Special Issue: Integrating ZooMS and Zooarchaeology: Methodological Challenges and Interpretive Potentials

Towards a Deeper Integration of ZooMS and Zooarchaeology at Paleolithic Sites: Current Challenges and Future Directions

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submitted: 15 October 2024; accepted: 16 October 2024

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Handling Editor in Chief: Erella Hovers

ABSTRACT

Advances in biomolecular methods, in particular the study of ancient proteins (paleoproteomics), have revolutionized how we can taxonomically identify archaeological bone fragments. Alongside traditional zooarchaeological assignments based on the visual inspection of morphological criteria, variations in collagen type I amino acid sequences can now be used to distinguish which animal a bone fragment belonged to. Using MALDI-ToF mass spectrometry, this method, known as Zooarchaeology by Mass Spectrometry (ZooMS), is now being applied regularly to archaeological faunal assemblages and, often at a large-scale, at Paleolithic sites. However, detailed explorations of how these ZooMS datasets can best be integrated with zooarchaeological and taphonomic data are only in their infancy.

To further advance this field, we hosted a workshop at the University of Kent in 2023, bringing together both zooarchaeologists and ZooMS specialists, to showcase and discuss various ways of integrating ZooMS and zooarchaeological data, especially within Paleolithic contexts. This special issue results from the papers presented at this workshop. In this introductory paper we reflect on the open discussion sessions that formed an essential part of the workshop. First, we discuss a series of methodological challenges; this includes the recording of zooarchaeology and taphonomy on morphologically unidentifiable bone fragments, ZooMS study design and sample

selection, pre-screening and sampling, pre-treatment and collagen extraction, and the acquisition, processing, and interpretation of MALDI data. Second, we delve deeper into the interpretive potential, and the wealth of future research directions, of a full contribution of ZooMS to a range of zooarchaeological research topics.

In concordance with the seven research papers in this issue, this introduction illustrates how a well-designed study, integrating zooarchaeological and taphonomic observations across both the morphological and ZooMS-identified fractions, cannot only increase the number of identifiable specimens at a site, but also provide novel insights into site formation histories, collection biases, carnivore behavior, environmental conditions, and past human subsistence, including site use, seasonality, carcass transport, prey preference, and butchery practices.

INTRODUCTION

Large quantities of bone fragments are regularly recovered from archaeological excavations and are ubiquitously present in many museum collections. Zooarchaeological studies of these bones, incorporating detailed observations of the processes that affected them after the animal's death (known as taphonomy), are key to fully reconstruct patterns of past human behavior. Central to this stands the taxonomic identification of these animal remains based on morphological criteria, which is usually achieved through in-depth comparisons with modern animal skeletons or other fossil examples. Linking these identified bones to past human behavior, including prey selection, carcass transport, and butchery practices, is not always straightforward, and is further hindered by large quantities of bone fragments that do not retain enough morphological characteristics to be assigned to a taxon. Recent advances in studies of ancient proteins have made it possible to taxonomically identify this non-diagnostic bone component through variations in their collagen, a method known as Zooarchaeology by Mass Spectrometry or ZooMS (Buckley et al. 2009). However, the methodological challenges and interpretive potential of integrating the morphological and ZooMS components of a faunal assemblage remain underexplored.

Recently, several papers have provided detailed overviews of the current contributions and limitations of the study of ancient proteins (paleoproteomics), including ZooMS (Richter et al. 2022; Warinner et al. 2022; Welker 2018). In this paper, we focus specifically on the challenges of integrating larger sets of ZooMS taxonomic identifications with existing zooarchaeological datasets and quantification indices. In light of this, we discuss various aspects of ZooMS analysis, from research design and sampling strategy, through to collagen extraction and spectral identifications. We follow this with a discussion on the wealth of interpretive potential and future research possibilities when fully integrating ZooMS with (zoo)archaeological data.

This paper builds on the fruitful discussion sessions held during a two-day workshop on integrating ZooMS and zooarchaeology organized by the authors in April 2023 at the University of Kent (UK). This workshop was attended by 27 researchers, including Master students, Ph.D. scholars, postdocs, and senior staff (Figure 1), who presented and discussed their on-going challenges with combining ZooMS and zooarchaeological data. The full program of the workshop and the abstracts of all talks can be accessed through ResearchGate.

ZOOARCHAEOLOGICAL FRAMEWORK

Obtaining insights into human behavior from archaeological bone assemblages, requires the detailed recording of a broad set of zooarchaeological, taphonomic, and metric observations. Many of these insights rest on our ability to provide secure taxonomic identifications for, often, large quantities of fragmentary bone. Traditionally, taxonomic and skeletal element identification is done through the use of reference collections of modern skeletal material, where available, or by using comparative osteological atlases (e.g., France 2009; Hillson 2016; Pales et al. 1971; Schmid 1972) or online archives of photos or 3D scans (archaeozoo.org, boneid.net, skull base, Max Planck 3D reference collection; Niven et al. 2009).

Assessing the quantity and size of the animal remains recovered at an archaeological site are key components to help understand the natural and behavioral mechanisms underlying their accumulation. Zooarchaeologists commonly use the number of identified specimens (NISP) per taxon to calculate a series of quantitative indices. This can include an estimate of how many skeletal elements (minimum number of elements [MNE]) and how many individual animals (minimum number of individuals [MNI]) are represented in the fragmented bone assemblage (Lyman 1994). Further quantification allows researchers to investigate whether variations in body part representation are related to the targeting of specific animal carcass resources by past human groups (e.g., minimum anatomical units [MAU], modified general utility index [MGUI], food utility index [FUI]: Grayson 1979; Jones and Metcalfe 1988; Lyman 1994, 2008; Metcalfe and Jones 1988).

Furthermore, secure species identifications allow us to investigate changes in overall species representation, richness, and homogeneity through various diversity indices (e.g., Shannon–Wiener and Simpson's: Faith and Lyman 2019; Grayson 1979; Lyman 2015; Reitz and Wing 1999). Such indices allow researchers to assess whether changes in taxonomic diversity are related to changes in site use (e.g., a carnivore den vs. human occupation). Further, they can be integrated with additional, more subtle, changes in faunal communities from additional biomolecular meth-

Figure 1. Group photo of the attendees at the first workshop on Integrating ZooMS and Zooarchaeology, held at the University of Kent (UK) in 2023.

ods, such as ancient sediment DNA (sedaDNA) (Smith et al. 2024; Zavala et al. 2021).

When taxonomic identification is uncertain or not possible, it is common to assign these fragments to relative categories based upon body size classes (based largely on live body weight: Brain 1981; Bunn 1986; Morin 2012). Bone fragments are assigned to these categories (e.g., small, medium, large) mainly through assessment of their overall size and the thickness of the cortical bone. In general, the portion of bone material that can be identified to species, or even to body size class, is often only a relatively small part of the overall bone assemblage. Especially in Paleolithic contexts, identification rates are often lower than 20%, meaning that for up to 80% of the bone fragments recovered we do not know which animal or human species they belonged to (e.g., Discamps et al. 2019; Gaudzinski-Windheuser et al. 2014; Niven et al. 2012; Pothier-Bouchard et al. 2020; 2024; Sinet-Mathiot et al. 2023; Smith et al. 2021; 2024). At these sites, the recovered bone fragments are often highly fragmented due to post-depositional, carnivore, and human processes. Especially at sites that are very rich in bone material, the smaller bone fraction (ca. less than 2cm, often recovered through screening) can remain almost completely unstudied from a zooarchaeological and taphonomic perspective (also see Raymond et al. 2024). Therefore, it remains to be explored how the identifiable and unidentifiable bone components relate, and if this partial bone identification has a direct influence on our interpretation of certain aspects of human behavior; or, in fact, whether some of the fragmentation patterns identified for different species at a site are the result of specific human and/or non-human behaviors.

COLLAGEN FINGERPRINTING OR ZOOMS

Collagen fingerprinting now permits researchers to extract taxonomic data from archaeological bone fragments that cannot be identified solely based on morphological criteria. Collagen type I is the most abundant protein in ancient skeletal tissues and can preserve for millions of years (e.g., Rybczynski et al. 2013). Collagen type I (COL1) is a quaternary structure composed of two COL1α1 chains and one COL1α2 chain in mammals, birds, reptiles, and amphibians. In fish, one of the COL1α1 chains is instead a COL1α3 chain, adding complexity to the analysis of fish COL1 (Richter et al. 2011). This COL1 triple helix is highly abundant, stable, and phylogenetically informative as, since it is a protein sequence, its amino acid sequence is encoded by DNA. Small differences occur in the sequence of nucleotides in the DNA of different taxa, resulting in slightly different amino acid sequences of collagen type I of different

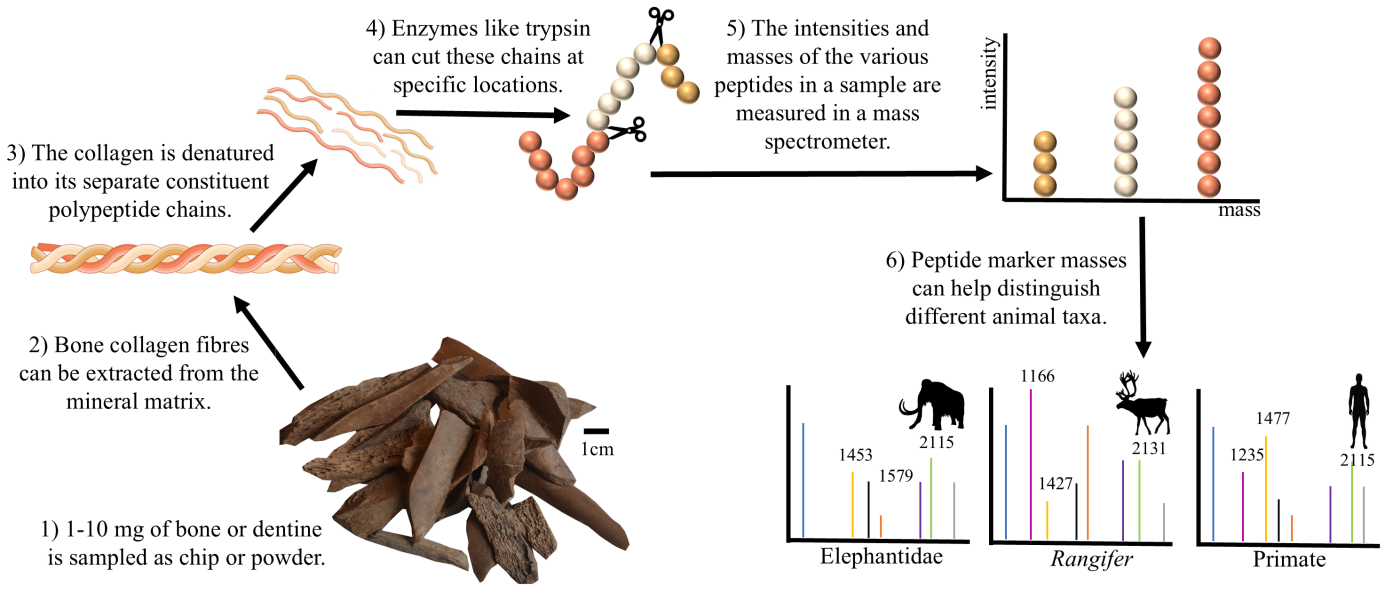

Figure 2. The main principles underlying the method of Zooarchaeology by Mass Spectrometry (ZooMS).

taxa. This is also reflected in variations in the amino acid sequences, and therefore mass, of the collagen peptides. As a result, taxa that are evolutionarily distinct have unique peptide mass fingerprints (PMF). ZooMS uses a set of distinct peptide markers, which occur at specific locations in these PMFs, to provide taxonomic identifications (Buckley et al. 2009).

Because of its relatively low cost, small sample requirements, and widely available equipment, ZooMS is relatively straightforward to implement (Figure 2). After taking a small sample of animal tissue (ca. 1–10mg), an acid or ammonium bicarbonate (AmBic) protocol can be applied and soluble collagen is extracted from its matrix through gelatinization (van Doorn et al. 2011). This collagen extract is then digested using an enzyme, usually trypsin, which cleaves the peptide bonds at specific locations. These peptides are then concentrated and desalted, and spotted on a steel target plate co-crystallized with a matrix, usually α-Cyano-4-hydroxycinnamic acid (CHCA). Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS) these peptides are ionized and propelled down a tube where their time-of-flight is measured. These measurements are then converted into a spectrum of mass-to-charge (m/z) ratios vs. intensity, which can be assessed for the presence of peptide marker peaks at specific mass locations (see Figure 2). For mammals, taxonomic identification is done through comparison with existing peptide marker reference libraries (Buckley et al. 2009; Welker et al. 2016). Due to the slow rate of collagen evolution, the taxonomic resolution of ZooMS is often restricted to the family (e.g., Elephantidae) or genus (e.g., *Rangifer*) level (see Figure 2).

