From Bones to Proteomes: Gaining a Deeper Understanding of the Middle to Upper Paleolithic Transition

Von Knochen zu Proteomen: Wege zu einem tieferen Verständnis des Übergangs vom Mittel- zum Jungpaläolithikum

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Abstract: *The Middle to Upper Paleolithic transition (MUPT) is a complex period in European prehistory during which Neanderthals go extinct, modern humans first enter the continent, and both hominin populations potentially interact in a variety of ways. For the MUPT, research has traditionally focused on several so-called "transitional" technocomplexes in the context of hominin behavior, chronology, and hominin biology (i.e., identity). Although researchers today, as compared to several decades ago, have acquired a better understanding of hominin behavior and chronology by employing new excavation and analytical methods utilizing state-of-the-art approaches, the hominins themselves remain elusive. This is because of a general absence of hominin fossil remains associated directly with the MUPT, and is largely the result of high bone fragmentation rates, making hominin bones that are present unidentifiable using standard morphological approaches. Recent research now demonstrates conclusively that collagen peptide mass fingerprinting (also named ZooMS, short for Zooarchaeology by Mass Spectrometry) is a suitable approach for efficiently identifying such hominin bones. Associated with high identification success rates across Europe, this approach has the potential to change our biological understanding of the MUPT significantly. Furthermore, advances in shotgun proteomic analysis of entire ancient hominin proteomes has now become feasible, allowing researchers to identify, among other elements, individual hominin bones as Neanderthals, Denisovans, or modern humans, even in the absence of ancient DNA preservation. As a result, paleoproteomics provides a biomolecular solution to the unknown biological 'maker' of archaeological assemblages across the MUPT.*
Keywords: *MUPT, Neanderthals, modern humans, paleoproteomics, ZooMS*

Zusammenfassung: Der Übergang vom Mittel- zum Jungpaläolithikum stellt eine komplexe und relativ kurze, vielleicht nur einige tausend Jahre währende Phase in der europäischen Urgeschichte dar, in welcher die Neandertaler ausstarben, moderne Menschen erstmals den Kontinent betraten und beide Menschenformen wahrscheinlich auf verschiedene Weisen miteinander in Wechselwirkung standen. Bei der Untersuchung dieses Überganges hat sich die Forschung in der Regel auf verschiedene so genannte „Übergangs"-Industrien konzentriert, die in verschiedenen Regionen Europas auftreten und die mittelpaläolithische sowie jungpaläolithische Elemente in sich vereinen. Meist werden hierunter Technokomplexe wie das Châtelperronien, das Uluzzien, das Szeletien und das Lincombien-Ranisien-Jerzmanowicien (LRJ) verstanden. Obwohl sie miteinander in Verbindung stehen, können Fragen zum Übergang zwischen Mittel- und Jungpaläolithikum im Einzelnen biologische Aspekte (wer lebte wo?), Aspekte des Verhaltens (wie haben sich die Menschen verhalten?) sowie chronologische Aspekte (wann

haben sie gelebt?) betreffen. Während das menschliche Verhalten und die Chronologie im Vergleich zu den vergangenen Jahrzehnten inzwischen besser verstanden werden, was vor allem auf neue Ausgrabungs- und Analysemethoden unter Nutzung modernster Ansätze zurückzuführen ist, bleiben die Menschen selbst schwer fassbar, da menschliche Fossilreste, die unmittelbar mit dem Übergang vom Mittel- zum Jungpaläolithikum verbunden sind, im Allgemeinen fehlen. Dies beruht größtenteils auf der hohen Fragmentierungsrate der Knochen, was zur Folge hat, dass die vorhandenen Knochen mit herkömmlichen morphologischen Ansätzen unbestimmbar sind.

Jüngste Forschungen belegen nun schlüssig, dass die Methode des Peptidmassenfingerprints (*collagen peptide mass fingerprinting*), nach der englischen Bezeichnung *Zooarchaeology by Mass Spectrometry* (Zooarchäologie durch Massenspektrometrie) auch ZooMS genannt, einen geeigneten Ansatz darstellt, um solche Menschenknochen effizient zu identifizieren. Diese Methode kann auf Hunderte oder gar Tausende einzelner Knochenfragmente angewendet werden und wurde kürzlich bei paläolithischen Inventaren eingesetzt, um nach menschlichen Knochenbruchstücken zu suchen, die morphologisch nicht bestimmbar sind. In Verbindung mit den hohen Identifikationsraten für Fundinventare aus verschiedenen Teilen Europas, hat dieser Ansatz das Potenzial, unser biologisches Verständnis des Überganges vom Mittel- zum Jungpaläolithikum maßgeblich zu verändern, auch wenn ZooMS über die Identifizierung der Knochensplitter als von Menschen stammend hinaus keine Zuweisung zu einer konkreten Menschenform erlaubt. Zusätzlich sind jedoch inzwischen große Fortschritte bei der Analyse vollständiger Proteome früher Menschen auf Basis der Methode der Schrotschuss-Proteomik (*shotgun proteomic analysis*) möglich geworden. Das erlaubt es, neben weiteren Aussagen, einzelne Menschenknochen ganz konkret Neandertalern, Denisova-Menschen oder anatomisch modernen Menschen zuzuweisen, selbst wenn bei ihnen keine frühe DNS erhalten ist. Im Ergebnis liefert die Paläoproteomik eine biomolekulare Lösung zur Identifikation der biologisch bisher unbekannten Erzeuger archäologischer Fundinventare aus der Übergangphase zwischen Mittel- und Jungpaläolithikum. So konnten beispielsweise durch ZooMS mehrere vorher nicht ansprechbare Knochensplitter aus dem Châtelperronien der Grotte du Renne in Burgund zunächst als menschlich identifiziert werden, anschließende paläoproteomische Analysen haben gezeigt, dass es sich um Knochensplitter von Neandertalern handelt.

