Species identification of archaeological marine mammals using collagen fingerprinting

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ABSTRACT

Throughout human history, coastal and marine resources have been a vital part of human subsistence. As a result archaeological faunal assemblages from coastal sites often contain large quantities of skeletal remains indicative of human interaction with marine mammals. However, these are often hard to identify due to a unique combination of factors regarding the procurement, utilisation, morphological and physical characteristics of marine mammal bones. These factors often result in a large number of archaeological cetacean and pinniped specimens fragmented beyond visual recognition, being labelled 'whale' or 'marine mammal'. In this paper we report the development of a **Zoo**archaeology by **M**ass **S**pectrometry (ZooMS) method of collagen fingerprinting, for efficient and low cost discrimination of a wide range of marine mammal species including cetaceans and pinnipeds. We apply the technique to more than fifty archaeological specimens from seven different North Atlantic sites ranging from the Mesolithic until the Early Modern period.

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1. Introduction

Coastal activities have been important to hominids from the earliest times, with studies on Neanderthals at different sites across Gibraltar providing evidence that marine mammal exploitation predates modern humans (Erlandson, 2001; Sabin, 2005; Stringer et al., 2008). These species continue to be significant to present day populations, especially to coastal communities and most importantly as a source of food. Marine mammals comprise the cetaceans (whales, dolphins and porpoises), pinnipeds (earless (true) seals, eared seals (sea lions and fur seals) and walruses) and sirenians (sea-cows). These groups are not related and differ markedly in ecology and behaviour. However, due to geographical constraints, only cetaceans and pinnipeds are discussed further in this paper. Pinnipeds are distinguished from cetaceans by their ability to move on land as well as water and have limbs configured

to allow this transition. Cetaceans never leave the water and have non-weight bearing forelimbs modified to act as flippers and hindquarters represented only by vestigial pelvises. As a result of this evolutionary commitment, post-cranial cetacean bones are mostly composed of cancellous bone with a thin cortical layer, fewer distinct morphological features and a lower density of mineral in their bones to aid buoyancy (Gray et al., 2007). This lower mineral density reduces the likelihood of archaeological bone preservation in relation to those of terrestrial mammals.

There is a large body of evidence for marine mammal exploitation in communities living on the coasts of the North-Eastern Atlantic, North Sea and Baltic since the prehistoric period (Storå and Lõugas, 2005). The manner of this exploitation can provide important insights into the cultural and technological achievement of a society, partly because of the relative difficulty in exploiting the various cetacean species (Erlandson, 2001; Mulville, 2002). The regular occurrence of cetacean bones at archaeological sites (e.g., Clark, 1947; Hallén, 1994; Herman and Dobney, 2004; MacGregor, 1985) has fuelled a long-running debate about their procurement (Erlandson, 2001; Savelle, 1997). In particular, there is considerable interest in whether cetaceans were obtained as an occasional "windfall" due to natural stranding events, or were actively hunted

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(e.g. Clark, 1947; Erlandson, 2001; Gardiner, 1997; Mulville, 2002) and, if deliberately procured, were they captured with harpoons from the shore, from small boats, or was a more active strategy of hunting whales at sea used?

Whilst marine mammal remains are present on sites from prehistory onwards, the earliest written reference for the exploitation of marine mammals comes from the late 7th century AD text by Adomnán on St Columba, who lived in the 6th century AD. Later, an early Christian text written by Bede in 731 AD mentions the hunting of both seals and cetaceans whilst later still there are accounts of herding, stranding and slaughter of small whales in the Western and Northern Isles of Scotland and in Iceland (Fenton, 1997; Kristjánsson, 1986). Overall, however, there is a paucity of detailed information about marine mammal exploitation in the early written records (Szabo, 2008). It is therefore important to examine archaeological bone assemblages if we are to understand the prehistoric and early historic human interaction with these mammals.

Cetaceans provide food, hide, blubber, sinew, fuel, containers, tool-making material and structural elements (Clark, 1947, 1952; Erlandson, 2001; Mulville, 2002; Savelle, 1997). Toothed whales also provide additional material for carving or ornamentation. Seals supply food, hide, sinew, storage containers from stomach and pericardium, teeth for decoration and blubber (Clark, 1952; Grigson, 1981). Although it is possible that complete seal carcasses may be processed at a settlement, it is unlikely that the bones of larger cetaceans would be transported from the site where an animal was accidentally beached, or a carcass was landed, unless there was some specific reason (Erlandson, 2001; Mulville, 2002; Savelle, 1997). Indeed many cetacean bones recovered from archaeological excavations tend to show evidence of modification, burning or *in situ* structural use. Whale bones were used as building material, for tools such as chopping blocks, and as craft items such as gaming pieces (Childe, 1931; Erlandson, 2001; Mulville, 2002; Savelle, 1997; Smith and Kinahan, 1984; Whitridge, 2002; Harrison et al., 2008; Kristjánsson, 1986; Mehler, 2007). This subjects them to further fragmentation and makes their identification through visible inspection more difficult (Eldjárn, 2000; Erlandson, 2001; Mulville, 2002).

Determining the numbers and species of marine mammal remains found at archaeological sites has an important part to play in resolving questions about their procurement and use. However archaeological cetacean bone is relatively fragile, due to its low mineral content, and has often been worked for various purposes. Consequently many archaeological specimens are fragmented beyond morphological recognition, often being labelled 'whale' or 'marine mammal' (e.g. Harrison et al., 2008; McGovern, 2009; Mulville, 2002; Pálsdóttir, 2008). Additionally, not all countries or regions have museum collections with sufficient numbers of cetacean and pinniped skeletons to use for species identification making it even more important to establish relatively cheap alternative methods to visual inspection of comparative morphology.

Three examples illustrate the difficulty of using comparative morphology for cetacean identification from archaeological sites. Firstly, in a collection from seven Western Isles archaeological sites, ranging from the later Bronze Age to the Norse period, only 30 (5.3%) of 568 cetacean bone fragments could be identified to species when compared with the marine mammal collection held at the Natural History Museum, London (Mulville, 2002). Secondly, only seven (5.2%) of 134 cetacean bone fragments recovered from the Iron Age site of Brest Ness in Orkney, could be identified to species using the comparative collection at the National Museums Scotland (Fraser, unpublished). Thirdly there were few specific identifications among an extensive list of cetacean bone recovered

from archaeological sites in the Baltic, North Sea and North-West Atlantic (Clark, 1947).

