# Integrating palaeoproteomics into the zooarchaeological analysis of Palaeolithic bone assemblages

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Cover design: Human toddler hands holding bone fragments in front of a landscape with *Bison priscus*, *Cervus elaphus* and *Equus ferus.* Design by Virginie Sinet-Mathiot and Anna Goldfield, Drawing by Anna Goldfield.

*“Fossils are the remains of single organisms,*
*but they are also pieces of much larger,*
*more complex,*
*and fascinating puzzles:*
*the ecosystems of the past.”*

**Behrensmeyer, 1980, “Fossils in the making”**

# Contents

Chapter One

# Introduction

After *Homo sapiens* moved out of Africa they spread across the globe, reaching western Europe around 50,000 years ago. About 10,000 years later, traces from local populations of Neanderthals, who had been occupying these territories for more than 400,000 years, disappeared from most of western Europe (Higham et al., 2014; Hublin, 2015). Understanding the complex patterns of interactions between these populations are central to assessing the development of modern groups and the reasons why local hominin groups went extinct, while considering that interactions may have ranged from mutual exclusion to admixture, including several episodes of interbreeding (Fu et al., 2015). In Europe, the period of transition between the Middle Palaeolithic cultural complexes (MP), biologically assigned to Neanderthal groups, and the Upper Palaeolithic, starting with the Initial Upper Palaeolithic (IUP) technologies and biologically assigned to *Homo sapiens* is denominated as the Middle to Upper Palaeolithic transition (MUPT). The spread of Upper Palaeolithic *Homo sapiens* groups in western Europe alongside the progressive extinction of Neanderthals and the genetic interactions between these two populations has led researchers to address this transitional phase, seeking to explore potential behavioural and cognitive differences eventually leading to the predominance of one taxon over the other (Clark & Speth, 2013). The emergence of behavioural complexity and changes in the lithic technology have possible consequences on resources procurement. Investigating shifts and prey selection variability within the context of transitional periods during human evolution contributes to a better apprehension of past hominin behaviour and potential differences between human groups. However, Late Pleistocene bone assemblages are most often highly fragmented, as the result of taphonomic processes or the action of accumulation agents, limiting the amount of morphologically identifiable material for interpretation.

Obtaining a clear understanding of human subsistence practices requires the assessment of bone assemblages in their *entirety*. Fragmented and morphologically unidentifiable bone components have constituted, up to now, an extensive taxonomically uninformative proportion of Pleistocene bone assemblages. Nonetheless, this component potentially contains information about hominin behaviour not previously included in interpretations related to subsistence strategies, possibly restraining our understanding of behavioural patterns occurring at certain sites. The recent development of biomolecular methods, for example proteomic analysis through Zooarchaeology by Mass Spectrometry (ZooMS), provides the opportunity to assign taxonomic information to bone remains for which this has

not been possible using traditional methods. Up to now, ZooMS screening studies have largely focused on the identification of additional hominin specimens, generally representing less than 1% of bone remains found on an archaeological site. The other 99% of specimens identified have been used most often to answer ecological questions, trying to improve the faunal spectrum or documenting the spread of domesticated species (Buckley et al., 2017; Coutu et al., 2021; Welker et al., 2016; Welker et al., 2015). However, investigating the relationship between faunal composition and bone fragmentation, in particular, remains to be explored and what such patterns might mean in terms of past human subsistence behaviour during transitional periods such as the MUPT in Europe.

This first chapter will briefly introduce background information on zooarchaeology and human subsistence during the MUPT, and will present basic concepts of Zooarchaeology by Mass Spectrometry (ZooMS) as well as outlining the aims of this dissertation.

# 1. Reconstructing human subsistence and diet

Since the eighteenth century, ancient animal remains have raised questions about human (pre)history (Grayson, 1983). The discovery of artifacts and human bones associated with extinct fauna led to new perspectives on the antiquity of the human species (de Perthes, 1847; Lubbock, 1865), and by the mid-nineteenth century, vertebrate remains started to play a major role in the reconstruction of past environments and human diet. The analysis of archaeofaunal remains aims to contribute to the archaeological investigation of human evolution (Steele, 2015) and to provide evidence of past population subsistence behaviour (Reitz & Wing, 2008)). Thus, subsistence has become crucial to understanding past human group's relationships with their environments, and the technologies they create to exploit it, as well as their relation to each other on a social and economic level. Humans respond to interactions between themselves and their environment in a variety of ways resulting in different subsistence strategies (Peres, 2010). The study of animal remains from archaeological sites aims to address changes in subsistence behaviours through time and space, and to explore the patterns that influenced them. More specifically, the faunal record offers an invaluable resource for examining human responses to climate changes and the possible impact of technological variation on hunting and processing strategies (Clark & Speth, 2013).

