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Dulias, Katharina, Birch, Steven, Wilson, James F. et al. (7 more authors) (2019) Maternal relationships within an Iron Age burial at the High Pasture Cave, Isle of Skye, Scotland. *Journal of archaeological science*. 104978. ISSN 0305-4403

<https://doi.org/10.1016/j.jas.2019.104978>

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1 **Research Paper**

2 **Title:** Maternal relationships within an Iron Age burial at the High Pasture Cave, Isle of Skye,
3 Scotland

4

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33 Declaration of interest: none.

34 **Highlights**

- 35 • Rare Iron Age cave burial assemblage of an adult female with foetal remains
- 36 • First report of complete ancient mitochondrial DNA sequences from foetal-aged bone
- 37 fragments
- 38 • Shot-gun sequencing and mitochondrial genome analysis for maternal lineage
- 39 identification
- 40 • Female could be excluded as being the mother of one the infants

41

42 **Abstract**

43 Human remains from the Iron Age in Atlantic Scotland are rare, which makes the assemblage
44 of an adult female and numerous foetal bones at High Pasture Cave, on the Isle of Skye,
45 particularly noteworthy. Archaeological evidence suggests that the female had been deposited
46 as an articulated skeleton when the cave entrance was blocked off, marking the end of use of
47 the site. Particularly intriguing is the deposition of disarticulated remains from a foetus and
48 perinate close to the adult female, which opens the possibility that the female might have been
49 the mother of both of the infants. We used shotgun genome sequencing in order to analyse
50 the mitochondrial genomes of all three individuals and investigate their maternal relationship,
51 and we report here, for the first time, complete ancient mitogenomes from foetal-aged bone
52 fragments. While we could not exclude the possibility that the female was the mother of, or
53 maternally related to, the foetus, we could definitely say that she was not the mother of the
54 perinate buried alongside her. This finding is contrary to the standard archaeological
55 interpretation, that women in such burials most likely died in childbirth and were buried
56 together with their foetuses.

57 Keywords: ancient DNA, foetus, mitochondrial DNA, shot-gun sequencing

58

59 **Introduction**

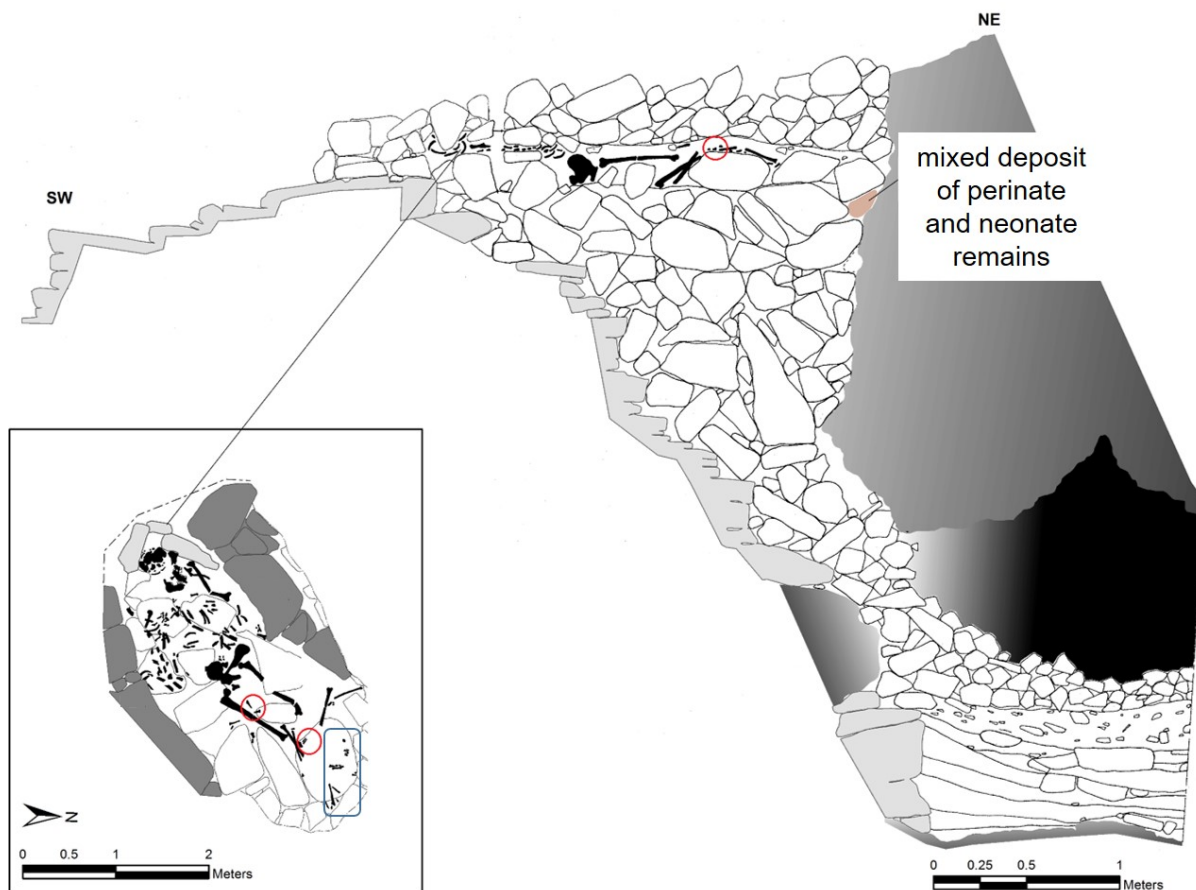
60 High Pasture Cave (Uamh an Ard Achadh) is situated in the Parish of Strath on the Isle of
61 Skye in Scotland. The wider environment surrounding the site revealed a diverse
62 archaeological landscape of monuments and sites relating to prehistoric and historic periods,
63 including a number of funerary monuments and hut-circles, combined with well-preserved field
64 systems (Birch and Wildgoose, 2013). Radiocarbon dates of samples from different layers
65 within the cave, and from the complex deposits outside the cave entrance, indicate periodic
66 activity at High Pasture Cave from the 19th century cal. BC to cal. AD 1st century (Birch and
67 Wildgoose, 2013). This, and the diversity of small finds, indicates a complex occupation history
68 with several phases of intense activity during the Iron Age (Birch and Wildgoose, 2013).