Pinniped identification is often hampered by the remarkable level of intra-specific variation in bone morphology (Amorosi, 1992; Hodgetts, 1999) and this has led to the widespread practice of identifying only a limited number of elements (e.g., cranium, mandible, humerus, ulna and femur) to the species level. Although other elements of the post-cranial skeleton can sometimes be identified, there is often overlap between species, so that many identifications are only made to 'seal' (Hodgetts, 1999). Since Clarks seminal publication many archaeological sites have consistently yielded small but significant proportions of cetacean and pinniped bone but few of these have been ascribed to species, illustrating the difficulties of identification using morphological characteristics. For example, in the Mesolithic shell midden at Cnoc Coig, Oronsay, Argyll and Bute, a number of grey seals were identified but as many again could only be recorded as *probable* grey seal (Grigson and Mellars, 1987); there were similar difficulties in identifying a common seal. At the same site a range of small cetacean bones could not be identified to species, but the authors suggested these probably derived from common porpoise or common dolphin based on size and present day distribution (Grigson and Mellars, 1987). At the site of Northton on the Isle of Harris, neither pinnipeds nor cetaceans could be identified to species. In the Neolithic assemblage, seven bone and tooth fragments were allocated to seal, which was 1% of those recovered. At the Beaker phase 1 of the site, 19 bones (3%) were allocated to seal and two bones (0.3%) were unidentified cetaceans (Finlay, 2006). At the Neolithic and Early Bronze Age period from Tofts Ness in Orkney, 172 bones were allocated only as 'seal', 1.4% of the total collection for that period, and a further 36 bones (0.3%) as cetacean (Nicholson and Davies, 2007).

Specific identification of cetacean remains has particular value in the understanding of species distributions before the time of large-scale commercial whale hunting in the early modern period (Roman and Palumbi, 2003). Zooarchaeological data has also been used in arguments for and against modern whaling (Mulville, 2005), thus the importance of accurate identification and thorough understanding of the nature of whale exploitation has become particularly significant in recent years. Extraction of DNA provides a means to identify such fragments and can also yield considerable information on species, number of individuals at the archaeological site and even in the source population (Foote et al., 2012; Nichols et al., 2007), however it is a relatively time-consuming and expensive process. In this paper we report the development of a **Zoo**archaeology by **M**ass **S**pectrometry (ZooMS) method, initially designed to separate sheep from goat (Buckley, 2008; Buckley et al., 2010), as a tool that can distinguish a wide range of marine mammal species. As described here, it can be used to separate cetaceans and pinnipeds at least to subfamily levels and down to species level in some groups of cetaceans.

1.1. Species identification using collagen peptide mass fingerprinting (ZooMS)

Depending on the conditions, as collagen (Type 1 collagen; COL1) loss in bone is sensitive to temperature, the preservation of collagen molecules in fossils can be sustained for hundreds of thousands or even millions of years; studies have used collagen peptide mass fingerprints from Mediterranean sites >10 Ka (Buckley et al., 2009; Buckley and Kansa, 2011), British Pleistocene fossils ~1.5 Ma (Buckley and Collins, 2011) and Arctic Pliocene fossils from ~3.5 Ma (Rybczynski et al., 2013) for species determination. This long term survival has been linked to the intimate

organization between the collagen fibrils and the bone apatite. The bone apatite grows into the fibrils, and it has been proposed that the radial compression that is applied by the intergrowth of bone mineral reduces the rate of collagen gelatinisation (Hofreiter et al., 2012). In colder regions, the preservation of both the bones and the proteins within the tissue allow collagen to become a valuable biomolecular tool, which can store information beyond the length of time for which DNA remains useful (Hofreiter et al., 2012).

This longevity is extremely useful for identification of extinct animals because it gives collagen the potential to be used as a molecular marker for analysing the remains of diverse taxa (Buckley et al., 2009; Collins et al., 2010), but over much greater periods of time than ancient DNA. Not only does the fibrous protein degrade at a slower rate than DNA, but it can also be sampled directly from bone, avoiding the risk of contamination during the amplification process usually carried out for DNA analysis. Although long considered a highly conserved protein with slow evolutionary rates, Buckley et al. (2009) found that one of the three polypeptide chains that make up the collagen triple helix (the COL1α2 chain) present higher sequence variability than previously assumed. This allows the discrimination of closely related genera and makes collagen peptides useful in zooarchaeological identification (Buckley et al., 2010; Collins et al., 2010), where it offers an inexpensive alternative to ancient DNA.

Our aim here is to show that the technique can be used to distinguish between the range of marine mammals that are likely to be found in archaeological sites around the North Atlantic ocean, at least to a taxonomic level that will provide useful information in this context, and to demonstrate its efficacy on a range of relevant archaeological bone remains.

2. Materials and methods

2.1. Materials

Hydrochloric acid (HCl) and acetonitrile (ACN) were obtained from Merck (UK) and Fisher Scientific (UK), respectively. Sequencing-grade trypsin was purchased from Promega (UK) and trifluoroacetic acid (TFA), ammonium bicarbonate (ABC), mass spectrometric standards (calibration peptides) and α-cyano-4-hydroxycinnamic acid were purchased from Sigma–Aldrich (UK). The C18 solid phase extraction pipette tips were purchased from Varian (UK). Bone samples from modern cetaceans and pinnipeds were obtained from Cardiff University (CU), National Museums Scotland (NMS), the Yorkshire Museum (YM) and the Norwich Castle Museum (NCM) (Table 1). Over 50 archaeological specimens were sampled from sites ranging from the Mesolithic period to the Early Modern period throughout the North Atlantic (Fig. 1). The samples were removed as powder using a diamond-tipped dremel drill.

2.2. Background information for archaeological sites included in this study

West Voe, Sumburgh, Shetland Islands. HU 39205 10199. Two shell middens exposed by coastal erosion and separated by a layer of sand were sampled. A shell from the lower midden was dated to 4320–4020 cal BC (Melton and Nicholson, 2004). Subsequent excavations (Melton and Nicholson, 2007; Melton, 2008, 2009) established that this midden comprised a sequence of mollusc species: initially oyster (*Ostrea edulis*), then limpet (*Patella vulgata*), and finally mussel (*Mytilus edulis*). Large numbers of bones from seals, mainly juveniles, and seabirds were also present. Additional radiocarbon dates established that the lower midden encompassed the Mesolithic–Neolithic transition. The Mesolithic

Table 1
Details of standards used in this study, listing the source and common, family, genus and species names.