Schlagwörter: Übergang vom Mittel- zum Jungpaläolithikum, Neandertaler, moderne Menschen, Paläoproteomik, ZooMS

Introduction

The Middle to Upper Paleolithic transition (MUPT) is a relatively short time period, probably lasting just several thousand years on a continent-wide scale, that has attracted a disparately large amount of scientific interest. The issue of the MUPT directly relates to the extinction of Neanderthals, the arrival or colonization of modern humans on the European continent, potential cognitive and behavioral differences between both hominin populations, as well as behavioral transfer through either direct or indirect contact between them (Churchill and Smith 2000; McBrearty and Brooks 2000; Green et al. 2010; Higham et al. 2014). This debate is particularly pronounced in the context of several so-called "transitional" industries that occur across Europe and that display, according to some specialists, behavioral features that combine Middle Paleolithic or Mousterian and Upper Paleolithic or Aurignacian elements. Normally the "transitional" technocomplexes include the Châtelperronian, the Szeletian, the Lincombian-Ranisian-Jerzmanowician (LRJ) and the Uluzzian. Although interlinked, questions around the MUPT can be separated based on biological issues (who is where?), behavioral issues (what is their behavior?), and chronological issues (when are they present?). It is only once a good understanding of these three main components is achieved separately that a comprehensive picture emerges of the processes that have shaped the MUPT on local, regional, and continental scales.

In recent years, developments in radiocarbon dating of bone and charcoal, OSL dating, tephrochronology, and the Bayesian modeling of such dates in site and regional

contexts have provided a rigorous framework that vastly improves our understanding of the MUPT chronology (Bird et al. 1999; Higham et al. 2006; Bronk Ramsey 2009; Talamo and Richards 2011; Lowe et al. 2012). They also allow us to revisit issues of taphonomic mixing that potentially cause associations between MP and UP components within a single archaeological horizon. Similarly, the direct dating of hominin fossils spatially associated with transitional technocomplexes enables us to make an empirical assessment of the likelihood that they chronologically belong to that technocomplex. This is important, as a number of direct radiocarbon dating studies have shown Paleolithic hominins to be intrusive from younger layers, sometimes by tens of thousands of years (Terberger et al. 2001; Conard et al. 2004; Hoffmann et al. 2011; Benazzi et al. 2014b). Combined, a mature set of analytical approaches is now available for establishing accurate chronological frameworks.

Hominin behavior observed in the MUPT technocomplexes is probably the component that is historically the best understood, as each of the technocomplexes is formally defined on technological characteristics in the lithic component. This includes the adoption of blade and bladelet production, which are common components of Upper Paleolithic technological systems (Roussel et al. 2016). Beyond lithic technology, some of the transitional industries contain sparse examples of (potential) ornamental bone technology such as perforated or grooved teeth and shell (Gambassini, 1997; Granger and Lévêque 1997; d'Errico et al. 2003; Flas 2011), as well as bone tools (d'Errico et al. 2012; Peresani et al. 2016). In contrast, few studies have characterized faunal hunting strategies employed during the MUPT, as only a subset of sites contain reasonably sized bone assemblages, particularly because of high bone fragmentation (Fig. 1). This is further

Fig. 1: A typical bone assemblage from a Paleolithic site. Photo: Frido Welker.
Abb. 1: *Ein typisches Knocheninventar von einer paläolithischen Fundstelle. Foto: Frido Welker.*

complicated by the methods used to collect and store bone assemblages from archaeological sites excavated in the first half of the 20th century. As a result, relatively few high-quality zooarchaeological datasets are available on the transitional period, limiting our behavioral information on human-animal interactions (Ruebens et al. 2015). Furthermore, some authors have argued that late Neanderthals or early modern humans in Europe adopted behavioral practices from the other hominin population, either through

direct or indirect contact (Hublin et al. 2012). It therefore follows that our behavioral understanding of the MUPT technocomplexes cannot be used to argue from a biological standpoint on the "maker" of a particular technocomplex. The biological component therefore requires independent evidence in the form of hominin fossils.

Only a limited number of hominin fossils are directly associated with the MUPT (Churchill and Smith 2000). In all cases, their associations have been questioned based on chronological or taphonomic issues. Specimens include the Spy Neanderthals, indirectly associated with the LRJ (Semal et al. 2009), the Kent's Cavern maxilla (Higham et al. 2011), the Saint-Césaire Neanderthal (Hublin et al. 2012; Lévêque and Vandermeersch 1980), the Grotte du Renne Neanderthals (Hublin et al. 1996), two hominin teeth from Grotta del Cavallo (Benazzi et al. 2011), an undiagnostic hominin tooth from Grotta di Fumane (Benazzi et al. 2014a), and a now lost molar from Dzeravá skala Cave (Hillebrand 1914). Some of these have been directly dated, while others have not. In addition, except for the Fumane tooth, all specimens were excavated before modern excavation techniques became commonplace.