69 Stonework found in the surface trench was associated with a series of entrance arrangements
70 leading down a stone stairwell into the cave, whose system provided direct access to an
71 underground stream. Although the archaeological material found in the cave indicated a fairly
72 typical domestic assemblage (comprising bone, stone and iron tools, ceramics, and residues
73 of metalworking), their depositional context, including the restricted morphology of the cave,
74 raises questions about this interpretation. A well-preserved faunal assemblage was recovered,
75 including animal and fish bones, and shellfish, as well as charred plant remains. Among the

76 animal bones, a high percentage were assigned to domesticated pig, which suggests a
77 feasting deposit (Livarda and Madgwick, 2017), and the bones displayed evidence of unusual
78 butchery practices, such as deliberate division of skeletons into left and right parts, and
79 unusual cut-marks across several ribs (Drew, 2005). The majority of the remains found at this
80 site displayed distinct ritual aspects, such as the burial of animals after butchery, and the
81 presence of both disarticulated human remains and complete human inhumations, including
82 foetal and infant burials (Birch and Wildgoose, 2013; Livarda and Madgwick, 2017).

83 A century or so after the stairwell was back-filled, with the passage being deliberately blocked
84 with boulders and sediments, a final act of closure at High Pasture Cave was carried out (Birch
85 & Wildgoose, 2013). Human remains from three individuals were deposited into the top of the
86 blockage (**Figure 1**), consisting of an adult female, aged 25–40 years and 1.55m tall, together
87 with two infants: one foetus that died between 12 and 26 weeks of gestation, and one perinate
88 that died during the last month of foetal development or during the first two weeks of life. The
89 woman had been laid out in the top of the backfilled stairwell that originally provided access
90 to the natural cave below, and large stones had been placed on top of her. The remains were
91 badly fragmented in the head, chest, abdominal area, and the right upper and lower arm, and
92 left lower arm regions, consistent with blunt force trauma from the stones. This happened
93 either at, or around, the time of deposition, precluding any chance to ascertain if the injuries
94 were the cause of her death.

95



96

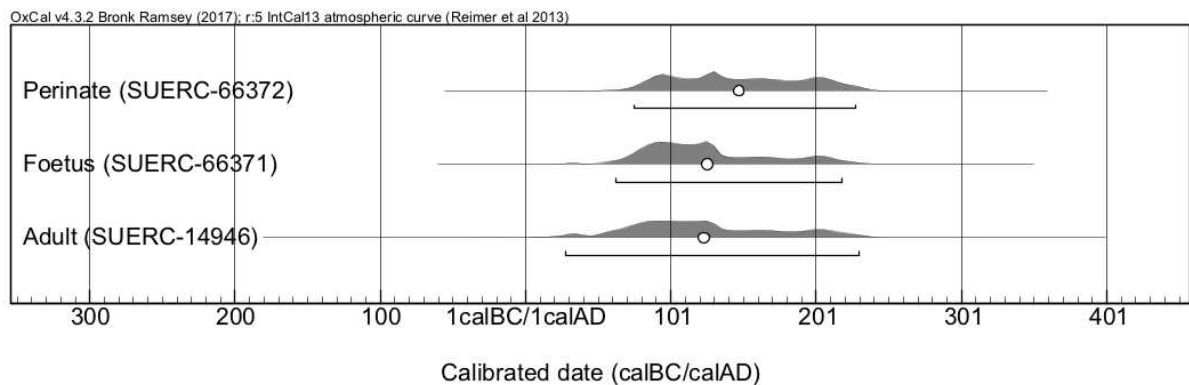
97 **Figure 1.** Burial schematic at High Pasture Cave, showing the position of the human remains
 98 within the stairwell blockage and, inset, the location of the adult, infant and animal bones. The
 99 remains of the foetus and perinate are indicated by red circles, with the foetal bones closer to
 100 the female's pelvis than the perinate. The blue rectangle shows the location of the foetal pig
 101 and perinatal dog, and also included a mixed deposit of perinate and neonate remains, as
 102 shown in the section of the stairwell.