Since its development in 2009, ZooMS has been applied to a wide array of archaeological bone remains to identify the type of animal (or human) they belonged to (Buckley et al. 2009; 2017; Welker et al. 2015; 2017). Besides targeted ZooMS studies to identify special objects (Bradfield et al. 2019; Dekker et al. 2021; Desmond et al. 2018; Evans et al. 2023; Hansen et al. 2024; Martisius et al. 2020; McGrath et al. 2019; Surovell et al. 2024) or find human remains (Brown et al. 2016; Devièse et al. 2017; Hublin et al. 2020; Mylopotamitaki et al. 2024; Welker et al. 2016), ZooMS is now also being applied untargeted, aimed at identifying large portions of the non-diagnostic fauna in a Paleolithic assemblage (Brown et al. 2021c; Holloran et al. 2024; Pothier-Bouchard et al. 2020; 2024; Raymond et al. 2024; Ruebens et al. 2022; 2023; 2024; Sinet-Mathiot et al. 2019; 2023; Xia et al. 2024). These large-scale proteomic analyses of morphologically unidentifiable bone remains are generating vast amounts of taxonomic and complementary data. While it is clear that these identifications can enhance our understanding of human subsistence practices at a site, its quantitative integration with zooarchaeological and taphonomic data remains underexplored, partly due to several unresolved methodological challenges (also see Wang et al. 2024 in this issue).

METHODOLOGICAL CHALLENGES

RECORDING ZOOARCHAEOLOGY AND TAPHONOMY

Depending on the site-specific characteristics of the faunal assemblage and the research questions at hand, anatomical and taphonomic attributes may or may not be recorded for both the morphologically identifiable and unidentifiable bone fragments. The relatively recent development of ZooMS has meant that many of the current ZooMS applications have been conducted *after* previous zooarchaeological sorting and analysis. ZooMS taxonomic data is then often compared solely with existing species data for the morphologically identifiable bones, and taphonomic data is often not recorded for the ZooMS-identified bones. While

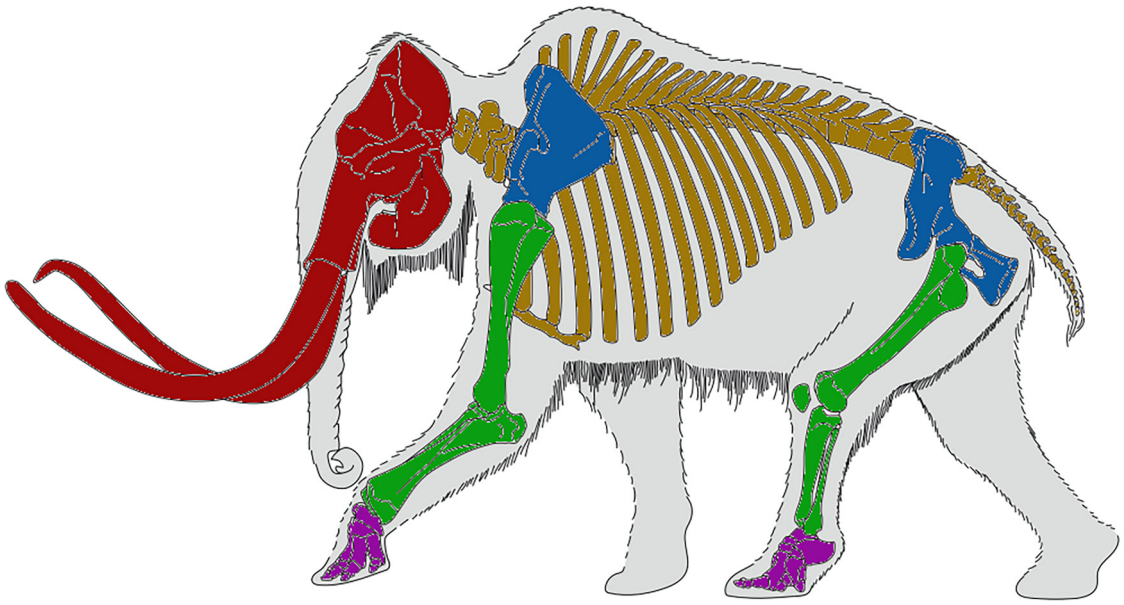

Figure 3. Body region categories to which ZooMS fragments can be assigned. 1) cranial (red), 2) long bone (green), 3) flat bone (scapula/pelvis, blue), 4) foot (carpal/tarsal, purple), 5) axial (vertebra and rib, brown); skeleton outline downloaded and modified from archaeozoo.org.

this does not diminish the value of these studies, it could, potentially, be more efficient and effective if this occurred *alongside* zooarchaeological analysis where possible, allowing for recording and sampling decisions to be taken in consultation with (zoo)archaeologists. Moreover, this allows for the standardized recording of zooarchaeological, metric, and taphonomic attributes across the entire faunal assemblage, facilitating in-depth comparisons between both identifiable and unidentifiable fractions.

In order to discuss issues of site formation, human behavior and site use it is important to record taphonomic attributes for all sampled ZooMS fragments. This will allow for comparisons between the morphological and ZooMS-identified fractions for qualitative measures of bone preservation (e.g., weathering, surface preservation, etc.), alongside more specific markers of biomolecular preservation (e.g., glutamine deamidation). The latter point is of particular interest to zooarchaeologists considering studies to date have shown limited correlation between observational preservation and glutamine deamidation (Brown et al. 2021b; Ruebens et al, 2023; 2024; Smith et al. 2024; but see also Xia et al. 2024).

Zooarchaeological Observations

Alongside taxonomic identification, zooarchaeologists record a range of information about the type of bone(s) present in their archaeological assemblages. Naturally, this is problematic for most ZooMS studies as these are focused on the, already, unidentifiable portion of the bone assemblages (Discamps et al. 2024; Morin et al. 2023). In order to maximize the potential of these fragmentary assemblages it is important to also record basic zooarchaeological information for all ZooMS fragments, including tissue type (e.g., cortical or trabecular), body region, bone element (where possible) and bone fusion (e.g., fused or unfused) (see Supplementary Information 1). This can help us reconstruct more fine-grained body part profiles and provide crucial insights into site formation history, differential preservation, human transport, and butchery practices.

Differentiating between various body portions (e.g., cranial vs. long bone) focuses on differences in bone fragment size and structure (e.g., bone cortical thickness, presence and proportion of spongy bone, shape) and diagnostic signatures, such as muscle attachments and nutrient foramen. ZooMS fragments, by their nature, contain limited, if any, characteristics to differentiate skeletal portions. Thus, a first step is to assign, where possible, fragments to more general body region categories, such as cranial, long bone, flat bone (e.g., scapula and pelvis), axial (e.g., vertebrae and ribs) and foot (including carpal and tarsal remains) (Figures 3 and 4). The inclusion or exclusion of dental remains should be considered on a site by site basis (for an example see Holloran et al. 2024). However, most often dental remains should be separated from other cranial fragments in subsequent analyses as they risk skewing body part profiles.

It is possible that zooarchaeologists are able to recognize and assign a bone fragment to a specific element (e.g., metatarsal, see Figure 4D) and this should be recorded to as much detail as possible, including information on bone portion (e.g., proximal epiphysis, mid-shaft, distal epiphysis, rib shaft, vertebral spine, etc.) and side (left/right) (see Supplementary Information 1). Fusion data should also be recorded, where possible, as this could provide further information on the presence of foetal, juvenile, and/or subadult individuals in the collection (see Supplementary

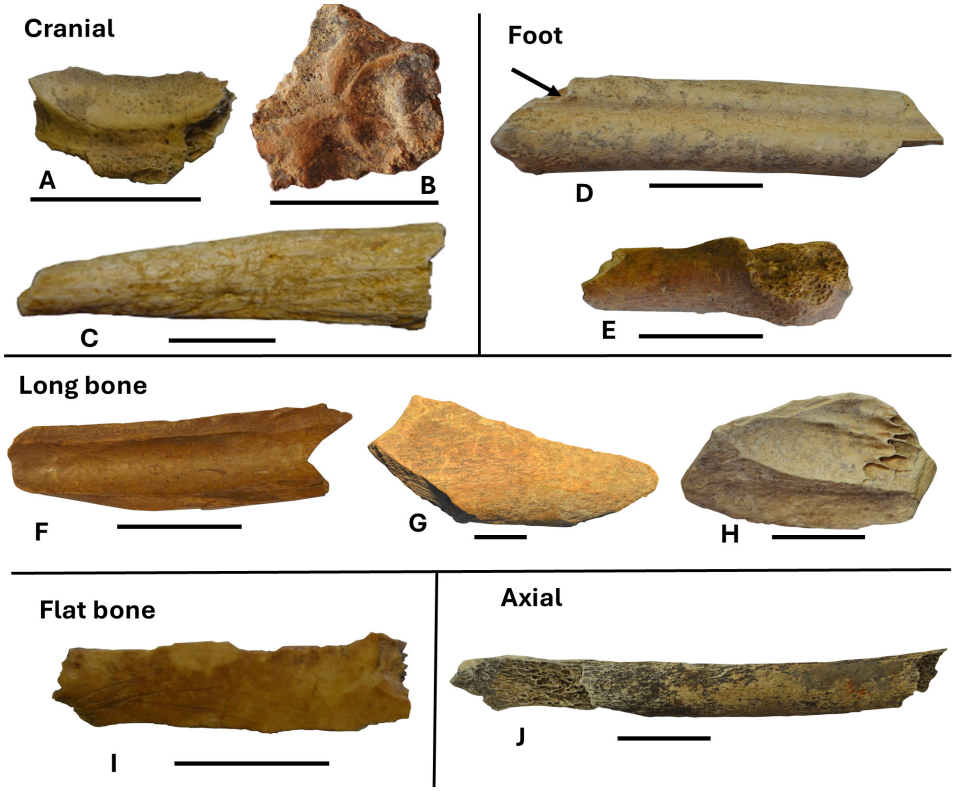

Figure 4. Examples of morphologically unidentifiable bone fragments selected for ZooMS and their identified body region. A) fragment of zygomatic bone (eye socket), B) specimen with suture consistent with cranial fragment, C) antler tine tip fragment, D) metatarsal fragment from ungulate (the groove running up the bone midline is a clear identifier), E) distal epiphysis from a phalanx (foot bone), F–H) non-diagnostic fragments of long bones, I) flat bone, J) rib fragment. For each bone fragment the scale bar equates to 2cm.

Information 1). Subsequently, these data can be used to investigate further questions on site occupation, seasonality, and hunting strategies (Arenas-Sorriqueta et al 2024; Torres-Iglesias et al. 2024).

Taphonomic Alterations

The physical appearance and preservation of archaeological bone can be affected by a broad range of processes, including environmental conditions (such as temperature, soil pH, and moisture), animal modifications (e.g., rodent gnawing and carnivore digestion), and human influences (including butchery marks and breakage). In general, in zooarchaeology the observation of these taphonomic processes are recorded through a large set of attributes including, for example: weathering (Figure 5), root-etching, water transport, bone surface modifications (rodent, carnivore, human, Figure 6), burning (see Figure 5), bone breakage (see Figure 5), and metrics (bone fragment length, width, thickness, weight) (see Supplementary Information 1 for descriptions and references for these various attributes). A recurring issue throughout zooarchaeology, and ZooMS studies, is to ensure consistency in the recording of these taphonomic attributes, which by their nature vary depending on experience, training and analyst (see, for example, Abe et al. 2002; Dominguez-Rodrigo et al. 2017; Fisher 1995). Although many faunal assemblages tend to be dominated by a small number of bone types, mainly long bone and rib shafts, the in-depth recording of zooarchaeological and taphonomic attributes can provide deeper insights into site formation and diagenesis alongside carnivore and human subsistence behavior (Brown et al. 2021c; Holloran et al. 2024; Ruebens et al. 2023; 2024; Wang et al. 2024).

Metric Measurements

It is standard practice throughout zooarchaeology to record a variety of metric measurements on each bone fragment to gain insights into breakage patterns. The maximum length and maximum width of the fragment can be measured using (digital) callipers (in mm), or fragments can be assigned to broader size categories (e.g., 1–2cm) using a template with concentric circles of known diameter. Additionally, depending on the research questions at hand, the thickness of the cortical portion of the bone can also be measured, to provide further insights into the physical characteristics of the bone. The weight of each fragment (before sampling) can be recorded using a pocket scale (in g). This requires only a minimal investment in additional recording time but has shown to be of great use when trying to quantitatively integrate both the morphologically identified and ZooMS identified assemblages (Discamps et al. 2024).