The lack of hominin fossils becomes even more apparent in the context of recent advances in ancient genomics. The sequencing of ancient genomes belonging to modern humans, Neanderthals, and the newly discovered Denisovans has painted a complicated genomic picture of biological interactions between hominins (Krause et al. 2010; Reich et al. 2010; Meyer et al. 2012, Slon et al. 2017; Hajdinjak et al. 2018). These or similar events may have taken place during the MUPT in Europe, but the absence of undisputed MUPT fossils makes this difficult to demonstrate genomically.

Therefore, despite over a century of research into this topic, the high bone fragmentation rates that are characteristic of Paleolithic bone assemblages are responsible for limited insights into 1) the ecological setting and hominin-animal interactions during the MUPT, and 2) reduced numbers of directly associated hominin bone specimens. The

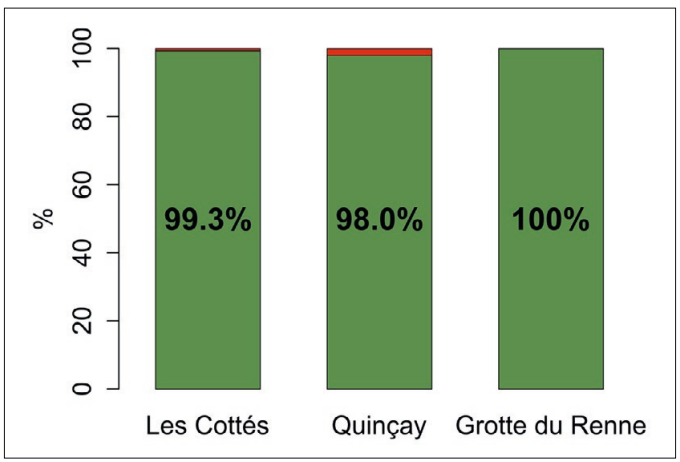

Fig. 2: ZooMS success rates for three Late Pleistocene MUPT bone assemblages. Primary data from Welker et al. 2015b, 2016, and 2017b.

Abb. 2: *ZooMS Erfolgsraten für drei spätpleistozäne Knocheninventare aus der Zeit des Übergangs vom Mittel- zum Jungpaläolithikum. Primärdaten aus Welker et al. 2015b, 2016 und 2017b.*

biological population responsible for most bone assemblages is therefore unknown, few direct hominin radiocarbon dates for the MUPT are available, and little stable isotope data exist on hominin diet. ZooMS, short for Zooarchaeology by Mass Spectrometry, is a recently introduced taxonomic identification approach through MALDI-TOF-MS peptide mass fingerprinting of collagen type I (COL1) rich tissues. It can be applied to hundreds or thousands of individual bone specimens and has recently been applied to Paleolithic assemblages to screen for hominin bone specimens that are morphologically undiagnostic (Brown et al. 2016; Welker et al. 2016). Initial studies have indicated high success rates of bone identification, approaching 100% for most studied bone assemblages (Fig. 2). Simultaneously, this approach allows researchers to refine the fauna species component at the same site (Welker et al. 2015b), and to recognize potentially intrusive bone specimens (Welker et al. 2017b). ZooMS is thereby a suitable tool for addressing the biological and behavioral gaps of knowledge currently present in the MUPT literature.

ZooMS

Zooarchaeology by Mass Spectrometry, otherwise known as ZooMS or collagen peptide mass fingerprinting, is a general purpose tool used to identify taxonomically samples rich in collagen type I, the main protein in bone, dentine, antler and skin (Buckley et al. 2009). It is reliant on the presence of amino acid sequence variation between different species of both the *COL1α1* and *COL1α2* chains that together form the collagen type I triple helix. Such phylogenetically informative positions are termed single amino acid polymorphisms (SAPs; Welker 2018b), analogous to the genetic SNPs (single nucleotide polymorphisms) from which they derive. Furthermore, ZooMS takes advantage of the fact that COL1 is the dominant protein in bone (Wadsworth and Buckley 2014). In addition, COL1 preferentially preserves over time (Welker 2018a), making recovery of this protein possible into the Pliocene for extremely cold environments such as the Arctic (Rybczynski et al. 2013).

As per standard approaches in proteomics, the application of mass spectrometry for ancient protein analysis currently involves the digestion of preserved proteins into smaller peptides by the use of a digestive enzyme. In the case of ZooMS, this is always trypsin (Buckley et al. 2009). The generated peptide mixture will be dominated by peptides originating from COL1, but can also include peptides from other bone collagens or non-collagenous proteins. Next, these peptides are injected into a MALDI-TOF mass spectrometer, which measures the mass and abundance (intensity) of the peptides in the mixture. In this case, only peptide masses (MS1-level) are obtained, and no amino acid sequence information can be deduced directly (MS2-level). Instead, ZooMS utilizes *a priori* knowledge on COL1 SAP variation for a reduced set of peptides, termed peptide markers, to compare the observed masses of these peptide markers with the expected homologues peptide marker masses based on sequence information (Fig. 3, Fig. 4). In general, the observed mass combination of nine different peptide markers is used to assign a taxonomic identity to the studied sample (called P1, P2, A-G; Buckley et al. 2009, Kirby et al. 2013), but some authors have recently suggested using additional peptide markers for specific taxonomic clades (Buckley et al. 2016).