103

104 Bones of the foetus were recovered from between the knees of the woman, while two small
 105 bundles of bones by her feet contained the skeletal elements from the perinate, and the
 106 remains of a foetal pig and a perinatal dog (**Figure 1**). The reasons for the segregation of the
 107 bones from the human foetus and perinate, and piglet and puppy are difficult to explain, but
 108 this may have formed a major part of the overall internment process. Although the relationship
 109 between the infant remains and the adult woman in the burial were uncertain, radiocarbon
 110 dates indicate that all three are roughly contemporary. The adult female yielded a radiocarbon
 111 date of cal. AD 28–230 (2 σ ; SUERC-14946), which corresponds to the Iron Age in this region.
 112 The infant remains were radiocarbon dated to cal. AD 63–219 (SUERC-66371) for the foetus,
 113 and cal. AD 76–228 (SUERC-66372) for the perinate (**Figure 2**). The majority of the other
 114 human bones (including infants and foetuses) found at this site (**Figure 1**), belonged to
 115 different contexts, and were dated as being older than the here presented analysed individuals

116 (Supplementary Information and **Table S1**). Although one perinate (D) bone, found in the west
117 wall of the stairwell (**Figure 1**), was roughly contemporaneous with the three individuals
118 studied here (**Table S1**), it only consisted of a proximal right humerus, and the entire sample
119 had been used for radiocarbon dating as part of an earlier study (Birch and Wildgoose, 2013;
120 Shapland et al., 2016), and so was unavailable for DNA analysis.

121



122

123 **Figure 2.** Radiocarbon dates for the human remains analysed from the High Pasture Cave
124 stairwell, calibrated using OxCal v.4.3.2 (Bronk Ramsey, 2017) and the most recent calibration
125 curve IntCal 13 (Reimer et al., 2013).

126

127 Several of the bones from the extremities of the articulated adult woman were missing,
128 suggesting that excarnation was the most likely pre-treatment of the body prior to final
129 deposition (Birch and Wildgoose, 2013). This may also have been the case with the foetus,
130 perinate, foetal piglet and perinatal puppy, as no cut-marks were noted on any of the bone
131 elements, making it unlikely that the bodies were cut up or dissected but rather were left to
132 decompose naturally. This type of funerary tradition would have provided the opportunity for
133 disarticulated human remains to be recovered for curation, use, or deposition, and
134 disarticulated human remains have indeed been recovered from a large number of Iron Age
135 settlement sites (Tucker, 2010). Therefore, it is possible that the remains of either the human
136 foetus or perinate were curated and only interred after the woman had died.

137 There does not appear to have been a tradition of formal burial of the dead throughout the
138 majority of the Iron Age in Atlantic Scotland, and it has frequently been posited that an
139 archaeologically 'invisible' form of disposal of the dead, such as exposure, must have been
140 practiced (Armit and Ginn, 2007). Consequently, human remains from this period are rarely
141 recovered; on Skye, for example, just one other Iron Age site has produced human remains –
142 Fiskavaig, a rock-shelter with evidence of Middle Iron Age occupation, from which a single
143 perforated fragment of adult human cranium was recovered in 2009 (Birch 2009). However,

144 distinctive and specific burials for women, fetuses and infants during the Iron Age in Britain,
145 including Scotland, were rather common (Finlay, 2000; Armit and Ginn, 2007). The general
146 archaeological interpretation has been that the women must have died in childbirth and were
147 thus buried together with their fetuses (Millet and Gowland, 2015). However, to our
148 knowledge, this hypothesis has not, to date, been analysed genetically. In order to test this
149 assumption, we used whole mitochondrial genome (mitogenome) data to analyse the maternal
150 genetic relationship of the adult female to both the foetus and the perinate.

151

152 **Material and Methods**

153

154 **Sampling and DNA extraction from ancient remains**

155 We sampled a petrous bone from the female adult, and two infant bone fragments from the
156 perinate (ischium) and the foetus (scapula) for genetic analysis. We carried out the sampling
157 at the Ancient DNA Facility at the University of Huddersfield under dedicated clean-room
158 conditions. Laboratory researchers wore full body suits, hairnets, gloves and face masks
159 throughout the drilling, extraction and library preparation processes. We constantly cleaned
160 all tools and surfaces with LookOut® DNA Erase (SIGMA Life Sciences), as well as with
161 bleach, ethanol and long exposures to UV light.