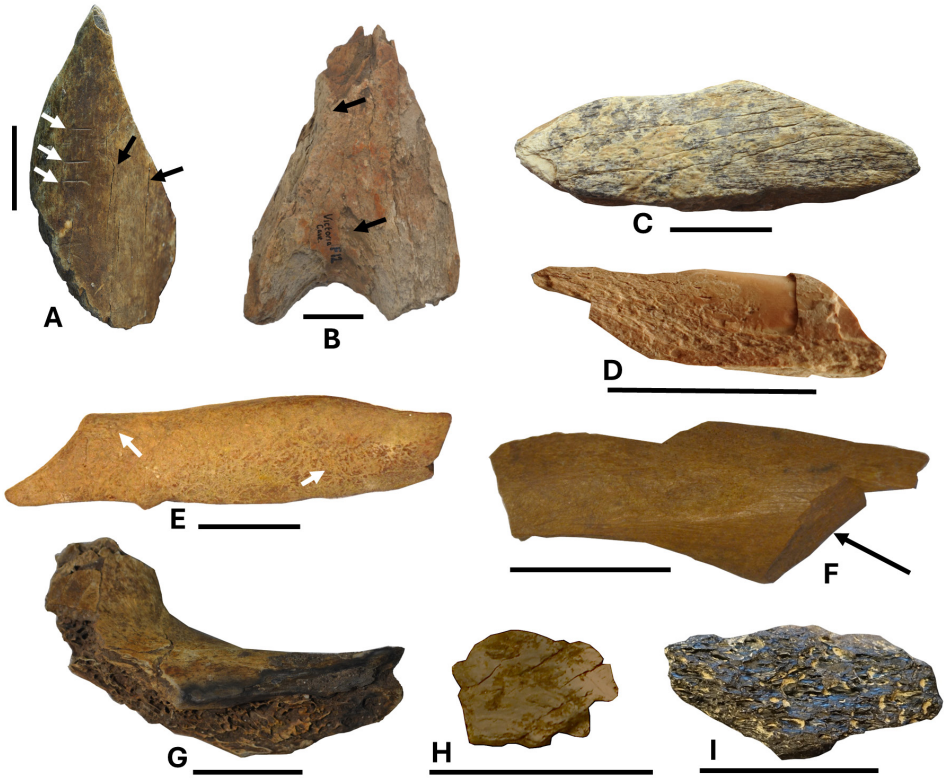

Figure 5. Examples of ZooMS fragments with different types of taphonomic alterations. A) low weathering with cracking on bone surface (Stage 2; black arrows) and note cut marks (white arrows), B) heavily weathered and exfoliated surface (Stage 4 or 5), C) long bone shaft fragment with some surface weathering illustrated by visible cracks (Stage 3), D) bone fragment with exfoliated surface (Stage 4 or 5), E) root etching across most of the bone surfaces, note the dendritic morphology indicative of root action on surface (white arrows), F) fresh bone fracture (black arrow), note the spiral and smooth fracture surface, G) bone charred (black) at its edge, H) calcined bone as indicated by the grey and white discoloration, I) burnt bone fragment. For each bone fragment the scale bar equates to 2cm.

STUDY DESIGN AND SAMPLE SELECTION

The development of ZooMS has helped to overcome certain methodological limits brought by taphonomic processes impacting a death assemblage over time, including human activity and bone fragmentation. However, the selection of the material for analysis, and particularly the criteria defining this selection, play an important role in how the results can be interpreted.

Research Design: Targeted vs. Untargeted Approaches

The selection and number of unidentifiable bone fragments sampled is often dependent on the research question, the nature of the archaeological site, and the resources available. Targeted ZooMS studies focus on identifying a specific set of samples. This includes special objects made out of bone, ivory, or antler (Dekker et al. 2021; Desmond et al. 2018; Martisius et al. 2020; Tomasso et al. 2018), closely related species that are difficult to identify morphologically (Buckley et al. 2011; Evans et al. 2016; Jeanjean et al. 2023) or verifications of specific morphological identifications (Morin et al. 2023). Conversely, untargeted studies use ZooMS as a tool to identify large quantities of bone fragments, with limited prior selection (Brown et al. 2021c; Holloran et al. 2024; Pothier-Bouchard et al. 2020; 2024; Raymond et al. 2024; Ruebens et al. 2022; 2023; 2024; Sinet-Mathiot et al. 2019; 2023).

In general, researchers must weigh the trade-off between sampling (almost) everything (e.g., Brown et al. 2021; Ruebens et al. 2023; 2024; Smith et al. 2024) or selecting a representative sample (e.g., Arenas-Sorriqueta et al. 2024; Holloran et al. 2024; Pothier-Bouchard et al. 2024; Raymond et al. 2024; Sinet-Mathiot et al. 2019; 2023, Wang et al. 2024; Welker et al. 2015). Opting to sample everything may provide a comprehensive dataset, but could be resource-intensive and time-consuming, especially in large or diverse assemblages. Conversely, selecting a representative sample dataset that best captures the overall diversity of the assemblage (e.g., all material from a speficic excavation square) could also already permit the correlation with the faunal component identified through morphology (Wang et al. 2024). Other selection criteria can target informative characteristics, such as anthropogenic traces and/or anatomical identification (e.g., skeletal element or fusion, Pothier-Bouchard et al. 2024). For example, the targeted sampling of morphologically unidentifiable foetal and newborn remains (Pothier-Bouchard et al. 2024; Torres-Iglesias et al. 2024) or axial elements (Arenas-Sorriqueta et al. 2024) can help to refine seasonality data and carcass transport dy-

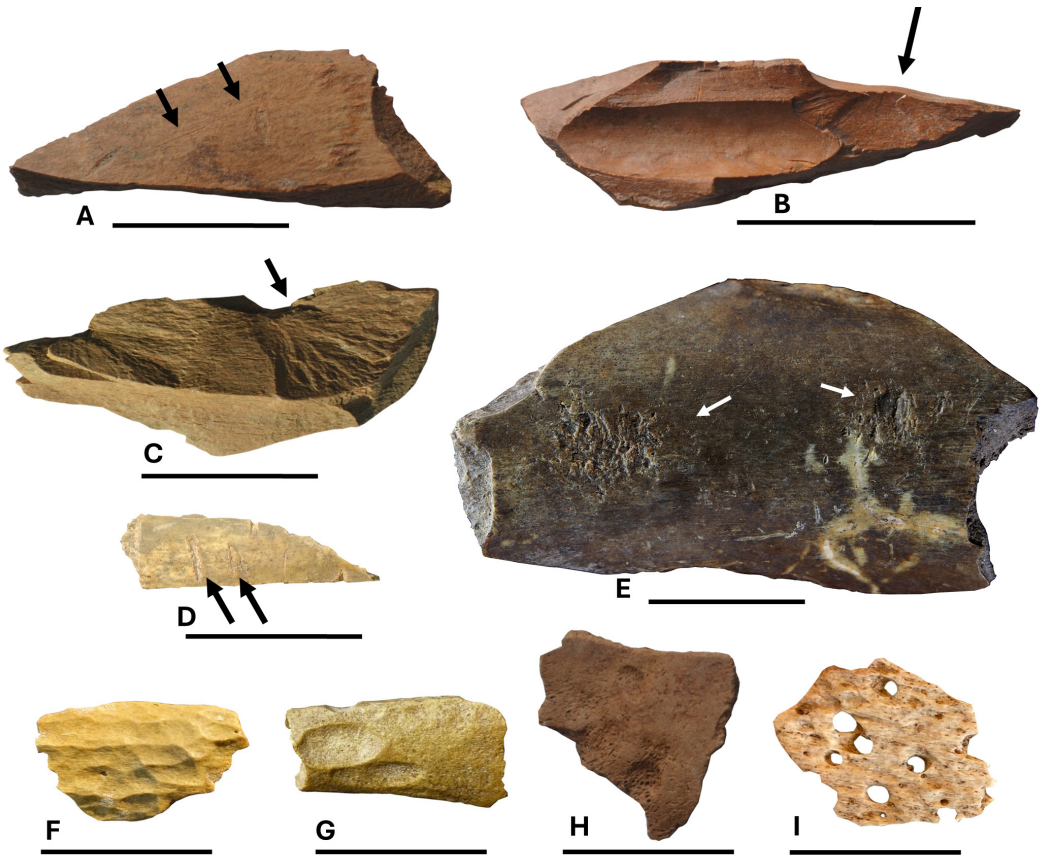

Figure 6. Examples of ZooMS fragments with different types of carnivore and human bone surface modifications. A) scrape marks, B–C) marrow fractures with impact point with associated negative in the internal cavity, D) cut marks, E) long bone fragment used as a retoucher, F–G) carnivore scalloped, H) carnivore tooth pits on bone surface, I) digested bone fragment. For each bone fragment the scale bar equates to 2cm.

namics. These targeted approaches balance efficiency with the need for robust and meaningful results, while being directly correlated with a specific research question.

Sample Selection Criteria

During the archaeological excavation of a Pleistocene site, the bone material is often categorized in various ways to facilitate finds processing, most often based on the size of the specimen. A size threshold of 20–25mm is frequently applied during excavation, although the definition of this criterion can be variable depending on the excavator and age of the site (McPherron 2005). In the context of Paleolithic archaeology, specimens equal to, or larger than, 20–25mm are often assigned individual specimen identification numbers linked to 3D coordinates; these are known as piece-plotted specimens (Dibble and McPherron 1988). Conversely, smaller specimens are recovered through sediment sieving through different meshes and, along with other smaller specimens from the same area, are associated with the coordinates of the sediment bucket or spit, and are known as screened material. The smaller fragments, often limited in their potential to retain informative taphonomic traces due to the preserved surface size, can pose challenges in addressing the taphonomic history behind their retrieval, thereby restricting interpretative possibilities (Raymond et al. 2024). On the other hand, the use of a strict 20mm fragment size cut-off has the potential to, inadvertently, exclude the identification of smaller animals and introduce another layer of taphonomic and taxonomic bias to our analysis. However, this threshold is generally used to ensure enough bone material remains available for further proteomic, isotope, ^{14}C, and/or aDNA analyses. More recently, it has been suggested that a weight rather than maximal dimension cut-off may be more applicable (e.g., >70 milligrams (mg) in Wang et al. 2024).

In the selection process, certain specimens, particularly burnt bones, are routinely excluded to optimize collagen retrieval and taxonomic identification. The exclusion of burnt fragments is based on the recognized impact of heat on collagen preservation (Collins et al. 2002; Faillace et al. 2020; Yates 2013). However, there are now several studies that have been successful in retrieving collagen peptides marker masses from bone specimens exposed to mild temperatures, as evidenced either visually or through FTIR (Hansen et al. 2024; Raymond et al. 2024; Wang et al. 2023). Nonetheless, the influence of heat on specimens subjected to higher stages of burning remains an area that requires further exploration.

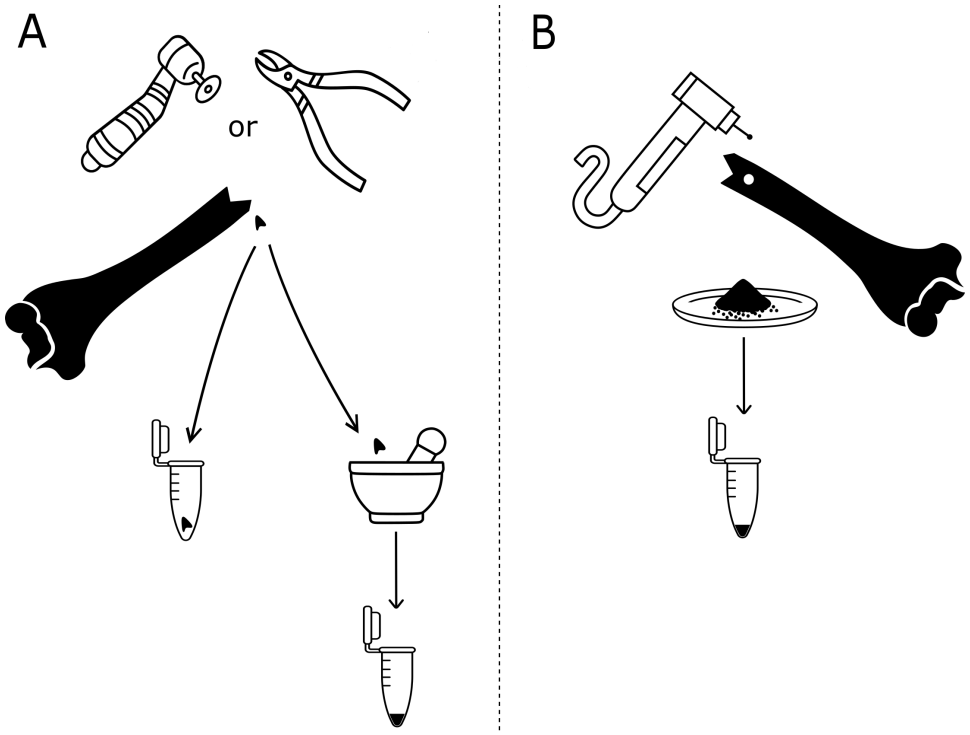

Figure 7. ZooMS sampling procedures. A) fragment sampling using cutting wheels or pliers, B) powder sampling using mortar and pestle or drilling.

PRE-SCREENING AND SAMPLING

Screening Collagen Preservation

Diagenetic processes can lead to significant degradation of collagen over time, including through hydrolysis, cross-linking, and microbial activity. The mechanisms underlying bone diagenesis are complex, not yet fully understood, and are largely affected by the depositional environment (Collins et al. 1995; 2002). Therefore, there can be large variations in collagen preservation between, and within, Paleolithic faunal assemblages, including among stratigraphic layers and across different areas within a site (e.g., in front of, or within, a cave).

Before undertaking large-scale ZooMS analysis, a small-scale pilot study can help determine the degree of collagen preservation in case this is relatively unknown for the material at hand (e.g., when there is no prior ^{14}C or stable isotope results). Furthermore, recent years have seen the development and application of several spectroscopy and tomography (Tripp et al. 2018) methods that can provide a more detailed assessment of biomolecular bone preservation and collagen content. Fourier Transform Infrared Spectrometry (FTIR) requires a small powder sample (ca. 1–5mg) and calculates the Amide I to phosphate ratio (CO/P) as an indicator of relative collagen abundance (Chowdhury et al. 2021; Pothier-Bouchard et al. 2019; 2024; Presslee et al. 2020). Near-Infrared Spectroscopy (NIR) is entirely non-destructive, as it uses light to penetrate the bone surface. The acquired near-infrared spectra are then analyzed using a calibration model to calculate a predicted collagen percentage (Fewlass et al. 2019; Iliopoulos and Stathopoulou 2023; Lugli et al. 2021; Malegori et al. 2023; Ruebens et al. 2023; Sponheimer et al. 2019; Vincke et al. 2014). Both of these techniques can be done with portable instruments, meaning that screening can be done during excavations or in museums. This pre-screening for collagen content is especially of use for heavily degraded Paleolithic faunal material to avoid sampling large numbers of fragments with not enough collagen for a ZooMS identification.