## 1.1. Vertebrate zooarchaeology: definitions and role

In 1865, Sir John Lubbock used for the first time the term “zoologico-archaeologists” to designate specialists studying animal remains (Lubbock, 1865). Defined in 1971 by Olsen (Olsen, 1971), the term ‘zooarchaeology’ refers to the analysis and interpretation of faunal remains from archaeological deposits. Vertebrate zooarchaeology consists of integrating the analysis of vertebrate remains, such as mammals, fish and birds, into a multi-methodological approach to answer questions about human-animal interactions (Reitz et al., 1999). The work presented in this thesis will focus on mammals as they represent the most extensively exploited class of animals by human groups and are, generally, the most prevalent remains on archaeological sites.

Zooarchaeological analysis of faunal remains provide a wealth of information related to site formation and chronology, relationship between humans and other species with their environment, and behavioural strategies related to food procurement and raw material exploitation (e.g., Behrensmeyer, 1983; Broughton, 2015; Gifford-Gonzalez, 2018). These inferences are based on primary data such as taxonomic identification, skeletal identification (bone element or body part), age at death, health and sex of the animal, as well as the presence and type of bone surface modifications. Through the interpretation of secondary data (e.g. relative species frequencies, patterns of butchery, proportions of bone accumulators), the analysis of bone assemblages provides substance for the understanding of how past human groups were selecting and acquiring resources from the surrounding environment, and how these assemblages have been modified during burial.

## 1.2. From the biosphere, through the lithosphere to present time

Due to their high inorganic mineral content, bones, teeth and antlers can survive over time and represent some of the most abundant finds from archaeological sites. The accumulation of faunal remains resulting from various agents (e.g. carnivores, humans, environment) form the faunal assemblages. However, due to the impact of these various agents faunal assemblages may not accurately reflect either the living faunal community around an archaeological site during human occupation or human subsistence behaviour itself. Indeed, the formation of these assemblages pass through different stages (*life, death, deposited, fossil* and *sampled assemblage;* **Figure 1**), increasing the sample bias over time (Behrensmeyer, 1984; Brain, 1981; Grayson, 1984; Katzenberg & Grauer, 2018; Kidwell & Flessa, 1995; Lawrence, 1968; Lyman, 1994b). Understanding these biases provide a clearer picture of the site formation process and the human involvement. The initial stage is the *life assemblage* represented by the taxa available in the immediate environment of

the archaeological site. This assemblage is defined by the social structure of the herds (age and sex) and their distribution around the year, influenced by the climatic and vegetation conditions. The *death assemblage* is represented by the community of animals hunted or scavenged by human groups and carnivores. The composition of this assemblage relies directly on the hunting choices taken by humans and carnivores. The *deposited assemblage* corresponds to faunal remains processed and deposited at a site. The deposition can be selective and depend on a number of factors including the accumulation agents and the intensity of their action on faunal remains. The *fossil assemblage* represents the deposited component that was preserved over time until its discovery during the excavation. The survivorship and preservation of the remains are determined by the burial environment and a number of 'diagenetic parameters'. These involve both mechanical and chemical factors, such as porosity and crystallinity, potentially altering the nature of the specimen, as well as biomolecular information it may contain, e.g. the collagen content (Hedges, 2002; Hedges et al., 1995). Moreover, not all skeletal elements nor every taxa will preserve in the same way, which is also called 'differential preservation'. The processes leading to the formation of the fossil assemblage can generate a bias in the composition of a faunal assemblage that can be independent from the death and deposited assemblages. Assessing these processes provides the possibility to untangle hominin behavioural signatures. Finally, the *sampled assemblage* refers to the material excavated, recorded, washed, stored, manipulated and analysed by one or several zooarchaeologists/ palaeontologists. The degree of representativity of the initial fossil assemblage depends on the methods of excavation and recording of the finds (Brain, 1969; Lyman, 1994b; Lyman, 2004). In addition, the biomolecular preservation of the remains also depend on post-discovery practices, e.g. consolidation treatment processes or surface cleaning using chemicals or organic glues and varnishes possibly altering the collagen content of the specimen (Le Cabec & Toussaint, 2017).

