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Species identification of Late Pleistocene bat bones using collagen fingerprinting

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Abstract

Bats form the second most diverse mammalian order (Chiroptera), after rodents, and vary widely in their physiology and ecology. Those species that live in temperate climates are generally insectivorous and nocturnal or crepuscular, sheltering in tree hollows, caves, or buildings during the day. They are potentially valuable ecological indicators, due to their dependence on suitable roosting sites and arthropod food, both of which are commonly affected by human activities. Identification of bats from ancient assemblages that are found in caves could therefore provide useful data for palaeoenvironmental reconstructions and show the effect of habitat loss. Here, we apply the recently developed approach of collagen fingerprinting by soft ionisation mass spectrometry to the identification of ancient bat remains in an archaeological assemblage from Pin Hole Cave (Derbyshire, England). Our results show that a simple set of markers can distinguish all seven genera of bats known to be present in either modern or ancient Britain (Myotis, Nyctalus, Pipistrellus, Barbastella, Plecotus, Eptesicus, and Rhinolophus). Further analysis indicates that species-level determination is possible in some of these taxa, but it would more readily be achieved using the more advanced methods of collagen sequence analysis by liquid chromatography coupled with tandem mass spectrometry. Within our assemblage yielding ~6,800 ancient bone collagen fingerprints, we identified only ~1% that derived from chiropterans, and these were predominantly derived from Myotis (two apparent Brandt’s bat fingerprints but the majority indistinguishable between the whis- kered, Daubenton’s and Natterer’s bats), Barbastella (the western barbastelle being the only member of this genus known within Europe), and Rhinolophus (identified as the lesser horseshoe bat R. hipposideros rather than the rare greater horseshoe bat R. ferrumequinum). We infer that the site was likely used by roosting bats throughout the year, and the accumulation of these remains was probably not the result of predator activity. More importantly, the peptide biomarkers provided here could prove valuable in the more systematic analysis of microfaunal remains across many European archaeological and palaeontological sites, preferably those that are collected with well curated stratigraphical information and chronological frameworks.

KEYWORDS

ancient bats, caves, Chiroptera, collagen fingerprinting, hibernation

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Bats are the second most diverse order of mammals, after rodents, with over a thousand extant species recognised and found throughout much of the world (Wilson & Reeder, 2005). They are the only mammals that are capable of active flight, made possible by modification of the limbs to form a membranous wing, most of it supported by the elongated finger bones. Although some species of megachiropteran bats can weigh as much as a kilogram when adult, for example, the golden-capped fruit bat Acerodon jubatus (Stier & Mildenstein, 2005), most bats are considerably smaller, typically weighing less than 50 g (Barclay & Brigham, 1991). However, despite their limited size, they still show great ecological and physiological diversity (Hutson, Mickleburgh, & Racey, 2001). Their ability to fly, together with their presence on every continent but Antarctica, has led to a wide range of feeding and roosting habits, as well as social behaviours (Jones, Jacobs, Kunz, Willig, & Racey, 2009).

Bats from the temperate regions of the Northern Hemisphere feed exclusively on insects and other arthropods, consuming a third or more of their own body mass each night in summer (Schober & Grimmberger, 1989). This level of consumption suggests that some species of bat may have a role in the suppression of arthropod populations (Kunz, Braun de Torrez, Bauer, Lobova, & Fleming, 2011). Bats from temperate regions roost during the day time in natural enclosures such as tree hollows, rock crevices, and caves (Lewis, 1995), as well as man-made structures such as kilns and buildings (Jones et al., 2009). Different types of roost site are occupied in different seasons, due to the changing needs of the bats (Schober & Grimmberger, 1989). For example, caves are frequently occupied in winter as the stable microclimates of these sites are favourable for hibernation. Given their reliance on suitable roosting sites, habitat infrastructure and invertebrate prey, all of which are likely to be affected by human activities, bats are sensitive to environmental changes and are therefore potentially important bioindicators (Jones et al., 2009). Many approaches are available for the study of modern bat populations, and this has led to a wealth of information regarding their present status; however, less is known about their past distributions and numbers. Information about prehistoric populations of bats can be obtained through the study of microfaunal assemblages, such as bone remains recovered from caves, and can provide useful comparative palaeoenvironmental data (e.g., Royer et al., 2017; Stoetzel et al., 2018).

Accumulations of bat remains from caves may be the result of natural deaths of the animals, especially when they are likely to be at their weakest towards the end of hibernation, but may also be deposited there by predators. A range of other vertebrates feed on bats, but the main predators of temperate bats are owls, with some species that are also vulnerable to falcons and hawks (Speakman, 1991). Given that owls may also roost in caves, the presence of bat remains in microfaunal assemblages could well be attributed to predation, with limestone cave environments offering better preservation of skeletal remains than open sites for various reasons that include more alkaline conditions and more stable typically cooler temperatures (Geigl, 2002; Weiner & Bar-Yosef, 1990).

Many of the morphological features used to identify bats are based on features of soft tissue, particularly the ears and muzzle (Schober & Grimmberger, 1989). These characters are not present in the case of ancient bat remains, as soft tissues do not typically survive for as long as skeletal elements. Although all but one of the 17 species that are presently found in the British Isles can generally be identified from skeletal characteristics, this relies mostly on features of the dentition, and only a few of the postcranial elements can be identified to genus or in a very few cases to species (Stebbins, Yalden, & Herman, 2007).

