossils.

2. Materials and methods

The river terrace deposits of the Thames Valley are of exceptional importance within north-west Europe for containing one of the longest and most complete sequences of Pleistocene sediments, including many important archaeological and fossiliferous localities (Bridgland, 1994; Gibbard, 1994; Bowen, 1999; White et al., 2006; Bridgland et al., 2013). For this study, 50 elephantid teeth (32 mammoth, both *Mammuthus primigenius* and *Mammuthus trogontherii*, and 18 straight-tusked elephant, *Palaeoloxodon antiquus*), 21 horse (*Equus ferus*) teeth and 15 bison (*Bison priscus*) teeth were sampled (Table 1). Specimens were sampled from collections at Oxford University Museum of Natural History (OUMNH) and from the British Geological Survey (BGS) (Supplementary Table 6). All specimens originate from Quaternary deposits in the Thames basin (Fig. 1), which is geographically subdivided into three regions: Upper, Middle and Lower. The Upper Thames consists of sites situated upstream of the Chiltern Hills from Goring Gap, the Middle Thames extends from the Goring Gap to central London, and the Lower Thames covers sites downstream of London, to the estuary that flows into the North Sea (Bridgland, 1994). Correlation between sites within the basin, as well as correlation with the stages of the marine oxygen isotope record, is largely based on biostratigraphy, lithology, uplift modelling and through numerous dating methods, including amino acid geochronology of molluscan material (Miller et al., 1979; Bowen et al., 1989; Penkman et al., 2013) (see Table 1). However, correlation of the Thames terrace deposits upstream of the Goring Gap has been problematic for some of the higher terrace remnants, in part due to the lack of deposits, as well as difficulties modelling uplift in this region (Bridgland and Schreve, 2009). Nonetheless, the relative chronological order of these deposits is relatively well understood.

The taxa were selected because their teeth are abundant at many of the sites in the Thames Valley and at other Eurasian Quaternary sites

Table 1

Summary of samples analysed and their independent evidence of age resulting in correlation to marine oxygen isotope stages (MIS). OSL = optically stimulated luminescence. More detail is provided in Supplementary Table 1.

Site	Species and number of specimens analysed for this study	Likely MIS correlation	Brief summary of independent evidence of age
Waltham Cross (Lea Valley)	*M. primigenius* (1)	MIS 3-2	Radiocarbon dating (Allison et al., 1952; Stuart, 1982; Lister, 2022) and biostratigraphy (Lister and Sher, 2001)
Hardwick (Upper Thames)	*M. primigenius* (4)	MIS 4-2	Biostratigraphy (Briggs et al., 1985) and terrace stratigraphy (Bridgland, 1994)
Cassington/Yarnton/Oxey Mead/Worton (Upper Thames)	*M. primigenius* (5) *E. ferus (1)* *B. priscus* (2)	MIS 5a-3	Terrace stratigraphy (Bridgland, 1994), OSL dating (Maddy et al., 1998), biostratigraphy (Maddy et al., 1998; Scott and Buckingham, 2021) and IcPD dating of shell and opercula (Penkman et al., 2007, 2013)
Tuckwell's Pit (Upper Thames)	*M. primigenius* (2) *E. ferus (1)* *B. priscus* (3)	MIS 5a-3 or MIS 1	Biostratigraphy (Eeles, 2009) and OSL dating (Scott, 2018). Also present at the site are Middle Iron Age Causeways and a Late Neolithic/Early Bronze Age 'burnt-mound' site (Eeles, 2009).
Sutton Courtenay - Northmoor Gravel (Upper Thames)	*M. primigenius* (4) *E. ferus (1)* *B. priscus* (5)	MIS 3	Biostratigraphy (Briggs et al., 1985; Lister, 2022), terrace stratigraphy (Bridgland, 1994) and radiocarbon ages of ca. 36 ka and 34 ka BP on mammoth bone and tusk (Lister, 2022).
Sutton Courtenay – underlying channel (Upper Thames)	*P. antiquus* (1)	MIS 5e	
Eynsham (Upper Thames)	*E. ferus* (2)	MIS 6/7 or post-MIS 5e	The *Equus* teeth from Eynsham (OUMNH PAL-Q.15205 and PAL-Q.15206) are unmarked and the exact provenance of the teeth is uncertain. The Eynsham Gravel has been correlated with MIS 5e due to the presence of *H. amphibius* (Currant and Jacobi, 2001); however, the occurrence of horse teeth in these gravels is unlikely due to the well-known absence of horse in Britain during MIS 5e (Schreve, 2001).
Stanton Harcourt Channel (Upper Thames)	*M. trogontherii* (10) *P. antiquus* (9) *E. ferus* (8) *B. priscus* (5)	MIS 7	OSL (Rees-Jones and Tite, 1997), ESR/U-series dating of tooth material (Zhou et al., 1997), U-series dating of bone (Pike et al., 2002; Lewis et al., 2006), IcPD dating of shell and opercula (Penkman et al., 2007, 2013) and biostratigraphy (Scott and Buckingham, 2021).
Latton (Upper Thames)	*M. trogontherii* (6) *E. ferus* (2)	MIS 7	Biostratigraphy (Scott and Buckingham, 2021; Scott, 2001; Lewis et al., 2006), U-series dating of bone (Lewis et al., 2006) and IcPD dating of shell (Penkman et al., 2007).
Wolvercote Channel (Upper Thames)	*P. antiquus* (1) *E. ferus* (3)	MIS 9	Biostratigraphy (Sandford, 1924) and terrace stratigraphy (Bridgland, 1994)
Little Thurrock (Lower Thames)	*P. antiquus* (1)	MIS 9	Terrace stratigraphy (Bridgland, 1994; Bridgland et al., 2013)
Grays Thurrock (Lower Thames)	*P. antiquus* (1)	MIS 9	Terrace stratigraphy (Bridgland, 1994; Bridgland et al., 2013), biostratigraphy (Schreve, 1997, 2001) and IcPD analysis of opercula (Penkman et al., 2011, 2013)
Ebbsfleet (Lower Thames)	*P. antiquus* (1)	MIS 11-2	OSL dating, biostratigraphy (Wenban-Smith et al., 2020) and IcPD analysis of shell and opercula (Penkman et al., 2013)
Long Hanborough (Upper Thames)	*P. antiquus* (4) *E. ferus* (2)	MIS 13-11	Terrace stratigraphy (Bridgland, 1994; Bridgland and Schreve, 2009) and biostratigraphy (Bridgland and Schreve, 2009)

spanning the Late and Middle Pleistocene. Stanton Harcourt, a site correlated with MIS 7 (Scott and Buckingham, 2021) was selected for a more detailed study because it enables comparison between different taxa. The site has a very high abundance of mammoth as well as some straight-tusked elephant teeth (enabling us to test for differences between these more closely related taxa), as well as horse and bison. MIS 7 is a climatically complex interval with multiple substages, but the coincidence of *P. antiquus* and *M. trogontherii* in MIS 7 is unlikely to be an artefact of conflation of substages, as the evidence points to contemporaneity at sites like Stanton Harcourt (Scott and Buckingham, 2021). Elephantid amino acid data from sites in the Thames basin, as well as modern *Elephas maximus* data analysed in Dickinson et al. (2019), have been included for comparison. Additionally, modern *Equus ferus* enamel was analysed to assess the initial amino acid composition.

2.1. Drilling and oxidative treatment

Preparation and analysis for the IcPD followed the methods developed in Dickinson et al. (2019). An enamel sample was removed from each tooth with a precision drill using a diamond coated abrasive cutting disc, and the outer layer was removed with a diamond coated abrasive bit burr, usually a 2 mm ball or flame shaped bit. Care was taken to remove any visible dark discolouration that was present, which is assumed to be due to iron or manganese staining (Białas et al., 2021). To prevent contamination of enamel samples with the powders of other dental components, chips were sonicated for 3 min in HPLC-grade water (Sigma-Aldrich) and then ethanol (VWR, analytical-grade), and air-dried before being finely powdered using an agate pestle and mortar. 15–30 mg of powdered enamel was weighed accurately into a 2 mL plastic microcentrifuge tube (Sarstedt), and NaOCl (12%, Fisher Scientific, analytical grade, 50 µL mg^{-1} of enamel) was added. Samples were exposed to NaOCl for 72 h whilst being continuously agitated to ensure complete penetration. The NaOCl was removed, and the powdered enamel was washed five times with HPLC-grade water. To remove any remaining NaOCl, powders were washed with methanol (Sigma-Aldrich, HPLC-grade) before the sample was left to air dry overnight.

2.2. Hydrolysis and biphasic separation

Two subsamples were analysed from each bleached portion of enamel: one subsample was directly demineralised, and the 'free' amino acids (FAA) analysed and the second was treated to release the peptide-bound amino acids, thus yielding the 'total hydrolysable' amino acid fraction (THAA).