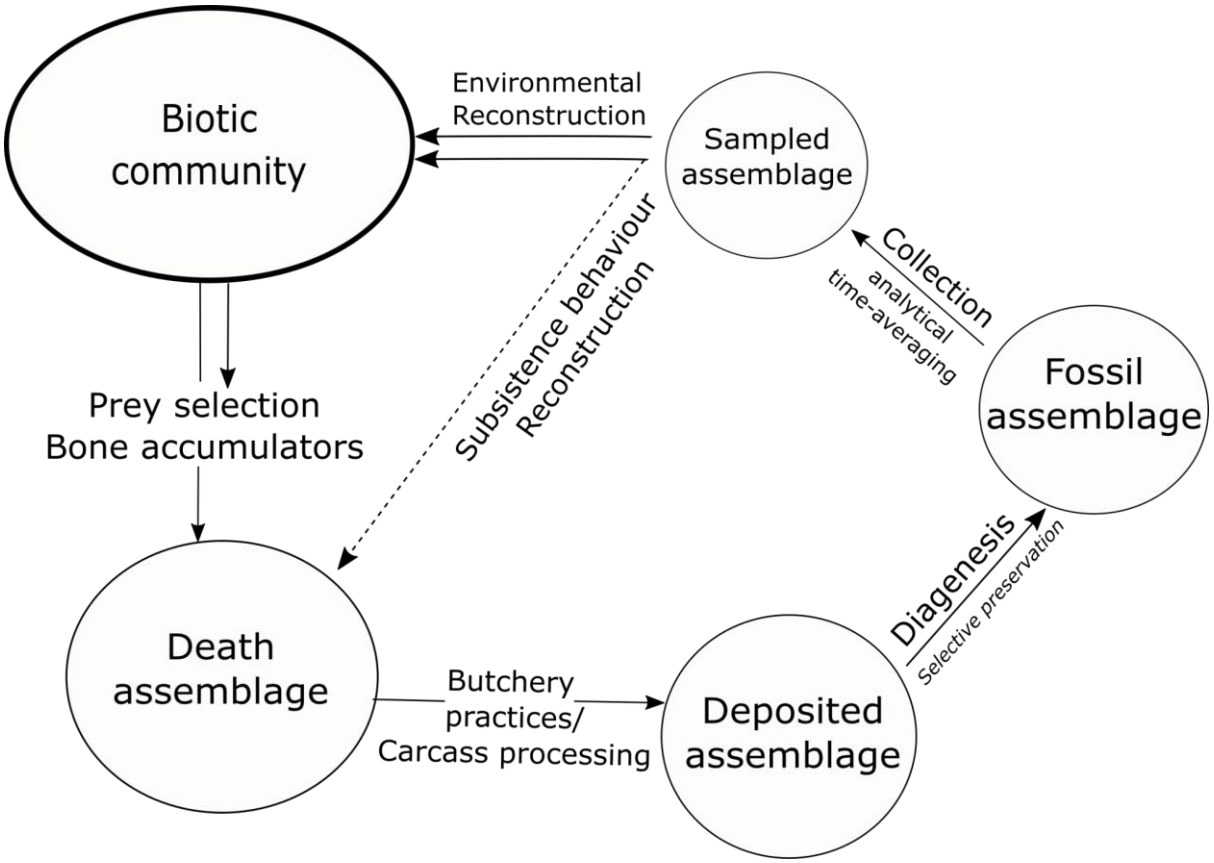


**Figure 1:** Simplified schematic representation of the taphonomic processes affecting the integrity of Palaeolithic faunal assemblages. Modified after Kidwell & Flessa, 1995, and Lyman, 1994b.

## 1.3. Bone modification agents

Identifying the agents of surface modification and bone accumulation is key to relate these bone specimens to past human activity and behaviour. Several organic and inorganic processes affect the preservation of faunal remains from the death of the animal until studied by zooarchaeologists (Brain, 1981; Rogers, 2000; Villa & Mahieu, 1991). Zooarchaeology relies heavily on taphonomic analysis. Initially defined as a subfield of palaeontology (Efremov, 1940), taphonomy is the discipline studying all events occurring between the death of an organism and the eventual discovery of the fossil remains or traces of this organism (Lyman, 1994b). It refers to the study of the action of agents affecting the remains of past living organisms constituting the prehistoric record (Fernandez-Jalvo & Andrews, 2016). These taphonomic agents generate modifications of the specimens (Lyman, 1994a) which can inform on the archaeological context and conditions during the decay and burial of the remains. Indeed, each agent generates diagnostic traces specific to their type of interaction with bone specimens. However, different agents can lead to

equifinality in signatures, often resulting in debates among taphonomists (Behrensmeyer et al., 1986; Blumenschine et al., 1996; Domínguez-Rodrigo et al., 2017; Olsen & Shipman, 1988) Determining the taphonomic history of a bone assemblage provides a better understanding of the formation of the archaeological record and of the impact of these processes on the integrity of the faunal assemblage. The identification of the causal agents behind the bone modifications is key for the recognition of potential sources of biases for subsequent measures and interpretations (Binford, 1978; Gifford-Gonzalez, 1991; Lyman, 1987; Upex & Dobney, 2020).