Source	Common name	Family	Genus	Species
NCM	Walrus	Odobenidae	*Odobenus*	*rosmarus*
NMS	Northern fur seal	Otariidae	*Callorhinus*	*ursinus*
NMS	Bearded seal	Phocidae	*Erignathus*	*barbatus*
NMS	Ringed seal	Phocidae	*Pusa*	*hispida*
NCM	Hooded seal	Phocidae	*Cystophora*	*cristata*
NCM	Grey seal	Phocidae	*Halichoerus*	*grypus*
NCM	Common seal	Phocidae	*Phoca*	*vitulina*
YM	Harp seal	Phocidae	*Phoca*	*groenlandica*
NMS	Bottlenose dolphin	Delphinidae	*Tursiops*	*truncates*
NMS	Common dolphin	Delphinidae	*Delphinus*	*delphis*
NMS	White-beaked dolphin	Delphinidae	*Lagenorhynchus*	*albirostris*
CU	Euphrosyne dolphin	Delphinidae	*Stenella*	*coeruleoalba*
CU	Risso's dolphin	Delphinidae	*Grampus*	*griseus*
NMS	Pilot whale	Delphinidae	*Globicephala*	*melas*
NMS	False killer whale	Delphinidae	*Pseudorca*	*crassidens*
NMS	Orca	Delphinidae	*Orcinus*	*orca*
NMS	White-sided dolphin	Delphinidae	*Lagenorhynchus*	*obliquidens*
NCM	Porpoise	Phocoenidae	*Phocoena*	*phocoena*
NCM	Narwhal	Monodontidae	*Monodon*	*monoceros*
YM	Beluga whale	Monodontidae	*Delphinapterus*	*leucas*
CU	Sperm whale	Physeteridae	*Physeter*	*catodon*
NMS	Sowerby's whale	Ziphidae	*Mesoplodon*	*bidens*
NMS	Bottlenose whale	Ziphidae	*Hyperoodon*	*ampullatus*
CU	Minke whale	Balaenopteridae	*Balaenoptera*	*acutorostrata*
NMS	Fin whale	Balaenopteridae	*Balaenoptera*	*physalus*
NMS	Humpback whale	Balaenopteridae	*Megaptera*	*novaeangliae*
NMS	Blue whale	Balaenopteridae	*Balaenoptera*	*musculus*
NMS	Sei whale	Balaenopteridae	*Balaenoptera*	*borealis*
NMS	Right whale	Balaenidae	*Eubalaena*	*glacialis*

'oyster' and 'limpet' phases spanned the period ca. 4300–3700 BC, and the Neolithic 'mussel' phase, in which domesticated species (cattle and sheep) and ceramics were also present, dated to ca. 3700–3500 BC. The single cetacean bone recovered, and included in this study, was found in the primary 'oyster' phase of midden deposition.

Links of Noltland, Westray, Orkney. HY 428 493. A late Neolithic settlement site, excavated on behalf of Historic Scotland in order to record the prehistoric buildings and the associated finds, soils, field boundaries and landscape features before severe and continuing erosion exposes the remains to disintegration and dispersal (Moore and Wilson, 2011; RCAHMS).

Rowiegar, Rousay, Orkney. HY 373 298. A Neolithic stalled burial cairn excavated by W.G. Grant in 1937 (RCAHMS).

Knap of Hower, Papa Westray, Orkney. HY 483 518. An early Neolithic farmhouse settlement, radiocarbon dated to the later 4th millennium BC, consisting of two interconnecting buildings built on a pre-existing midden. Excavated by W. Trail and W. Kirkness in the 1930's and Dr A. Ritchie in the 1970's (RCAHMS; Ritchie, 1983).

Cladh Hallen, South Uist, Western Isles. NF 731 220. A multi-stage site, from a late Bronze Age cemetery through to the occupation of a series of roundhouses and figure of eight houses from the 12th to 10th century BC to the 7th to 6th century BC. Excavated by the Sheffield Environment Archaeological Research Campaign in the Hebrides (RCAHMS).

A'Cheardach Mhor, South Uist, Western Isles. NF 757 413. A settlement comprising five phases of occupation from a middle Iron Age roundhouse through to the late medieval period. This site was excavated on behalf of the Scottish Office Ministry of Works in 1956 (RCAHMS; Young and Richardson, 1959).
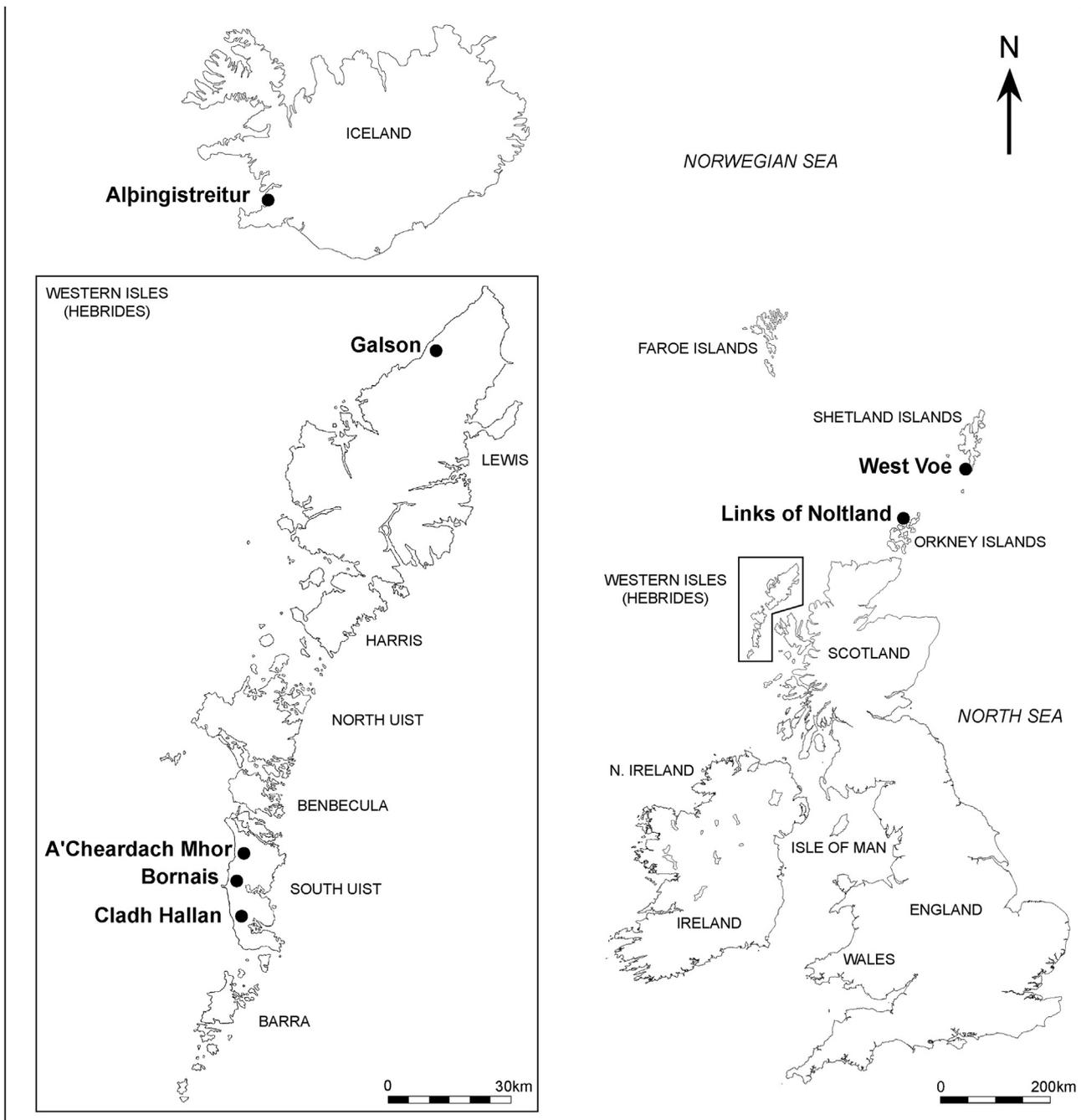
Fig. 1. Locations of the archaeological sites included in this study.