FAA samples were demineralised in HCl (1 M, 25 µL mg^{-1}) in a sterile 0.5 mL plastic microcentrifuge tube (Eppendorf); if all visible signs of undissolved material had not disappeared, samples were sonicated for up to 3 min to aid in dissolution. THAA samples were placed in sterile 2 mL glass vials (VWR) and dissolved in HCl (7 M, 20 µL mg^{-1}). The vials were purged with N$_2$ to prevent oxidation, sealed, and heated at 110 °C for 24 h. The acid was removed by centrifugal evaporation. THAA samples were re-dissolved in HCl (1 M, 20 µL mg^{-1}) and transferred into a sterile 0.5 mL plastic microcentrifuge tube (Eppendorf). For both the FAA and THAA samples, the pH of the solution was raised with KOH and then centrifuged for 5 min at 13,000 rpm, whereupon a biphasic solution formed (Dickinson et al., 2019). The supernatant was extracted and dried via centrifugal evaporation.

2.3. Reverse phase-high performance liquid chromatography (RP-HPLC) analysis

Samples were rehydrated with a solution containing an internal standard (L-homo-arginine; 0.01 mM), sodium azide (1.5 mM) and HCl (0.01 M), to enable quantification of the amino acids. Separation of chiral amino acid pairs was achieved using an Agilent 1100 Series HPLC fitted with a HyperSil C18 base deactivated silica column (5 µm, 250 ×

3 mm) and fluorescence detector, using a modified method outlined by Kaufman and Manley (1998) (modifications in Penkman, 2005). The column temperature was controlled at 25 °C, and a tertiary system containing sodium buffer (23 mM sodium acetate trihydrate, sodium azide, 1.3 µM EDTA, adjusted to pH 6.00 ± 0.01 with 10 % acetic acid and sodium hydroxide), acetonitrile and methanol was used as the mobile phase. During preparation of samples, asparagine and glutamine undergo rapid irreversible deamination to aspartic acid and glutamic acid, respectively (Hill, 1965). Therefore, it is not possible to distinguish between these amino acids and their derivatives and they are reported together as Asx and Glx.

Both FAA and THAA samples were prepared in duplicate from bleached powders, with standards and blanks prepared and analysed alongside samples.

2.4. Scanning electron microscopy (SEM) and energy-dispersive x-ray (EDX)

To investigate mineral diagenesis, two of the samples (OUMNH PAL-Q.11705 and OUMNH PAL-Q.11710) were selected for SEM and EDX analyses. Sections of the enamel samples were prepared parallel to the tooth enamel surface with a Struers Minitom with a diamond wafering blade (IsoMet). The samples were polished with an alumina suspension solution (MetPrep, 0.05 µm) and sputter-coated with copper to a thickness of 3 nm (JEOL, JFC-2300HR). The microstructure of enamel and the local distribution of elements in the enamel were then analysed by SEM (JEOL 7800F Prime) in combination with an energy-dispersive X-ray (EDX) probe (Oxford Instruments EDX detectors, Ultim Max), Energy range: 20 keV, number of channels: 1024 Lsec: 31s) at an accelerating voltage of 15 kV.

3. Results and discussion

We first compare the extent of IcPD for samples from four mammalian taxa from the Stanton Harcourt Channel (Bridgland, 1994), a single-age deposit, to identify potential taxonomic differences in the degradation of the amino acids. We then compare results from multiple taxa from Sutton Courtenay where we have analysed samples from deposits of different ages. Finally, we construct relative amino stratigraphies for these four taxa from several sites in the Thames Valley, including data from Dickinson et al. (2019).

3.1. Taxonomic differences

The rates of amino acid racemisation in molluscs have been shown to vary between taxa (Miller and Hare, 1975; Lajoie et al., 1980; Ortiz et al., 2013), which is thought to be due to differences in the original structures of proteins (e.g. Sakalauskaite et al., 2021). Reported peptide sequence differences in mammalian tooth enamel (e.g. Fincham et al., 1983; Cappellini et al., 2019) mean that it is possible that similar taxonomic differences would be observed for enamel; it is therefore important to determine which taxa have significantly different protein and amino acid decomposition patterns, to establish whether they can be used in unified amino acid geochronologies.

The taxonomic differentiation may not follow a systematic or hierarchical trend, so it is therefore difficult to predict *a priori* which taxa can be compared and thus used in comparisons for geochronological purposes. To better understand potential taxonomic differences, four different species of mammal (*M. trogontherii*, *P. antiquus*, *E. ferus* and *B. priscus*) were analysed from Stanton Harcourt, and their differences in amino acid composition and extent of breakdown were evaluated.

3.1.1. Evidence for closed system behaviour in enamel

In a closed system, where all of the products of decomposition are retained, the FAA and THAA D/L values should be highly correlated (Collins and Riley, 2000). Divergence from this trend can indicate that
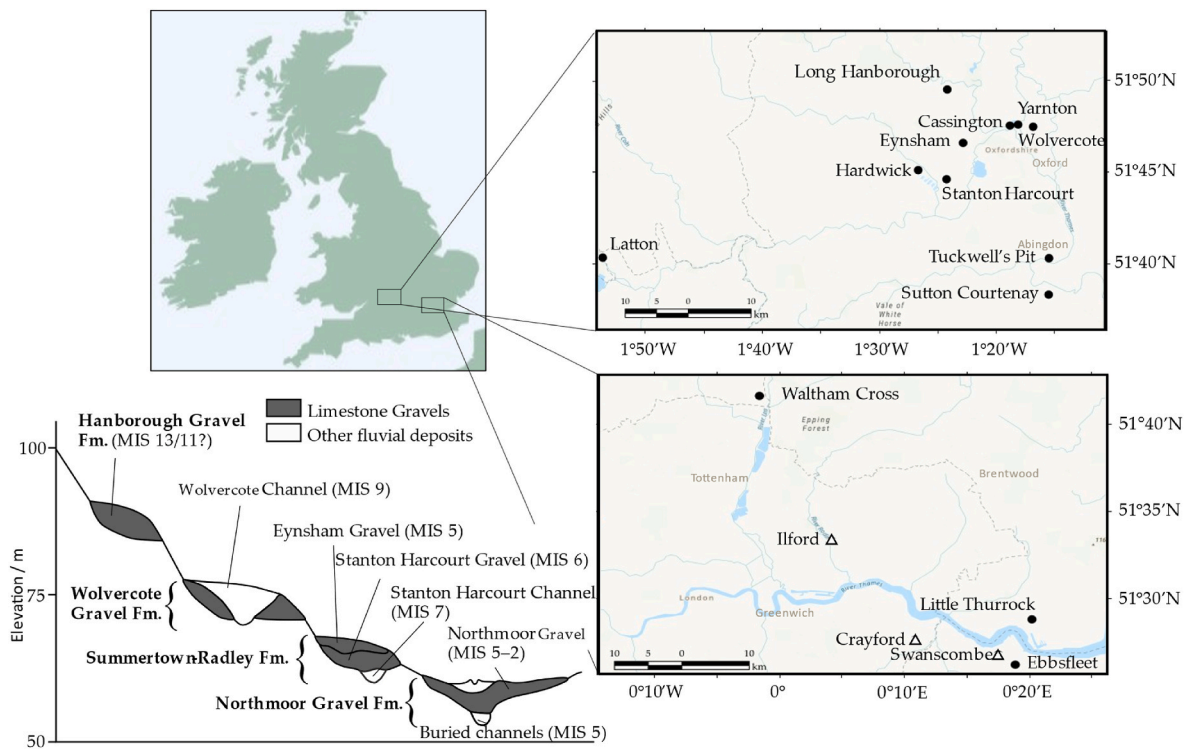
Fig. 1. Location of British sites from which teeth have been analysed for this study (circles), as well as sites from Dickinson et al. (2019) which have been used as part of the comparison (triangles). Upper Thames sites (top right) and Lower Thames sites (bottom right). Composite transverse profile through the terrace deposits of the Upper Thames (bottom left), based on Bridgland (1994).

the amino acids have been exposed to open system conditions, and thus could have been subject to external factors that could influence protein/amino acid decomposition, leaching of amino acids, and/or contamination (Collins and Riley, 2000; Preece and Penkman 2005). The relationship between the FAA and THAA D/L values for all the material from Stanton Harcourt is generally consistent with closed system behaviour (Fig. 2). However, one *P. antiquus* sample, OUMNH PAL-Q.11705, does not follow the expected covariance trend between FAA and THAA D/L values, with the Glx, alanine (Ala) and phenylalanine (Phe) FAA D/L values much higher than for the rest of the Stanton Harcourt material.

The non-concordance observed in the amino acid data for this sample is most likely caused by taphonomic or diagenetic mineralogical changes to the structure of the enamel; this tooth had substantial visible staining of the enamel (Supplementary Fig. 3). Furthermore, during preparation of the sample for RP-HPLC analysis, upon addition of HCl during hydrolysis of the sample proteins, the solution turned orange, probably due to a high concentration of Fe. Scanning electron microscope (SEM) imaging of enamel, in combination with energy-dispersive X-ray spectroscopy from sample OUMNH PAL-Q.11705, revealed areas which were higher in Fe and S, and lower in Ca and P, when compared to the surrounding mineral (Fig. 3). These features were more common in regions around the enamel rods (interprismatic matrix). SEM imaging was also conducted on another tooth from Stanton Harcourt (OUMNH PAL-Q.11710), which conformed to the expected patterns of amino acid decomposition; these Fe/S rich regions were not observed for this sample (e.g. Supplementary Fig. 5). These mineralogical changes could have implications for the preservation of closed system amino acids and might therefore be the cause of the non-concordance in the patterns of IcPD for sample OUMNH PAL-Q.11705.