The analysis of DNA sequences has been widely applied in wildlife studies (e.g., Cronin, Palmisciano, Vyse, & Cameron, 1991; Foran, Crooks, & Minta, 1997) with even guano being used as a resource for bat identifications (Clare, Lim, Fenton, & Hebert, 2011). However, ancient DNA studies are relatively expensive and time-consuming and have limited success rates. This is particularly the case with smaller sample sizes and for those dating back to the Pleistocene (Höss, Jaruga, Zastawny, Dizdaroglu, & Paabo, 1996). An alternative biomolecular approach to species identification, collagen fingerprinting was more recently developed and circumvents some of these limitations. Collagen fingerprinting is where type 1 collagen, the dominant protein in bone, is extracted into solution and enzymatically digested into peptides, which can be measured by soft ionisation mass spectrometry. The simplest approach of this is through matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry and has been applied to the study of domesticated animals (Buckley et al., 2010; Buckley, Collins, Thomas-Oates, & Wilson, 2009) as well as wild fauna (Buckley & Kansa, 2011), including marine mammals (Buckley et al., 2014) and rodents (Buckley, Gu, Shameer, Patel, & Chamberlain, 2016). Since the initial publications using peptide mass fingerprinting (PMF) of archaeological bone (Buckley et al., 2009), several subsequent studies have been conducted that establish the validity of this approach to species identification; these are not limited to bone (e.g., van der Sluis et al., 2014) but have also been applied to a range of other archaeological substrates including antler (von Holstein et al., 2014), hair (Solazzo et al., 2011), skin (Kirby et al., 2011), and eggshell (Stewart et al., 2016). Here, we present new markers for the identification of bat bones, concentrating on those present in Britain, determined through application of the technique to the analysis of >6,800 collagen fingerprint spectra retrieved from Pin Hole Cave (Creswell Crags, Derbyshire, UK) faunal assemblage (Buckley, Harvey, & Chamberlain, 2017).

Pin Hole Cave (SK5333742) is a particularly important archaeological site in the British Isles because it acts as the type site for the mammal assemblage of marine isotope stage 3 fauna, so it is of potential importance in palaeobiostatigraphy (Currant & Jacobi, 2001). The Creswell Crags, a limestone gorge within which Pin Hole Cave is located, is also known for Britain’s earliest cave art, yielding insights
into one of the three known phases of ancient human occupations in the area, beginning with Neanderthals. The Creswell area is dominated by a north–south linear outcrop of Upper Permian Cadeby Formation limestone (formerly known as Lower Magnesian Limestone), which consists of a prominent west-facing escarpment from which the surface of the outcrop slopes gently to the east. The Creswell Crags constitutes a narrow fluvial gorge running west–east through this limestone outcrop with the entrances on both the north and south sides of the gorge. The Pin Hole Cave has an entrance in the north side of the Creswell Crags gorge and measures 31 m long by ~1–2 m wide. Similar to today, it is likely that fine-grained sediments washed down into the cave through small fissures in the limestone, mainly in Devensian times (~50 to 10 Ka). Excavations in the late 19th century and early 20th century revealed that there were at least two principal sediment bodies dating to the Pleistocene period, an upper red cave earth and a lower yellow cave earth but with faunal remains and lithic artefacts found throughout both (Jenkinson et al., 1984). Excavations were carried out in the early 1980s, focussing on two small areas approximately 30 m into the cave, with one ~1.5 × 1.0 m at the top of the sequence and the other ~1.0 × 0.5 m in much earlier deposits at the base (see Buckley et al., 2017) to more carefully obtain microfaunal remains through the use of sieving. These remains from the 1980s excavations were the source of the collagen fingerprints analysed here.