Sampling Procedure

The choice between fragment and powder sampling strategies in ZooMS analysis (Figure 7) involves careful consideration of several factors. Fragment sampling, often achieved with tools like pliers or cutting wheels, results in obtaining a small piece of material for analysis. This method is advantageous for preserving morphological integrity, allowing for potential future morphological examinations. However, it may be less suitable for highly fragmented or delicate specimens. Conversely, powder sampling, achieved through methods like drilling or using a mortar and pestle, involves the extraction of collagen from powdered material. This strategy is particularly useful for poorly preserved or fragmentary specimens, as it maximizes the surface area for collagen extraction. Powdered samples are also advantageous for their homogeneity, aiding in consistent results across analyses. However, studies comparing collagen extraction protocols for stable isotope and ^{14}C analyses indicate the potential for higher collagen yields from whole bone fragments compared to powder (Talamo et al. 2021), but this has not yet been tested within a proteomic context. Overall, the choice between these strategies would there-

fore often depend on the preservation state of the specimens and the specific aims of the study.

In ZooMS analyses, the question of sample weight can also be a critical consideration for obtaining reliable results. The literature usually mentions the use of 10mg to 30mg as starting material, but the optimal amount of material to be sampled typically falls within the range of 2mg to 5mg (van Doorn et al. 2011; Wang et al. 2021), depending on the archaeological context and biomolecular preservation of the specimen. This range is usually identified as sufficient for successful collagen extraction and subsequent mass spectrometry analysis. However, establishing a standardized weight for ZooMS samples would facilitate comparisons between different specimens or datasets and therefore promote reproducibility and robustness in the interpretation of ZooMS data.

SAMPLE PRE-TREATMENT AND COLLAGEN EXTRACTION

Reducing Contaminants and Interferences

Extraction protocols typically contain an incubation step prior to demineralization or denaturation, for example, in water or AmBic at room temperature overnight, in an effort to remove some of the soluble contaminants (van Doorn et al. 2011). However, the efficiency of these decontamination processes on the resulting PMFs has not been assessed so far, though shotgun proteomic research suggests that the impact of such incubation steps are likely to be minimal (Fagernäs et al. 2024). Furthermore, extraction batches should, wherever possible, contain extraction blanks processed identically alongside archaeological material, to verify that no COL1 contamination is likely to have occurred in the laboratory or mass spectrometry environments.

In archaeological contexts, it needs to be considered that the humic acids present in a sample can potentially interfere with mass spectrometry measurements. Humic substances are usually brown in color, are naturally formed during the decomposition of organic materials, and are difficult to separate from proteins in solution. Several approaches have been tested to remove humic substances from bone (Cleland 2018; Schroeter et al. 2019). For example, sodium hydroxide (NaOH) is often used to remove humics before ^{14}C dating or isotopic analyses (Szpak et al. 2017) and can also be incorporated into the ZooMS protocol, if needed (Brown et al. 2020; Ebsen et al. 2019). However, caution is needed when applying NaOH to poorly preserved samples as they could damage the remaining collagen (Szpak et al. 2017; van der Haas et al. 2018).

Collagen Extraction

Different extraction techniques can be used for ZooMS, individually or combined, depending on the molecular preservation, to obtain suitable peptide mass fingerprints. Most commonly, collagen molecules are extracted from the mineralized tissues using hydrochloric acid (HCl, Buckley et al. 2009; Welker et al. 2015). This approach, called acid demineralization, is usually performed on poorly preserved samples. In this cold acid protocol, the specimen is pretreated with HCl acid (ca. 18 hours or several days depending on sample type), which is then removed, and the collagen is extracted from the demineralized fragment. This protocol can be used in conjunction with the acid soluble protocol which extracts soluble collagen from the acid solution used for demineralization (Buckley et al. 2009; van der Sluis et al. 2014).

An alternative and less destructive method consists of extracting the soluble collagen from the surface of the osseous fragments by unfolding the molecule in an ammonium bicarbonate (AmBiC) buffer using heat, typically at 65°C for one hour (Buckley et al. 2009; van Doorn et al. 2011). The advantage of this semi-destructive AmBic buffer extraction is that it only causes minimal damage to the bone sample, allowing for subsequent analysis or duplication of the extraction (Brown et al. 2021c; Sinet-Mathiot et al. 2023).

Subsequently, digestion with trypsin, operated at 37°C for optimal activity, is used to cleave the sequence into peptides of different length and mass depending on the taxa. Although there is no complete consensus across the field, many paleoproteomic protocols include an overnight trypsin digestion of approximately 18 hours. However, the reduction of digestion duration from 18 hours to 3 hours has been shown to have no effect on the success rate for taxonomic identification obtained through ZooMS and SPIN (Le Meillour et al. 2024). In a final step, peptides are acidified to neutralize the enzyme and purified through solid-phase extraction (SPE) techniques allowing for the removal of salts, impurities, and contaminants, and to concentrate them.

This collagen extraction and purification protocol can be applied to samples in individual tubes or in 96-well plates. Individual processing can be beneficial for the analysis of limited or precious samples, whereas plate processing is best for the analysis of large quantities of samples. This only requires limited specialist instrumentation (e.g., HyperSep™ Universal Vacuum Manifold) to facilitate a faster, semi-automatic, processing of the samples.

ZooMS protocols published by Brown et al. (2020) on platforms like protocol.io offer accessible and standardized approaches, contributing to the growing repository of best practices. In addition, comparative studies of protocols, such as those by Wang et al. (2021) and Mylopotamitaki et al. (2023), help to refine and standardize methodologies, thereby ensuring more reliable and consistent results. Recent work by Jensen et al. (2023) addresses the challenges of sample preservation in harsh environments by developing a protocol designed for analyzing poorly preserved collagen samples, thus permitting the analysis of degraded specimens. Overall, all steps of the ZooMS protocol should be tailored specifically towards the sample material that needs to be studied.

DATA ACQUISITION AND ANALYSIS

MALDI Data Acquisition and Export

After SPE concentration and elution of the resulting peptide

mixture, 0.5–1µl of the peptide extracts are spotted onto a specialized MALDI target plate, normally in triplicate, with 0.5–1µl of matrix, commonly α-Cyano-4-hydroxycinnamic acid (CHCA). The matrix can be added before the peptide mixture, after the peptide mixture, or the two can be mixed together prior to spotting onto the MALDI target plate. Subsequently, the matrix will co-crystallize with the peptide mixture and allow for ionization during mass spectrometry measurements. The crystallization typically happens at room temperature, and should result in an even distribution of similarly-sized crystals. In addition, the MALDI target plate will contain a number of predetermined calibration spots, where a mixture of the matrix and reference peptides with known masses is placed.

A range of MALDI-ToF MS instruments has been utilized by the ZooMS community, including Bruker Ultraflex, Autoflex, and RapifleX instruments, a Bruker MALDI timsTOF fleX instrument (Ásmundsdóttir et al. 2024), a Shimadzu MALDI-8020 mass spectrometer (Holloran et al. 2024), an AB Sciex MALDI-ToF 5800 instrument (Raymond et al. 2024; Ruebens et al. 2024), as well as MALDI-FT ICR MS instrumentation (Bruker Tesla SolariX XR: Bray et al. 2023; Raymond et al. 2024). Based on mass resolution, both MALDI-FT ICR MS and the timsTOF instrumentation would provide superior resolution. However, it is currently unclear whether the spectra produced by these various mass spectrometry instruments has a significant impact on the acquired taxonomic identifications.

Normally, and regardless of the MALDI MS instrument used, one has to decide on a number of MS instrument parameters, including the number of shots, laser intensity, and laser movement pattern. These settings have a significant impact on the total ion count and mass resolution, and therefore on the ultimate quality of the acquired MALDI spectrum. Likewise, one commonly has the option to perform several kinds of internal calibration during data acquisition, based on the known mass of the peptides located on the calibration spots. After raw data acquisition, spectral information, containing continuous mass information without deisotoping procedures applied, is typically exported to a more readable data format (such as .txt, .msd, .mzxml, or .mzml), and during this transformation most vendor software will allow one to perform additional calibration, spectral alignment, and spectral correction. It should therefore be realized that the spectral data utilized for subsequent taxonomic identification is, normally, neither raw data, nor uncalibrated, also not at the level of the individual replicate spectra. Significantly, many of the instrumentation parameters remain unreported in the ZooMS literature.

Data Processing

After spectral data acquisition and export, an attempt is commonly made to assign a taxonomic identity directly to these spectra individually, or based on a merged version of three independent replicates. Several approaches for the processing and merging of spectral replicates have been published, normally based on the MALDIquant R package (Gibb and Strimmer 2012), including subsequent steps for automated taxonomic identification (Hickinbotham et al. 2020; Végh and Douka 2024), or restricted to the merging of the replicates (Le Meillour et al. 2024).

Advanced processing of the individual replicates prior to and after merging is advisable, since this allows one to remove as much as possible of the baseline, reduce noise, and therefore increase the signal-to-noise ratio of the monoisotopic peaks. In addition, such processing minimizes the mass error associated with peptide peaks confidently observed (Figure 8). In terms of the signal-to-noise-ratio (SNR), several studies report values of around 3 to 10 as useful cut-offs. However, resulting thresholds of minimum SNR and minimum absolute ion intensity are instrument-specific and possibly run-specific. Therefore, care must be taken to optimize these parameters on a run-by-run basis.

Spectral Quality and Taxonomic Identification

Subsequently, the resulting spectrum/spectra are inquired manually or computationally for the presence of monoisotopic peaks that correspond to the peptide masses of a number of peptide markers for a range of animal taxa (Figure 9, Table 1). Since identifications are based on the correct observation of the monoisotopic peaks, it should be remembered that these monoisotopic peaks may not be the most intense peaks of a peptide's isotopic distribution, and that the relative intensity will shift across the isotopic distribution (to +0.984 Da) in relation to deamidation (Wilson et al. 2012). Manual analysis is often performed in the free, open-source software mMass (see GitHub; Brown 2021; Strohalm et al. 2010). Correspondence between the observed peptide marker series of a MALDI spectrum and one or several species in the reference list (Buckley et al. 2009; Végh and Douka 2024; Welker et al. 2016) then allows one to assign a taxonomic identity, even when one or several peptide markers are not observed experimentally (Table 2).

Some studies have noticed that in worse preservation contexts, or associated with certain sampling methods, the masses in the higher m/z range tend to be absent (m/z over 2,500), while peptide masses in the lower mass range (m/z up to 1,500) tend to remain present (Martisius et al. 2020; McGrath et al. 2019; Sinet-Mathiot et al. 2021). The absence of peptide masses in the higher range of the ZooMS mass range typically observed might therefore not prevent a taxonomic identification, but can result in a more general, and therefore less informative, one (e.g., Caprinae rather than *Capra* sp.).

Although the MALDI-ToF MS spectra are normally dominated by collagen type I peptides, peptide masses are regularly present deriving from trypsin autolysis products (e.g., at 2,211 Da), as well as bovine albumin, (human) hair, and skin keratins (e.g., at 1,208 Da; Keller et al. 2008). Masses of such peptides might overlap, or be located close to, the mass of relevant peptide marker masses. For example, a keratin-derived peptide frequently results in the presence of a monoisotopic mass of 3,017 Da. Since this corresponds to mass values of peptide marker α2 757, the presence of 3,017 should only be used for taxonomic identifications if