162 We decontaminated all of the bone surfaces by UV radiation for 30 minutes on each side,
163 followed by cleaning with 5µm aluminum oxide powder using a compressed air abrasive
164 system. For the adult sample, we excised the densest part of the petrous bone (Pinhasi et al.,
165 2015) using a circular saw. We obtained bone powder by crushing this excised petrous portion,
166 and the complete bone fragments from the two infants, in a MixerMill (Retsch MM400). We
167 extracted DNA from 150mg of the petrous powder and 50–100mg of each infant bone,
168 following the protocol by Yang et al. (1998), with modifications by MacHugh et al. (2000). We
169 included blank controls throughout the extractions, library preparation and amplification
170 reactions to monitor for possible modern DNA contamination.

171

172 **Library preparation and sequencing of ancient DNA**

173 We constructed next-generation sequencing libraries from the ancient DNA extracts using the
174 method described in Meyer and Kircher (2010), with modifications outlined in Gamba et al.
175 (2014) and Martiniano et al. (2014). Briefly, the changes were as follows: we used T4 DNA
176 polymerase buffer (Thermo Scientific) instead of Tango buffer in the blunt-end repair step, and
177 we heat inactivated *Bst* polymerase activity by incubating the libraries for 20 minutes at 80°C.
178 We set up indexing amplifications using Accuprime *Pfx* Supermix (Life Technology), primer
179 IS4 (0.2µM), a specific indexing primer (0.2µM) and 3µl of sample library, with a total reaction
180 volume of 25µl. We performed all DNA purification steps using the QIAQuick MinElute
181 purification kit (Qiagen) following the manufacturer's protocol, with the modification of adding
182 0.05% Tween 20 (Fisher BioReagents) to the elution buffer.

183 We prepared single-end libraries for both of the infant samples. We ran amplification under
184 the following thermal cycling conditions: 5 minutes at 95°C; 11 cycles of 15 seconds at 95°C,

185 30 seconds at 60°C and 30 seconds at 68°C; with a final extension step of 5 minutes at 68°C.
186 We purified the resulting amplification product using the QIAQuick MinElute purification kit
187 (Qiagen).

188 As the adult was part of an earlier study, four dual-index libraries (Kircher et al., 2012) were
189 made from this individual. We set up amplification using AmpliTaq Gold (5U/μl), Thermopol
190 reaction buffer (10x), dNTPs (10mM each), both indexing primers (10μM each) and 10μl DNA
191 sample library. The following thermal cycling steps were used for amplification: 12 minutes at
192 95°C; 10 cycles of 20 seconds at 95°C, 30 seconds at 60°C, and 40 seconds at 72°C; with a
193 final extension step of 5 minutes at 72°C. We purified the amplification product using the
194 QIAQuick MinElute PCR purification kit. For the second amplification round, we used
195 Accuprime *Pfx* SuperMix (Life Technology), together with primers IS5 (10μM), IS6 (10μM) and
196 2.5μl of sample library. We carried out amplification under the following thermal cycling
197 conditions: 30 seconds at 98°C; 10 cycles of 20 seconds at 98°C, 30 seconds at 60°C, and
198 40 seconds at 72°C; with a final extension of 5 minutes at 72°C. We purified the resulting
199 amplification product as previously described.

200 We quantified all amplification reactions using a Qubit® ds-DNA High Sensitivity assay kit on
201 the Qubit® 3.0 Fluorometer, and additionally we checked the quality of each library using an
202 Agilent 2100 Bioanalyzer High Sensitivity DNA kit prior to pooling equimolarly for next-
203 generation sequencing (NGS) purposes. For the adult, we sent four dual-indexed libraries for
204 100-bp (base-pair) single-end sequencing on a HiSeq2500 (NBAF Liverpool, United
205 Kingdom), whereas we assigned the infant remains one single-indexed library each, and sent
206 them both for 100-bp paired-end sequencing on a HiSeq4000 (Macrogen, South Korea).

207

208 **Analysis of ancient sequence data**

209 We trimmed the NGS reads and aligned to the human mitochondrial genome revised
210 Cambridge Reference Sequence (rCRS, NC_012920; Andrews et al., 1999) and the human
211 reference genome (UCSC hg19). We trimmed the reads using cutadapt (v.1.13; Martin, 2011),
212 allowing a minimum overlap of 1bp between read and adapter (adapter sequence:
213 AGATCGGAAGAGCACACGTCTGAA-CTCCAGTCAC), discarding reads shorter than 30bp.
214 We used BWA aln (v.0.7.12-r1039; Li and Durbin, 2009) to map reads to both the rCRS and
215 hg19, filtering by base quality 15, and disabling seed length, as recommended for ancient DNA
216 data (Schubert et al., 2012), with a maximum edit distance of 0.01 and maximum number of
217 gap opens set to 2. We used SAMtools v.1.3 to sort and filter reads, and to remove PCR
218 duplicates, and assessed DNA damage patterns using mapDamage 2.0 (Jónsson et al., 2013)
219 and bamdamage (Malaspinas et al., 2014). We used picard tools (v.2.18,