2 MATERIALS AND METHODS

The collagen fingerprints of 6,805 specimens were collected previously as part of an earlier study that focussed on megafaunal bone fragments (Buckley et al., 2017). All of the bone remains were analysed as intact specimens that were small enough to fit within the 96-well microtiter plates used for high-throughput analyses. Given this constraint, megafaunal remains were clearly fragments, but microvertebrate remains mostly comprised intact skeletal elements. They were acquired using a relatively nondestructive approach in which 0.3 M hydrochloric acid (HCl) was added to the samples for only 3 hr prior to being removed and filtered into 50 mM ammonium bicarbonate (ABC) in which the collagen extracted from each sample was digested with the enzyme trypsin overnight for 18 hr. Spotted with 2 μl α-cyano hydroxycinnamic acid matrix, the air-dried droplets were analysed using a Bruker Ultraflex II MALDI-ToF mass spectrometer (see Buckley et al., 2017). Modern reference samples were also analysed following the above criteria. These included specimens of greater horseshoe bat (Rhinolophus ferrumequinem), lesser horseshoe bat (Rhinolophus hipposideros), whiskered bat (Myotis mystacinus), Natterer's bat (Myotis nattereri), Daubenton's bat (Myotis daubentoni), greater mouse-eared bat (Myotis myotis), barbastelle (Barbastella barbastellus), serotine (Eptesicus serotinus) sampled from the National Museums Scotland, and brown long-eared bat (Plecotus auritus), noctule (Nyctalus noctula), and common pipistrelle (Pipistrellus pipistrellus) sampled from the University of Sheffield's Department of Archaeology modern reference collections. Where available to us, specimens known to have come from the British Isles were used, in order to minimise the risk of variation in collagen sequences among the new and ancient samples. The peptide digest aliquot of the M. daubentoni, M. mystacinus, M. nattereri, N. noctula, B. barbastellus, E. serotinus, P. pipistrellus, and P. auritus was subject to LC-Orbitrap Elite tandem mass spectrometry following Buckley et al. (2015) in order to assist with peptide sequence identification. For sequence comparison, the little brown bat (Myotis lucifugus) sequences for both COL1A1 (G1QDY4_MYOLU) and COL1A2 (G1PSJ6_MYOLU) were BLAST searched against “Chiroptera” using National Center for Biotechnology Information, with only taxa that resulted in sequence matches retained for analysis here. Peptides of interest in the PMF, determined through visual comparison of spectra from the collagen digests of different species, were associated with peptide sequence information where possible, evaluating them against expected precursor mass without the proton (i.e., −1 Da). Phylogenetic analyses were also carried out following Buckley et al. (2015), in which maximum

### TABLE 1 Collagen peptide mass fingerprint biomarkers observed through matrix-assisted laser desorption/ionisation analysis

| Family            | Species          | 2t85   | 2t21   | 2t26   | 2t76   | 2t45   | 2t69   | 2t67   |
|-------------------|------------------|--------|--------|--------|--------|--------|--------|--------|
| Vespertilionidae  | Myotis           | 1,247.7| 1,277.7| 1,548.8| 1,578.8| 1,594.8| 2,147.1| 2,969.4|
| Vespertilionidae  | Plecotus         | 1,247.7| 1,277.7| 1,522.8| 1,534.8| 1,594.8| 2,147.1| 2,999.4|
| Vespertilionidae  | Nyctalus         | 1,247.7| 1,277.7| 1,562.8b| 1,534.8c| 1,582.8| 2,147.1| 3,027.4|
| Vespertilionidae  | Pipistrellus     | 1,247.7| 1,277.7| 1,534.8c| 1,534.8d| 1,582.8| 2,163.1| 3,027.4|
| Vespertilionidae  | Eptesicus        | 1,247.7| 1,277.7| 1,548.8c| 1,534.8d| 1,582.8| 2,147.1| 2,999.4|
| Vespertilionidae  | Barbastella      | 1,247.7| 1,277.7| 1,503.8| 1,534.8d| 1,594.8| 2,163.1| 2,999.4|
| Rhinolophidae     | Rhinolophus      | 1,253.7| 1,267.7| 1,503.8| 1,534.8d| 1,594.8| 2,163.1| 2,999.4|
| Rhinolophidae     | ferrumequinum    | 1,291.7| 1,267.7| 1,503.8| 1,534.8d| 1,594.8| 2,163.1| 2,999.4|

*Myotis brandti A–S substitution described in text.
*Every other species here has a different peptide also with this m/z.
*Value observed in others (e.g., Rhinolophus, Myotis, and Eptesicus) but useful for separating Barbastella from Plecotus due to absence in the latter. Peptide labels following Buckley et al. (2016); see Table S1 for peptide biomarker sequences.
*By sequence similarity based on E. fuscus.
likelihood analyses were performed using PhyML with a JTT + I + G amino acid substitution model used and 10,000 generations/bootstrap reiterations to give branch support.

3 | RESULTS

3.1 | Taxon discrimination

A combination of markers that we found useful for distinguishing bats from other taxa were m/z 1,435.7, 1,453.7, and 1,459.7. All seven genera could also be discriminated using a combination of other markers (Table 1 and Figures S1 and S2). Our marker at m/z 1,246.6 (known elsewhere as peptide A, Buckley et al., 2009, or the 85th tryptic peptide of the alpha-2(I) chain—2t85), which readily deamidates to m/z 1,247.6 reflects all members of the suborder Vespertilioniformes that were examined (Myotis, Plecotus, Nyctalus, Pipistrellus, Eptesicus, and Barbastella), with Rhinolophus of the other suborder the Pteropodiformes reflected at m/z 1,253.6. The peptide marker 2t76, which we have not described before, appears most valuable for discriminating within the Vespertilioniformes themselves, but peptide 2t67 is also particularly useful, this latter being homologous to a marker used to separate sheep from goat bone (Buckley et al., 2010). Another marker that we have previously described for other taxa (2t85 reflecting marker A elsewhere; Buckley et al., 2009) remains useful for separating at the family level, along with a further marker described here (2t21). Intriguingly, the previously published “D” marker (2t69) was useful at separating the noctule bats from the pipistrelles.