When considering Palaeolithic faunal assemblages, various taphonomic agents can cause bone modifications. These agents include natural processes such as weathering, geological or other mechanical forces, soil pH and water content, insects, plants and fungi, temperature changes, and biological agents. In addition, human butchering and processing practices can generate modification, resulting notably in the presence of cut marks, scrape marks, percussion traces, burning, and non-human activity from carnivores or rodents (Backwell et al., 2022; Binford, 1981; Blumenschine et al., 1996; Fisher, 1995; Lyman, 1994b; Olsen & Shipman, 1988; Soulier & Costamagno, 2017). These modifications can consist of alteration to the surface or the shape of the specimen, but also penetrate the bone tissue and cause erosion (Fernandez-Jalvo & Andrews, 2016). Bone modifications provide evidence for the reconstruction of human activities on a site through the identification of traces associated with human action. As an example, body part representation, the occurrence and location of cut marks, and percussion traces can inform about specific transport decisions and if certain resources were particularly targeted (Morin & Ready, 2013). The involvement of human groups in the formation of the bone assemblage and interpretations around subsistence behaviour and diet is made possible through the recognition of hominin behavioural signatures and the attempt to extricate the potential relative contribution of humans, carnivores and other bone accumulating agents (Domínguez-Rodrigo et al., 2014; Grayson & Delpech, 2003; Lyman, 2004; Marean et al., 2000; Patou-Mathis, 2000).

The effect of these taphonomic factors on bone preservation can ultimately lead to bone fragmentation. Addressing the fragmented portion of a bone assemblage requires the implementation of bone surface modification analysis on every fraction of a bone assemblage in order to have a better understanding of the origin of all components of the archaeological record.

## 1.4. Bone fragmentation in Palaeolithic faunal assemblages

Several pre- and/or post-depositional taphonomic processes, which are not mutually exclusive, can lead to high bone fragmentation particularly in the context of Palaeolithic bone assemblages. The characterisation of the fragmentation patterns through zooarchaeological analysis aims to recognize the taphonomic processes involved and to identify the bone accumulators and potential human activities.

Such patterns of fragmentation may be caused by natural actions among which high stages of weathering, effects related to the environmental conditions prior to burial of the remains (Behrensmeyer, 1978; Madgwick & Mulville, 2012), gelifraction or the impact of frost (Pokines et al., 2016), sediment compression (Müller et al., 2018), and can provide details about site formation and depositional processes (Bartram et al., 1999; Bonnichsen, 1979; Lam & Pearson, 2005; Madgwick & Mulville, 2015; Nielsen-Marsh et al., 2007; Stiner, 1994). Bone breakage can be induced by non-human biological agents (e.g. carnivores/other animals) through trampling and gnawing (Marean, 1991), but also resulting from excavation, transport and storage of the remains (Behrensmeyer et al., 2000; Brugal, 2017; Durocher et al., 2022). Moreover, fragmented assemblages can infer a wide range of anthropogenic activities and human behaviours notably related to butchery practices: the production of worked bones (Gummesson et al., 2019; Smith & Poggenpoel, 1988), the percussion of bones for marrow extraction (Vettese et al., 2020), and the use of bone as fuel or skeletal fat rendering (Bovy et al., 2019; Morin, 2010, 2020; Morin & Soulier, 2017; Outram, 2001; Théry-Parisot et al., 2005; Todd & Rapson, 1988). Due to their high calorific value, bone fats play an important role in subsistence economies. The exploitation of bone fat usually requires the breakage of the medullary cavity for the extraction of bone marrow from the long bone shafts, and separation of the epiphyses for the extraction of grease from the spongious bone, while the bone is relatively fresh. Thus, the intensity of the exploitation can be reflected by the degree of fragmentation related to fat exploitation activities (Outram, 2002). Certain taxa such as reindeer store large quantities of bone marrow fat relative to their body size (West, 1996). As a result, these fragmented and morphologically unidentifiable fragments may carry information about bone marrow and grease exploitation, and can be of high interest for the interpretation of past subsistence strategies related to bone fat exploitation (Outram, 2001)

However, such highly fragmented bones are less likely to contain morphologically diagnostic features, affecting their anatomical and taxonomic identification (Lyman, 2002; Morin et al., 2017a). Three attributes can be identified on faunal specimens: the skeletal element from which the fragment originates, the taxonomic group, and possible surface

modifications retracing its taphonomic history (Driver et al., 2011). The attribution of the bone fragment to an anatomical part will be necessary to assign the identified element to a taxonomic group. However, fragmentation of long bones or ribs, notably, tends to generate diaphysis fragments which do not contain many characteristic features, thus leading to undiagnostic specimen morphology. Without the possibility to identify a bone specimen, they are categorised as unidentifiable or assigned to body size classes (Lyman, 2002). In addition, differences of identifiability between anatomical parts and taxa can ultimately affect measures of quantification, representing another source of bias for the interpretation of Palaeolithic faunal assemblages (Morin et al., 2017a).

Late Pleistocene bone assemblages are most often highly fragmented (Villa et al., 2004) resulting in a small proportion of remains identifiable using traditional methods through visual comparison of the bone morphology, for example at Pech de l'Azé IV (France) (Niven, 2013), at Abri Peyrony (Martisius et al., 2015), or Les Pradelles (Costamagno et al., 2006) (other examples include Gaudzinski, 1996; Niven, 2007; Terlato et al., 2019). Previous studies of past human behaviour relies on morphologically identifiable fauna which, in many cases, represent a small portion of the complete sampled bone assemblages uncovered in a site, leaving a potentially incomplete picture of human subsistence (Dirrigl & Frank, 2002; Marean, 1991; Marean & Kim, 1998; Morin, 2004; Morin et al., 2017a, 2017b; Pickering et al., 2006). Thus, bone fragmentation leads to a loss of taxonomic identification but also of hominin behavioural information and with the interpretation based on a relatively small proportion of identifiable remains.