The tooth OUMNH PAL-Q.11705 was located just above the Oxford Clay surface and is thought to have been buried by silt as the energy conditions of the river channel changed. As a result, the lower part of the tooth has been stained differently to the upper part of the tooth

(Supplementary Fig. 3) and this could reflect a difference in preservation of the enamel. The data from sample OUMNH PAL-Q.11705 was therefore excluded from subsequent data analysis and statistical testing.

3.1.2. Mammuthus vs palaeoloxodon

The British Middle–Late Pleistocene faunal record contains three species belonging to the family Elephantidae (Schreve, 2001; Lister, 2022): two species of mammoth (the steppe mammoth, *Mammuthus trogontherii*, and the woolly mammoth, *Mammuthus primigenius*) and the straight-tusked elephant, *Palaeoloxodon antiquus*. *Mammuthus* first appeared in Europe between 3.5 and 3 million years ago; *Palaeoloxodon* did not appear until around 0.75 million years ago (Lister et al., 2005; Lister and Sher, 2015). To examine whether the genera *Mammuthus* and *Palaeoloxodon* have similar enough IcPD patterns to enable direct comparison in a combined aminostratigraphy, t-tests with Bonferroni-corrected confidence intervals were undertaken. We statistically tested the D/L values for the four most commonly used amino acids for enamel IcPD analysis (Dickinson et al., 2019; Cappellini et al., 2019; Welker et al., 2020) and assessed the amino acid compositions. Due to limited material, the number of samples compared is lower than optimal for a robust statistical test (Supplementary Table 4), but they still provide useful information.

The FAA vs THAA D/L values for the *M. trogontherii* and *P. antiquus* material from Stanton Harcourt plot in broadly similar regions (Figs. 2 and 4). When the differences in mean THAA D/L values are tested statistically using a Welch two-tailed two sample t-test, no significant difference for Asx, Glx, Ala and Phe are observed (Supplementary Table 4). For the FAA fraction, there is no statistical difference between the means of Asx and Ala D/L values. However, there are differences in the FAA D/L values of Phe, with *M. trogontherii* being slightly higher.

The most abundant amino acids in the THAA fraction in the *M. trogontherii* and *P. antiquus* enamel are Glx (~16 %) and glycine (Gly) (~27 %), with most other amino acids constituting around 5–10 % each. There is very little difference in the average THAA composition of
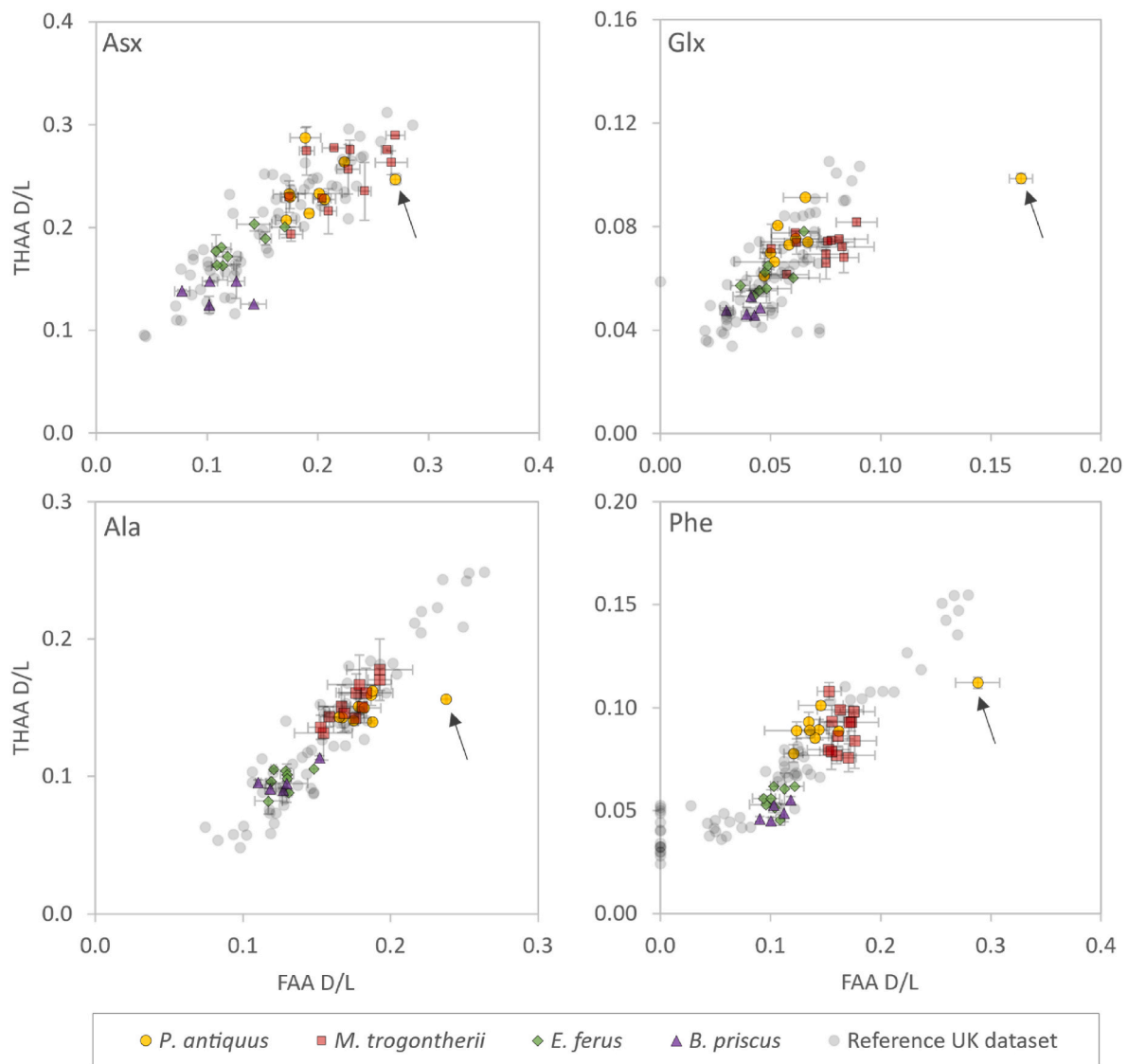
Fig. 2. *Comparison of FAA vs THAA intra-crystalline racemisation in* P. antiquus, M. trogontherii, E. ferus *and* B. priscus *enamel from the MIS 7 deposits at Stanton Harcourt for four different amino acids: Asx, Glx, Ala and Phe. A reference dataset (greyed out data points) of elephantid,* E. ferus *and* B. priscus *samples from other UK sites* (Table 1 *and* Dickinson (2018)) *has been included to illustrate the trend between FAA and THAA values for closed system enamel. The sample from OUMNH PAL-Q.11705 (indicated by the arrow) does not fall on the same trend as the rest of the material analysed. The error bars depict 1σ about the mean based on replicate sub-samples.*

M. trogontherii and *P. antiquus* (Fig. 5). This indicates the composition of the peptides that survive into the fossil record for these two species are likely to be very similar.

[Ser]/[Ala] values have also been used as a measure of the extent of decomposition, often to corroborate other measures of amino acid decomposition (Preece et al., 2009; Penkman et al., 2010). Ser is a relatively unstable amino acid, undergoing a dehydration decomposition pathway while peptide bound (Bada and Mann, 1980). It is therefore expected that within a closed system, where there is no loss of decomposition products, older specimens would have lower [Ser]/[Ala] values, although there is a higher degree of variability in these values when compared to other decomposition metrics (Penkman et al., 2008; Dickinson 2018). For the *M. trogontherii* and *P. antiquus* enamel there is very little difference in the [Ser]/[Ala] ratios (difference of ~5%; Supplementary Fig. 2), which is consistent with samples of a similar age, degrading at comparable rates.

The similar D/L values and amino acid compositions between the two elephantid species indicate that the amino acids and proteins are likely to be breaking down at a very similar rate. It is likely that *M. primigenius* and *M. trogontherii* will also break down in a comparable

way, given their closer phylogenetic distance. We therefore propose that these three species can be used together in a combined amino-stratigraphy. Using both genera, we can apply this elephantid-based geochronology to a wider range of Quaternary deposits than would be possible using just one species. It is also likely that the southern mammoth, *M. meridionalis,* which was present in Europe in the Late Pliocene and Early Pleistocene, will also be able to be included in this framework, but will require further testing.