3.2 | Sequence analysis

In addition to Myotis, the only taxa with sequence information relevant to this study are Eptesicus and Rhinolophus (Table 2 and Appendix S1). With predicting species biomarker values, peptide 2t85 (the 85th tryptic peptide of the COL1A2 chain, Table S2, a previously published biomarker; Buckley et al., 2009) is NGHPGVVGPAGIR (also m/z 1,246.7 as nondeamidated form) in the serotine (confirmed in the fingerprint) but TGHPGVVPAGIR in the horseshoe bats, which results in an expected peak at m/z 1,235.7 in Rhinolophus. Unfortunately, this is masked by m/z 1,235.7, which represents another peptide of sequence GEAGAAGPAGPAGPR (see Table S3). We also can assume that the 2t21 marker at m/z 1,277.7 in most Myotis (Myotis davidii, M. lucifugus, M. daubentoni, M. nattereri, and M. mystacinus) with sequence GI PGP AGAAGPR where underlined residues reflect hydroxylated proline (Myotis brandtii reportedly has GI PGP SAGAAGPR, which would be reflected by a +16 Da shift in the PMF) to be identical in Eptesicus (GI PGP AGAAGPR) but substantially different in Rhinolophus, with a sequence GI PGP AGAAGPR.

TABLE 2 Number of amino acid substitutions between known COL1A1 and COL1A2 bat sequences (the latter shaded)

| Species                  | Myotis lucifugus | Myotis davidii (49a) | Myotis brandtii (8a) | Eptesicus fuscus | Rhinolophus sinicus (61a) | Rousettus aegyptiacus | Miniopterus natalensis | Hipposideros armiger | Pteropus alecto | Pteropus vampyrus (17b) | Sorex araneus |
|--------------------------|------------------|----------------------|---------------------|------------------|--------------------------|-----------------------|------------------------|---------------------|----------------|--------------------------|---------------|
| Myotis lucifugus         | X                | 2                    | 1                   | 4                | 27                       | 35                    | 11                     | 25                  | 27             | 28                       | 26            |
| Myotis davidii           | 8                | X                    | 1                   | 4                | 26                       | 34                    | 10                     | 25                  | 26             | 16                       | 25            |
| Myotis brandtii          | 3                | 7                    | X                   | 3                | 25                       | 33                    | 9                      | 24                  | 25             | 27                       | 24            |
| Eptesicus fuscus         | 15               | 16                   | 15                  | X                | 26                       | 35                    | 13                     | 24                  | 27             | 29                       | 25            |
| Rousettus sinicus (23a)  | 65               | 64                   | 65                  | 63               | X                        | 29                    | 24                     | 19                  | 24             | 26                       | 31            |
| Rousettus aegyptiacus    | 69               | 68                   | 69                  | 64               | 48                       | X                     | 33                     | 32                  | 13             | 76                       | 38            |
| Miniopterus natalensis   | 37               | 37                   | 40                  | 34               | 63                       | 58                    | X                      | 25                  | 26             | 28                       | 27            |
| Hipposideros armiger (21b)| 71               | 69                   | 70                  | 69               | 24                       | 55                    | 67                     | X                   | 26             | 28                       | 39            |
| Pteropus alecto (54a)    | 66               | 65                   | 66                  | 62               | 22                       | 13                    | 52                     | 30                  | X              | 2                        | 31            |
| Pteropus vampyrus (26a)  | 68               | 67                   | 68                  | 62               | 44                       | 15                    | 54                     | 53                  | 4              | X                        | 33            |
| Sorex araneus            | 88               | 91                   | 89                  | 82               | 87                       | 76                    | 77                     | 94                  | 71             | 77                       | X             |

Note. The number of uncertain amino acids per sequence follow species name, which were not included. See the Supporting Information for sequences.

*aOverlap of five uncertain residues with those from Myotis davidii.

*bOverlap of 21 uncertain residues from Hipposideros armiger with Rousettus sinicus and 23 from Rousettus sinicus with Pteropus alecto.
resulting in an expected peak at \( m/z \) 1,283.7. However, interestingly, we observe this at \( m/z \) 1,267.7 due to the substitution at one of the proline hydroxylation sites. Perhaps one of the most valuable regions of the spectra is at \( m/z \) 1,534.8 and 1,620.8, respectively (as observed in the fingerprints). Peptide 2t69 at GLPGVSGVGEPGGPLGIAGPPGAR reflects an A-S transition at the penultimate (A) residue but with only these two forms observed in our fingerprinted taxa. Most significantly, the peptide at \( m/z \) 1,594.8 (2t45; GPPGESGAVGPSGPTGPQGLLGAPGILGLPGSR) and at \( m/z \) 2,597.3 (2t60; GENGVGVGPTGPVGAAGPSGPNGPPGPAGTR) appears to contain species-specific differences from \( M. \) myotis, with sequences in \( M. \) davidii being GPPGESGAVGPSGPTGSR and GENGVGVGPTGPVGAAGPSGPNGPPGPAGTR, respectively (in the latter, not chosen as a required marker in this study). Eptesicus has the sequence GENGVGVGPTGPVGAAGPSGPNGPPGPAGTR, reflecting a gain of 14 mass units (Daltons), and \( R. \) rhinolophus, a substantially different sequence GENGPVGPAGPVGAAGPSGPNGPPGPAGTR, which is consistent with the expected markers for \( M. \) brandtii in comparison with \( M. \) davidii).