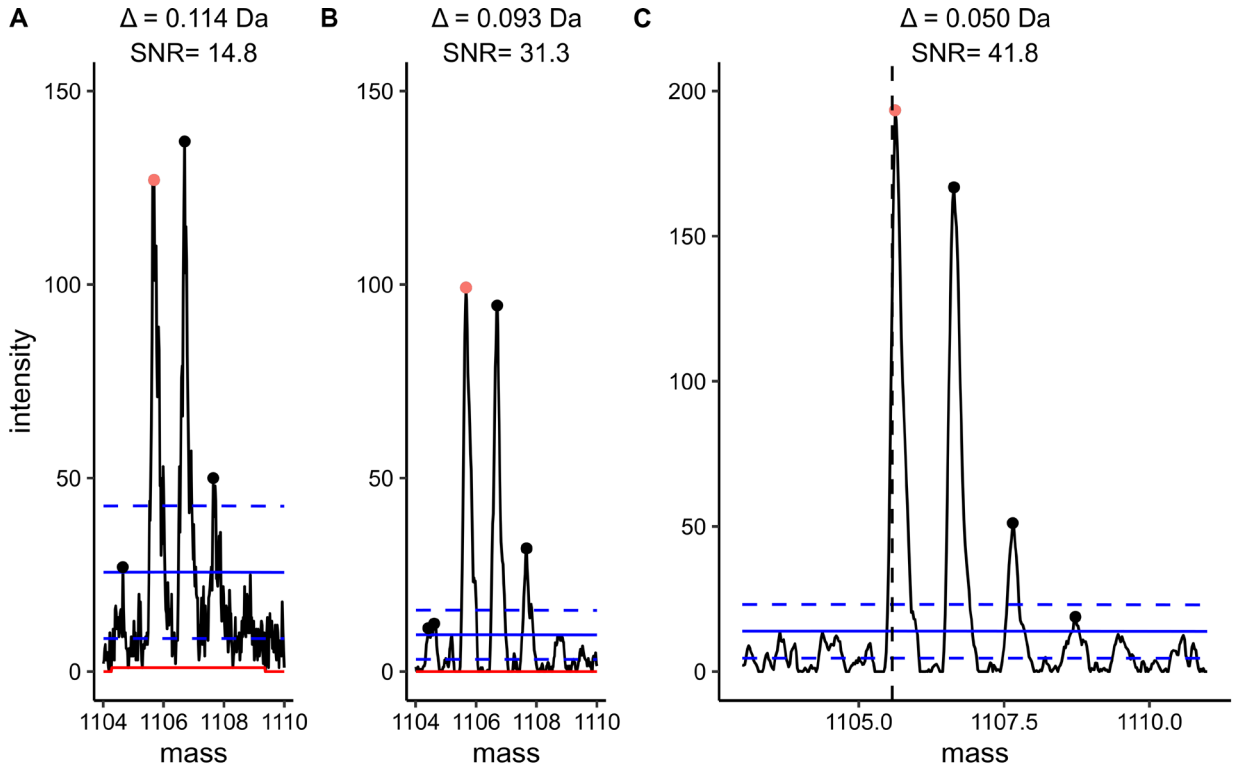

Figure 8. A) Close-up of a MALDI-ToF MS spectrum centred around COL1a1 508–519 after exporting from a Bruker AutoFlex instrument. B) The same spectrum after spectral smoothing (method="MovingAverage," halfWindowSize=2), baseline removal (method="TopHat," halfWindowSize=14), and spectral alignment between the three replicates (halfWindowSize=7, peaksMethod="SuperSmoother", SNR=3). C) The same spectral dataset after merging of the three independent replicates (method="sum"), followed by another round of baseline removal. SNR= signal-to-noise ratio. Δ = mass deviation in Da between the experimental monoisotopic peak and the theoretical mass of COL1a1 508–519. Peaks with a SNR of 3 or above are indicated by a black dot, while monoisotopic peaks are indicated with a red dot. The red line indicates the local baseline, the dashed blue lines a SNR of 1 and 5, respectively, and the solid blue line indicates a SNR of 3. The vertical dashed line in C indicates the theoretical mass of COL1a1 508–519 (m/z=1105.5748 Da). Note differences in y-axis.

the accompanying ion at 3,033 Da is also observed (α2 757 (+16); Brown 2021). Furthermore, peaks might be present deriving from glues, including protein-based ones (van der Sluis et al. 2023). Some of these manifest as a repeating series of peaks at regular intervals, for example 44 Da apart for polyethylene glycol (PEG), and 62 Da apart for polyvinyl chloride (PVC) (Keller et al. 2008). In addition to including extraction blanks, researchers can therefore also cross-check their lists of observed monoisotopic peaks with the known masses of a range of common contaminating proteins and/or interfering compounds (Martisius et al. 2020).

Assessing Biomolecular Preservation

MALDI-ToF MS spectral data have been used to access information about collagen preservation in several ways. Firstly, one can compare the number of ZooMS peptide markers observed. A larger number of observed peptide markers would then be interpreted as indicating better preservation (Hansen et al. 2024). The same could be done for the total number of monoisotopic peaks confidently identified (Le Meillour et al. 2024; Wang et a. 2021). Supporting quantitative data in this context would relate to the SNR of the observed peptides—they are expected to be higher for better-preserved COL1 peptides.

Secondly, the ZooMS field has attempted to quantify the extent of glutamine deamidation of a number of COL1 peptides in several ways (Chowdhury et al. 2019; Nair et al. 2023; Wilson et al. 2012). The resulting information on how often it is possible to calculate this value has been taken to indicate general COL1 preservation as well (Ruebens et al. 2023). The deamidation values themselves similarly indicate the extent to which glutamine has been modified into glutamic acid. In general, deamidation is expected to be more advanced in older archaeological assemblages, and/or assemblages that have accumulated higher thermal ages (Smith et al. 2003). Some experimental datasets indicate that this is indeed the case within single archaeological sites, although chronological resolution is, as expected, generally low (Bray et al. 2023; Brown et al. 2021b; Silvestrini et al. 2022; Welker et al. 2017; Xia et al. 2024). Moreover, care should be taken to assess that extraction methods have not (negatively) influenced the deamidation rates themselves (Procopio and Buckley 2017).

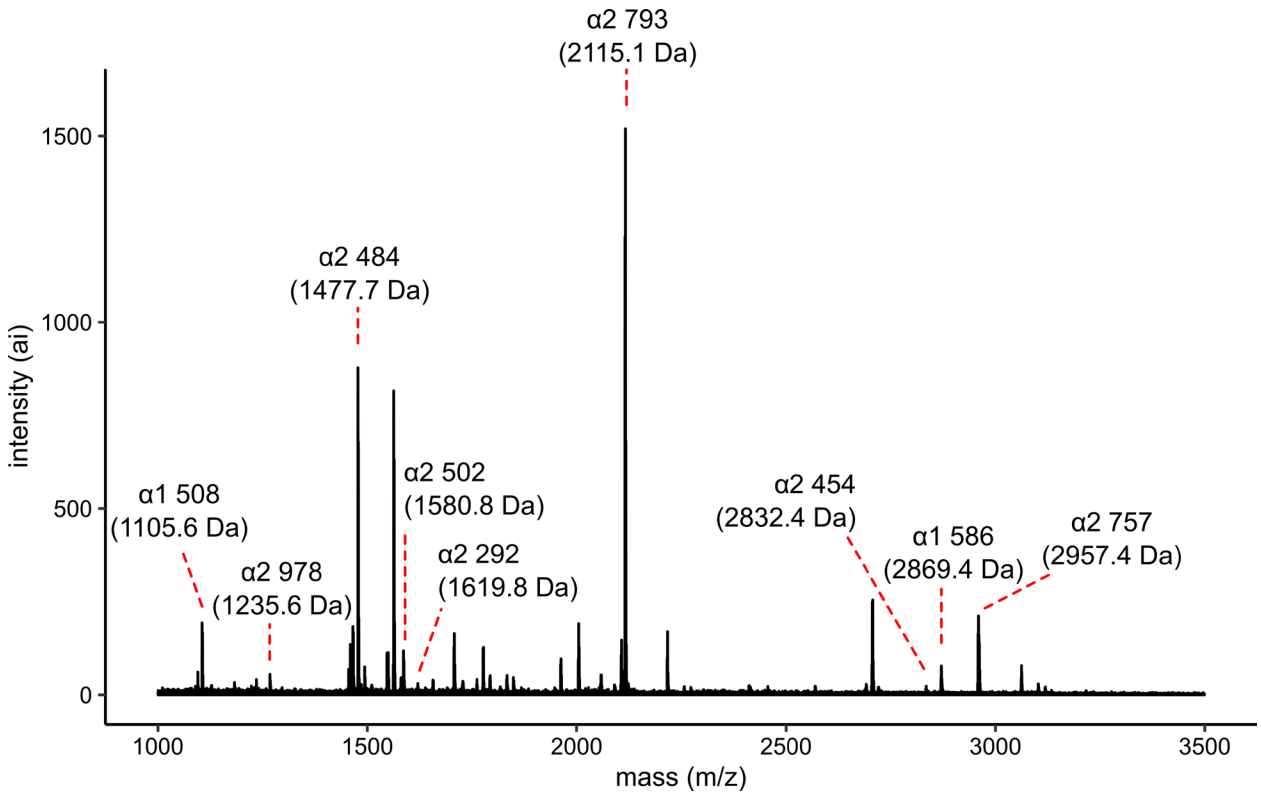

Figure 9. A complete MALDI-ToF MS spectrum in the mass range of 1,000-3,500m/z, after replicate processing and spectral merging. The nine "common" peptide markers used for taxonomic identification are annotated. Based on the observed mass values of these nine peptide markers, the spectrum would be identified as representing a hominin (Lanigan et al. 2020).

Data Standardization and Availability

Recent guidelines are available to label the peptide markers used for ZooMS taxonomic identification in a uniform way (Brown et al. 2021a), helping to standardize the ever-growing reference libraries. This nomenclature marks the position of the peptide marker in the collagen type I sequence and lists its first and last amino acid, counted from the start of the helical region (e.g., COL1α1 508–519, shortened as α1 508; Brown et al. 2021a). Further, when reporting ZooMS identifications, taxonomic writing rules should be applied. Family names are capitalized (e.g., Cervidae). Genus and species names are always italicized, with the genus capitalized and the species lower case (e.g., *Rangifer tarandus*). When the species cannot be specified the abbreviation "sp." is used, which in itself is not capitalized (e.g., *Aves* sp.).

In general, ZooMS focuses on nine main peptide markers to identify samples to taxa (see Table 1; Buckley et al. 2009). However, depending on the preservation state of the

TABLE 1. PEPTIDE MARKER SERIES FOR SOME OF THE MAIN PREY SPECIES AT EUROPEAN PALEOLITHIC SITES.*

	α1 508	α2 978 (+16)		α2 484	α2 502	α2 292	α2 793	α2 454	α1 586 (+16)		α2 757 (+16)	
Bos/Bison	1105.6	1192.7	1208.7	1427.7	1580.8	1648.8	2131.1	2792.3	2853.4	2869.4	3017.5	3033.5
Capra sp.	1105.6	1180.6	1196.6	1427.7	1580.8	1648.8	2131.1	2792.3	2883.4	2899.4	3077.5	3093.5
Cervid/Saiga	1105.6	1180.6	1196.6	1427.7	1550.8	1648.8	2131.1	2792.3	2883.4	2899.4	3017.5	3033.5
Elephantidae	1105.6	x	x	1453.7	1579.8	x	2115.1	2808.3	2853.4	2869.4	2999.5	3015.5
Equidae	1105.6	1182.6	1198.6	1427.7	1550.8	1649.8	2145.1	2820.4	2883.4	2899.4	2983.5	2999.5
Rangifer	1105.6	1150.6	1166.6	1427.7	1580.8	1648.8	2131.1	2792.3	2883.4	2899.4	3077.5	3093.5
Rhinocerotidae	1105.6	1182.6	1198.6	1453.7	1550.8	1623.8	2145.1	x	2869.4	2885.4	2983.5	2999.5

*An X in reference tables like this indicates that, for the relevant species, the mass of a given peptide marker is unknown. Some peptide markers have variable hydroxylation status, indicated by "+16," implying that for the relevant species one or both of the possible mass values can occur in a MALDI-ToF MS spectrum.

TABLE 2. EXAMPLES OF TAXONOMIC LABELS FOR SPECIFIC GROUPS OF PEPTIDE MARKERS.

ZooMS identification	α1 508	α2 978 (+16)	α2 484	α2 502	α2 292	α2 793	α2 454	α1 586 (+16)	α2 757 (+16)
Empty	x	x	x	x	x	x	x	x	x
Indeterminate	1105	x	1427	x	x	x	x	x	x
Bovidae/Cervidae	1105	x	1427	x	1648	x	x	x	x
Bovidae/Cervidae/Equidae	1105	x	1427	1550	x	x	x	x	x
Cervid/*Saiga*/*Capreolus*	1105	1180+1196	1427	1550	1648	2131	2792	2883+2899	x
Cervid/*Saiga*	1105	1180+1196	1427	1550	1648	2131	2792	2883+2899	3033
Carnivora	1105	x	1453	1566	x	x	x	x	x
Hyaenidae/*Panthera*	1105	1207	1453	1566	x	2147	x	2853+2869	2999
Bovidae/*Rangifer*	1105	x	1427	1580	1648	x	x	x	x
Caprinae/*Rangifer*	1105	x	1427	1580	1648	x	x	2883	x
Bos/*Bison*/*Ovibos*	1105	1208	1427	1580	1648	x	x	x	3017
Caprinae	1105	1180+1196	1427	1580	1648	2131	2792	2883+2899	x
Caprinae (not *Capra* sp.)	1105	1180+1196	1427	1580	1648	2131	2792	2883+2899	3033

samples, not all markers are always present. When none of the relevant peptide markers can be identified, a spectrum can be labelled as empty. When only one or two of the markers are clear, it can be assigned as indeterminate. Once three of the markers are present, a more precise taxonomic identification can generally be assigned (see Table 2).

When identifying spectra, it is important to keep in mind that not all species are present in the reference library, and a more cautious identification might be advisable, for example, at the family level (e.g., Suidae instead of *Sus*). Often, it is possible to narrow down the potential taxa by assessing which species are present among the contemporaneous morphologically identifiable fraction, both at a site and regional level. A broadly applied example of context-based inference relates to the Cervidae family. ZooMS can currently not distinguish between certain Bovid and Cervid taxa that were present in Paleolithic Europe (see Supplementary Information 2). This includes saiga antelope (*Saiga tatarica*), fallow deer (*Dama dama*), red deer (*Cervus elaphus*), giant deer or Irish elk (*Megaloceros giganteus*), or moose or elk (*Alces alces*). However, certain of these taxa were absent during specific time periods and/or specific geographic regions (e.g., fallow deer in southwest France or reindeer in Montenegro [Morin et al. 2023]). A recent paper reported on an additional marker peptide that helped to distinguish red deer from moose at a Danish Neolithic site (Jensen et al. 2020), illustrating the potential for more fine-grained identifications within the Cervidae in the near future.