The general ZooMS approach allows individual samples, normally under 10 mg, to be identified taxonomically. As a result, ZooMS is a quantitative approach providing basic

NISP counts of faunal assemblages. This is a distinction compared to sedimentary or bulk bone metabarcoding approaches in the field of ancient DNA (Haile et al. 2009; Murray et al. 2013; Grealy et al. 2015; Pedersen et al. 2015). Such genetic methods also provide information on species presence, but are less quantifiable and do not allow us to refer to a particular bone specimen *post*-identification. Hence, any subsequent multidisciplinary analysis of the studied samples is not possible through these approaches (see below).

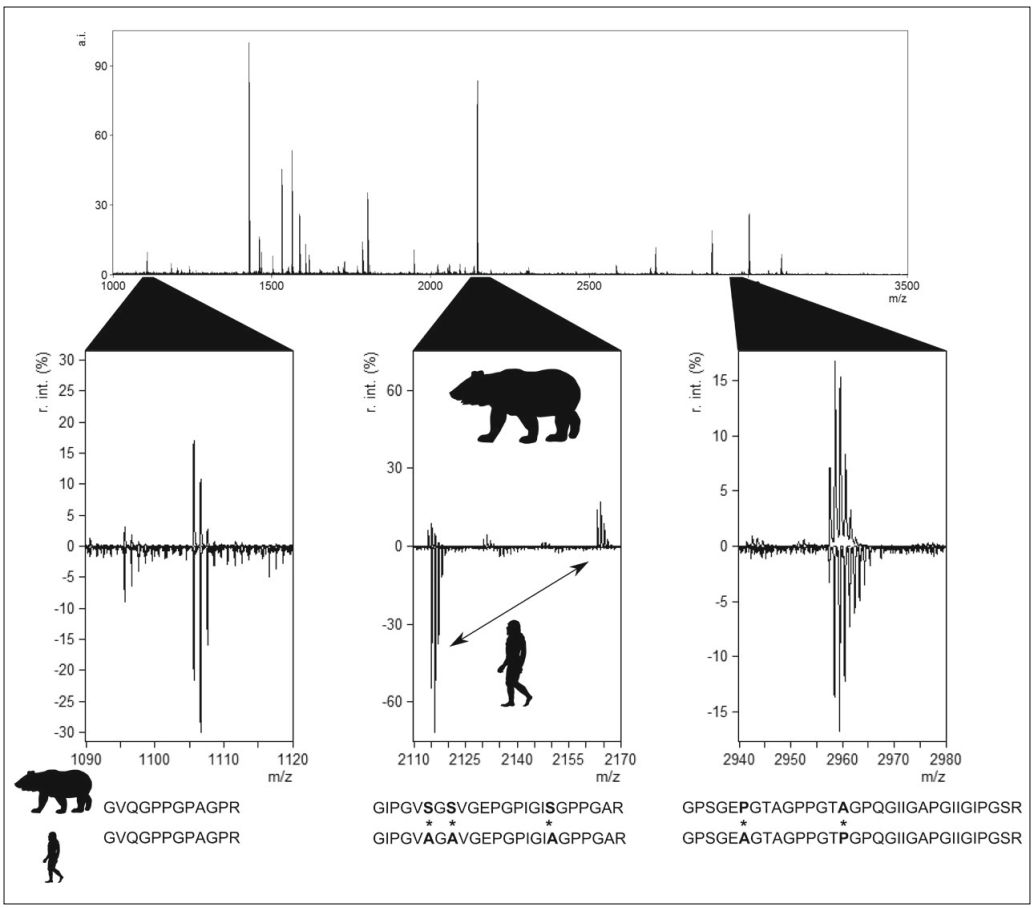

Fig. 3: Collagen type I sequences and peptide marker masses. The top shows a typical MALDI-TOF-MS collagen type I fingerprint (extinct *Equus hydruntinus*). Below are three mass ranges shown spanning three separate peptide marker positions of a cave bear (top) and a human (bottom) at m/z ≈ 1105, m/z ≈ 2115 or 2163, and m/z ≈ 2957. Below each window is the homologous sequence alignment shown for both species. Highlighted in bold are amino acids that differ between the cave bear and the human amino acid sequence. All examples taken from Welker et al. 2016.