3.3 | Identifications from Pin Hole Cave

The bats (Figure 2) represent a very small proportion of the overall microfaunal assemblage from the site (fewer than 800 of the ~6,800 good quality fingerprints were identified as megafauna (Oryctolagus, Lepus, “mustelid,” Lutra, Meles, Lynx, Panthera, Crocuta, Ursus, Alopex, Vulpex, bovine (Bos/Bison), Ovibos, Ovis, Capra, Sus, cervid, Rangifer, Equus, rhinocerotid, Castor, and Mammuthus; Buckley et al., 2016), whereas the remaining microfauna were dominated by cricetid rodents (predominantly Dicrostonyx followed by Lemmus and then several species of Microtus; Buckley et al., 2017). Among the bats (Figure 3), lesser horseshoe bats (R. hipposideros) predominated (n = 34), followed closely by mouse-eared bats (Myotis; n = 23), with the most additional spectrum that does not match any of

**FIGURE 1** Maximum likelihood tree of the concatenated bat COL1A1 and COL1A2 sequences (showing bootstrap support for 10,000 replicates); see Appendix S1 for sequences.
our reference material but most closely resembles *Nyctalus*. Representative spectra for the bat taxa that were identified are shown in Figure 2, together with spectra from the putative representatives of *M. brandtii* and *Nyctalus leisleri*. Note that it is difficult to distinguish *Plecotus* from *Barbastella*, as this is reliant upon the presence of the 2t76 marker (see Tables 1 and S2). The differences observed at the species level in some taxa are supported by differences observed in both *Rhinolophus* species analysed (Figures S1 and S2) in addition to the sequence differences discussed above.
4 | DISCUSSION

We have demonstrated that collagen fingerprinting can distinguish all seven genera of bats known to be present in either modern or ancient Britain (Myotis, Pipistrellus, Barbastella, Plecotus, Eptesicus, Nyctalus, and Rhinolopus). Many ancient bat remains will not be readily identifiable from their morphology, especially in the case of postcranial elements, exacerbated in cases of predation where acid erosion damages surface morphology. Although ancient DNA-based approaches are technically challenging, expensive, and highly destructive in the case of micromammals like bats, the collagen fingerprinting approach offers a more reliable and robust form of analysis, albeit at a reduced level of taxonomic resolution, but one much less affected by degradative processes such as the acid erosion that occurs in the digestion system of predators such as owls. Although the technique developed here clearly needs further refinement in terms of available reference spectra (particularly for the less common species from which samples have proved more difficult to obtain rare species), it could nevertheless provide a new opportunity to use bat remains from archaeological sites for palaeoenvironmental reconstructions, including the identification of cryptic species, which could be valuable given the sensitivity of bats to environmental changes and their potential importance as bio-indicators (Jones et al., 2009). As it has been proposed for megafaunal remains that are difficult to distinguish such as sheep from goat (Buckley et al., 2010), a rapid biomolecular approach such as collagen fingerprinting could also be used to support morphological criteria for species identification of bat elements.

The collagen fingerprint spectra reflect the patterns of peptide sequence evolution within the order Chiroptera, as described above, whereas the maximum likelihood tree for the concatenated COL1A1 and COL1A2 sequence data (Figure 1) is in accordance with recent phylogenetic reconstructions (e.g., Agnarsson, Zambrana-Torrelio, Flores-Saldana, & May-Collado, 2011). The two monophyletic groups recovered here (Hipposideros, Rhinolophus, Rousettus, and Pteropus, alongside Myotis, Eptesicus, and Miniopterus) conform with the division of the bats into the Yinpterochiroptera and Yangochiroptera (e.g., Teeling et al., 2002, 2005). Although this split of the order Chiroptera was initially controversial, and rejects the longstanding view that the Microchiroptera is a monophyletic group, it has subsequently been confirmed from genomic data and is now generally accepted (Lei & Dong, 2016; Tsagkogeorga, Parker, Stupka, Cotton, & Rossiter, 2013). The relative dates of the divergence between Yinpterochiroptera and Yangochiroptera, ~52–58 Ma (Jones, Bininda-Emonds, & Gittleman, 2005; Teeling et al., 2005), are reflected in the differences between their collagen sequences with 90–99 amino acid substitutions between the Rhinolophus sequence and the three Myotis sequences. Within the Yinpterochiroptera, Rhinolophus and Hipposideros are more closely related to each other than to the fruit bats Rousettus and Pteropus, and within the Yangochiroptera, the three Myotis species are closer to each other than to the other vespertilionid Eptesicus and the more distantly related Miniopterus (Figure 1), reflecting previous phylogenetic reconstructions (e.g., Agnarsson et al., 2011).