Galson, Lewis, Western Isles. NB 437 595. Iron Age site with middens and some stone structures, excavated in the 1920's (RCAHMS).

Gurness, Mainland, Orkney. HY 382 286. An Iron Age broch surrounded by outbuildings, with subsequent Dark Age and Viking activity. Excavations first took place in 1929 (RCAHMS).

Bornais, South Uist, Western Isles. NF 729 302. A late Iron Age and early 10th to 15th century Viking site (RCAHMS).

Alþingisreitur, Reykjavík, Iceland. Loc. 64.1468, −21.94195. Excavated in 2008−2012, the site is divided into four main phases based on tephrochronology and radiocarbon dates. Phase IV dates to 871−1226 AD, phase III to 1226−1500, phase II to 1500−1800

and phase I from 1800 to the modern day (Garðarsdóttir, 2010). Animal bone preservation ranged from excellent to very poor, in some parts of the site bones were completely encrusted in soil, brittle and hard to identify but the majority of the bone material was well preserved. A portion of the bone from the site had vivianite crystals on the surface (Pálsdóttir, 2010, 2013); vivianite is a mineral which is formed in soil when iron, phosphate and water are present (McGowan and Prangnell, 2006). Icelandic soil is generally very iron rich and the location of the site in an area with a high water table and close proximity to the Reykjavík pond allowed for the formation of vivianite, which can affect the results of DNA analysis and conservation of bone (McGowan and Prangnell, 2006).

2.3. Methods

2.3.1. Peptide isolation and extraction

From each of the specimen samples, ∼50 mg of bone powder was demineralised prior to collagen extraction using 1 mL 0.5 M HCl, overnight at 4 °C. The samples were centrifuged (13,000 × *g*, 5 min) and the supernatant was discarded. The remaining acid-insoluble pellet from each sample was resuspended using 500 μL of 50 mM ABC (pH 7.4) and gelatinised at 70 °C for 3 h. Subsequent to gelatinisation, the sample was centrifuged (13,000 × *g*, 15 min), precipitating the ungelatinised protein from the supernatant. The supernatant was then removed for tryptic digestion where 2 μL of 1 μg/μL sequencing-grade trypsin solution was added to the supernatant and incubated at 37 °C for 18 h.

To produce Peptide Mass Fingerprints (PMFs), C18 pipette tips were used to purify (desalt and concentrate the peptides) and fractionate the generated peptide mixture. The pipette tips were firstly equilibrated for sample binding, washing, and elution. This was done with two bed volumes of 50% ACN/0.1% TFA, followed by two bed volumes (100 μL) of 0.1% TFA. Post digestion, the samples were centrifuged (13,000 × *g*, 15 min) and acidified to 0.1% TFA. The samples were then loaded onto the activated C18 pipette tips by aspirating and dispensing with 10 cycles. The pipette tips were then washed twice with 100 μL 0.1% TFA and a stepped gradient of increasing ACN concentration was applied to the tips to fractionate and elute the peptides (100 μL 10% ACN and 50% ACN with 0.1% TFA). The eluant was aspirated and dispensed 10 times, dried using a centrifugal evaporator and resuspended with 10 μL 0.1% TFA.

2.3.2. MALDI analysis

1 μL of the sample solution was spotted onto a Bruker ultraflex 384 target plate, mixed together with 1 μL of α-cyano-4-hydroxycinnamic acid matrix solution (1% in ACN/H2O 1:1v/v) and dried to air. Each of the collagen digest fractions were analysed by MALDI-MS in reflectron mode using a Bruker ultraflex II MALDI-TOF/TOF mass spectrometer equipped with a Nd:YAG smart beam laser. MS spectra (e.g., Fig. 2) were acquired over a mass range of *m/z* 700–3700 using 1000 laser acquisitions. Final mass spectra were externally calibrated against an adjacent spot containing five calibrant peptides. To confirm the homology of peptides between different species, tandem MS (MS/MS) was carried out on selected peptide markers (precursor ion selected with 500 laser acquisitions, up to 4500 laser acquisitions were used for the fragment ions, and argon was used as the collision gas). In some cases, particularly the more abundant (i.e., precursor ion count >1000) and variable peptides (particularly those discussed in Buckley et al., 2009), the peptide sequences could be deciphered (labelled peptide B & D in this study; see Inline Supplementary Fig. S1 & Supplementary Fig. 2) by manually interpreting the spectra produced from collision-induced dissociation product ion (MS/MS) of these selected peptides (i.e., *de novo* sequencing).

Inline Supplementary Fig. S1 can be found online at http://dx.doi.org/10.1016/j.jas.2013.08.021.