Abb. 3: *Kollagen Typ I-Sequenzen und Massen der Peptidmarker. Der obere Teil zeigt einen typischen MALDI-TOF-MS Kollagen Typ I Fingerabdruck (ausgestorbener* Equus hydruntinus*). Darunter sind drei Massebereiche abgebildet, die drei separate Peptidmarker-Positionen bei einem Höhlenbären (oben) und einem Menschen (unten) umfassen, und zwar bei m/z ≈ 1105, m/z ≈ 2115 oder 2163 und m/z ≈ 2957. Unter jedem Fenster ist die entsprechende Reihung für beide Spezies gezeigt. Fett hervorgehoben sind Aminosäuren, die sich zwischen der Aminosäuresequenz des Bären und der des Menschen unterscheiden. Alle Beispiele stammen aus Welker et al. 2016.*

ZooMS taxonomic limits

Because ZooMS is reliant on a set of peptide markers present in both the *COL1α1* and *COL1α2* chains, not all the phylogenetically-relevant sequence variants in both chains are used to deduce the taxonomic identity of a studied specimen. As a result, most species that have identical peptide marker series group together at the (sub)family level. Nevertheless, in some cases more specific identities can be obtained through ZooMS directly. For example, because of a unique SAP, red fox (*Vulpes vulpes*) can be separated from all other Canidae (Fig. 4). These other Canidae cannot be separated from each other, however, as no useful SAPs are present in the peptide markers. Their complete COL1 sequences do accurately reflect their phylogenetic relationships though, nicely illustrating the loss of information that accompanies the use of selected peptide markers (Fig. 4).

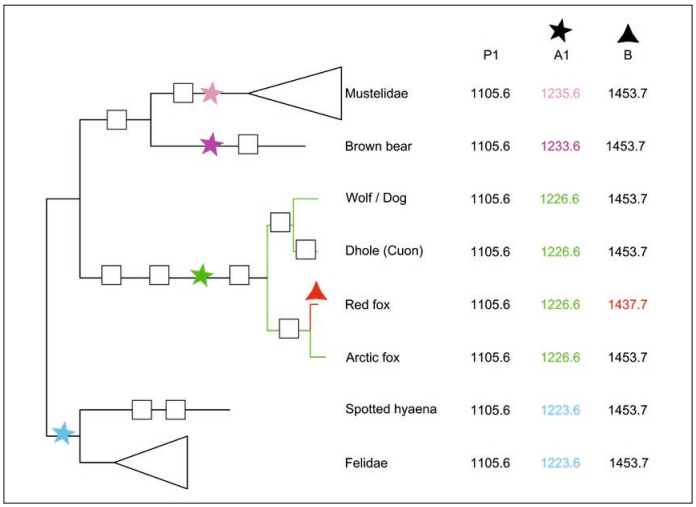

Fig. 4: Example of peptide marker series identification within Carnivora. Phylogenetic tree based on entire triple helical regions of *COL1α1* and *COL1α2*. Due to a unique substitution on the lineage leading to *Vulpes vulpes* (red fox), this species can be distinguished from other Canidae, which cannot be distinguished from each other, based on peptide marker B (triangle). In addition, peptide marker A allows us to differentiate between the major Carnivore families (stars), while peptide marker P1 has an identical amino acid sequence/mass between the carnivore species shown. Some available species and peptide markers are excluded for clarity. Open squares indicate protein sequence variation not used or observable in peptide marker identification of MALDI-TOF-MS spectra. Phylogenetic tree and peptide marker masses are based on publicly available resources (Welker et al. 2016), while SAP variations projected onto the tree are indicative only. Full color version available online: mgfuopenaccesas.org.

Abb. 4: *Beispiel für die Identifizierung von Peptidmarkerreihen innerhalb der Carnivora (Raubtiere). Das Kladogramm basiert auf vollständigen Dreifachhelix-Regionen von* COL1α1 *und* COL1α2. *Aufgrund einer einzelnen Ersetzung auf der Linie, die zu* Vulpes vulpes *(Rotfuchs) führt, kann diese Spezies auf der Basis des Peptidmarkers B (Dreieck) von anderen Canidae (von lat.* Canis = *Hund) abgegrenzt werden, welche untereinander nicht unterscheidbar sind. Darüber hinaus erlaubt es Peptidmarker A, zwischen den Haupt-Raubtierfamilien (Sterne) zu unterscheiden, während Peptidmarker P1 eine identische Aminosäuresequenz/Masse bei den gezeigten Raubtierarten hat. Einige vorhandene Arten und Peptidmarker wurden aus Gründen der besseren Erkennbarkeit ausgeschlossen. Offene Quadrate bedeuten Proteinsequenzvariationen, die nicht bei der Identifizierung der Peptidmarker von MALDI-TOF-MS-Spektren benutzt wurden oder beobachtbar sind. Das Kladogramm und die Peptidmarkermassen beruhen auf öffentlich zugänglichen Quellen (Welker et al. 2016), während die SAP-Variation, die auf den Baum projiziert ist, nur ein Beispiel darstellt. Die farbige Version ist online verfügbar: mgfuopenaccess.org*

Based on additional information known from species distributions, ZooMS taxonomic classifications might be further reduced. An example would be the Cervidae. Deer are notoriously difficult to separate based on ZooMS. One can identify reindeer or roe deer individually, but distinguishing Old World deer (fallow deer, red deer, giant deer) from elk is not possible. Often, these four deer species do not co-occur, and one could argue for excluding one or more species as possibly represented based on temporal or geographic arguments.

ZooMS species composition

As ZooMS is primarily a taxonomic identification tool, it is surprising that most studies utilizing ZooMS focus on the identification or exploration of a particular species. As a result, in most studies the entirety of the bone assemblage identified through ZooMS is neither discussed nor interpreted. One reason for this might be that bone specimens studied through ZooMS are expected to be highly fragmented, as compared to the morphologically identified component of the same site, preventing valid direct comparisons. Another might be that, intuitively, the fragmented bone component at archaeological sites is expected to represent a subset of the species identified through morphological means.