With regard to the bat remains from the Pin Hole Cave assemblage, although these are relatively few in number they show some interesting similarities and contrasts with the bat fauna nowadays present in this part of the British Isles. Horseshoe bats (Rhinolopus hipposideros) were most common, constituting slightly more than half of the good quality fingerprints that were obtained (Figure 3). Cave sites are frequently used by overwintering horseshoe bats (Schober & Grimmberger, 1989; Schofield & McAney, 2008), and the accumulation is in that sense not surprising. Although the species does not appear to be present in this area now (National Biodiversity Network, 2018), its range is known to have contracted substantially towards the south and west, since the beginning of the last century (Schofield & McAney, 2008). Lesser horseshoe bats were previously dominant among the bat species identified from Pin Hole Cave (Jenkinson, 1984) and were also numerous among the remains identified from the Neolithic material of Dowel Cave, further west in Derbyshire (Yalden, 1986), in keeping with this observation. The contraction in the species’ range has been attributed to the loss of both woodland and roosting sites in abandoned mines (Harris, Morris, Wray, & Yalden, 1995), and the disappearance of the lesser horseshoe bats from these cave sites may offer some tentative support to the former explanation.

Mouse-eared bats (Myotis spp.) were also relatively common in the assemblage (Figure 3). At least five species of Myotis presently occur in central England (M. mystacinus, M. brandtii, M. alchathoe, M. daubentoni and M. nattereri) and generally use cave sites both for winter hibernation and for social interaction in the autumn (Berge & Jones, 2008a, 2008b; Jan et al., 2010; Richardson, Waters, & Waters, 2008; Smith & Rivers, 2008). It is therefore not surprising that many of the remains were identified as Myotis, with at least two species apparently present. Indeed, three species of mouse-eared bat (M. mystacinus, M. nattereri, and M. daubentoni) were identified from the earlier excavation of Pin Hole Cave, as were two species (M. mystacinus and M. nattereri) from Dog Hole Fissure, another cave in the Creswell Crags (Jenkinson, 1984), and at least two species (M. nattereri and M. daubentoni) from Dowel Cave (Yalden, 1986). In addition, although Bechstein’s bat (Myotis bechsteinii) has only recently been recorded so far north in England, around 70 km to the west of Creswell (National Biodiversity Network, 2018), we cannot rule out that this species may have been more common earlier in the Holocene, when its deciduous woodland habitat was more extensive (Yalden, 1986). Given that this is the one species listed above that we have not been able to rule out with either reference material or available sequences, it is a plausible candidate for our unconfirmed identification of two specimens.

Remains from barbastelles (Barbastella) also appeared to be present in the assemblage (Figure 2), where a few individuals of this species were previously identified among the Pin Hole Cave and Dog Hole Fissure microfauna (Jenkinson, 1984). Although nowadays rare and generally restricted to more southerly parts of Britain, this species has recently been recorded in the area of the site (Cook, 2018; National Biodiversity Network, 2018). Overwintering barbastelles will enter cave sites during colder weather and are therefore not unexpected here (Schober & Grimmberger, 1989; Greenaway, 2008).
presence of relatively numerous barbastelle remains at the site, despite the current rarity of these bats, may again relate to their preference for old woodland (Greenaway, 2008), much of which has now been lost through human activity. On the other hand, it might equally reflect the severity of the winter weather at the time, as these bats respond to such conditions by entering caves (Harris et al., 1995).

Long-eared bats (*Plecotus*) were notably absent from the identified microfaunal remains. Brown long-eared bats (*P. auritus*) often hibernate in caves, and the species is common throughout most of Britain (Schober & Grimmberger, 1989; Entwistle & Swift, 2008; National Biodiversity Network, 2018). These bats are generally associated with tree cover (Entwistle & Swift, 2008), so they are likely to have been at least as common in the past, when deciduous woodland was more widespread (Yalden, 1986). Furthermore, long-eared bats have previously been recorded from both Pin Hole Cave and Dog Hole Fissure at Creswell (Jenkinson, 1984); however, this material was from the earlier excavations that encompassed much of the length of Pin Hole Cave. It may therefore originate from bats that occupied more exposed situations near the cave entrance, whereas the material identified in the present study was excavated from a single location at the rear of the cave.

No bats from the genera *Pipistrellus*, *Eptesicus*, and *Nyctalus* were confidently identified among the fingerprints, with the possible exception of *N. leisleri*. A single uncertain bat spectrum was found to most closely match *N. noctula* but had several peak differences, although the site is within the known range of both of these *Nyctalus* species (National Biodiversity Network, 2018). However, none of the above bats are generally associated with underground sites in Britain (although they may roost in rock crevices in some locations; Schober & Grimmberger, 1989; Hutson, 2008; Jones & Racey, 2008; Mackie & Racey, 2008; Shiel, Jones, & Waters, 2008), so it is unlikely that they would be present in the assemblage unless they were the victims of predators. Notably, one pipistrell and two Leisler's bats (*N. leisleri*) were previously recorded from the Pin Hole Cave microfauna (Jenkinson, 1984) making our interpretation of the unknown fingerprint more plausible, but it should be emphasized that these were from the earlier excavations and therefore may have come from more exposed locations near to the entrance or be the result of predator activity. Likewise, a single Leisler's bat that was identified from Dowel Cave was attributed to avian predation (Yalden, 1986).

Overall, the bat fauna recovered from the cave suggests that it was used for hibernation, as all of the species that were present use underground sites in winter. It is therefore conceivable that the remains are from bats that died there, when they were at their weakest towards the end of hibernation.