3. Results

3.1. Fractionation using 10% and 50% acetonitrile

Fractionation was used to separate and identify some of the most variable sequences in collagen, reducing the signal to noise ratio for the *m/z* peak values. Fig. 2 highlights the stronger signal achieved for the larger and more hydrophobic peptides from the tryptic digest with higher *m/z* peak values in the 50% acetonitrile. Although the majority of samples showed little improvement in the signal of the larger, hydrophobic peptides, it has previously been vital in the detection of such peptides, and has been particularly useful in quickly identifying peptides D and G in other analyses (Buckley et al., 2010; Buckley and Collins, 2011). For example, the fractionation is particularly useful in distinguishing between sheep
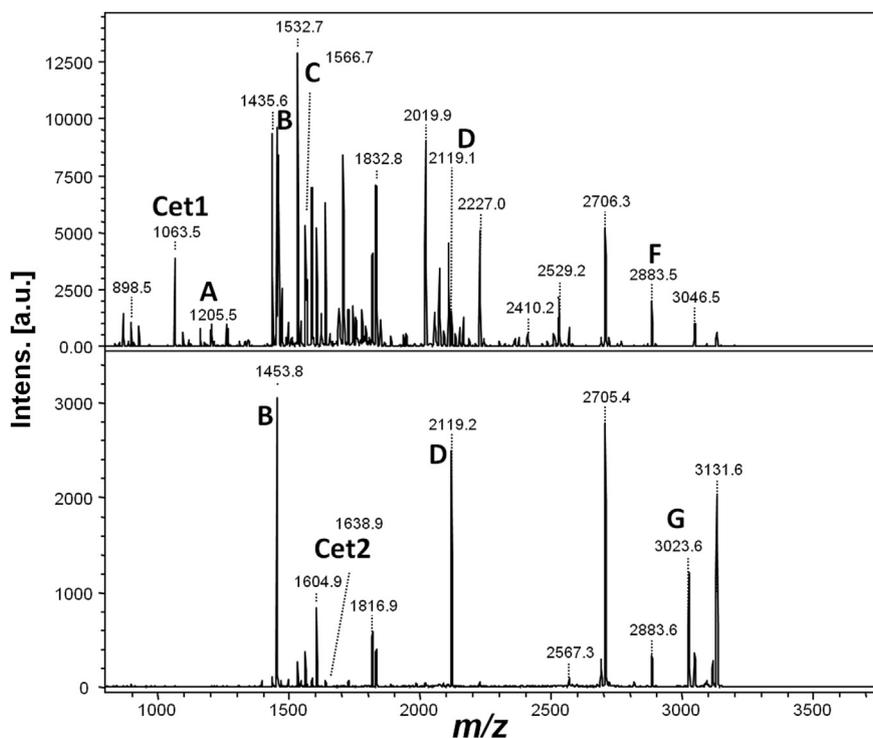
Fig. 2. Example MALDI-ToF-MS spectra showing 0–10% (top) and 10–50% (bottom) ACN fractionation of collagen extracted from Risso's dolphin bone following digestion with trypsin and purification using C18 solid phase extraction. Proposed peptide biomarkers are labelled A–D, F, G, Cet1 and Cet2 (Table 2).

and goat collagen solely by improving the signal intensity of the two peptide G variants (Buckley et al., 2009, 2010).

3.2. Collagen fingerprinting in marine mammals

It has been well established that collagen sequences can be used in the discrimination of mammals at the family (Buckley et al., 2011), genus (Buckley et al., 2009) and occasionally species level (Rybczynski et al., 2013) depending upon the species being studied. Although more than 100 peaks representing collagen peptides can be seen in the mass spectra (e.g., Fig. 2; Supplementary Figs. 3–28), six of the seven specific peptide markers previously reported in Buckley et al. (2009), labelled peptide A–G (peptide E was not frequently observed in the archaeological collagen fingerprints and is not included in this study), and three additional peptide markers were needed for confident species separation with the modern standards (Figs. 2 and 3).

The cetaceans and pinnipeds can easily be separated using peptides Cet1 and A (Fig. 3; Table 2), which are at m/z 1063/1079 and m/z 1205 in cetaceans and m/z 1105 (all other known mammals) and m/z 1221 (pinnipeds) respectively. The three families of pinnipeds, Odobenidae, Otariidae and Phocidae can be separated using peptide D (m/z 2121 in the former two, m/z 2171 in the latter family) and peptide G (m/z 3003 in the former, m/z 2957 in the latter two families). Distinction within the earless ('true') seals (Phocidae) can be made using peptide F (m/z 2869) to separate the Phocini tribe (all species with these markers were formerly named within the genus 'Phoca') from all other pinnipeds (m/z 2853). Bearded seal can be identified by its unique Cet1 peptide (m/z 1121) and ringed seal by its unique P peptide (m/z 2231).

The markers for the cetacean group were more species-specific but also more complex to systematically describe. Peptide D is by far the most diagnostic peptide (Table 3; Supplementary Fig. 2) although highly conserved within the baleen whales (m/z 2135; all except blue whale) and dolphins (m/z 2119). However, the baleen whales can be separated to species level using peptides B, C, D and F (although unable to separate bowhead whale from right whale). Within the dolphins the two main sub-families taxa could be separated using a combination of Cet1 and Cet2. The Monodontidae family is represented by a diagnostic peptide B peak at m/z 1443 (m/z 1453 in most cetaceans; m/z 1441 in Minke and beaked whales) and also have unique sequences for marker peptide D (within cetaceans) at m/z 2089 and m/z 2121 for narwhal and beluga whale respectively. Sperm whale (Physeteridae) can also readily be identified using the peptide D with a peak at m/z 2133.

The Delphinidae family only showed distinctive biomarkers at the subfamily level, with *Delphinus*, *Tursiops*, *Stenella* all being members of the Delphininae subfamily, and *Globicephala*, *Grampus* and *Pseudorca* being members of the Globicephalinae subfamily. *Lagenorhynchus* and *Orcinus* are typically considered basal groups within this family (McGowen, 2011), but share the same markers as those for the Delphininae subfamily, perhaps indicating the derived nature of the amino acid substitution in the Globicephalinae in the Cet1 marker.

In the cetacean species, only peptide A sequences were conserved throughout all the species sampled with peptide F conserved in all except the Humpback whale, with an m/z 2869; peptides B, D and G showed multiple variants within the cetaceans for each peptide. This high level of variability is contrasted to the marine carnivores, where only peptide D, F and G showed any differences in their amino acid composition.

3.3. Collagen fingerprinting in archaeological samples

Over 50 archaeological samples, mostly from unidentifiable fragmentary material, were tested for the presence of collagen in order to establish the potential for this technique in a sample age range spanning over 6000 years. Most of the material studied was originally only identified to 'marine mammal' or 'cetacean', yet the ZooMS results were able to provide identification to at least the subfamily/tribe level. For many samples, specifically the whales, it was possible to get the identification down to the level of genus and species (Table 4; Fig. 4). Within our sample, large whales make up the bulk of unidentified material, presumably because bones from smaller animals are less likely to be broken up or worked by humans.