3.1.3. Elephantidae vs Equus and Bison

Based on the results of Welch two sample *t*-tests, there are significant differences in the mean FAA and THAA D/L values for Asx, Glx, Ala and Phe between *Equus* and Elephantidae, and for *Bison* and Elephantidae for the samples from Stanton Harcourt (Figs. 2 and 4 and Supplementary Table 5). The rate of racemisation is slower for *Equus* and *Bison* than for Elephantidae. The composition of elephantid enamel amino acids is different from the composition of *Equus* and *Bison*, while there are fewer differences between *Equus* and *Bison*. The most abundant amino acids in *Equus* and *Bison* are Gly (~21 %), which is slightly lower than for the Elephantidae, and Glx (~20 %) which is slightly higher. The abundance
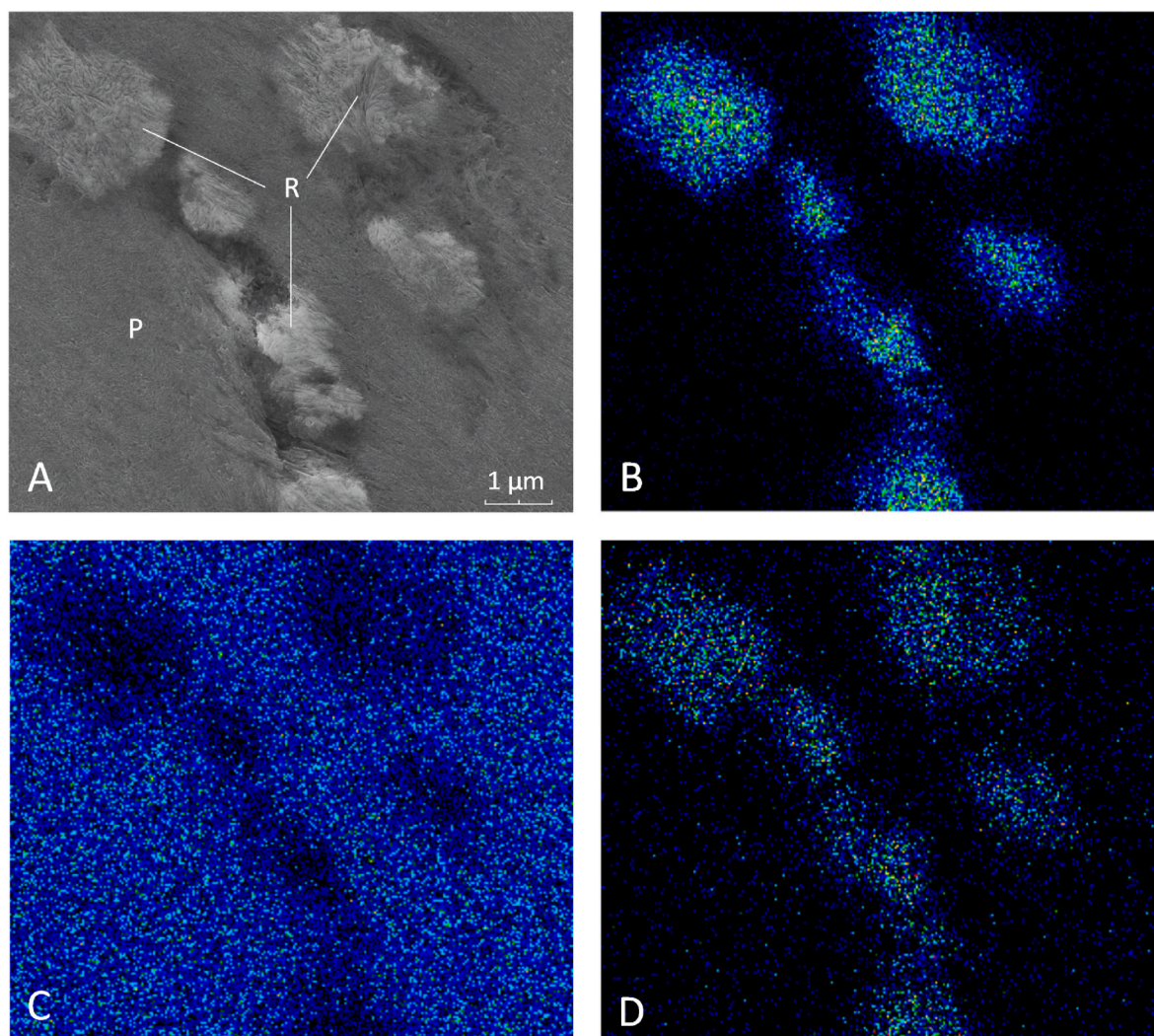
Fig. 3. (A) Scanning electron micrograph of tooth enamel from P. antiquus (OUMNH PAL-Q.11705) from Stanton Harcourt. Enamel prism (P) (Fernandes and Chevitarese, 1991) with regions of alteration (R) predominantly affecting the interprismatic matrix. EDX elemental mapping of the local distribution of sulphur (B), calcium (C) and iron (D). The iron and sulphur content are higher in the same alteration regions visible in the scanning electron micrograph, and calcium content is lower.

of Ser was also higher in the *Equus* and *Bison* (~10 %, compared to ~5 % for Elephantidae). The differences in the THAA compositions may reflect a difference in the original enamel proteomes (e.g. Hasegawa et al., 2000; Weikard et al., 2006; Delgado et al., 2008), which could be responsible for the differences in the rates of racemisation between the taxa. The THAA composition of modern Asian elephant (*E. maximus*) and horse (*E. ferus*) also indicates a difference in the original enamel proteome, but the compositional differences are not the same as those observed in the fossil specimens (Fig. 5). For example, the THAA composition of modern horse has a higher proportion of Gly (~30 %) than modern Asian elephant (23 %), which is the opposite to the diagenetic trends indicated by the fossil material.

The higher contribution of Ser and a higher [Ser]/[Ala] ratio in the *Equus* and *Bison* samples could also be indicative of a lesser degree of protein decomposition. However, it is not clear whether this difference is due to differences in starting composition (i.e. at time = 0 when the tissue/biomineral was formed), or whether this is due to a faster rate of breakdown over time.

3.1.4. Equus vs bison

Negligible differences between *Equus* and *Bison* are observed for mean THAA D/L values of Ala and Phe, and the FAA D/L values of Asx,

Glx, Ala and Phe, in the samples from Stanton Harcourt (Fig. 2 and Supplementary Table 5). Significant differences are observed in the THAA D/L values of Asx and Glx (Fig. 4). Given their phylogenetic distance (divergence at least 60 Ma: Carlisle et al., 2023), it would not necessarily be expected that the rates of IcPD for *Equus* and *Bison* would be the same. The extent of IcPD in *Equus* and *Bison* enamel is low in the samples studied; so, it is possible that differences in other amino acids may be more apparent in more degraded samples. The similar amino acid compositions of the two genera may explain why some of the rates of racemisation are progressing, at least initially, at a similar rate. Until more comparative data for these two species is generated, given the differences in THAA D/L for Asx and Glx, we recommend construction of separate aminostratigraphies for each genus (*Equus* and *Bison*) as well as for elephantids.

3.2. Comparison of different age specimens from a single locality – Sutton Courtenay

The faunal material from Sutton Courtenay is derived from two deposits. The upper unit consists of several metres of the Northmoor Gravels (correlated with MIS 3 on the basis of biostratigraphy (Briggs et al., 1985; Lister, 2022) and radiocarbon dating (Lister, 2022)) and
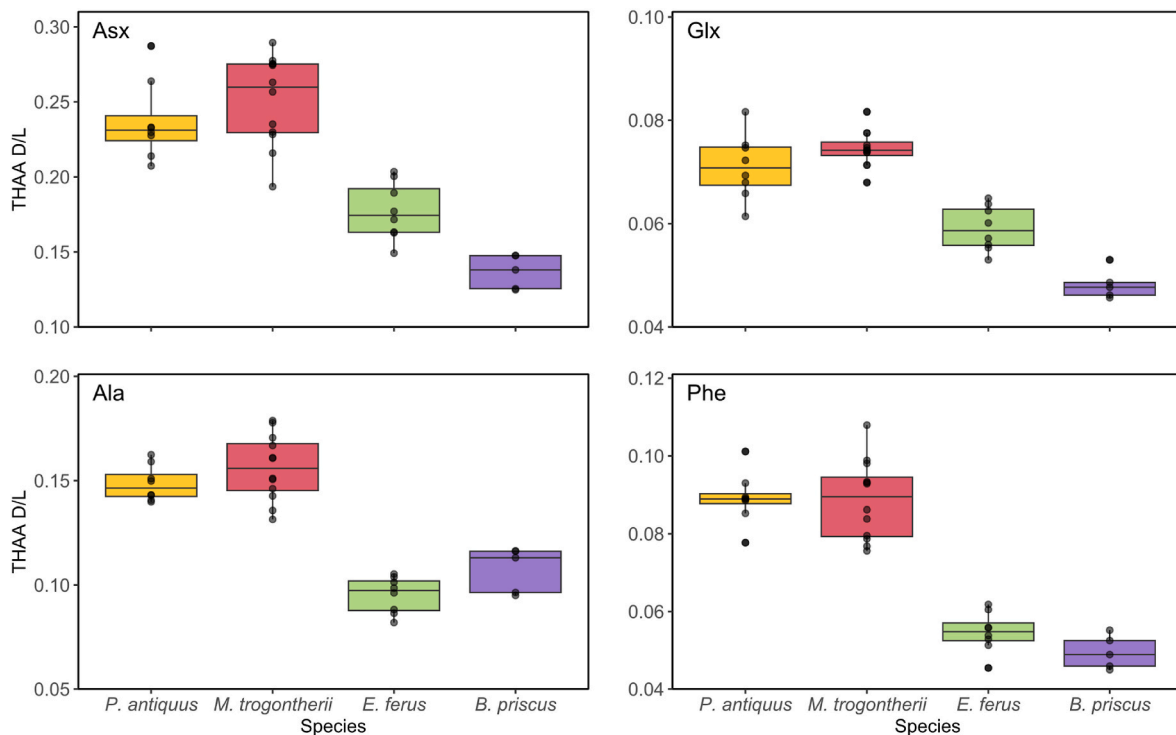
Fig. 4. *Median Asx (top left), Glx (top right), Ala (bottom left) and Phe (bottom right) D/L values with quartile intervals, for ~200 ka enamel samples of* P. antiquus, M. trogontherii, E. ferus *and* B. priscus *from the MIS 7 deposits at Stanton Harcourt. Individual data points have also been plotted.*
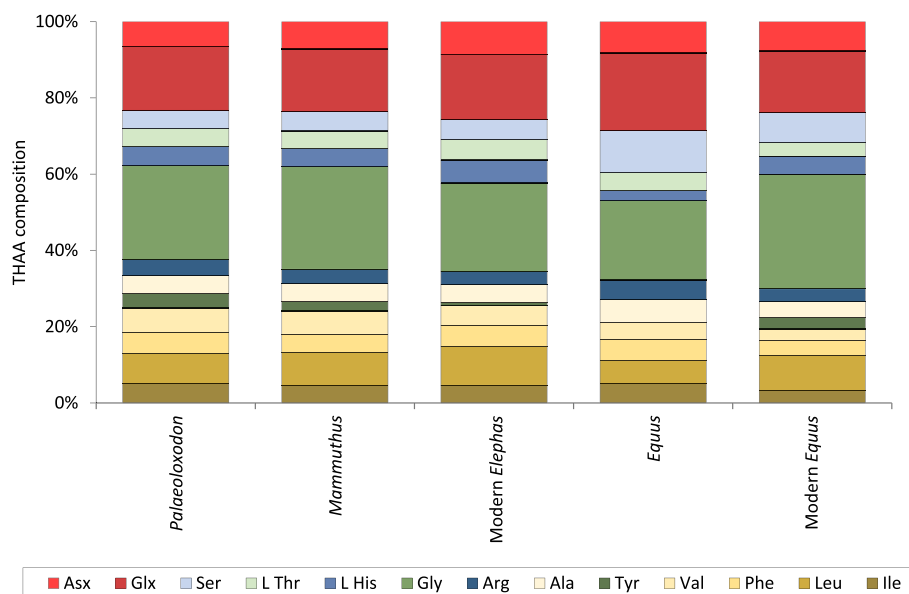