## 5 CONCLUSIONS

Past microfauna provide an opportunity to infer the effects of changing environments and, in this context, the potential importance of bat remains from archaeological sites in Britain has been recognised for some time (Yalden, 1986). However, their study has been hampered by the challenges in identifying isolated bones from their morphological attributes alone. Here, we demonstrate the successful use of collagen peptide mass fingerprinting to distinguish genera of bats that are present in Britain. The application of the procedure to an assemblage from a cave site in England highlights changes that have occurred in the local bat fauna, which can to some extent be related to the effects of past human activity on the landscape.

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## REFERENCES

Agnarsson, I., Zambrana-Torrelio, C. M., Flores-Saldana, N. P., & May-Collado, L. J. (2011). A time-calibrated species-level phylogeny of bats (Chiroptera, Mammalia), PLoS Currents, 3(3), RRN1212.

Barclay, R. M., & Brigham, R. M. (1991). Prey detection, dietary niche breadth, and body size in bats: Why are aerial insectivorous bats so small? The American Naturalist, 137(5), 693–703.

Berge, L., & Jones, G. (2008a). Whiskered bat *Myotis mystacinus*. In S. Harris, & D. W. Yalden (Eds.), *Mammals of the British Isles: Handbook* (4th ed.) (pp. 310–315). Southampton: The Mammal Society.

Berge, L., & Jones, G. (2008b). Brandt’s bat *Myotis brandtii*. In S. Harris, & D. W. Yalden (Eds.), *Mammals of the British Isles: Handbook* (4th ed.) (pp. 315–319). Southampton: The Mammal Society.

Buckley, M., Collins, M., Thomas-Oates, J., & Wilson, J. C. (2009). Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, 23(23), 3843–3854. https://doi.org/10.1002/rcm.4316

Buckley, M., Farina, R. A., Lawless, C., Tambusso, P. S., Varela, L., Carlini, A. A., ... Martinez, J. G. (2015). Collagen sequence analysis of the extinct giant ground sloths Lestodon and Megatherium. *PLoS ONE*, 10(11), e0139611. https://doi.org/10.1371/journal.pone.0139611

Buckley, M., Fraser, S., Herman, J., Melton, N., Mulville, J., & Pålsséttir, A. (2014). Species identification of archaeological marine mammals using collagen fingerprinting. *Journal of Archaeological Science*, 41, 631–641. https://doi.org/10.1016/j.jas.2013.08.021

Buckley, M., Gu, M., Shameer, S., Patel, S., & Chamberlain, A. (2016). High-throughput collagen fingerprinting of intact microfaunal remains; a low-cost method for distinguishing between murine rodent bones. *Rapid Communications in Mass Spectrometry*, 30, 1–8.

Buckley, M., Harvey, V. L., & Chamberlain, A. T. (2017). Species identification and decay assessment of Late Pleistocene fragmentary remains from Pin Hole Cave (Creswell Crags, UK) using collagen fingerprinting. *Boreas*, 46(3), 402–411. https://doi.org/10.1111/bor.12225

Buckley, M., & Kansa, S. (2011). Collagen fingerprinting of archaeological bone and teeth remains from Domuztepe, South Eastern Turkey.
Archaeological and Anthropological Sciences, 3(3), 271–280. https://doi.org/10.1007/s12520-011-0066-z

Buckley, M., Kansa, S. W., Howard, S., Campbell, S., Thomas-Oates, J., & Collins, M. (2010). Distinguishing between archaeological sheep and goat bones using a single collagen peptide. Journal of Archaeological Science, 37(1), 13–20. https://doi.org/10.1016/j.jas.2009.08.020

Clare, E. L., Lim, B. K., Fenton, M. B., & Hebert, P. D. (2011). Neotropical bats: Estimating species diversity with DNA barcodes. PLoS ONE, 6(7), e22648. https://doi.org/10.1371/journal.pone.0022648

Cook M. 2018. The Nottinghamshire Barbastelle Project. Inside Ecology. https://insideecology.com/2018/01/22/the-nottinghamshire-barbastelle-project/

Cronin, M. A., Palmisciano, D. A., Vyse, E. R., & Cameron, D. G. (1991). Mitochondrial DNA in wildlife forensic science: Species identification of tissues. Wildlife Society Bulletin (1973–2006), 19(1), 94–105.

Cuff, A., & Jacob, R. (2001). A formal mammalian biostratigraphy for the Late Pleistocene of Britain. Quaternary Science Reviews, 20(16), 1707–1716. https://doi.org/10.1016/S0277-3791(01)00035-X

Entwistle, A. C., & Swift, S. M. (2008). Brown long-eared bat Plecotus auritus. In S. Harris, & D. W. Yalden (Eds.), Mammals of the British Isles: Handbook (4th ed.) (pp. 364–370). Southampton: The Mammal Society.

Foran, D. R., Crooks, K. R., & Minta, S. C. (1997). Species identification of teeth. Mitochondrial DNA in wildlife forensic science: Species identification and DNA sequence retrieval from ancient tissues. Archaeometry, 44(3), 337–342. https://doi.org/10.1111/1475-4754.00066

Greenaway, F. (2008). Barbastelle Barbastella barbastellus. In S. Harris, & D. W. Yalden (Eds.), Mammals of the British Isles: Handbook (4th ed.) (pp. 362–364). Southampton: The Mammal Society.