Of the six 'marine mammals' identified by morphology, three turned out to be bovine, whilst of the 27 'cetacean' samples, only one was found not to be cetacean, instead being identified here as walrus; these examples highlight the problems that fragmentation
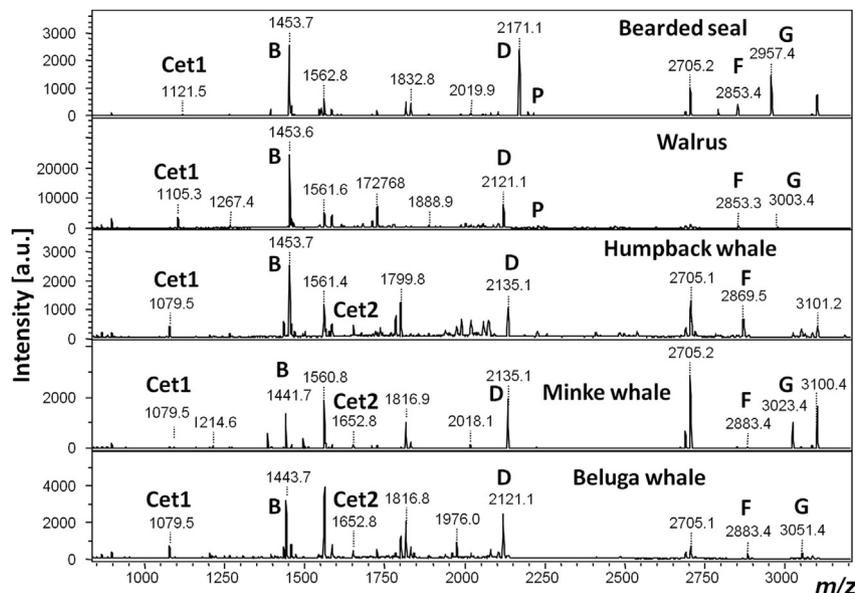

Fig. 3. MALDI-ToF-MS spectra of collagen peptide digest (10–50% ACN fraction) from selected modern pinnipeds and cetaceans showing the main peptide markers used in this study. The complete set of spectra and markers (e.g., markers A, C, Cet2 and P are more noticeable in the 0–10% ACN fractions) is given in the Supplementary Material.

Table 2
List of *m/z* markers for marine mammals where bottlenose dolphin (*Tursiops truncatus*) is the only cetacean species with a genome-derived collagen sequence.

m/z	Walrus	Northern furseal	Bearded seal	Ringed seal	Hooded seal	Common/Grey/Harp seal	Common/Bottlenose/White-beaked/Euphrosyne dolphin	Risso's dolphin/Pilot whale/False killer whale	Orca/White-sided dolphin	Porpoise	Narwhal	Beluga whale	Sperm whale	Bottlenose/Sowerby's whale	Minke whale	Fin whale	Humpback whale	Blue whale	Sei whale	Right whale
1063								Cet1						Cet1						
1079							Cet1		Cet1	Cet1	Cet1	Cet1	Cet1		Cet1	Cet1	Cet1	Cet1	Cet1	Cet1
1105	Cet1	Cet1		Cet1	Cet1	Cet1														
1121			Cet1																	
1205							A	A	A	A	A	A	A	A	A	A	A	A	A	A
1221	A	A	A	A	A	A														
1441														B	B				B	
1443											B	B								
1453	B	B	B	B	B	B	B	B	B	B			B			B	B	B		B
1550										C	C	C	C					C	C	
1566	C	C	C	C	C	C	C	C	C					C						C
1626																				
1638							Cet2	Cet2						Cet2						
1652	Cet2	Cet2	Cet2	Cet2	Cet2	Cet2			Cet2	Cet2	Cet2	Cet2	Cet2		Cet2	Cet2	Cet2	Cet2	Cet2	
1682																				Cet2
2089*											D									
2091*														D						
2105*																		D		
2119*							D	D	D	D										
2121*	D	D										D								
2133*													D							
2135*															D	D	D		D	D
2171			D	D	D	D														
2215		P	P		P	P														
2231				P																
2246	P																			
2853	F	F	F	F	F															
2869						F											F			
2883							F	F	F	F	F	F	F	F	F	F		F	F	F
2957		G	G	G	G	G														
3003	G																			
3023							G	G	G	G				G	G	G	G	G	G	G
3039													G							
3051											G	G								

*Sequences for peptide G₁₈₆₁–R₁₈₈₄ are given in Table 3. Two new markers (Cet1 and Cet2) are useful for separating cetaceans, and one (P) is useful for separating pinnipeds.

Table 3
Sequences for Peptide D obtained by MS/MS sequencing (except* which was also publicly available and used in Buckley et al., 2009). Underline represents hydroxylated proline residues, amino acid substitutions are coloured red. In sperm whale 'I' is assumed by sequence similarity to published (UniProt) sequences of other mammals, despite being isobaric with 'L'.

Species	MH+	Peptide D Sequence
Dolphins* (ENSTTRG00000003717)	2119.1	GLPGVAGSVGEPGPLGIAGPTGAR
Beluga	2121.1	GLPGVAGSMGEPGPLGIAGPAGAR
Narwhal	2089.1	GLPGVAGSVGEPGPLGIAGPAGAR
Sperm Whale	2133.1	GLPGVAGSIGEPGPLGIAGPTGAR
Baleen Whales	2135.1	GLPGVAGSVGEPGPLGISGPTGAR
Blue Whale	2105.1	GLPGVAGSVGEPGPLGISGPAGAR
Beaked Whales	2091.1	GLPGAAGSVGEPGPLGIAGPTGAR
Dog*(O46392)	2131.1	GLPGVAGSVGEPGPLGIAGPPGAR
Walrus & Eared Seals	2121.1	GLPGVSGSVGEPGPLGIAGPSGAR
True Seals	2171.1	GLPGVSGSVGEHGPLGIAGPPGAR

can have on morphological interpretation. The newly proposed marker (Cet1) for cetaceans at *m/z* 1163/1179 is highly conserved amongst most terrestrial mammals at *m/z* 1105, including cattle, sheep, goats and horses. Although the more degraded collagens yield fingerprints with lower signals for higher *m/z* peaks (e.g., Buckley and Collins, 2011), all of the proposed marine mammal markers are low *m/z* peaks that are easily recognisable following adequate collagen extraction and fingerprint analysis. All of the ten 'Phocidae' samples were identified as belonging to the Phocini group. However, all three common seal samples could only be identified to Phocini using the presented markers, highlighting that when possible, the morphological information can be much more informative. However, it should be noted that in all three cases this identification was based on the easily diagnostic distal humerus, suggestive of the potential biases that relying solely on morphology could have on the interpretation of an archaeological assemblage. Although the identification of walrus was corroborated with the molecular results, a bearded seal specimen was reassigned as also deriving from walrus, despite being from a nearly complete pelvis. Overall the species found within this study are not substantially different from those previously reported in the North Atlantic area. Most notable is the presence of a number of right whales (*Eubalaena*) as this species was absent from all Scottish assemblages reviewed by Mulville (2002). This has been attributed to problems with identification, perhaps due to the paucity of comparative material from this recently extinct population.

4. Discussion

Previous research has demonstrated that collagen fingerprinting has three main advantages. Firstly it can provide molecular data from historical, archaeological and palaeontological remains that originate further back in time than other methods (e.g., up to ∼3.5 Ma; Rybczynski et al., 2013). Secondly it can work on poorly preserved bone (Buckley et al., 2009). Thirdly it is ideal for the analysis of fragmentary material (Buckley and Collins, 2011). We demonstrate here that the method can be used to not only separate terrestrial from marine mammals (using the Cet1 marker), but to distinguish between cetaceans and pinnipeds, which will allow important inferences to be made about the exploitation and evolutionary histories of such species. Cetacean exploitation in human populations has been recorded in a variety of studies (Cortes-Sanchez et al., 2011), but research on their procurement in human communities has been limited by the difficulties of identification using traditional methods.