**Figure 2**: Example of morphologically unidentifiable bone remains. Bone material from Layer 1 Collection Gilles, Abri du Maras, France. Credit: Virginie Sinet-Mathiot.

## 2. Hominin subsistence during the Middle to Upper Palaeolithic Transition (MUPT)

The Middle to Upper Palaeolithic Transition, starting around 47,000 years BP (Fewlass et al., 2020; Hublin et al., 2020), is marked by the replacement of local populations of Neanderthals by modern *Homo sapiens* groups across western Eurasia. This crucial period during recent human evolution in Europe has been perceived as a period of changes marked by important biological and behavioural changes and the development of innovations, both cultural and technological (Mellars, 1989). The abrupt aspect has been smoothed out in favour of a progressive emergence of modern behaviours (d'Errico, 2003; d'Errico et al., 2009; Teyssandier et al., 2010; Zilhão et al., 2010). While it is now widely accepted that modern humans are not derived from local archaic forms, interactions between the two populations have been attested by the identification of a gene flow from Neanderthals into modern *Homo sapiens* (Fu et al., 2015; Prüfer et al., 2014). Some scholars considered the MUPT as a period of "human revolution" (Bar-Yosef, 1998; Binford, 1985; Klein, 1989, 1995, 2008; Mellars, 1996b; Mellars, 1989; Noble & Davidson, 1991; Tattersall, 1995; Trinkaus, 1989), while others have proposed alternative models emphasising the emergence of so-called "behavioural modernity" in the late Middle Stone

Age of Africa and possibly in the late MP of Europe. Central to these debates lies a critical difference between the two continents: Europe witnessed a major population replacement (Neanderthals by modern *Homo sapiens*), while in Africa there is essentially a biological continuity of the human peopling.

The archaeological record from this period has played a key role in discussions related to the detection of signs of behavioural modernity (d'Errico, 2003; Henshilwood & Marean, 2003; Klein et al., 1999; Mcbrearty & Brooks, 2000; Wadley, 2001). Such evidence is illustrated by the multiplication of symbolic behaviours and archaeological manifestations of complexity, such as the manufacture of bones and teeth for personal ornaments and tools (Martisius et al., 2022; Vanhaeren & d'Errico, 2006), the proliferation of a symbolic culture with figurative art (both parietal and portable), geometric signs or musical instruments (Conard et al., 2009; White et al., 2012), or the change in lithic technological complexity and appearance of stereotypical lithic artefacts and long distance projectiles used for hunting (O'Driscoll & Thompson, 2018). The implications of these findings have been widely discussed regarding their inference with various aspects of behavioural complexity, such as the use of symbols and abstract thinking (d'Errico & Henshilwood, 2011; Mcbrearty & Brooks, 2000; Nowell, 2010; Wadley, 2006, 2010) or the appearance of language (d'Errico et al., 2009; d'Errico & Henshilwood, 2011). Some scholars attribute cultural changes associated with Neanderthals to a parallel development towards "cultural modernity", independently of any cultural changes observed among modern *Homo sapiens* throughout their expansion across Europe (d'Errico, 2003). However, cultural interactions between local populations of Neanderthals and modern *Homo sapiens*, introducing new behaviours and techniques across Europe through their progressive migration from the Levant/Africa, might have influenced the development of certain cultural changes (Hublin, 2012, 2015; Hublin et al., 1996; Roussel, 2013; Soressi & Roussel, 2014).

Behavioural variability has historically been linked to distinct lithic production systems, and understanding what influences these changes is essential to assessing cultural variation and population dynamics among Pleistocene groups. Regional-specific transitional technocomplexes are defined on the base of their chronostratigraphic intermediate position between Middle Palaeolithic Mousterian and Upper Palaeolithic Aurignacian assemblages, but chronologically overlapping with the Initial Upper Palaeolithic and the beginning of the Aurignacian complex. The Initial Upper Palaeolithic relates to the first known dispersal of modern *Homo sapiens* out of Africa and takes its origin in southwest Asia. The transitional notion of these lithic industries rely on the identification of shared features from both of these assemblages, although to varying degrees (Ruebens et al., 2015). These techno-complexes are distributed in limited territories across Europe: the Châtelperronian (present

across central-east of France until northwestern Spain), the Uluzzian (present in Italy and on the west coast of Greece), the Szeletian (present in Czech Republic and Hungary), the Lincombian-Ranisian-Jermanowician (present across northern Europe including the south of the United Kingdom), the Bohunician and the Bachokirian (both present in eastern Europe, notably in the Balkans). The direct association of these industries with a particular biological maker is rare, and has only been described for the Châtelperronian with the identification of Neanderthals remains at Grotte du Renne (Bailey & Hublin, 2006). The Châtelperronian assemblage is characterised by Châtelperron points and the presence of personal ornaments and worked bones (d'Errico et al., 2003; Granger & Lévêque, 1997). The recent dating between 46,790 and 42,810 cal BP of *Homo sapiens* remains associated with an Initial Upper Palaeolithic industry at Bacho Kiro Cave extend the period of contact between Neanderthals and modern *Homo sapiens* in eastern Europe (Hublin et al., 2020).