220 <http://broadinstitute.github.io/picard>) to add read groups to the sequencing reads aligned to
221 hg19, and assessed the sequencing quality using FastQC (v.0.11.5; Andrews 2010) and
222 qualimap (v.2.2.1; Okonechnikov et al., 2015). We used samtools mpileup and bcftools (v.1.3)
223 to create a consensus FASTQ file of the alignment to the rCRS, and SEQTK (v.1.2-r102-dirty)
224 to convert the FASTQ file into FASTA format. We used schmutzi (Renaud et al., 2015) to
225 detect possible modern mitochondrial contamination, and we assigned haplotypes using
226 Haplogrep 2 (Weissensteiner et al., 2016) and confirmed them manually using the Integrative
227 Genomics Viewer (IGV v.2.4.13; Robinson et al., 2011; Thorvaldsdóttir et al. 2013). The only
228 difference in the treatment of the paired-end reads compared to single-end reads was our use
229 of AdapterRemoval (v.2.1.7; Schubert et al., 2016), which trimmed the adapters from the read
230 pairs and merged the reads.

231

232 **Mitochondrial haplotype calling for ancient samples**

233 We created the mitochondrial genome consensus sequences using SAMtools mpileup and
234 BCFtools. As the coverage of the foetus and perinate mitogenomes was very low (see Results
235 section), we also used GATK (v.3.7) to call the variants against the rCRS for the two infant
236 samples. This approach allowed a maximum output of variants in order to determine the
237 mitochondrial haplogroup of the studied individuals, and also allowed us to compare both
238 methods. All other bioinformatic steps were the same for both read types.

239 We called the mitochondrial haplotypes of the newly sequenced modern samples using
240 HaplotypeCaller, with 'Downsampling' set to 250, 'Ploidy' set to 100 and 'Standard Call
241 Confidence' set to 30, and, for SNP filtering, we set the 'Minimum Coverage Filter' to 2. In
242 order to filter the mutations and heteroplasmies, we used bcftools v.1.3 (Li and Durbin, 2009;
243 Li, 2011). We considered mutations with a read frequency ≥ 0.7 as variants, whilst mutations
244 with an allele frequency > 0.3 and < 0.7 were considered heteroplasmies. We checked all
245 heteroplasmies manually in Geneious® (version 6.1.8). We excluded variants at positions
246 around 302-315 including 309.1C(C) and 315.1C, AC insertions and deletions at 515-522,
247 16182C, 16183C, and 16193.1C(C) from analyses. We checked the final haplotype
248 classifications with HaploGrep 2 (Weissensteiner et al., 2016) and converted these into
249 FASTA files with HaploFasta.

250

251 **Modern mitochondrial genomic sequences and SNP data**

252 We sequenced a total of 13 modern samples belonging to H7a1 (Germany, $n = 9$; Scotland,
253 $n = 3$; Wales, $n = 1$) to accompany the published data (Denmark, 15; England, 2; Finland, 1;

254 Germany, 1; Italy, 3 [including 1 Sardinian]; Russia, 2; Scotland, 1; United Kingdom, 1; USA,
255 2; unknown geographic origin, 9; Bulgaria ancient, 1) (**Table S2; Figure S1**). The four newly
256 generated British samples originated from a selection of samples collected by BritainsDNA
257 (now owned by Source BioScience). An overall mitochondrial SNP (single nucleotide
258 polymorphism) dataset of anonymised samples was available to us, also from
259 BritainsDNA/Source, consisting of 5,337 British and Irish individuals. We used these SNP data
260 for mitochondrial haplogroup frequency estimates.

261

262 **Analysis of mitochondrial DNA data**

263 We carried out polymerase chain reactions (PCR) for modern samples in the modern genetics
264 lab at the University of Huddersfield. To amplify the complete mitogenome (16,568 bp), we
265 used two fragments for each sample following the method in Brandini et al. (2017). We purified
266 the PCR products using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA)
267 with an elution volume of 50µl. We assessed the DNA concentration of each fragment using
268 1µl of the purified PCR product using a Qubit® 3.0 fluorometer. After quantification, we pooled
269 both fragments equimolarly with a final volume of 40µl and a concentration of 1ng/µl. We then
270 sent the samples for sequencing at the Earlham Institute (Norwich, UK) on a MiSeq250 (PE-
271 Nano x1). We used EAGER (Peltzer et al., 2016) for assessment of quality control, removal
272 of duplicates, mapping and SNP calling, using the following settings: AdapterRemoval for the
273 removal of adapters and read merging; bwa-mem to align sequences to the rCRS (Andrews
274 et al., 1999); and removal of duplicates using DeDup. We aligned published sequences to the
275 rCRS using Sequencher™ version 5.1 (<http://www.genecodes.com>), and converted these to
276 variants using mtDNAGeneSyn (Pereira et al., 2009). We verified haplogroup classifications
277 using Haplogrep 2 (Weissensteiner et al., 2016). We also used HaploGrep2 to call
278 haplogroups from the SNP data of the 5,337 British and Irish individuals.