One of the great advantages of ZooMS, is that it allows zooarchaeologists to return to the morphologically unidentifiable bone fragments afterwards. This opens up the possibility to further refine broader-level ZooMS identifications by assessing the characteristics of the bone (e.g., overall size and cortical thickness) and its estimated body size (Morin et al. 2023). For example, this could exclude small (e.g., roe deer) or large (e.g., elk or giant deer) deer species from the list of potential taxa grouped under *Cervid*/*Saiga*/*Capreolus*. However, this needs to be done with caution as ZooMS studies have shown that body size assignments are not always reliable (Holleran et al. 2024; Ruebens et al. 2023; 2024; Sinet-Mathiot et al. 2019; 2023; Torres-Iglesias et al. 2024) and that Pleistocene taxa, overall, were often larger than modern day reference material.

During our workshop it was discussed how making raw datasets freely available online is good practice, as it encourages data transparency, inclusivity, accessibility, and reproducibility. There are a range of data repositories available that permit the hosting of large sets of MALDI-ToF data files, such as Zenodo, Mendeley Data, and the Open Science Framework (OSF). To facilitate several types of further analyses, it is best to make the individual data files available unprocessed, in the format as obtained from the MALDI (e.g., .txt or .mzml), including a separate metadata file with information on the different instrument runs.

INTERPRETIVE POTENTIALS

In this section, we want to highlight the enormous potential of ZooMS to provide new insights into patterns of site formation, past environmental conditions, and human subsistence practices. Our focus is predominantly on the Paleolithic, illustrated mainly, but not exclusively, by examples presented in the various papers throughout this special issue.

SITE FORMATION

Excavation and Preservation Biases

ZooMS can help address and understand the effect of previous excavation and sampling strategies. For example, it allows to identify more post-cranial remains when the

initial excavators prioritized the collection of teeth (Holloran et al. 2024). Further, in this issue, Wang et al. (2024) apply ZooMS to a subset of bones recovered from backdirt of 1930s excavations at Vogelherd (Germany) and integrate these new data with various existing zooarchaeological datasets. They highlight that while, overall, the patterns of faunal representation are similar, recent excavations of the backdirt revealed a large component of smaller material (microfauna, worked material, ivory beads, and smaller unidentifiable bone remains). This helped refine previous interpretations, which emphasized the presence of larger body size classes at the site. This shows how ZooMS can help counter potential biases and overrepresentations in material recovered from early excavation campaigns.

Further, ZooMS can help assess patterns of density-mediated attrition. In general, numerous zooarchaeological studies have suggested that variation in bone mineral density between skeletal elements directly influences, and therefore biases, their survival in the archaeological record (Grayson 1989; Karr and Outram 2012; Kreutzer 1992; Lam et al. 2003; Lyman 1984, 1994; Stiner 2002). In this issue, Pothier-Bouchard et al. (2024) illustrate through a multivariate taphonomic and ZooMS analysis that this is indeed the case at the site of Riparo Bombrini (Italy), as suggested by low cranial-to-tooth ratios and fracture freshness indices. However, at the same site, the ZooMS sample itself is also biased since mainly bones towards the exterior of the rock shelter had enough collagen preserved for a ZooMS identification. Finally, Pothier-Bouchard et al. (2024) assess how small assemblages can impose bias on taxonomic richness, by assessing the relationship between sample size and the number of taxa (NTAXA). All these studies illustrate the various ways in which ZooMS can help assess and overcome assemblage biases.

Biomolecular Preservation

Through ZooMS we can obtain fresh, complementary, insights into site formation by investigating differences in collagen preservation, more specifically peptide deamidation (van Doorn et al. 2012). If a specific taxon has a different signal of biomolecular preservation, this can give an indication that their carcasses underwent a different amount, or type, of diagenesis. For example, at Fumane (Italy), cervid specimens analyzed through ZooMS showed a deamidation distribution significantly different from the other main taxa at the site, indicating they underwent a distinct degree of molecular diagenesis (Sinet-Mathiot et al. 2019). At Salzgitter-Lebenstedt (Germany) fewer deamidation values could be obtained for the mammoth fragments, showing they were less well-preserved compared to the reindeer remains (Ruebens et al. 2023). These observations correlated with previous suggestions that the reindeer were hunted by Neanderthals, while the mammoths mainly formed part of a natural background fauna (Gaudzinski and Roebroeks 2000). At Quinçay (France), glutamine deamidation values were assessed over a broader spatial and temporal resolution (Welker et al. 2017). This study illustrated a limited chronological resolution in the data, but was able to identify bone specimens that had undergone different site formation histories in terms of duration or type. Deamidation outliers were identified, which could represent intrusive material, and increased deamidation values were obtained for specimens closer to the cave dripline. These studies highlight the potential of ZooMS to provide additional insights into site formation, identifying spatial patterns across a site, chronostratigraphic trends, as well as diagenetic differences between taxa.

Carnivore Behavior

A key question at Paleolithic sites relates to the competition between large carnivores and human groups, both for site locations and resources. Previously, many bone remains intensively modified by carnivores (digested, gnawed, etc.) could not be identified to a specific taxon. However, despite these modifications, collagen seems often well enough preserved for a ZooMS identification. For example, Ruebens et al. (2024 this issue) identified 334 carnivore-digested bone fragments, including remains of mammoth, for which hyaena involvement is often difficult to identify. In addition, they identified a much larger involvement of carnivores with the accumulation of the rhinoceros remains compared to the cervid fragments. Further, Holloran et al. (2024 this issue) integrate ZooMS, taphonomy, and zooarchaeology to assess Neanderthal-carnivore interactions at Picken's Hole (UK). Their study confirms the primary use of the site as a hyaena den, but also identified larger portions of the post-cranial skeleton. They were able to highlight differences in taphonomic history between taxa, and distinguish between phases of intense intra-clan competition and periods of prey abundance. This approach helps to better understand the local palaeoecological community and potential seasonal variation or broader climatic and environmental change. Similarly, Pothier-Bouchard et al. (2024 this issue) illustrate reduced carnivore activities in the Proto-Aurignacian deposits, compared to the underlying Mousterian levels at Riparo Bombrini (Italy).

ENVIRONMENTAL RECONSTRUCTIONS

Since ZooMS is aimed at providing taxonomic identities, at a most basic level, ZooMS can enhance our understanding of the range of animals that were present around an archaeological site, refining past environmental reconstructions. For example, in existing faunal assemblages additional cold-adapted species can be identified. While taxa such as reindeer and musk ox have a distinct set of peptide markers that enable genus-level identification, mammoth and woolly rhinoceros can only be assigned to their family level. Therefore, the archaeological context is essential for accurately assigning Elephantidae and Rhinocerotidae to their cold-adapted taxa (such as woolly mammoth and woolly rhinoceros)..

The high resolution paleoenvironmental archive from Europe allows for a high degree of certainty in both the local and regional distribution of species. Nevertheless, the identification through ZooMS of taxa previously unrecognized (e.g., beaver, Arenas-Sorriqueta et al. 2024) or un-

derrepresented (Raymond et al. 2024; Ruebens et al. 2022; Sinet-Mathiot et al. 2023) can help better understand the degree of heterogeneity in past environments (e.g., a more varied range of deer taxa). For example, the ZooMS identification of a wild boar specimen at the site of Les Cottés in France shows that there were at least patches of forest in an otherwise open, cold steppe landscape (Welker et al. 2015).

The current limitation of ZooMS in differentiating closely related taxa, especially within the Cervidae family, raises the possibility of missing individuals from taxa of similar body size (e.g., moose vs. giant deer). However, the targeted application of LC-MS/MS to these specimens can help distinguish these species and potentially further increase our resolution of species abundance and distribution (Jensen et al. 2020; Mylopotamitaki et al. 2024; Rüther et al. 2022). This can provide additional insights into ecological conditions, as, for example, *Bison* is indicative of an open and cold steppe environment (Smith et al. 2024).

PAST HUMAN SUBSISTENCE

Species Representation

ZooMS allows for larger portions of a faunal assemblage to be identified to taxon, including more precise identifications of fragments that previously could only be assigned to body size classes. While several ZooMS studies have illustrated the complex relationship between bone fragment thickness and body size classes (Discamps et al. 2024; Ruebens et al. 2024; Torres-Iglesias et al. 2023; Wang et al. 2024), revisiting reference material for additional analyses may help redress some of these inconsistencies. This approach could potentially provide a clearer understanding of species representation, as well as correct for potential misidentifications or classifications (Silvestrini et al. 2022; Sinet-Mathiot et al. 2023). By taxonomically identifying a larger proportion of bone remains we can help guard against "Reindeer blindness," where fragments of a certain size are assigned to a particular size or taxonomic class based largely on the predominance of a particular taxa (e.g., reindeer) within the assemblage.

For example, Sinet-Mathiot et al. (2023) illustrate distinct differences in taxonomic representation between morphological and ZooMS identified remains, potentially related to the ease of identification of some taxa (reindeer and Ursidae) compared to more fragmented remains (*Bos*/*Bison* and Equidae), resulting in an underestimated representation of large ungulates (Sinet-Mathiot et al. 2023; but see also Discamps et al. 2024). Arenas-Sorriqueta et al. (2024 this issue) incorporates diversity indices in combination with skeletal quantifications and biomass estimates to illustrate changes in human subsistence and diet. This illustrates a decrease in ungulate biomass and taxonomic diversity during the cold event at 8.2 ka cal BP, which is offset by an increase in marine resources, which remains stable after 8.2 ka cal BP, but with human subsistence again dominated by ungulate biomass.

ZooMS identifications can also be used to redefine or clarify archaeological layers within a site post-excavation (Discamps et al. 2019; 2023; Ruebens et al. 2024). At Cassenade (France), Ruebens et al. (2024) integrate the ZooMS identified fauna into existing spatial analysis illustrating limited change in the overall pattern of the main species though some novel differences in rarer species (reindeer, red deer, and mammoth). Previously, reindeer and red deer were differentiated within the sequence, potentially related to changes in climate, but ZooMS analysis highlights the co-occurrence of both taxa throughout the sequence. Finally, this study also highlights a concentration of mammoth remains in the middle of the deposits that was not recognized during excavation or subsequent morphological analysis. Taken together all these case studies highlight the potential of ZooMS to refine our understanding of species representation and faunal changes through time.

Species Abundance

Large-scale ZooMS studies often significantly increase the number of identifiable specimens (NISP) at a site. However, it is generally unclear how this relates to the number of individual animals at a site. The question of "How can we reduce chances of 'double' counting multiple fragments from the same animal?" remains key to future integration of zooarchaeological and ZooMS data. During the workshop, both Saunders and Discamps proposed methods to adjust NISP based on fragment weight (Discamps et al. 2024; Saunders et al. 2023). These offered different approaches based on either weight estimates of live animals, or weight of the archaeological bone fragment. Correlating archaeological bone remains with live animal weight offers the potential to be applied across a number of sites but requires significant input in terms of acquiring live weight figures. In contrast, Discamps et al. (2024 this issue) discuss the potential of scaling the weight of the ZooMS fragments relative to the weight of the morphologically identified remains, proposing the calculation of a ZooMS equivalent NISP (ZooMS-eNISP). While this ZooMS-eNISP metric is site-specific, it can be calculated across a range of sites and the scaled data compared. While there remain problems with both approaches, it is exciting that the field is trying to tackle the issue of integration to provide finer detail on species abundance and preservation. Future studies should focus on refining these methods and combining them with element representation, where possible.

Site Use and Seasonality

Targeted ZooMS studies can enhance our understanding of human site use patterns by investigating the seasonal timing of prey deaths and providing insights into the specific seasons during which humans were present at the site. Traditionally, zooarchaeology has focused on a combination of dental eruption and wear, alongside fusion information from post-cranial remains. Often foetal remains are recovered but have insufficient morphological information to be assigned to species. ZooMS can now be used to identify more foetal and newborn bone specimens to species, enabling us to obtain a larger dataset to estimate the season of death for these animals. For example, most deer spe-

cies give birth in spring, so the presence of newborn and juvenile deer remains at a site show that they died during spring or summer (Rendu et al. 2019; Smith et al. 2021). The presence of foetal or newborn cave bear remains can indicate that they died during hibernation (Kindler 2012; Stiner 1998). In this issue, Arenas-Sorriqueta et al. (2024) applied ZooMS to a selection of foetal and newborn remains (including red deer, wild boar, and ibex) and identified human site use throughout both spring and summer.

3.3.4 Carcass Transport and Mobility

The identification of specific skeletal elements can provide insights into whether an animal was processed in its entirety at a site, or if only specific body parts (e.g., not the skull and feet) were brought back. Previous morphological analysis often struggled with the large numbers of bone fragments that could only be assigned to body portions (cranial, long bone, etc.), but for which no taxonomic identification was possible. Moreover, it is often difficult to attribute specific skeletal elements or body portions to taxa of similar body size. Several ZooMS studies have now focused on identifying cranial and post-cranial fragments from various species that could not be identified based on morphology alone, and were able to refine patterns of carcass transport (Arenas-Sorriqueta et al. 2024; Pothier-Bouchard et al. 2020; 2024; Ruebens et al. 2023; Sinet-Mathiot et al. 2023; Torres-Iglesias et al. 2024). Moreover, with a ZooMS identification in hand, zooarchaeologists are able to revisit certain bone fragments and successfully assign a skeletal element (Silvestrini et al. 2022).