The emergence of behavioural complexity and changes in the lithic technology occurs in a period of fluctuating climatic and environmental conditions. The Late Pleistocene corresponds to the last 100 ka-climatic cycles, comprising several Marine Isotope Stages (MIS 5-2) and is marked by an alternation of abrupt warming and cooling events (Sánchez Goñi, 2022). During the Middle to Upper Palaeolithic transition (MIS 3), a climatic deterioration occurs during the Upper Palaeolithic (Banks et al., 2013; Sánchez Goñi et al., 2013), as arctic species slowly replace temperate woodland and cold steppic species in the archaeological record (Discamps et al., 2011; Rendu et al., 2019). Considering that primary consumers, such as herbivores, depend on the availability of vegetal resources, climatic shifts can cause significant ecological changes (Rivals et al., 2022). Understanding how these climatic fluctuations might have impacted human ecology can enable us to determine whether the groups changed their species procurement or made deliberate choices related to subsistence strategies. Although the contemporaneity between climatic and archaeological events does not necessarily imply that one was the cause of the other, understanding subsistence behaviour during a period of environmental change is key to address changes of hunting strategies and the emergence of new cultures.

Faunal exploitation is related to a wide range of behaviours and cognitive capacities such as mobility, social organisation and technological development. Thus, investigating shifts and prey selection variability lead to the assessment of past hominin behaviour. Ungulates, such as wild horse, reindeer, large bovine, red deer and fallow deer, or occasionally caprines, wild ass, saiga and woolly mammoth, were the basis of the economy of these groups. These taxa were not consistently hunted in the same proportions, and the regional variability in human diet seen during this time period seems to be more related to changes in the abundance of large herbivores rather than shifts in the spectrum of hunted prey

(Morin et al., 2016; Yravedra-Sainz de los Terreros et al., 2016). Such consideration has led some authors (Klein 1998) to describe a change in some of the ungulate abundance as an indicator of hunting skills.

Even though methodological frameworks have improved, the question of how Late Pleistocene hominins lived and subsisted continues to be debated, notably in relation to the identification and the timing of the emergence of 'behavioural modernity' (Bar-Yosef, 2004; Burke, 2000; Burke, 2004; d'Errico, 2003; Discamps et al., 2011; Gaudzinski, 2006; Grayson et al., 2001; Grayson & Delpech, 2002, 2006; Marean & Assefa, 1999; Mcbrearty & Brooks, 2000; Mellars, 1996b, 2004; Münzel & Conard, 2004; Speth, 2004; Stiner, 2001; Straus, 2013). In terms on subsistence, several signatures have been proposed to indicate a behavioural modernity (d'Errico, 2003; Henshilwood & Marean, 2003; Klein, 1989; Mcbrearty & Brooks, 2000; Mellars, 1996a; Mellars, 1989) such as the emergence of specialised hunting (Mellars, 1973, 1989; Mellars, 2004), the use of throwing weapons (Shea, 2009), a more diverse diet with the exploitation of small game and marine resources (Álvarez-Fernández, 2011; Marean et al., 2007; Richards et al., 2001; Stiner et al., 2000), or the optimised exploitation of animal resources through seasonal hunts and a more exhaustive exploitation of carcasses (Binford, 1984; Klein, 1995). Many scholars have discussed the occurrence of these criteria among Middle Palaeolithic and Upper Palaeolithic faunal assemblages (Costamagno et al., 2006; Gaudzinski, 2000, 2006; Gaudzinski & Roebroeks, 2000; Gaudzinski-Windheuser et al., 2014; Grayson & Delpech, 2002, 2006; Klein, 1995, 2003, 2008; P. Mellars, 1973, 1989, 1996b; Mellars, 2004; Morin, 2004, 2008, 2012; Rendu, 2007, 2010; Rendu et al., 2012, 2019; Smith, 2015; Smith et al., 2021).