Fig. 5. *Mean THAA compositions for ~200 ka P. antiquus (n = 9), M. trogontherii (n = 12), E. ferus (n = 8), and B. priscus (n = 5) enamel from MIS 7 of Stanton Harcourt with comparisons to modern E. ferus and E. maximus (E. maximus from* Dickinson et al., 2019*).*

includes a varied fauna including numerous specimens of *Bison, Equus* and *M. primigenius* (Briggs et al., 1985; Currant and Jacobi, 2001; Lister, 2022). Below these deposits, in depressions in the Oxford Clay, were remnants of river sediments correlated with MIS 5e (based on biostratigraphy) with a more limited fauna, including *P. antiquus* and *Hippopotamus* (Currant and Jacobi, 2001).

The extents of racemisation in the three taxa (*M. primigenius, E. ferus* and *B. priscus*) from the Northmoor Gravels are analogous with the trends observed with the material from Stanton Harcourt: i.e., in samples of the same age, *Mammuthus* is more degraded than *Equus* and *Bison*,

while *Bison* and *Equus* have comparable levels of racemisation (Fig. 6). The extent of racemisation in Asx, Glx, Ala and Phe for the *P. antiquus* from the lower deposits is greater than for the *M. primigenius* material from the Northmoor Gravels, indicating a greater antiquity, which agrees with the current understanding of the stratigraphic context of the material. This corroborates the ability of IcPD dating of enamel to distinguish samples from deposits of different ages (MIS 3 and MIS 5e). This also indicates that IcPD analysis could be used to identify reworked mammalian material at sites with complex sample contexts, as well as to potentially provide background to museum specimens that lack

provenance.

3.3. Thames enamel IcPD geochronology

Given the taxonomic differences observed (Section 3.1), three enamel-based IcPD geochronologies have been built for the Thames deposits: Elephantidae (combining *Palaeoloxodon* & *Mammuthus*), *Equus*, and *Bison*.

3.3.1. Elephantidae aminostratigraphy

3.3.1.1. Devensian sites – Hardwick, Sutton Courtenay, Cassington (Yarnton/Oxey Mead), Tuckwell's pit and Waltham Cross Pit. The extent of Asx, Glx, Ala and Phe racemisation in elephantid teeth from Hardwick, Sutton Courtenay (Northmoor Member) and 3 of the 4 teeth (OUMNH PAL-Q.09167, PAL-Q.09766 and PAL-Q.09767) from Cassington are comparable, which is consistent with their expected similar age (Table 1). These specimens all exhibit lower levels of IcPD than the other analysed specimens from the Upper Thames, consistent with their stratigraphic position in the terrace sequence. The extent of racemisation is also similar to the specimens from Waltham Cross Pit, a terrace remnant in the Lea valley, which is thought to correlate with MIS 3-2 (Lister, 2022).

One of the *M. primigenius* teeth (OUMNH PAL-Q.09759) (Fig. 7 and Supplementary Table 6) from Cassington (Oxey Mead) has higher racemisation values for Asx, Glx, Ala and Phe, indicating that this tooth is potentially older than the other teeth correlated to MIS 3-2. Most of the large faunal remains at Cassington (Yarton/Oxey Mead), which are dominated by reindeer and bison, are thought to originate from deposits dated to late MIS 5 (Maddy et al., 1998; Penkman et al., 2013). However, the mammoth remains from Cassington are generally paler in colour and less well preserved than the rest of the large vertebrates, which has led to the suggestion they originate from the sands and gravels that overlie deposits dated to MIS 5a (Maddy et al., 1998). The state of preservation of the OUMNH PAL-Q.09759 sample appears to be different to the other mammoth specimens from Cassington (Supplementary Fig. 6). It is more complete, and appears better preserved, which could indicate the sample is from a different stratigraphic context to the other mammoth

specimens sampled from the site. Unfortunately, most of the sampled specimens from Cassington (Yarnton/Oxey Mead) lack detailed stratigraphic context because they were surface collected. If the specimen OUMNH PAL-Q.09759 is from an older deposit than the rest of the mammoth material analysed or if the deposits are mixed, the extent of IcPD indicates the sample is not older than MIS 7 and would be most consistent with dating to MIS 4/5.

The faunal remains at Tuckwell's Pit are thought to correlate with MIS 5a–3 (Scott, 2018; Eeles, 2009). The faunal assemblage at Tuckwell's Pit, comprising reindeer, bison, wolf and bear, resembles that of Cassington and is associated with molluscs, beetles and vegetation indicative of a temperate environment, which is thought to date to c. 90 - 78 ka, indicating equivalence to MIS 5a (Scott, 2018). However, the mammoth and horse specimens are likely to be derived from other, younger deposits present at the site (Supplementary Table 1).

The extent of racemisation in the *Mammuthus* enamel from Tuckwell's Pit is generally similar to sample OUMNH PAL-Q.09759 from Cassington and to the *P. antiquus* from Sutton Courtenay (correlated with MIS 5e; Fig. 7), which indicates that this material might be slightly older than the material correlated with MIS 3–2 and indicates a possible MIS 4/5 age.

It is not clear whether the temporal resolution of this IcPD geochronology based on elephant enamel is high enough to distinguish MIS 3 from MIS 4–5. However, it hints that at both Tuckwell's Pit and Cassington there may be mammoth present in the deposits correlating to MIS 4–late MIS 5, and at Cassington this geochronology may be able to distinguish between fossils from the two ages.