Identification of marine mammals on archaeological sites can not only provide useful biogeographical information on the species identified, but the distribution of the species can further reveal insights into the ecological and palaeoenvironmental conditions of the region in the past (Murray, 2008). The ability to positively identify previously indistinguishable marine mammal samples can allow us to reveal species range expansions and contractions in correlation to changing climates, and can provide information on the ability of humans to adapt and exploit different resources. This study and further work will provide an invaluable technique for marine mammal species identification that is relatively inexpensive when compared to existing molecular tools such as DNA sequencing, yet substantially more reliable than morphological identification alone. Identification of bone samples to family and in some cases genus and species level provides an opportunity to further understanding of marine mammal exploitation by human societies. For example species characteristics can help to distinguish seasonality and acquisition strategy and this technique can furnish important information for the debate on whether cetaceans and pinnipeds were acquired through natural standings, opportunistic capture or organised and specialist hunting (Clark, 1947, 1952; Erlandson, 2001; Hallén, 1994; MacGregor, 1985; Mulville, 2002).

We have shown here that cetaceans, in this case porpoise, were exploited at West Voe, one of the most remote Mesolithic communities in north-western Europe. Although the hunting-stranding point cannot be addressed from this single fragment of cetacean bone, the contrast with the numbers of seal bones at the site is suggestive that whilst the latter were hunted, cetaceans were not. Material from the later periods has added new detail to our interpretation of species from previously unidentified cetacean remains. The majority of whales represented here belong to two of the commonly hunted larger baleen whales, the slow-moving coastal northern right whale (*Eubalena glacialis*) and the humpback whale (*Megaptera novaeangliae*) both of which rarely swim faster than 12 km/h. There are additional records from the smaller minke whale (*Balaenoptera acutorostrata*), which can be trapped by sealing off inland bays with nets, and then dispatching the animals by hand. This focus on the deliberate selection of easily captured species adds weight to the argument that these communities took part in whale hunting, however sample sizes remain small. The wide range of species identified from Alþingisreitur seems to point more towards the utilisation of stranded whales and perhaps some opportunistic hunting.

Table 4
Morphological identifications and identifications from collagen fingerprinting (ZooMS) of the archaeological samples.

Sample no.	Site	Age	Bone condition	Morphological ID	ZooMS ID
WV02/WV3	West Voe	Late Mesolithic	Fragment	Cetacean	Porpoise
LON-09 (9009)	Links of Noltland	Neolithic	Fragment	Marine mammal	Dephininae
LON-08 (7302/sp2)	Links of Noltland	Neolithic	Fragment	Marine mammal	Bovine
LON-08 (7302/sp3)	Links of Noltland	Neolithic	Fragment	Marine mammal	Humpback whale
LON-08 (7302/sp4)	Links of Noltland	Neolithic	Fragment	Marine mammal	Humpback whale
1940.21.34	Rowiegar, Rousay, Orkney	Neolithic	Fragment	Cetacean	Humpback whale
1941.9.34(a)	Knap of Howar, Papa Westray	Neolithic	Fragment	Cetacean	Minke whale
2588/4174	Cladh Hallan	Late Bronze Age	Fragment	Mammal	Humpback whale
721	Cladh Hallan	Late Bronze Age	Fragment	Cetacean	Humpback whale
2211/4014	Cladh Hallan	Late Bronze Age	Fragment	Cetacean	Sperm whale
1900	Cladh Hallan	Late Bronze Age	Worked fragment	Cetacean	Sperm whale
2A	A' Cheardach Mhor	Iron Age	Fragment	Cetacean	Humpback whale
2B	A' Cheardach Mhor	Iron Age	Fragment	Cetacean	Humpback whale
1940.20.34(a)	Galson, Lewis	Iron Age	Fragment	Cetacean	Right whale
1940.20.34(b)	Galson, Lewis	Iron Age	Fragment	Cetacean	Fin whale
1940.11.34(b)	Gurness (Aikerness), Orkney	Iron Age	Fragment	Cetacean	Fin whale
2771	Bornais	Late Iron Age	Worked fragment	Cetacean	Right whale
1785	Bornais	9 to 10th century	Worked fragment	Cetacean	Right whale
1607	Bornais	7th to 11th century	Fragment	Cetacean	Right whale
1072/75G	Bornais	7th to 9th century	Fragment	Cetacean	Sperm whale
213.8/5552	Bornais	7th to 11th century	Fragment	Cetacean	Fin whale
1190	Bornais	7th to 11th century	Fragment	Cetacean	Phocini
2012-32-3916 (1049)	Alþingisreitur	871–1226	Vertebra with chop marks.	Cetacean	Globicephalinae
2009-32-2419 (1052)	Alþingisreitur	871–1226	Vertebra	Cetacean	Walrus
2012-32-3913 (1044)	Alþingisreitur	871–1226	Possibly walrus ivory, knife mark.	Marine mammal	Bovine
2012-32-3916 (1048)	Alþingisreitur	871–1226	Vertebra with chop marks.	Cetacean	Globicephalinae
2008-32-1217 (1072)	Alþingisreitur	871–1226	Skull fragment	Phocidae	Phocini
2008-32-1396 (1073)	Alþingisreitur	871–1226	Skull fragment	Phocidae	Phocini
2008-32-709 (1051)	Alþingisreitur	871–1226	'Heavy' skull fragment	Walrus	Walrus
2012-32-3916 (1069)	Alþingisreitur	871–1226	Distal humerus, chopped	Phoca vitulina	Phocini
2012-32-4061 (1043)	Alþingisreitur	871–1226	Skull fragment	Marine mammal, possibly walrus	Globicephalinae
2008-32-1395 (1067)	Alþingisreitur	871–1226	Tibia shaft fragment	Phocidae	Phocini
2012-32-3840 (1047)	Alþingisreitur	871–1226	Fragment	Cetacean	Dephininae
2012-32-3797(1046)	Alþingisreitur	871–1226	Fragment	Cetacean	Humpback whale
2012-32-3916(1032)	Alþingisreitur	871–1226	Distal humerus, chopped	Phoca vitulina	Phocini
2008-32-2371 (1066)	Alþingisreitur	1226–1500	Fragment of scapula, chopped	Phocidae	Phocini
2008-32-1166 (723)	Alþingisreitur	1226–1500	Distal humerus	Phoca vitulina	Phocini
2008-32-685 (711)	Alþingisreitur	1226–1500	Nearly complete pelvis, chop marks	Bearded seal	Walrus
2008-32-685 (1062)	Alþingisreitur	1226–1500	Tibia shaft	Phocidae	Phocini
2009-32-1947 (1058)	Alþingisreitur	1226–1500	Vertebra, chop marks	Large cetacean	Fin whale
2008-32-14409 (1070)	Alþingisreitur	1226–1500	Unfused pelvis	Phocidae	Phocini
2008-32-285 (1065)	Alþingisreitur	1226–1500	Fragment of pelvis, chopped	Phocidae	Phocini
2008-32-217 (1064)	Alþingisreitur	1226–1500	Juvenile (unfused), knife mark	Phocidae	Phocini
2009-32-2083 (1071)	Alþingisreitur	1226–1500	Scapula, chopped	Phocidae	Phocini
2009-32-1787 (1061)	Alþingisreitur	1500–1800	Mandible fragment	Small cetacean	Dephininae
2009-32-1596 (1054)	Alþingisreitur	1500–1800	Occipital fragment, scorched	Cetacean	Dephininae
2009-32-1453 (1059)	Alþingisreitur	1500–1800	Rib, part of articulated vertebral column, chopped	Cetacean	Dephininae
2009-32-1787 (1057)	Alþingisreitur	1500–1800	Vertebral epiphysis fragment, scorched	Cetacean	Bottlenose whale
2009-32-1792(1053)	Alþingisreitur	1500–1800	Vertebral epiphysis	Cetacean	Globicephalinae
2012-32-1496(1045)	Alþingisreitur	1500–1800	Smoothed fragment	Marine mammal	Bovine
2009-32-1391(1060)	Alþingisreitur	1500–1800	Vertebra, chopped	Cetacean	Globicephalinae
2009-32-1686(1063)	Alþingisreitur	1500–1800	Tibia, unfused, juvenile	Phocidae	Phocini