Hass, M., Carver, M., Kitchener, A. C., & Walters, P. S. (2010). DNA damage and DNA sequence retrieval from ancient tissues. Nucleic Acids Research, 24(7), 1304–1307. https://doi.org/10.1093/nar/gnq070

Hutson, A. M. (2008). Serotine Eptesicus serotinus. In S. Harris, & D. W. Yalden (Eds.), Mammals of the British Isles: Handbook (4th ed.) (pp. 356–360). Southampton: The Mammal Society.

Hutson, A. M., Mickleburgh, S., & Racey, P. A. (2001). Microchiropteran bats: Global status survey and conservation action plan. Gland, Switzerland: IUCN. https://www.iucn.org/sites/default/files/pdf/2001/10/20010302_230510520.pdf

Jan, C. M. I., Frith, K., Glover, A. M., Butlin, R. K., Scott, C. D., Greenaway, F., ... Altringham, J. D. (2010). Myotis alcathoe confirmed in the UK from mitochondrial and microsatellite DNA. Acta Chiroptologica, 12(2), 471–483. https://doi.org/10.3161/15088110X538043

Jenkinson, R. (2004). Creswell Crags: Late Pleistocene sites in the East Midlands. In British Archaeological Reports British Series 122: 1–371. Oxford: Archaeopress.

Jones, G., Jacobs, D. S., Kunz, T. H., Willig, M. R., & Racey, P. A. (2009). Carpe noctem: The importance of bats as bioindicators. Endangered Species Research, 8(1-2), 93–115. https://doi.org/10.3354/esr00182

Jones, K. E., Bininda-Emonds, O. R., & Gittleman, J. L. (2005). Bats, clocks, and rocks: Diversification patterns in Chiroptera. Evolution, 59(10), 2243–2255. https://doi.org/10.1111/j.0014-3820.2005.tb00932.x

Kunz, T. H., Braun de Torrez, E., Bauer, D., Lobova, T., & Fleming, T. H. (2011). Ecosystem services provided by bats. Annals of the New York Academy of Sciences, 1233(1), 1–38.

Lei, M., & Dong, D. (2016). Phylogenomic analyses of bat subordinal relationships based on transcriptome data. Scientific Reports, 6, 27726. https://doi.org/10.1038/srep27726

Lewis, S. E. (1995). Roost fidelity of bats: A review. Journal of Mammalogy, 76(2), 481–496. https://doi.org/10.2307/1382357

Mackie, I. J., & Racey, P. A. (2008). Noctule Nyctalus noctula. In S. Harris, & D. W. Yalden (Eds.), Mammals of the British Isles: Handbook (4th ed.) (pp. 338–342). Southampton: The Mammal Society.

National Biodiversity Network 2018. NBN atlas. https://nbnatlas.org/

Shiel, C. B., Jones, G., & Waters, D. (2008). Leisler’s bat Nyctalus leisleri. In S. Harris, & D. W. Yalden (Eds.), Mammals of the British Isles: Handbook (4th ed.) (pp. 334–338). Southampton: The Mammal Society.

Steebings, R. E., Yalden, D. W., & Herman, J. S. (2007). Which bat is it? A guide to bat identification in Great Britain and Ireland (3rd ed.). London: The Mammal Society.

Stier, S. C., & Mildenstein, T. L. (2005). Dietary habits of the world’s largest bats: The Philippine flying foxes, Acerodon jubatus and Pteropus vampyrus lanesiens. Journal of Mammalogy, 86(4), 719–728. https://doi.org/10.1644/1545-1542(2005)086[0719:DHOTWL]2.0.CO;2

Teeling, E. C., Madsen, O., Stanhope, M. J., de Jong, W. W., Van den Bussche, R., & Springer, M. S. (2002). Microbat paraphyly and the convergent evolution of a key innovation in Old World rhinolophid microbats. Proceedings of the National Academy of Sciences, 99, 1432–1436.

Teeling, E. C., Springer, M. S., Madsen, O., Bates, P., O’Brien, S. J., & Murphy, W. J. (2005). A molecular phylogeny for bats illuminates biogeography and the fossil record. Science, 307(5709), 580–584. https://doi.org/10.1126/science.1105113

Tsaikogeorga, G., Parker, J., Stupka, E., Cotton, J. A., & Rossiter, S. J. (2013). Phylogenomic analyses elucidate the evolutionary relationships of bats. Current Biology, 23(22), 2262–2267. https://doi.org/10.1016/j.cub.2013.09.014

Weiner, S., & Bar-Yosef, O. (1990). States of preservation of bones from prehistoric sites in the Near East: A survey. Journal of Archaeological Science, 17, 187–196. https://doi.org/10.1016/0305-4403(90)90058-D

Wilson, D. E., & Reeder, D. M. (2005). Mammals of the world: A taxonomic and geographic reference (3rd ed.). Baltimore: Johns Hopkins University Press.

Yalden, D. W. (1986). Neolithic bats from Dowel Cave, Derbyshire. Journal of Zoology A, 210, 616–619.

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