Comparison of the three Châtelperronian bone assemblages studied so far, Les Cottés, Quinçay, and Grotte du Renne, shows that the second assumption is incorrect. ZooMS components of the same assemblages at each site are richer in species than the morphology-identified component (Fig. 5). Additional species are present in herbivores, carni-

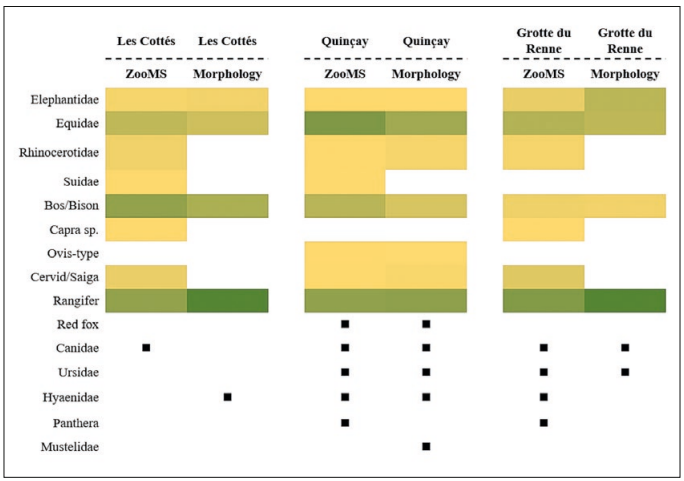

Fig. 5: Species composition of morphology-identified and ZooMS-identified components of the same bone assemblages. Colored cells indicate relative % for that species group per assemblage/identification method (only for herbivores), ranging from dark green (50%) to yellow (1%). Homininae identifications for the Grotte du Renne assemblage are excluded. Full color version available online: mgfuopenaccesas.org.

Abb. 5: *Artenzusammensetzung von morphologisch sowie auf der Basis von ZooMS identifizierten Komponenten derselben Knocheninventare. Die farbigen Felder geben, von Dunkelgrün (50%) bis Gelb (1%), den relativen Prozentanteil für die jeweilige Artengruppe pro Inventar/Identifizierungsmethode (nur für Herbivoren) an. Identifizierte Menschenreste aus der Grotte du Renne wurden ausgeschlossen. Die farbige Version ist online verfügbar: mgfuopenaccess.org.*

vores, smaller mammals and megafauna (for example, Rhinocerotidae at Grotte du Renne). Such additional species are always present at low frequencies, with just a small number of bone specimens. In line with this, in general, species frequencies observed so far are comparable between the morphology-identified and ZooMS-identified components at the three sites. We are dealing with a small number of published assemblages, however, and so it is unclear if these patterns are generally true. If not, as could be the case for bone assemblages where the heavily-fragmented bone component is very different in species composition or species frequency distribution from the morphology-identified component, new interpretative frameworks for ZooMS and zooarchaeology assemblages would then be required. This entails direct involvement of zooarchaeology within ZooMS analysis, and represents the next frontier in ZooMS applications.

ZooMS as a multidisciplinary research tool

ZooMS gives the most basic information about a bone possible – its taxonomic identity. As this can be done for hundreds or thousands of bone specimens in a relatively short time span, ZooMS allows the taxonomic identification of entire small to medium-sized bone assemblages. With this taxonomic information in hand, these previously uninteresting bone specimens now have the ability to become central to a series of research questions frequently asked in paleoanthropological contexts (Fig. 6).

For example, the growing need for large cohorts of ancient genomes at a high spatial or chronological density requires the availability of hominin or human bone specimens at sufficiently dense spatial or chronological scales. Often, such conditions cannot be met in prehistoric time periods because human bones are absent or scarce. In addition, when they are present, they are often deemed so unique that no destructive sampling is allowed. Furthermore, ethical reasons have been raised to stop large-scale sampling or "hoarding" of ancient human remains for future ancient DNA projects (Makarewicz et al. 2017). In such cases, ZooMS screening at sites with known hominin or human remains could be a way to resolve this issue on a collaborative level through the screening for hominin or human remains within the morphologically unidentifiable bone component.

In the archaeological sciences, ZooMS easily interacts with isotopic subdisciplines. For example, bone specimens identified through ZooMS can provide starting points for radiocarbon dating studies of hominins as well as fauna to test their extinction chronologies at more refined time scales (Talamo et al. 2016). This is not only relevant for the MUPT, but also applies to the extinction of megafauna at the end of the last ice age (Meiri et al. 2014; Fellows Yates et al. 2017). Similarly, the identification of entire fauna communities, including novel identifications of species present at low frequencies (Fig. 5), allows dietary modeling of fauna communities through stable isotope analysis at better resolved scales by the incorporation of such small components into the isotopic landscape.

This also works the other way round. Charlton et al. (2016) recently demonstrated that the extraction tubes used during collagen extraction in stable isotope analysis and radiocarbon studies frequently retain enough collagen adhering to their plastic surfaces for successful ZooMS analysis. This approach can therefore easily be implemented in existing studies that utilize collagen, for example to verify the taxonomic identity of bone specimens in a stable isotope dietary framework by molecular means and at no additional costs.