279

280 **Phylogenetic tree reconstruction**

281 We placed all newly generated sequences in a maximum-parsimony phylogenetic tree,
282 constructed by compiling published ($n = 38$) and unpublished ($n = 15$; 13 modern and the two
283 ancient) complete mitogenome data (**Table S2; Figure S1**). We also included data generated
284 at Huddersfield as part of a larger British Isles study. We used mtPhyl v.4.015 (Eltsov and
285 Volodko, 2011; <https://sites.google.com/site/mtphyl/home>) to reconstruct the phylogeny
286 following the classification of PhyloTree build 17 (<http://www.phylotree.org/tree/index.htm>),
287 and using the molecular clock developed by Soares et al. (2009) to date nodes. We calculated
288 maximum-likelihood (ML) estimates assuming the HKY85 mutation model with gamma-

289 distributed (32 categories) rates (plus invariant sites) and two partitions: coding region
290 (positions 00577–16023) and control region (positions 16024–00576) with baseml v.4.7 from
291 PAML package (Yang, 1997). We then converted the distances into years using a mutation
292 rate of one substitution in every 3,624 years (Soares et al. 2009), further accounted for
293 purifying selection. We obtained coalescent time estimates using rho (ρ) and sigma (σ) values
294 exported from the in-house developed founder analysis software, applying the same clock. All
295 ancient samples ($n = 3$) were excluded from the age calculations.

296

297 **Results**

298 We successfully extracted and sequenced DNA from all three ancient samples. The
299 endogenous DNA content of all individuals was quite low, at 11.9%, 8.0% and 7.6% for the
300 adult, perinate and foetus respectively. The nuclear genome of the adult female was
301 sequenced to a coverage of 0.28x, whilst her mitochondrial genome was sequenced to a
302 coverage of 58.07x. For the two infants, the nuclear genome coverage was 0.014x for the
303 perinate and 0.006x for the foetus, with mitochondrial genome coverages of 4.14x and 2.24x,
304 respectively. Due to the low coverage of the nuclear genomes of the foetus and the perinate,
305 and the extremely low number of overlapping SNPs retrieved (<10,000), no meaningful
306 autosomal analysis was possible. The genome-wide data of the adult female is currently being
307 further analysed in a broader Iron Age study (in preparation) and, since the focus of the present
308 study is the maternal relationship of the individuals analysed, and the coverage of the genome-
309 wide data retrieved from the two infant remains was too low to be useful, we do not present
310 any genome-wide analysis here.

311

312 **Contamination estimation**

313 Contamination estimates ranged from 0.165% to 0.185% for the adult female, and 0 to 0.005%
314 for both infants. We observed no exogenous contamination in extraction or library blanks,
315 supporting the authenticity of the results, and there were no matches between the
316 mitochondrial haplotypes from the three ancient samples and those of the researchers.

317

318 **Genetic sex identification**

319 We identified the genetic sex using the tool published by Skoglund et al. (2013). The
320 osteological determination that the adult was female was supported by the genetic sex
321 identification. We were unable to assign the genetic sex for the two infants due to their low
322 genomic coverage.

323

324 **Mitochondrial haplotype determination**

325 To securely determine mitochondrial haplotypes for the infants, we compared the results from
326 two bioinformatic approaches (that is, GATK Variant Call vs. calling the consensus sequence,
327 as discussed in the Methods section), and we assigned the same haplotypes to each individual
328 using both methods. Due to the low coverage in both infants' mitochondrial genomes, the

329 haplotypes initially generated presented a number of ambiguous base calls (**Table 1**). In order
330 to reduce the number of ambiguities, and thus simplify any haplotype comparison, we checked
331 each one of the ambiguous positions manually, and made a decision by combining
332 phylogenetic and molecular criteria. We provide a thorough overview of the rationale behind
333 every mutation disregarded in **Table S3**. Although the haplotypes finally reconstructed could
334 still contain some errors (**Table 1**), they show that only one of the infants, the foetus, shares
335 haplogroup (H7a1b) with the adult female and, could, therefore, possibly be related to her. We
336 assigned the perinate to haplogroup J1c3, thus excluding a direct maternal kinship.

337

338 **Table 1.** Comparison of mutations scored for all three ancient mitochondrial genomes using Haplogrep 2.

Sample	Haplogroup	Final Haplotype ^a	Initial Haplotype ^b	Gaps ^c
Adult female	H7a1b	263G 750G 1393A 1438G 1719A 4769G 4793G 8860G 15326G 16261T 16519C	-	-
Foetus	H7a1b	263G 750G 1393A 1719A 4769G 4793G (7025G) 8860G 16261T 16519C	263G <u>633M</u> 750G <u>911K</u> 1393A 1719A 4769G <u>4793R</u> <u>5239K</u> <u>6272R</u> <u>6359M</u> <u>6842Y</u> <u>7022K</u> <u>7025R</u> <u>8576K</u> 8860G <u>10674K</u> <u>10750R</u> <u>11629R</u> <u>12308R</u> <u>12766Y</u> <u>12822R</u> <u>12864K</u> <u>12873W</u> <u>13207W</u> <u>14864K</u> <u>15601K</u> 16261T 16519C	1438G 15326G
Perinate	J1c3	<i>73G 185A 228A 263G 295T 462T 489C 750G 2706G</i> 3010A 10398G 11251G 11719A 12612G 13708A 13934T 14766T 14798C 15326G 15452A 16069T 16126C	73G <u>152Y</u> 185A 228A 263G 295T 462T 489C <u>539Y</u> 750G 2706G 3010A <u>3276R</u> <u>4892M</u> <u>6243R</u> <u>9233Y</u> <u>9477R</u> <u>9814Y</u> 10398G <u>10567W</u> <u>11251R</u> 11719A <u>12210W</u> 12612G <u>12835R</u> <u>12998Y</u> 13708A 13934T 14766T 14798C 15326G 15452A <u>15854W</u> <u>16022Y</u> 16069T 16126C	-