Interestingly, Holloran et al. (2024 this issue) illustrate differential patterns in the identification rates (both taxonomic and bone element) at Pickens Hole (UK). They link these variations to competition for resources between large carnivores and human groups and suggest differential transport and consumption of body parts from various taxa at the site. Moreover, the ability of ZooMS to identify 'rarer' taxa represented by more intact skeletal elements, can provide further insights into faunal accumulation (e.g., carnivore accumulation) and illustrates the importance of maximizing the understudied fragmented bone material. Future work needs to focus on refining this further to provide more in-depth quantification with zooarchaeological indices such as MNE, MNI and MAU.

Prey Preference and Carcass Processing

Applying ZooMS to bone fragments with human modifications cannot only help understand which animals were processed by humans, but also assess differences in carcass processing strategies between taxa. For example, at Fumane Cave (Italy) Sinet-Mathiot et al. (2019), showed a previously unrecognized high proportion of bovinae remains among the morphologically unidentifiable material. This component reveals a higher proportion of percussion traces in relation to anthropogenic bone fragmentation, suggesting a specific processing strategy, such as marrow extraction, for the bovinae. Similarly, Raymond et al. (2024 this issue) identify an overrepresentation of *Bos*/*Bison* among their trabecular bone fragments, which could possibly be explained by the use of grease-rich bones for fuel.

With its low cost and high throughput, ZooMS opens up the potential for an unparalleled, in-depth study of *all* bones with human bone surface modifications offering, potentially, a more complete overview of species representation and subsistence behavior at Paleolithic sites. For example, in this issue, at Cassenade (France), Ruebens et al. (2024) identified cut marks on a range of species, including those already identified in the morphological component, but also on woolly rhinoceros, representing the first potential instance of exploitation of this species in a Châtelperronian context.

CONCLUSIONS AND FUTURE DIRECTIONS

Our workshop showcased how the ZooMS-Zooarchaeology field is finding creative ways to fully integrate ZooMS within zooarchaeological theoretical frameworks, moving beyond merely increasing NISP counts. This was aptly illustrated throughout the workshop's talks and discussion sessions, and across the seven papers within this special issue. They show that a well-designed ZooMS study, which includes recording of taphonomic, zooarchaeological, and metric attributes on all fragments, can provide novel insights into site formation and human behavior when integrated with data from the morphologically identifiable fauna.

Excitingly, we are very much still at the beginning of fully exploring various ways to integrate ZooMS with zooarchaeological datasets, but also with other biomolecules, such as stable isotopes and ancient DNA. The growth potential for this field of study was demonstrated by the long list of ideas for further work that was compiled at the end of the workshop. This included ways for advancing methodologies, broadening applications, and furthering interpretations, such as:

Expanding the reference database: ZooMS identifications are dependent on comparisons with a peptide marker database (e.g., the living document of published ZooMS markers curated by the University of York). There is still a lot of scope to expand this reference database, firstly by including more non-European taxa (e.g., China: Wang et al. 2023; Xia et al. 2024; Australia: Multari et al. 2023; Peters et al. 2021; Jordan: Jensen et al. 2023; Africa: Janzen et al. 2021; Nel et al. 2023; North American megafauna: Antonosyan et al. 2024). Secondly, the determination of additional peptide markers will be key to further distinguish taxa (e.g., Ursidae (Garcia-Vazquez et al. 2023); horse vs. donkey (Paladugu et al. 2023); North American birds (Codlin et al. 2022); red deer vs. moose (Jensen et al. 2020), and African micromammals (Nel et al. 2023). Thirdly, the current database has a strong focus on mammalian taxa and ongoing studies are set to add more species of fish (Baker et al. 2023; Buckley et al. 2021; Richter et al. 2011; 2020), birds (Codlin et al. 2022; Eda et al. 2020), reptiles (de Kock et al. 2024; Harvey et al. 2019), amphibians (Buckley and Cheylan 2020), and microfauna (Buckley et al. 2016) in the near future. Moreover, so far, few studies have focused on non-mammalian

taxa present at Paleolithic sites.

Optimizing lab protocols: While the standard ZooMS HCl protocol has been successfully applied to a varied range of faunal assemblages, there is scope to further optimize various aspects of this protocol and tailor it to the unique characteristics of the targeted archaeological bone material. Further work can be done on optimizing the demineralisation phase, sample pretreatments, collagen extraction (Mylopotamitaki et al. 2023; Wang et al. 2021), and enzymatic digestion (Le Meillour et al. 2024). This is especially important when trying to study more degraded samples, for example, from warmer (arid) contexts (Jensen et al. 2023; Le Meillour et al. 2018; Peters et al. 2023; Pothier-Bouchard et al. 2019) or from material that was affected by heat (e.g., boiling or burning, Díaz-Martín et al. 2019; Hansen et al., 2024; Raymond et al. 2024; Wang et al. 2023; Yates 2013). Moreover, integrating pre-screening methods can also help target specimens with better collagen preservation (e.g., Pothier-Bouchard et al. 2024).

Automatization: A time-consuming aspect of ZooMS is the manual identification of the MALDI-ToF spectra. However, recent work is trying to move the field toward semi-automated spectral identification, using purpose-developed software (Hickinbotham et al. 2020), machine learning (Baker et al. 2023; Gu and Buckley 2018) or computational algorithms (SpecieScan: Végh and Douka 2024). Recently, there have also been advances in automatizing parts of the ZooMS lab work, for example, through the use of liquid-handling robots (AutoZooMS: Oldfield et al. 2023).

Integration with shotgun proteomics: LC-MS/MS mass spectrometry can be used to reconstruct the sequences of both collagenous and non-collagenous proteins (NCPs). This cannot only provide higher-resolution taxonomic identifications, but also additional insights in, for example, tissue type and biological sex. Species by Proteome Investigation (SPIN: Rüther et al. 2022) has been developed recently to facilitate the medium-throughput analysis of bone proteomes for taxonomic identification purposes. While the SPIN reference database is still in development, it already enables the distinction of certain taxa that cannot be differentiated using the standard ZooMS protocol, such as *Bos* and *Bison*, or horse and donkey (Mylopotamitaki et al. 2024; Rüther et al. 2022). This is possible if the peptides containing the informative amino acid substitutions are represented within the sample. Additional LC-MS/MS approaches, whether using long or shorter LC gradients, are similarly amenable to provide taxonomic identifications through whole-proteome analysis. These approaches offer significant opportunities to improve on the taxonomic resolution beyond what is achievable with ZooMS alone (Bray et al. 2022; Gilbert et al. 2024; Goffette et al. 2024; Taniguchi and Miyaguchi 2023).

Although dental enamel contains a small proteome, its composition is highly distinct and survives across the Pleistocene (Madupe et al. 2023; Welker et al. 2020). Amelogenin is the major protein in dental enamel. Since amelogenin proteins are located on the X (AMELX) and Y (AMELY) chromosomes, with distinct protein sequences for some mammalian taxa, the detection of AMELY-specific peptide sequences is capable of providing genetic sex assignments (Porto et al. 2011; Stewart et al. 2016). Large scale proteomic sexing of faunal remains thus has the potential to provide unique insights into Pleistocene herd structure (Berezina et al. 2024; Rey-Iglesia et al. 2024), with implications for hunting decisions and seasonality.

Methodological integration: ZooMS can be used in concordance with a range of other methods from archaeological science. For example, ZooMS identifications can be achieved on collagen extracts obtained for ^{14}C dating (e.g., Mylopotamitaki et al. 2024) or stable isotope analysis (SIA: Charlton et al. 2016; McCormack et al. 2022; Smith et al. 2024). In relation to the latter, it can be used to check the identification of SIA outlier values that do not match the expected species. Further, it has been recently demonstrated that ZooMS can be used to identify bone fragments present within micromorphology thin sections (Bartsch et al. 2024), within coprolites (Runge et al. 2021), and digested by carnivores (Ruebens et al. 2023; 2024; Smith et al. 2024; Welker et al. 2015). Finally, ZooMS can also be combined with virtual histology using micro-CT scanning to gain insights into the tissue type (e.g., laminar structure of ivory: Williams et al. 2024), the type of taphonomic processes that affected the microstructure of the bone (e.g., bacterial bioerosion: Smith et al. 2024), or use-wear analysis (Dekker et al. 2024; Hansen et al. 2024).

Experimental studies: Blind tests are extremely useful for checking the reliability of various observed zooarchaeological phenomena such as cut marks (Blumenschine et al. 1996), as well as species and element identification (Hawkins et al. 2022; Morin et al. 2017a; b). Such an approach is now also being applied through ZooMS to check previous morphological identifications (Morin et al. 2023), but few studies so far have checked ZooMS MALDI data across different instruments or labs (Dekker et al. 2024; Ruebens et al. 2023). Additionally, a plethora of experimental studies are still possible to help us better understand taphonomic processes (e.g., differences in bone breakage patterns across body size classes) and how they correlate with collagen preservation (e.g., the effect of boiling bones or carnivore damage [Runge et al. 2021]). Moreover, there is also a lot of scope to undertake further experimental work to optimize both sampling strategies (e.g., sampling weight, fragments vs. powder), and extraction methods (e.g., demineralization duration, effect of demineralization on deamidation, trypsin digestion, sample and matrix crystallization and deposition onto MALDI plates).

Finally, we cannot overstate the importance of continued discussions and dialogues within the broader community to forward our understandings, applications, and integrations of various paleoproteomic and zooarchaeological datasets. The development of this community is witnessed by the recent establishment of the PAASTA community as a forum for sharing and exchanging ideas (https://paasta-community.github.io/), as well as the continued success of the Integrating ZooMS and Zooarchaeology workshops.

We look forward to seeing this field develop its full

potential in the coming years. These smaller-scale, discussion-led events are crucial to find ways to further advance the field of biomolecular zooarchaeology and increase the communication and integration between (zoo)archaeologists and paleoproteomic specialists. Overall, we hope that this special issue provides food-for-thought and illustrates the importance of maximizing the potential of fragmented bone remains from both new excavations and within existing museum collections.

ACKNOWLEDGEMENTS

We are grateful to all the participants of our 2023 workshop at the University of Kent (UK), presenting interesting case studies and providing thought-provoking remarks during two long discussion sessions. G.M.S. received funding from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 101027850. The workshop was funded by the same Marie Sklodowska-Curie grant and the University of Kent seed fund. K.R. is supported by project COEXIST, which was selected by the ERC and funded by the UKRI (Grant number EP/Y037448/1). F.W. received funding from the European Research Council under the European Union's Horizon 2020 research and innovation program (grant agreement number 948365). V.S.-M. was supported by a Fyssen Foundation postdoctoral fellowship (2023–2025). We would like to thank all the editors of *PaleoAnthropology* for the opportunity to publish this collection of papers as a special volume and thank all the reviewers for each paper for providing timely, thoughtful, and constructive comments.

DATA AVAILABILITY STATEMENT

This paper is an introduction to the special issue on the integration of ZooMS and zooarchaeology and has no primary data associated with it.

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Special Issue: Integrating ZooMS and Zooarchaeology: Methodological Challenges and Interpretive Potentials

Supplement 1: Towards a Deeper Integration of ZooMS and Zooarchaeology at Paleolithic Sites: Current Challenges and Future Directions

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SUPPLEMENT 1
This supplement includes: supplementary material text, tables, and references.

Example of a zooarchaeological recording scheme for ZooMS fragments

This scheme provides a starting point to record zooarchaeological, taphonomic, and metric observations on bone fragments *before* they are sampled for ZooMS. This scheme can be adjusted for the faunal material at hand and is a first step to facilitate integration with existing zooarchaeological data from morphologically identified remains. At many sites, existing zooarchaeological data may already be available, so a paired back system may be necessary focused on zooarchaeological observations only; this depends on the research questions of study. In this issue, a detailed overview of taphonomic variables and their interpretive potential is also presented in Pothier-Bouchard et al. (2024).

1. Zooarchaeological observations

1.1. Tissue type

Categories: *cortical, trabecular, both, dental, n/a*

Description: This relates to the major type of bone in the sample.

cortical	Also known as compact bone, is dense and solid, and forms the outermost layer of bones. Is especially prevalent in long bone shaft fragments.
trabecular	Also referred to as spongy or cancellous bone, is porous bone tissue and is found most commonly in long bone epiphyses (ends of bones), carpals and tarsals, and within flat bones, such as scapula and pelvis.
both	This is recorded when both cortical and trabecular bone are present in the sample, for example, if a long metaphysis is present; "; if both present can be recorded as cortical/trabecular or trabecular/cortical to denote which is most abundant.
dental	Refers to tooth fragments; to provide a taxonomic identification through ZooMS, sampling should only be from root or dentine as enamel does not contain collagen.
n/a	This should be recorded if the analyst does not know what tissue type the specimen represents, either due to an absence of bone element portions or intensive taphonomic modifications that removed the surface.

References: Gifford-Gonzalez 2018; Reitz and Wing 1999.