3.3.1.2. Post-hoxnian and pre-ipswichian – Stanton Harcourt channel, Latton, Ilford, Crayford, Wolvercote Channel, Little Thurrock, Grays Thurrock and Ebbsfleet. The extent of racemisation in sites correlated with MIS 7 (Stanton Harcourt, Ilford, Crayford and Latton) is similar (Fig. 7) and is therefore consistent with a comparable age for those sites. The IcPD in these MIS 7 sites is generally greater than for the sites correlated with MIS 3–2 (excluding the one specimen from Cassington, OUMNH PAL-Q.09759, Section 3.3.1.1), indicating a greater age, with consistent separation between D/L values observed in Asx, Ala and Phe. The lack of clear temporal resolution in D/L values for Glx is likely due
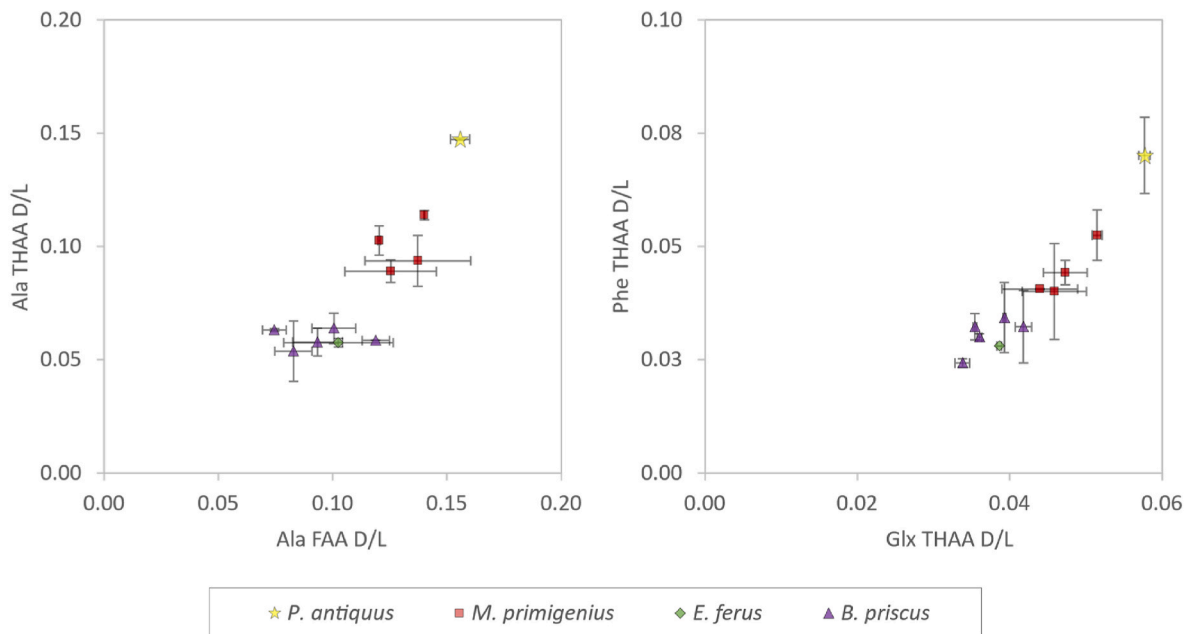

Fig. 6. *Comparison of intra-crystalline racemisation in* P. antiquus*,* M. primigenius*,* E. ferus *and* B. priscus *enamel from the different deposits at Sutton Courtenay for three different amino acids: Glx, Ala and Phe. Note: THAA Phe D/L values have been plotted against THAA Glx D/L values (instead of FAA Phe D/L values), because the concentrations of FAA D-Phe are too low for accurate determination for these samples.*
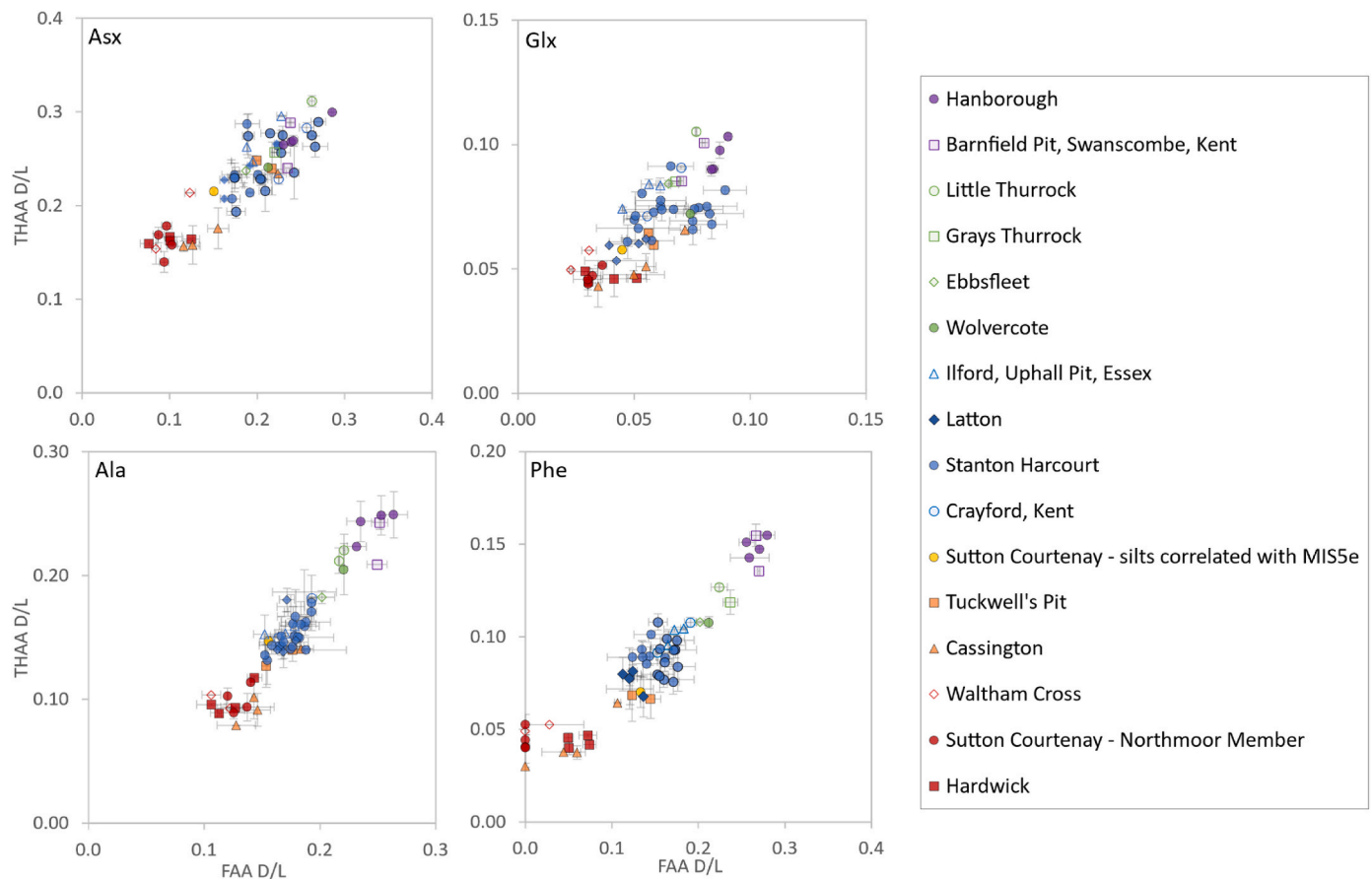
Fig. 7. *Comparison of FAA vs THAA intra-crystalline racemisation in elephantid (*P. antiquus, M. primigenius *and* M. trogontherii*) enamel from several Thames Middle and Late Pleistocene sites for four amino acids: Asx, Glx, Ala and Phe. Colours of the data points are chosen based on independent evidence correlating the material to MISs: red = MIS 3-2, orange MIS 4-5a, yellow = MIS 5e, blue = MIS 7, green = MIS 9, purple = MIS = 11/13. Error bars depict 1σ about the mean based on replicate sub-samples. Data plotting on the y-axis does not represent FAA DLs of 0; for those samples the concentration of D amino acids was too low for accurate quantification.*

to the slower rates of racemisation for this amino acid (Dickinson et al., 2019).

The *P. antiquus* sample from the Sutton Courtenay sediments correlated with MIS 5e, and samples from Tuckwell's Pit, have similar IcPD to some of the samples from sites correlated with MIS 7. The extents of racemisation for Phe are generally lower in the samples from MIS 3/4/5e sites than from MIS 7 sites, but there is no clear separation between the groups. Whilst amino acid geochronologies based on molluscan material can provide glacial-interglacial temporal resolution throughout the Late and Middle Pleistocene (Preece et al., 2009; Penkman et al., 2011), this may not be the case for enamel (Dickinson et al., 2019). Rates of racemisation in enamel are considerably slower than in mollusc opercula (Fig. 8) leading to lower levels of temporal resolution, but also a larger time range over which enamel based amino acid geochronologies may be applicable.

The extent of racemisation in sites correlated with MIS 9 (Wolvercote Channel, Little Thurrock, Grays Thurrock) is generally greater than for sites correlated with MIS 7, particularly for Ala and Phe. This suggests the potential for enamel IcPD to discriminate between MIS 7 and 9; however, there are only a limited number of samples from MIS 9 sites. It is therefore not possible without additional data to confidently resolve these two interglacials at present.

The extent of IcPD in the enamel of the *P. antiquus* sample from Ebbsfleet (GSM 5031) is slightly greater that those correlated with MIS 7 and is most similar to those correlated to MIS 9, indicating a similar age for this tooth.

3.3.1.3. Hoxnian/pre-hoxnian sites – Hanborough and Swanscombe. The Hanborough Terrace gravel deposits have yielded both a temperate climate mammalian assemblage (e.g. *P. antiquus*), as well as a cold-climate molluscan assemblage (Briggs and Gilbertson, 1973). The warmer taxa (such as *P. antiquus*) came from the base of the sequence, which has been interpreted as a lag deposit, and the cold climate molluscs were from thin silt beds interbedded with the gravels (Bridgland and Schreve, 2009). Bridgland and Schreve (2009) suggest the elephants were reworked from a pre-Anglian temperate-climate context (MIS 13), with cold climate molluscs more likely recording conditions during the deposition of the terrace sediments in the Anglian (MIS 12).