^a *Italics*: changes vs. the rCRS up to the H2 root; **bold**: H7 to H7a1b diagnostic mutations in the case of the adult and foetus, and JT to J1c3 diagnostic mutations in the case of the perinate; the dubious possible mutation seen in the foetus is shown in parentheses.

^b underlined: ambiguous positions

^c gaps: positions seen in the adult, but not covered in the foetal mitogenome

339

340

341 **Discussion**

342 Here we report for the first time complete ancient mitogenomes from foetal-aged bone fragments.
343 Previous studies have used PCR-based methods to determine the genetic sex of neonates
344 (Faerman et al., 1998; Waldron et al., 1999; Irish et al., 2008), but no mitogenomes or genome-
345 wide data have been published to date. Using next-generation sequencing to generate whole
346 mitogenomes results in a more detailed analysis of the haplotype than a PCR-based approach,
347 where typically only fragments of the non-coding region are amplified. Although we generated
348 whole mitogenomes from the two infants, we were unable to determine the molecular sex of either
349 the perinate or the foetus due to their low nuclear genome coverage.

350 Mitochondrial genomes are used to trace the female line of descent as they are passed on from
351 mother to child without recombination. The only changes that occur over generations are
352 naturally-occurring mutations, and these can be used for phylogenetic reconstructions over wide
353 geographic areas and long timespans. Both the female and the foetus belong to the very
354 distinctive and uncommon (among present-day populations) haplogroup H7a1b. In contrast, the
355 perinate belongs to haplogroup J1c3, and so can be ruled out as a child of the adult female. In
356 addition, as we saw no other defining mutations, we can say that the mitogenome of this individual
357 is basal to J1c3. For more details about haplogroup J1c, see Supplementary Information.

358 The main defining mutations of haplogroup H7a1b are the transitions at positions 4793, 1719,
359 16261 and 1393, which define H7, H7a, H7a1 and H7a1b, respectively. These are all found in
360 both individuals (shown in bold in **Table 1**). After all ambiguities were assessed (**Table S3**), the
361 sequence of the foetus displayed a single plausible difference compared to the adult female, the
362 A7025G transition (**Table 1**). The 7025 transition defines subclade H5a5, but has never been
363 observed in any H7 sequence to date. Further sequencing would be necessary to securely define
364 the base read at position 7025 for the foetal sequence. However, based on the current data, we
365 must recognise that, although the foetus and the adult female share the same rare haplogroup,
366 they might not share the same haplotype (if a mutation at np 7025 is accepted).

367 In our modern mtDNA SNP database, haplogroup H occurs in 41% of all modern British Isles
368 mitochondrial lineages (2247 out of 5537). However, haplogroup H7 is only a minor European
369 subclade, which is found at 1.6% (85 out of 5337) in our British Isles dataset, while its nested
370 subclade, H7a1b is even rarer, and is found at 0.7% (13 out of 1926) in Scotland, 0.3% (1 out of
371 374) in Ireland and 0.2% in England (4 out of 2304), while it has not been detected in Wales (0
372 out of 733). For more details about haplogroup H7, see Supplementary Information. The

373 subhaplogroup of H7a1b dates to c. 4300 years [2400–6300] and is seen in modern-day Scotland
374 and England, but also in Denmark, Finland and Sardinia (Behar et al., 2012; Li et al., 2014; Raule
375 et al., 2014). The apparently wide geographic distribution and young age of H7a1b suggest that
376 it may have been introduced into Britain more recently than the arrival of the Bell Beaker settlers,
377 c. 4450 years ago, when the genome-wide pattern of the British gene pool began to take on its
378 modern form (Olalde et al. 2018) – possibly in the Bronze Age or in the Iron Age itself. The two
379 individuals from High Pasture Cave are, nevertheless, the earliest known H7 sequences from
380 north-west Europe (**Figure S1**). The nodal haplotype represented by the female, and possibly by
381 the foetus, has survived in modern-day Scotland and Denmark.

382 The fact that the two ancient individuals share the same rare haplogroup, and possibly the same
383 haplotype, suggests that, even if they were not directly related, they belonged to a population that,
384 similarly to other Iron Age populations, was most likely small and in-bred. The possibility that the
385 two individuals might not share the same haplotype is potentially even more tantalizing, as it would
386 imply that, although small and geographically isolated, this population carried a level of diversity
387 (represented by the nodal haplotype +7025 mutation) that could have either arisen *in loco*, thus
388 implying some level of prolonged isolation, or been introduced from a more diverse, and possibly
389 larger, and interconnected, metapopulation.