1.2 Skeletal portion

Categories: *cranial, dental, flat bone, axial, long bone, foot, indeterminate*

Description: Records which body portion the specimen belongs to (see Figure 3).

cranial	Fragments from the cranial vault and mandible; cranial fragments are generally quite identifiable (e.g. Figure 4A), often due to the imprint of the brain on the internal surface (e.g. Figure 4B); mandibular fragments can be identified through the root sockets that are quite distinctive. This also includes horn and antler fragments (e.g. Figure 4C).
dental	Includes dentine and root fragments; as discussed in the main text it should be carefully considered and reported whether dental remains are included in further analysie to avoid issues of biassing.
axial	Bone remains from the axial portions of the skeleton, including the vertebral column, ribs (e.g. Figure 4J) and sternum; these are generally thinner fragments with a larger proportion of trabecular bone.
flat bone	Fragments from portions of the scapula and pelvic girdle; these bones can be quite thin and have an increased amount of trabecular; also the shapes of these bone fragments can be different from long bone fragments (e.g. Figure 4I).
long bone	Bone fragments from the forelimb (humerus, radius, ulna) and hindlimb (femur, tibia, fibula); often the most common fragments in ZooMS studies with large quantities of think cortical shaft fragments (e.g. Figure 4F-H); distinctive morphology near bone ends/growth plates with increased trabecular bone.
foot	Bones from the foot, including the carpals, metacarpals, tarsals, metatarsal and phalanges (e.g. Figure 4D-E); potential confusion with long bone fragments.

indeterminate	This category should be used when the fragment has very few identifying features and does not allow for a secure assignment to the broad categories above. Often this is the most abundant category in ZooMS studies.

References: Gifford-Gonzalez 2018; Reitz and Wing 1999; Stiner 1991a, 1991b; Smith et al. 2021, 2024.

1.3 Bone element

Description: This additional category can be used to record additional anatomical observations, including assignments to a specific skeletal element based on previously unrecognised morphological criteria (e.g. bone structure, shape, or diagnostic signatures, such as muscle attachments and nutrient foramen). This can be done through close comparison with reference collections of modern skeletal material, comparative osteological atlases or online archives of photos or 3D scans.

References: France 2009; Hillson 2016; Pales et al. 1971; Schmid 1972; archaeozoo.org, boneid.net, skull base, Max Planck 3D reference collection.

1.4 Bone fusion

Categories: *foetal, unfused, fusing, fused, indeterminate*

Description: Bone fusion can provide information on the age-structure kill off patterns, as well as seasonality and site occupation by both human and large carnivores.

foetal	These remains are often very porous in nature and lack the fusion areas at the ends of these elements, such as long bone epiphyses.
unfused	This illustrates some development (i.e. not foetal); if the growth plate is absent the spongy trabecular bone of the epiphysis will be visible.
fusing	Growth plate not fully fused, potentially illustrating a more sub-adult individual.
fused	Bone fully fused, indicating a completely adult individual.
indeterminate	No fusion data available due to absence of diagnostic portion.

References: Gifford-Gonzalez 2018; Reitz and Wing 1999.

2. Natural taphonomic observations

2.1 Bone readability

Categories: *100%; >50%; < 50; 0%.*

Description: This records how much of the original bone surface remains on the bone and is recorded in broad percentage categories. This provides a qualitative assessment of preservation across an assemblage and can be used to assess whether this has affected identification rates and identification of taphonomic modifications.

100%	All of the original bone surface present with no alteration from taphonomic processes
>50%	The majority of the bone surface retains the original surface.
<50%	Most of the bone surface has been removed through taphonomic processes.
0%	No original bone surface remains.

References: Ruebens et al. 2023; Sinet-Mathiot et al. 2023; Smith 2015; Smith et al. 2021, 2024.

2.1 Abrasion

Categories: *0%, <50%, >50%, 100%*

Description: Abrasion refers to the modification of the bone surface through movement pre-depositionally, but can also occur when material has been incorporated into deposits but subsequently re-exposed. This often results in the gradual removal of bone surfaces and also potentially in the rounding of fragment edges. It can be a good indicator of movement of specimens within a site or layer. It may not be necessary to record both readability and abrasion, as these can often overlap in the data they provide.

0%	None of the bone surface exhibits abrasion or removal.
<50%	A small proportion of the bone surface illustrates evidence for abrasive action; this could include the removal of bone surface and the rounding of some, previously sharp fracture edges
>50%	A large portion of the bone surface is covered by abrasion resulting in destruction of a surface and obscuring of other modifications; increased rounding on bone edges.
100%	All bone surface is abraded with all of the surface removed and potentially extensive rounding on edges.

References: Fernandez-Jalvo and Andrews 2016; Sinet-Mathiot et al. 2023; Smith et al. 2021, 2024

2.2 Weathering

Categories: *0, 1, 2, 3, 4, 5*

Description: Weathering relates to the in situ destruction of the bone surface through processes such as freeze/thaw, hot/cold, etc. This scheme was developed through actualistic work in Africa with modern material, and while a quantitative age range (in years) was suggested, it is mainly used in a qualitative fashion to assess exposure of bone and burial times at a site. References to modern carcass materials have been removed. Modified from Behrensmeyer. 1978, also see examples on Figure 5.

0	Bone surface shows no sign of cracking or flaking due to weathering
1	Bone shows cracking, normally longitudinal in long bones. Articular surfaces may show mosaic cracking of covering tissue, as well as in the bone itself.
2	Outermost concentric thin layers of bone show flaking with potentially some deeper and more extensive flaking follows, until most of the outermost bone is gone. Crack edges are usually angular in cross section.
3	Bone surface is characterised by patches of rough, homogeneously weathered compact bone, resulting in a fibrous texture. In these patches, all the external, concentrically layered bone has been removed.
4	The bone surface is rough with large and small splinters that may be loose enough to fall away. There are often large weathering cracks that penetrate into the inner portions of the bone.
5	Bone is falling apart in situ and is extremely fragile. Original bone shape may be difficult to determine.

References: Behrensmeyer 1978.

2.3 Root etching

Categories: *0% 1-25%, 26-50%, 51-75%, 76-100%*

Description: Plant roots can affect the surface of the bone leaving dendritic modifications across the surface; this can be scored in simple presence/absence, but some schemes can include percentage of surface affected. This can also be simplified and integrated with readability and bone abrasion schemes. For examples see Figure 5.

0%	No root etching visible on the bone surface.
1-25%	Root etching confined to a small portion of the bone surface; largely superficial, not deep in the surface.
26-50%	Root etching across a larger portion of the surface, perhaps with more intensive modification of the original surface.
51-75%	A majority of the surface is covered in root etching, with deeper and often overlapping modifications.
76-100%	The whole bone surface is covered by root etching with deep modifications and modifications overlapping and obscuring each other.

References: Fernandez-Jalvo and Andrews 2016; Smith 2015; Smith et al. 2021.

2.4 Break morphology

Categories: *fresh, dry, indeterminate*

Description: Break morphology can provide information about whether the bone remains were fresh (containing a biotic component) or dry when broken. This can inform zooarchaeologists about how long

these fragments were exposed at a site. Various schemes have been developed to record fracture morphology. These include recording basic information such as fresh or dry, to more complex attributes including shape, such as smooth, jagged or perpendicular. For examples see Figure 5.

fresh	A bone broken in this way will generally have a smooth surface; fresh bone breaks depend on the type of bone that has been fractured and how much of the biotic component remains; for example, long bones will always fracture spirally if fresh.
dry	A dry bone break is generally sharper and can be more ragged in cross section indicating a lack of biotic component remaining.
indeterminate	not possible to determine when the bone was broken.

References: Gifford-Gonazalez 2018; Lyman 1994; Marshall 1989; Reitz and Wing 1999; Smith et al. 2021.

3. Non-anthropogenic taphonomic modifications.
At a minimum, these are all recorded as absent (no) or present (yes).

3.1 Carnivore tooth pits
Description: This relates to carnivore modifications from the disarticulation and consumption of animal carcasses; these are generally circular or semi-circular, though this can depend on tooth type and type of carnivore; the diameter of the modifications can also be recorded to potentially infer which type of carnivore the tooth marks relate to.
References: Arriaza et al. 2019; Dominguez-Rodrigo and Piqueras 2003; Fernandez-Jalvo and Andrews 2016; Fisher 1995; Lyman 1994; Yravedra et al. 2018.

3.2 Carnivore digested bone
Description: This relates to the ingestion of bone fragments by large carnivores, particularly hyaenas, to extract bone nutrients. This leaves characteristic modifications across the bone surface, the main ones being frequent small holes across the surface and a shiny surface (see Figure 6 for examples).
References: Blasco et al. 2011; Fernandez-Jalvo and Andrews 2016; Fisher 1995; Lyman 1994.

3.3 Crenelation
Description: Characteristic jagged edges often around the epiphysis of bone fragments (where the bone is weakest), but if hyaenas are present they can also be found on denser long bone shaft portions.
References: Fernandez-Jalvo and Andrews. 2016; Fisher 1995; Lyman 1994.

3.4 Carnivore bone breakage
Description: Often identified on long bones these indicate the breaking of bones to extract and exploit bone marrow. This is done when the remains are fresh so will result in a fresh bone break; to assign this to carnivores requires presence of tooth pits and scratches around an impact on the bone surface along, with clear evidence for bone breakage through impact points from teeth.
References: Fernandez-Jalvo and Andrews 2016; Lyman 1994; Fisher 1995; Reitz and Wing 1999.

3.5 Rodent gnawing
Description: Rodents also chew bones to keep their continuously growing incisors small; this produces distinct modifications often in a line and often on the densest parts of the bone. While this may be partly for nutrition, it is mainly to keep their teeth from overgrowing in their mouths.
References: Fernandez-Jalvo and Andrews. 2016; Lyman 1994; Gifford-Gonzalez 2018.

4. Anthropogenic taphonomic modifications
At a minimum, these are all recorded as absent (no) or present (yes), but the exact numbers of modifications and their orientations can also be recorded. Examples are presented on Figure 6.

4.1 Cut marks
Description: These are marks left on the bone surface by human tools during all stages of carcass butchery and processing. These marks are, frequently, fine and v-shaped in cross section, can contain small microstriations at their base and a step related to the edge of the tool. There remains considerable debate surrounding the identification of these marks, and the number of attributes necessary to identify them.

References: Blumenschine 1996; Dominguez-Rodrigo et al. 2017, 2019; Fernandez-Jalvo and Andrews. 2016; Fisher 1995.

4.2 Chop marks

Description: Chop marks are bone surface modifications with a broader and deeper profile. They are often located on or around the joints of carcasses and thought to indicate dismemberment and disarticulation.

References: Fernandez-Jalvo and Andrews. 2016; Fisher 1995; Lyman 1994; Reitz and Wing 1999.

4.3 Scrape marks

Description: Often long, more shallow marks, which run almost parallel to the long axis of the bone. They are seen as indicative of removal of meat, or sometimes of removal of the bone periosteum, prior to the deliberate fracturing of bone remains for marrow.

References: Fernandez-Jalvo and Andrews 2016; Fisher 1995; Lyman 1994; Reitz and Wing 1999.

4.4 Marrow fractures

Description: Often identified on long bones these indicate the breaking of bones to extract and exploit bone marrow. This is done when the remains are fresh, so will result in a fresh bone break; in order to assign this to human action requires the presence of either an impact point (on bone surface) and a scar negative on the bone inner surface. There may also be ripple marks and hackle marks indicating the direction of impact. For examples see Figure 6 B-C.

References: Fernandez-Jalvo and Andrews. 2016; Fisher 1995; Lyman 1994; Reitz and Wing 1999.

5. Metric measurements

5.1 Fragment size

Measured using digital callipers (mm or cm), or using a pre-prepared sheet with concentric circles of known diameter e.g. 1cm, 2cm, 3cm, 4cm etc.

Description: The maximum length and width of the fragment can be measured to compare identification rates by fragment size and deamidation to see if this may have affected preservation. Fragment thickness can also be recorded, though most often length and width is sufficient.

References: Discamps et al. 2019; Ruebens et al. 2023; Wang et al. 2024.

5.2 Fragment weight

Measured using a (pocket) scale (mg or g).

Description: The weight of the bone fragment (before sampling) can be measured to compare identification rates by fragment weight and deamidation to see if this may have affected preservation. When integrating with weights of the morphologically identifiable fraction, this also allows for the calculation of an adjusted ZooMS-eNISP.

References: Discamps et al. 2024; Holloran et al. 2024; Wang et al. 2024.

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Special Issue: Integrating ZooMS and Zooarchaeology: Methodological Challenges and Interpretive Potentials

Supplement 2: Towards a Deeper Integration of ZooMS and Zooarchaeology at Paleolithic Sites: Current Challenges and Future Directions

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SUPPLEMENT 2
This supplement includes: supplementary material figure.

Example of the taxonomy of Cervidae (A) and Bovidae (B), listing the main taxa that are present at European Palaeolithic sites and the ZooMS peptide marker m/z or masses that can help distinguish them. Peptide marker masses given are α2 978 (+16), α2 502 and α2 757 (+16). Marker α1 586 (+16) is at m/z 2883+2899 for all listed taxa, except for *Bos* and *Bison*, and therefore the presence of this marker can help distinguish these from *Ovibos* (hence, this marker is also listed (in italics) for these three taxa). Note that additional peptide markers are commonly used to assign taxonomic identities, and recent work has indicated a marker that can set *Alces alces* apart from *Cervus elaphus* (Jensen et al. 2020).