Other species present include small dolphins and porpoises that are previously recorded as being hunted but also includes larger, faster and more elusive cetacea (Mulville, 2002). Historically, whalers neglected the rorquals (*Balaenoptera*) as their speed made them difficult to catch, for example the blue whale (*Balaenoptera musculus*) can reach up to 55 km/h and they have a tendency to sink once dead. The sperm whale (*Physeter macrocephalus*) was considered dangerous and pursuit of it did not begin until 1713 (Clark, 1947). Thus the presence of these species may relate to accidental strandings as all have been reported as beached on North Atlantic shores, however the greater range of species and proportion of whale bone on later sites (Norse onwards) on the Western

Isles has been interpreted as an increase in cetacean utilisation that reflects an expansion in procurement activities (Mulville, 2002). Further research to routinely characterise the fragmentary marine mammal bone remains from North Atlantic sites using this effective and inexpensive method will provide valuable information on hunting, whaling and scavenging strategies.

The identification of a number of whales is of particular importance, as some species were hunted close to extinction prior to the establishment of cetacean reference collections and are rare within museum collections. For example the north-eastern Atlantic population of the right whale (*E. glacialis*) was hunted to extinction and north-western Atlantic population was reduced
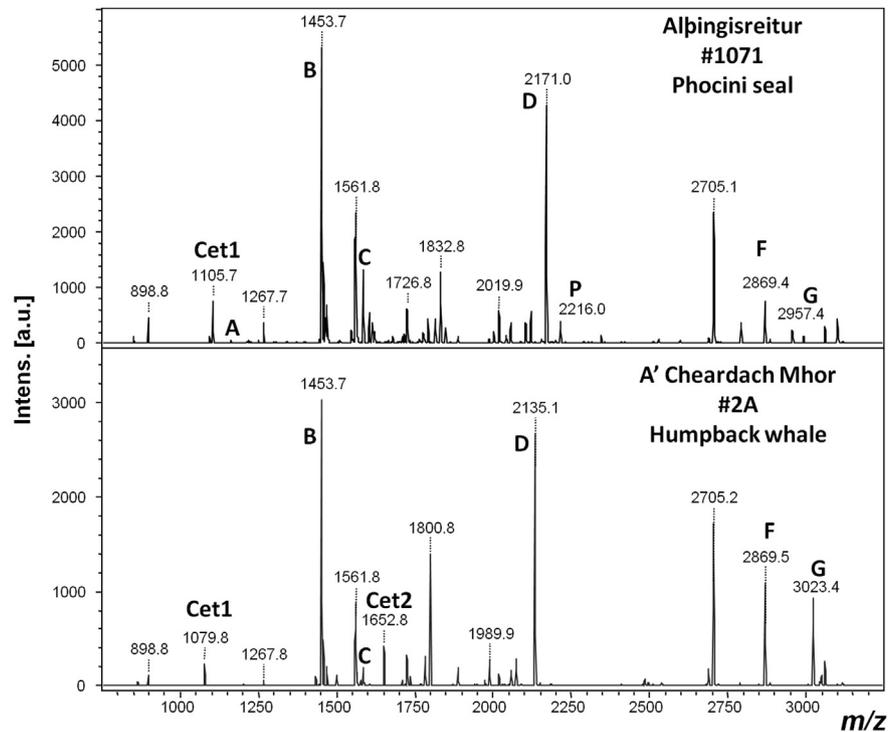
Fig. 4. Example MALDI-ToF-MS spectra of collagen peptide digest (10–50% ACN fraction) from selected archaeological samples showing an identified 'Phocini' seal (the group of seals formerly included in the genus *Phoca*, e.g., common, grey, harp) and a humpback whale showing the dominant peptide markers used in this study.

from c. 12,000 in the 11th century to around 300 by the 21st century (Waldrick et al., 2002). These species are therefore a challenge to identify within archaeological material but their remains are particularly valuable in reconstructing the genetic histories of heavily exploited species. This method has demonstrated a previously undocumented, but suspected, focus on right whales and these specimens now offer the potential for new research into the timing and intensity of the genetic bottleneck documented in this species and its relation to commercial whaling.

Accurate identification of seal bone may increase the number of vagrant seals identified. Thus, for example, in Orkney ringed, harp, bearded and hooded seals and walrus have all been noted as rare visitors to Orkney (Booth and Booth, 2005) but only possible walrus bones have been identified at archaeological sites in this area to date. Although only Phocini seals were identified in the unknown material in this study, the ability to distinguish between the above taxa, may prove useful in future research in this area.

The samples analysed here were selected to demonstrate the success of the technique as a means to distinguish morphologically unidentifiable remains across a useful range of times, from the Mesolithic to the present day. Although they are therefore insufficient to fully address the question of distribution and exploitation of marine mammals here, we have demonstrated the potential of the proposed ZooMS's routinely effective and inexpensive identification of fragmentary remains. This will assist with the resolution of many questions on their past distribution and exploitation, and could provide evidence on changing palaeoenvironmental conditions.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2013.08.021.

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