Thus, taxonomic abundance plays a major role for the investigation of diet variability, subsistence behaviour, prey selection and environmental adaptation. Faunal assemblages showing a single dominant taxon, based on the morphological identification of bone specimens, and denominated as monospecific, have been argued to result from "specialised hunting". In Upper Palaeolithic contexts, this concept refers to the communal hunting of large numbers of animals and the processing of their meat for storage (Costamagno et al., 2006; David & Enloe, 1993), and potentially imply a drastic change in hunting strategies and group organisation. Some scholars have argued that this specific hunting strategy has been brought by modern *Homo sapiens* through their expansion across Europe (Costamagno et al., 2006; Mellars, 1996b; White, 1989), hence the progressive multiplication of reindeer-dominated faunal assemblages throughout the MUPT. Specialised hunting is often used as a criterion to differentiate the MP generalised hunting from the UP specialised hunting. In numerous Mousterian sites, however, a single

taxon dominates the faunal assemblage, as it does at La Borde [Citation error], Mauran (Farizy et al., 1994), Coudoulous I (Jaubert et al., 2005), Jonzac (Airvaux, 2004) or Salzgitter (Gaudzinski & Roebroeks, 2000). Nonetheless, such specialised hunting strategies associated with Neanderthal occupations are still widely debated (Binford, 1982; Mellars, 1996a; Morin, 2004). The causal association between deliberate selection of a particular taxon and behavioural modernity has been questioned (Stanford, 1995). The difficulty to identify the stratigraphic signature of a mass kill event compared to the repetitive individual hunting episodes of a specific taxon (Speth, 2004) is problematic as they do not require the same set of skills and group organisation. Nonetheless, the formulation of such a hypothesis rests upon the ability to securely identify bone fragments to species using comparative bone morphology. Obtaining a secure interpretation of a faunal assemblage requires understanding the context of these bone assemblages and more specifically their formation and exhaustive taxonomic composition.

# 3. Palaeoproteomics in archaeology

Palaeoproteomics refers to the field dedicated to the identification and study of ancient proteins retrieved from archaeological, historical, palaeoanthropological, palaeontological remains and environmental samples (Hendy, 2021; Hendy et al., 2018; Richter et al., 2022; Warinner et al., 2022; Welker, 2018). Multiple approaches exist to identify ancient proteins such as amino acid analysis (Abelson, 1954), immunoassays (Child & Pollard, 1992), peptide mass fingerprinting (Ostrom et al., 2000), and liquid chromatography–tandem mass spectrometry (LC-MS/MS). The application of palaeoproteomics in archaeology and evolutionary anthropology ranges from the phylogenetic reconstruction of extinct species to the investigation of past human diets and lifeways, or can provide insights into prehistoric and historic material culture. Although ancient DNA has been and will remain a highly informative source of biological information, recent years have witnessed the development of alternative biomolecular methods for species identification which analyses proteins, more stable than DNA, that survive in organic remains (Cappellini et al., 2014; Collins et al., 2010).

## 3.1. Collagen: structure and interests

Taxonomic identification methods require biomolecules that differ between taxa, and their resolution will be determined by the degree of taxonomic variation. As proteins are coded by DNA, amino acid sequences of a protein are directly specified by the gene sequence, through the translation of a mRNA (copy of a portion of DNA corresponding to one or more genes of a biological organism) into amino acids in the ribosomes. Mutations of the gene sequences

over evolutionary time causing variation in the protein sequence, are of interest for taxonomic identification and phylogenetic analyses. Collagen type I represents the major protein in connective tissues (Henriksen & Karsdal, 2016) and consists of a triple helix made from three polypeptide α-chains (COL1α): two identical ones, COL1α1, and one slightly different in its chemical composition, COL1α2 (Cowan et al., 1955; Rich & Crick, 1955; Vuorio & de Crombrugghe, 1990). Both COL1α1 and COL1α2 result as protein sequences from two different genes. These triple helices are assembled into microfibrils, bundled together to form the fibrils which compose the collagen fiber (**Figure 3**). The high resistance of the structure of the protein is enhanced by the repeated amino acid motif in the sequence (Glycine-Proline-X or Glycine-X-Hydroxyproline, X being any various other amino acid), and through hydrogen bonding provided by hydroxyprolines (Némethy & Scheraga, 1986; Szpak, 2011). Collagen varies between organisms as its sequence possesses enough amino acid variation allowing for taxonomic discrimination. This long-term preservation biomolecule persists in archaeological samples and is routinely extracted for radiocarbon and stable isotope analyses (Buckley, 2018). Thus, the protein collagen type I is phylogenetically informative (Welker et al., 2015), easily accessible, and survives beyond the temporal preservation range of ancient DNA (Demarchi et al., 2016; Rybczynski et al., 2013), which strengthens its advantage for an application on Palaeolithic material.