Interdisciplinary research in archaeology, particularly the biomolecular analyses of hominin and human remains, has greatly advanced in the past two decades. To some extent, this has put pressure on available resources for destructive analyses. Although ZooMS does not resolve the destructive nature, either of paleoproteomic research, isotopic research, or ancient DNA research, it does provide an alleviation of this pressure by tapping into the previously and largely unused repository of morphologically unidentifiable bone specimens that are stored and curated in museums and universities worldwide. As a result, exactly because such bone specimens hold no or little morphological value, their true potential lies in the molecular "treasure trove" locked within them.

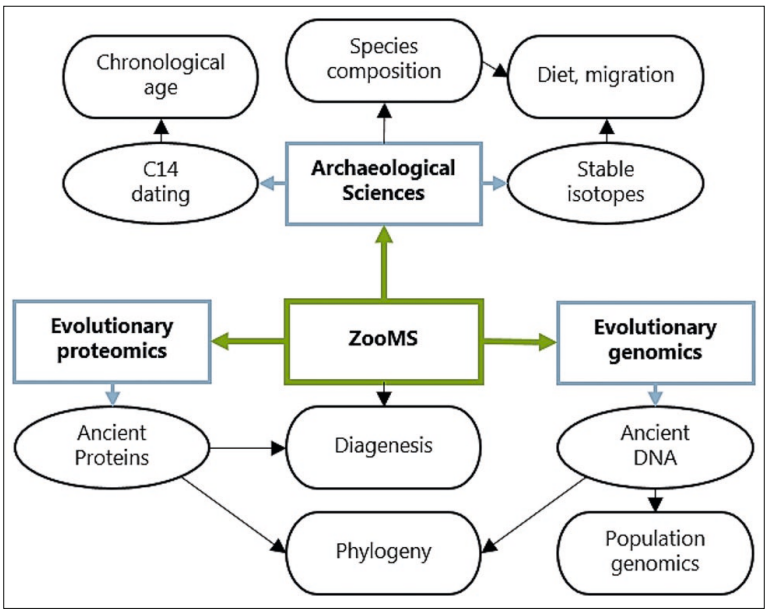

Fig. 6: ZooMS has the potential to play a central role in research projects across archaeological science, evolutionary genomics, and evolutionary proteomics.

Abb. 6: *ZooMS besitzt das Potenzial, eine zentrale Rolle bei interdisziplinären Forschungsprojekten der archäologischen Wissenschaften, der Evolutionsgenomik und der Evolutionsproteomik einzunehmen.*

Full proteome analysis

ZooMS utilizes a single protein, composed of two gene products, to obtain a taxonomic classification. This can be done at a large scale, with high success rates, and with minimal or no sample destruction, as shown above. Hence, ZooMS is now adopted in an ever-growing range of applications in archaeology. Nevertheless, COL1 is just one protein out of many that co-occur in the bone and dentine proteomes. Just like COL1, each of these other proteins has the potential to provide (additional) phylogenetic information.

Modern bone and dentine proteomes are nearly identical. They are both dominated by COL1 but, in addition, are composed of several hundreds of other collagens and non-collagenous proteins (NCPs; Alves et al. 2011; Jágr et al. 2012; Salmon et al. 2013). They facilitate biomineralization of both tissues, but also include proteins deriving from

cartilage, vascular systems, the neural system, and some involved in immune response. All such tissues or processes either occur within the living bone or are spatially associated with it. During life, the bone proteome is therefore a dynamic composition of a large number of interacting proteins (Fig. 7).