390 Infants and young children were often distinguished in burial customs from the rest of society
391 (Ucko, 1969). In early Iron Age Thrace, young or newborn children were sacrificed for rituals and
392 it was only in the late Iron Age that newborn and young animals were used instead (Nekhrizov
393 and Tzvetkova, 2010). In Roman and Anglo-Saxon periods in England, children who had not yet
394 teethed were, instead of being cremated, buried within building walls or under buildings (Stoodley,
395 2000; Gowland et al., 2014). Also, in Roman Dorset and Buckinghamshire, women who died in
396 pregnancy were only buried after the child had been removed from the womb (embryotomy
397 surgery) (Gowland et al., 2014).

398 The separation of infant burials from the rest of the population appears to have been widespread
399 across the Iron Age and into the Romano-British period (Redfern and Dewitte, 2011). In the British
400 Iron Age, women who died in childbirth, as well as fetuses and young infants, were often given
401 special burials, highlighting the importance of the link between woman and child (Millet and
402 Gowland, 2015). Human remains of infants were often placed into cave systems, and the number
403 and geographic spread of cave burials during this period in Scotland is relatively high – especially
404 given that burials from this period are generally not abundant (Saville and Hallén, 1994), and that

405 the deposition of individual articulated skeletons in Iron Age Scotland is rare (Tucker, 2010).
406 However, there is also evidence of infants being buried in unstable, abandoned buildings or
407 disused settlement sites. For example, an excavation at the site of Howe in Orkney revealed an
408 Iron Age burial of an adult male, aged 35–45 years, along with a perinate and an infant, all of
409 whom had been placed within accumulated rubble in a midden in a disused yard (Armit and Ginn,
410 2007). The bones showed gnaw marks, and their layout has been interpreted as an informal
411 burial; that is, they were deposited without ceremony (Armit and Ginn, 2007). Another example is
412 the Iron Age site at the Knowe of Skea, also in Orkney, where over one hundred, mainly infant,
413 bodies were found deposited in domestic rubble contexts (Armit and Ginn, 2007). Cremations and
414 inhumations of adult women, alongside foetal/neonatal remains, are also known from Ireland,
415 located in reused, separate burial areas (Finlay, 2000).

416 The context of the High Pasture Cave appears to mirror those burials in cave systems, with the
417 adult female being deposited as an articulated skeleton just prior to the closure of the cave.
418 However, the trauma evident on the female (whether inflicted before or after death), and the lack
419 of any wider tradition of inhumation in the region, has led to the suggestion that this burial was a
420 deviant one, or even a sacrifice connected to the closure of the cave stairwell, rather than a
421 ceremonial burial of a mother and her children (Armit, in press). The disarticulated remains of the
422 foetus and perinate appear to have been collected and purposefully deposited with her, raising
423 several questions, especially now that our results show that we can positively exclude a direct
424 maternal kinship for the perinate.

425

426 **Conclusions**

427 The results of our study show that one of the two infants buried alongside the adult female at High
428 Pasture Cave was definitely not her child, whereas the foetus could not be excluded as being
429 maternally related to her. This is an intriguing finding considering the common archaeological
430 understanding of this type of female/child burial, where the infants are assumed to be the children
431 of the women. So far, archaeological interpretations of these types of burial indicate either
432 mother–child relationships, or a midwife buried in the same place as infants. In the case of High
433 Pasture Cave, where the woman had potentially been crushed to death, archaeological questions
434 still remain. Why might she have been chosen for this extremely unusual form of treatment
435 before/after death? Why were the human bones of infants curated, collected, and deposited in
436 the cave with her, and why was she buried with an unrelated child?

437 Our genetic analysis shows that a general assumption of mother–child relations within such
438 burials can be misleading, and that genetic analysis can aid the analysis and interpretation of
439 such archaeological contexts.

440

441 **Acknowledgements**

442 KD is supported by a Leverhulme Doctoral Scholarship awarded to MBR and MP. P.S., M.B.R.
443 and M.P. acknowledge FCT support through project PTDC/EPH-ARQ/4164/2014, partially funded
444 by FEDER funds (COMPETE 2020 project 016899). P.S. is supported by FCT, European Social
445 Fund, Programa Operacional Potencial Humano and the FCT Investigator Programme and
446 acknowledges FCT/MEC for support to CBMA through Portuguese funds (PIDDAC)—PEst-
447 OE/BIA/UI4050/2014. The genetic research was supported by the UK Natural Environment
448 Research Council (NERC) Biomolecular Analysis Facility at the University of Liverpool, under
449 NBAF Pilot Scheme NBAF685, awarded to CJE whilst at the University of Oxford.

450

451 **Data availability**

452 Sequencing data deposited in GenBank, accession number xxxx.

453

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