Correlations of the Hanborough terrace with the Middle-Lower Thames terrace sequence have varied through time: earlier correlations were made with the downstream Boyn Hill/Orsett Heath terraces (Bridgland, 1994), while more recent correlations have been with the Winter Hill/Black Park terraces based on revised uplift modelling (Bridgland and Schreve, 2009; Westaway, 2011). These different correlations have a bearing on the age of the mammals recovered from the base of the Hanborough terrace. The earlier correlation implies a Hoxnian/MIS 11 age for the Hanborough elephants, because vertebrates of Hoxnian/MIS 11 age have been recovered from the Boyn Hill/Orsett Heath terraces, most notably at the site of Swanscombe in Kent (Schreve, 2001). In contrast, the correlation with the Winter Hill/Black Park terraces indicates an older age, since these terraces are known to have formed during the Anglian cold stage (MIS 12). Directly dating the Hanborough elephants provides an independent test of these different hypotheses for terrace correlation.
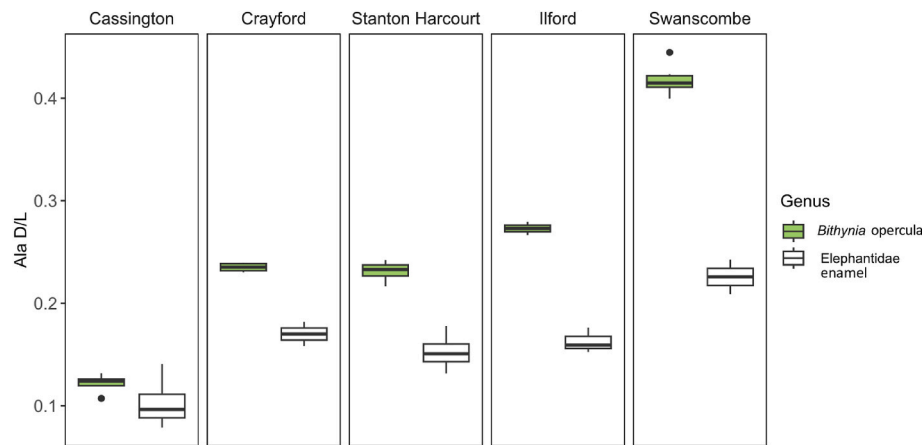
Fig. 8. *Comparison of Elephantidae enamel and* Bithynia *opercula THAA Ala DL ratios for sites where both are present in contemporaneous deposits.* Bithynia *opercula values have been taken from* Penkman et al. (2013). *The slower racemisation in the enamel results in poorer temporal resolution for these Early & Middle Pleistocene sites than for the opercula, but a longer temporal range, as equilibrium is not reached so quickly.*

The extent of racemisation in enamel from Hanborough is generally greater than the material from Wolvercote and other sites correlated with MIS 9, and is similar to the *P. antiquus* material from Swanscombe (MIS 11) (Fig. 7). Therefore, the *P. antiquus* material from Hanborough is most likely of a similar age based on the extent of enamel IcPD. However, due to the lack of older comparative material, an MIS 13 age cannot be dismissed. Future sampling of MIS 13 and older elephantids would help to test whether these interglacials can be distinguished based on enamel IcPD analysis.

3.3.2. Equus aminostratigraphy

For sites where both elephantids and *Equus* have been analysed, the extent of racemisation in the *Equus* enamel is lower than contemporaneous elephantid enamel, which is consistent with the trends observed in the material from Stanton Harcourt (Section 3.1). Nonetheless, the *Equus* geochronology is generally in agreement with the elephantid geochronology (Fig. 9).

3.3.2.1. Devensian and ipswichian sites – Sutton Courtenay (Northmoor Gravels), Cassington (Yarnton/Oxey Mead), and Tuckwell's Pit.
The precise provenance of the material from Tuckwell's Pit is largely unknown, and as such the material from this site could originate from several deposits of different ages (Supplementary Table 1). The *Equus* enamel analysed from Tuckwell's Pit (OUMNH PAL-Q. 10665) was from a near-complete horse skull, and due to its completeness and dark colour of preservation (consistent with deposition in peat), it is likely from the Iron Age peat near the location documented for the specimen (Eeles, 2009). It is therefore likely that this tooth is Iron Age. The extent of racemisation in the *Equus* enamel sample (OUMNH PAL-Q. 10665) from Tuckwell's Pit is similar to the *Equus* material from the Northmoor Gravels at Sutton Courtenay, indicating that because of the slow rates of racemisation, enamel IcPD dating is unlikely to be able to easily distinguish between more recent time periods (e.g. Iron Age and MIS 3) for horse specimens from this region.

Similar to the mammoth material, the *Equus* material from Cassington is also not thought to be derived from the deposits dating to MIS 5 because horse is believed to be absent in the British Isles during MIS 5 (Currant and Jacobi, 2001, 2011; Schreve, 2001). It has therefore been assumed that the *Equus* specimen from Cassington is from the younger deposits correlated with MIS 3/4. However, the IcPD in the *Equus* tooth (OUMNH PAL-Q.09346) is inconsistent with an MIS 4-3 correlation and is more comparable to the material from Stanton Harcourt and Wolvercote (Fig. 9). Very little of the faunal material from the series of sites in the Cassington area were excavated *in situ* and as such, for most of the fossils, precise provenance is unknown. The horse specimen (OUMNH

PAL-Q.09346) is pale in colour and incompletely preserved; this state of preservation has been linked to the MIS 4-3 gravels at Cassington by Maddy et al. (1998). The IcPD results suggest that this specimen instead derives from older deposits and may have been reworked. Near Cassington (Yarnton) there are deposits of the Summertown-Radley Terrace, which contains mammals of MIS 7 age, including horse as demonstrated at the site of Stanton Harcourt (Scott and Buckingham, 2021). One explanation for this unexpected IcPD result could be that the horse tooth was reworked from older sediments nearby and re-deposited at Cassington (Yarnton) during MIS 5-3 along with younger material.

3.3.2.2. Post-hoxnian and pre-ipswichian sites – Stanton Harcourt, Latton and Eynsham.
The samples from sites correlated with MIS 7 (Stanton Harcourt and Latton) cluster together, having higher D/L values than the samples from MIS 3/younger deposits, and lower D/L values than the samples from Wolvercote. This is consistent with an MIS 7 correlation for these sites and indicates the Wolvercote material is older, in agreement with correlation to MIS 9 (Schreve, 1997).

Prior to analysis, due to the lack of provenance and the number of potential different deposits exposed at Eynsham, it was unknown whether the horse teeth analysed here were post-Ipswichian (MIS 5e) or pre-Ipswichian (most likely MIS 7/6); an Ipswichian age would challenge the conventional biostratigraphy (Schreve, 2001), while the state of preservation of the teeth was equivocal for identifying reworking (Supplementary Table 6). There are cold climate gravel units above the Ipswichian Eynsham Gravels at Eynsham, probably dating to younger time periods (Sandford, 1924; Bridgland, 1994). In addition, there are exposures of the Summertown-Radley terrace near Eynsham, and it is possible that lower deposits correlating to MIS 7/6 (equivalent to those at Stanton Harcourt) have yielded mammal material of this age in this area. There is also evidence of reworked molluscan material in the Ipswichian Eynsham Gravel, e.g., an abraded shell fragment of *Corbicula fluminalis* at Radley (Bridgland, 1994), which raises the possibility of reworked mammalian material in this deposit too. The extent of racemisation in the *Equus* enamel from Eynsham is most similar to material thought to correlate with MIS 7, and is slightly lower than material from Wolvercote which is thought to correlate with MIS 9. This rules out a post-MIS 5e age for the Eynsham *Equus* teeth and indicates the teeth are pre-Ipswichian, likely correlating with MIS 7.