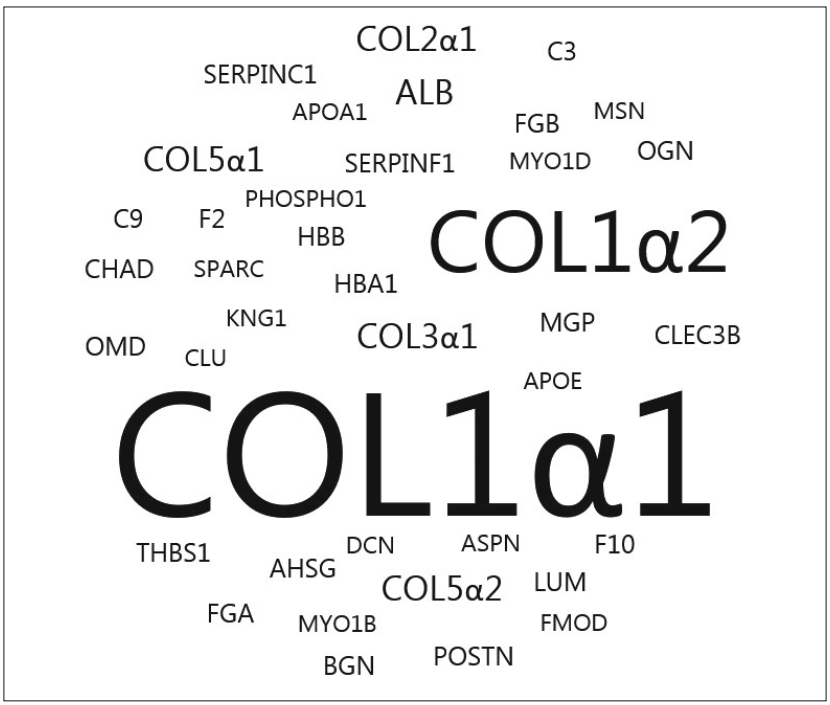

Fig. 7: Schematic depiction of a Pleistocene bone proteome composition. Compositionally, the bone, and dentine, proteome is composed of several hundreds of proteins in life. After molecular breakdown, Pleistocene bones tend to contain several dozens of surviving proteins. These include various collagens and non-collagenous proteins. Quantitatively, the bone proteome is dominated by *COL1α1* and *COL1α2*, the two protein chains that together form the collagen type I triple helix.

Abb. 7: *Schematische Darstellung einer Proteomzusammensetzung pleistozäner Knochen. Was den Aufbau angeht, werden das Knochen- und das Dentinproteom zu Lebzeiten aus mehreren hundert Proteinen gebildet. Nach dem molekularen Zusammenbruch enthalten pleistozäne Knochen in der Regel mehrere Dutzend fortbestehender Proteine. Diese umfassen verschiedene Kollagene und nicht-kollagenhaltige Proteine. Mengenmäßig wird das Knochenproteom von* COL1α1 *und* COL1α2 *dominiert, den beiden Proteinketten, die zusammen die Dreifachhelix des Kollagens Typ I bilden.*

In contrast to MALDI-TOF-MS, tandem mass spectrometry methods such as LC-MS/MS allow probing of the entire proteome in ancient mineralized tissues. In addition, LC-MS/MS enables us to determine individual peptide sequences generated during tryptic digestion (MS2-level). In the past decade or so, this approach has become central to paleoproteomics (Cappellini et al. 2014, 2018). Research has now demonstrated that although COL1 is a protein that survives over long periods of time, other non-collagenous proteins survive into the Pleistocene as well (Cappellini et al. 2012; Wadsworth and Buckley 2014). Furthermore, bioinformatics tools have become available that allow peptide and protein sequences to be determined that are outside the protein sequence

variation currently present in proteomic and genomic databases. This allows the phylogenetic reconstruction of extinct organisms, as demonstrated for ground sloths (Buckley et al. 2015), mammoths (Buckley et al. 2011), an extinct giant beaver (Cleland et al. 2016), the approximate evolutionary relationships of the rhinoceros genus *Stephanorhinus* (Welker et al. 2017a), and a large clade of extinct South American ungulates (Buckley 2015; Welker et al. 2015a).

Full proteome analysis is more costly in terms of lab consumables and sample preparation, LC-MS/MS running costs, and bioinformatics infrastructure than ZooMS and MALDI-TOF-MS. Shotgun proteomics is therefore not currently used to screen large numbers of samples for the presence of specific taxa; however, it is used when applied to particular samples of interest.

For example, Brown et al. (2016), Welker et al. (2016) and Devièse et al. (2017) demonstrate that ZooMS screening provides access to morphologically unidentifiable hominin bone specimens at Paleolithic sites. This directly addresses the need for additional hominins associated with the MUPT. However, ZooMS is not capable of determining whether a bone is a Neanderthal, Denisovan, modern human, or representative of some sort of hybrid or alternative population (Welker 2018a, b). Instead, the taxonomic identification limit for such populations is at the *Pan+Homo* generic level. Welker et al. (2016) went beyond this with LC-MS/MS analysis of one hominin bone identified through ZooMS screening at the important Châtelperronian site of Grotte du Renne (France). Through shotgun mass spectrometry, they recovered a suite of endogenous hominin proteins. For some of these, informative SAPs were identified that reliably separate between Neanderthals, Denisovans and modern humans based on (ancient) genomes available for these populations (Castellano et al. 2014, The 1000 Genomes Project Consortium 2015; Meyer et al. 2016). This paleoproteomic analysis indicated that at least part of the nuclear ancestry of the Grotte du Renne Châtelperronian hominins derived from Neanderthals (Welker et al. 2016; Welker 2018a, b). This was further supported by ancient mtDNA analysis of the same specimen. With additional stable isotope data and direct radiocarbon dating, the Grotte du Renne Châtelperronian hominins currently represent the only available MUPT hominins directly associated with archaeology that are both temporally and molecularly characterized.

Conclusions

Our understanding of the Middle to Upper Paleolithic transition in Europe is complicated by high bone fragmentation rates, greatly limiting our understanding of human-animal interactions as well as hominin presence in space, time, and in terms of cultural association. This problem has persisted for over a century, despite advances in excavation and analytical methods. Recently, fast and cheap taxonomic identification of fragmentary, morphologically unidentifiable bone specimens has become feasible through collagen type I peptide mass fingerprinting (ZooMS). While adopting mass spectrometry into paleoproteomics, which enables us to obtain amino acid sequences of entire hominin bone proteomes, ZooMS has now been demonstrated to be game changing in our biological understanding of the MUPT. ZooMS thereby provides access to new hominin MUPT fossils at sufficiently resolved spatial and chronological scales, making detailed (replacement) population dynamics understandable in the near future.

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