3.3.2.3. Hoxnian/pre-hoxnian sites - Hanborough.
The *Equus* material from Hanborough has a greater extent of IcPD than the Wolvercote material, which is consistent with the elephantid data and corroborates a greater antiquity. Due to the lack of pre-Hoxnian *Equus* data, this data cannot currently refine the age assignment any further. However, given
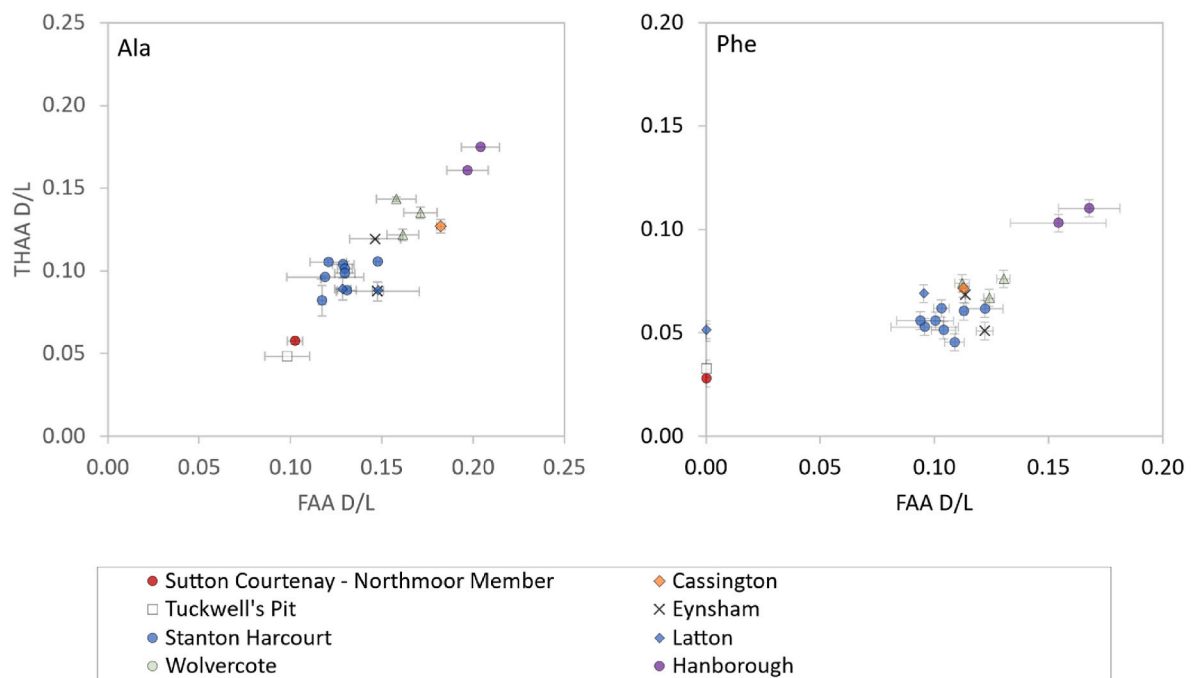
Fig. 9. *Comparison of FAA vs THAA intra-crystalline racemisation in* E. ferus *enamel from several Thames Middle and Late Pleistocene sites for two amino acids: Ala and Phe. These amino acids have been selected because they give the best separation between sites for this time period. Colours of the data points are chosen based on correlation of the material to MISs. Error bars depict 1σ about the mean based on replicate sub-samples. Data plotting on the y-axis does not represent FAA D/Ls of 0, for those samples the concentration of D amino acids was too low for accurate quantification.*

the degree of IcPD in the Hanborough *Equus* samples, it is likely that they correlate with MIS 13-11.

3.3.3. Bison aminostratigraphy

IcPD analysis has been conducted on *Bison* enamel from four deposits in the Upper Thames Valley: Sutton Courtenay – Northmoor Gravels, Tuckwell's Pit, Cassington, and Stanton Harcourt. The *Bison* aminostratigraphy is consistent with the relative order of the ages of the deposits based on the independent evidence of age. The samples from the Northmoor Gravels at Sutton Courtenay are the least degraded (Fig. 10), consistent with their position in the terrace sequence and correlation with MIS 3. The samples from Tuckwell's Pit are more degraded than the material from the Northmoor Gravels at Sutton Courtenay, but less degraded than the material from Stanton Harcourt. This is consistent with correlation with MIS 5, but lack of comparative data precludes assignment to a specific substage. The material from Cassington also plots between material from the Northmoor Gravels at Sutton Courtenay, and Stanton Harcourt, and is also consistent with a MIS 5 correlation. These two sites, Cassington and Tuckwell's Pit, are known to have stratigraphic layers dominated by remains of bison and reindeer that are thought to date to MIS 5a (Maddy et al., 1998; Eeles, 2009; Scott, 2018). A similar bison-reindeer fauna famously occurs at the Early Devensian site of Banwell Bone Cave, which is also now thought to date to MIS 5a (Currant and Jacobi, 2011; Stevens and Reade, 2021), and several other sites have been assigned to the Banwell Bone Cave mammal assemblage-zone including Picken's Hole, Stump Cross Caverns, Isleworth and Wretton (Scott, 2018; Lundberg et al., 2020). The new IcPD results provide independent support for the assignment of the bison-reindeer strata at Cassington and Tuckwell's Pit to the Early Devensian. The samples from Stanton Harcourt have higher IcPD values that the rest of the bison material analysed, which is consistent with the MIS 7 correlation. Where comparison is possible, *Bison* is less degraded than the Elephantidae (Fig. 10), which is consistent with the trends observed in the specimens from Stanton Harcourt (Section 3.1).

4. Conclusions

Significant differences in the rates of IcPD are observed between elephantids and both *Equus* and *Bison*, confirming suspected taxonomic differences in enamel IcPD (Cappellini et al., 2019), and highlighting the importance of building IcPD geochronologies based on tested taxonomic groups. We recommend that the genera *Palaeoloxodon* and *Mammuthus* can be used to build a single geochronology, and the genera of *Equus* and *Bison* should be considered separately. These geochronological frameworks can be used to correlate other Middle-Late Pleistocene elephantid, *Equus* and *Bison* specimens from British palaeontological sites to the marine oxygen isotope record, providing direct relative dates on mammalian material.

These enamel-based IcPD geochronological frameworks for the Thames Valley terraces agree with the terrace stratigraphy and biostratigraphical models for the region, demonstrating a robust relationship between extent of IcPD and age of specimens. Several horse specimens with uncertain provenance from sites with horizons of multiple ages did not indicate an MIS 5 age, bolstering biostratigraphical interpretations of horse absence in Britain during MIS 5. The temporal resolution of the enamel-based IcPD technique is not as high as that observed for molluscan opercula (Penkman et al., 2013), which is likely due to the slower rates of decomposition in the enamel (Dickinson et al., 2019). However, good temporal resolution is observed between specimens correlated with MIS 3 and 7, and the enamel-based technique may be able to resolve MIS 5 material from MIS 3 in Britain. Additionally, although the material is limited, the data suggests it may be possible to separate specimens correlated with MIS 7 from MIS 9. While enamel-based IcPD generally appears capable of resolving at an interglacial level for this time period in Britain, it seems unlikely that it can distinguish substages.

These amino acid stratigraphies based on tooth enamel demonstrate the viability of directly dating mammalian fossils from Late and Middle Pleistocene deposits in Northern Europe, and the potential for expansion to other regions. This is of particular value for archival material; many teeth have been collected for centuries and been stored in museums and
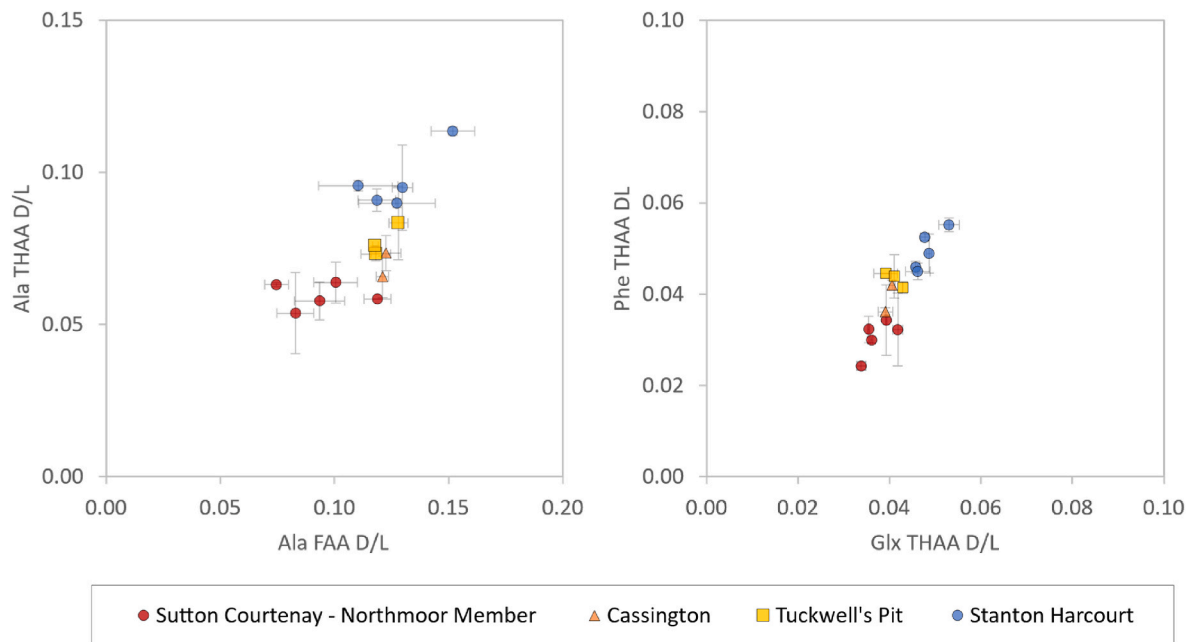
Fig. 10. *Comparison of FAA vs THAA intra-crystalline racemisation in* B. priscus *enamel from several Thames Middle–Late Pleistocene sites for four amino acids: Asx, Glx, Ala and Phe. Colours of the data points are chosen based on correlation of the material to MISs. Error bars depict 1σ about the mean based on replicate sub-samples.*

private collections, often lacking stratigraphical information. This technique can now be applied to these teeth, providing direct dates on these important specimens, thereby increasing their scientific value.

CRediT authorship contribution statement

M.R. Dickinson: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **K. Scott:** Writing – review & editing. **N.F. Adams:** Writing – review & editing. **A. M. Lister:** Writing – review & editing, Conceptualization. **K.E.H. Penkman:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Kirsty E. H. Penkman reports financial support was provided by Natural Environment Research Council. Marc R. Dickinson reports financial support was provided by Natural Environment Research Council. Kirsty E. H. Penkman reports financial support was provided by European Research Council. Marc R. Dickinson reports financial support was provided by European Research Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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