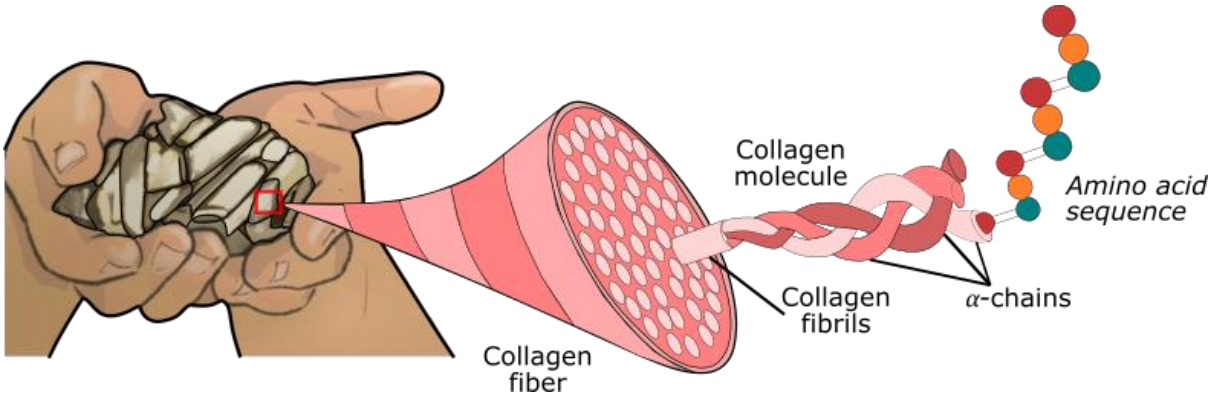


**Figure 3:** Structure of collagen type I. Drawing “bones in hands” credit: Anna Goldfield

## 3.2. Zooarchaeology by Mass Spectrometry (ZooMS)

Peptide mass fingerprinting (PMF) is a technique of protein identification using soft-ionization mass spectrometry such as Matrix Assisted Laser Desorption Ionization - Time of Flight Mass Spectrometry (MALDI-TOF MS), developed in the 1990’s (Aebersold & Goodlett, 2001; Ostrom et al., 2000; Pappin et al., 1993), and primarily used to identify organic glues and milk or egg-based paint blinders in art work (Hynek et al., 2004; Kuckova et al., 2007). This

method was then developed as a taxonomic screening tool to identify fragmentary and morphologically unidentifiable remains, or so-called Zooarchaeology by Mass Spectrometry (ZooMS) (Buckley et al., 2009). ZooMS relies on the abundance of collagen type I in the vertebrate kingdom as well as the archaeological record (Shoulders & Raines, 2009), and its variation between organisms.

ZooMS was first developed slightly more than a decade ago (Buckley et al., 2009) and the use of this analytical technique is increasingly growing in the field of archaeology, ecology and cultural heritage (Brown et al., 2021; Richter et al., 2022). This method consists of the analysis of collagen type I through MALDI-TOF MS technology, with the aim to assign some level of taxonomic identity, normally via comparison to a reference list of peptide marker masses of possible species (PMF). It is a minimally destructive method, as it requires only a small amount of collagenous material (<20 mg), and can be performed with a low analytical cost per sample, allowing for rapid large-scale taxonomic investigations and providing robust taxonomic identities (Richter et al., 2022).

Different ZooMS extraction techniques can be used to obtain suitable peptide mass fingerprints. After a minimally destructive bone/dentin fragment is taken from each specimen (van Doorn et al., 2011; Welker et al., 2016; Welker et al., 2017), collagen molecules can be extracted from the mineralised tissues using hydrochloric acid (Buckley et al., 2009). This approach, called acid demineralisation (Buckley et al., 2009), is usually performed on poorly preserved samples. 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 buffer using heat. The advantage of this semi-destructive ammonium-bicarbonate buffer extraction (AmBic; van Doorn et al., 2011; Welker, Soressi, et al., 2015) is to cause minimal damage to the bone sample allowing for subsequent analysis or duplication of the extraction (von Holstein et al., 2014; Welker, Soressi, et al., 2017). This step will prepare the primary amino acid structure of the protein for enzymatic digestion (**Figure 4**). Digestion with trypsin is used to cleave the sequence into peptides of different length and mass depending on the taxa. Peptides are then acidified to neutralise the enzyme and purified through C18 filters. Samples are then spotted onto a specialised plate, normally in triplicate, with a matrix which will co-crystallize with the peptides. Once placed inside the MALDI-TOF mass spectrometer, a laser will excite the matrix which will vaporise the peptides and ionise them with a charge of +1. Based on their time-of-flight to the spectrometer´s detector, the peptide mass(es) can be determined and converted into mass-to-charge ratio (m/z). A single mass spectrum is produced for each collagen sample, and peptide markers will be identified by associating an intensity peak with a characteristic m/z. The combination of the identification of nine selected peptide markers will allow the taxonomic identification of

the sample specimen through comparison to a database of peptide marker series for all European Pleistocene medium- to large-sized mammals (Welker et al., 2016). The obtained taxonomic identifications are generally in the range of subfamily or genus.

Details of the protocols and the methodology used in this thesis are described in chapters two, three and four, but also published elsewhere (Buckley et al., 2009; Naihui et al., 2021; van Doorn et al., 2011; Welker, Soressi, et al., 2015).

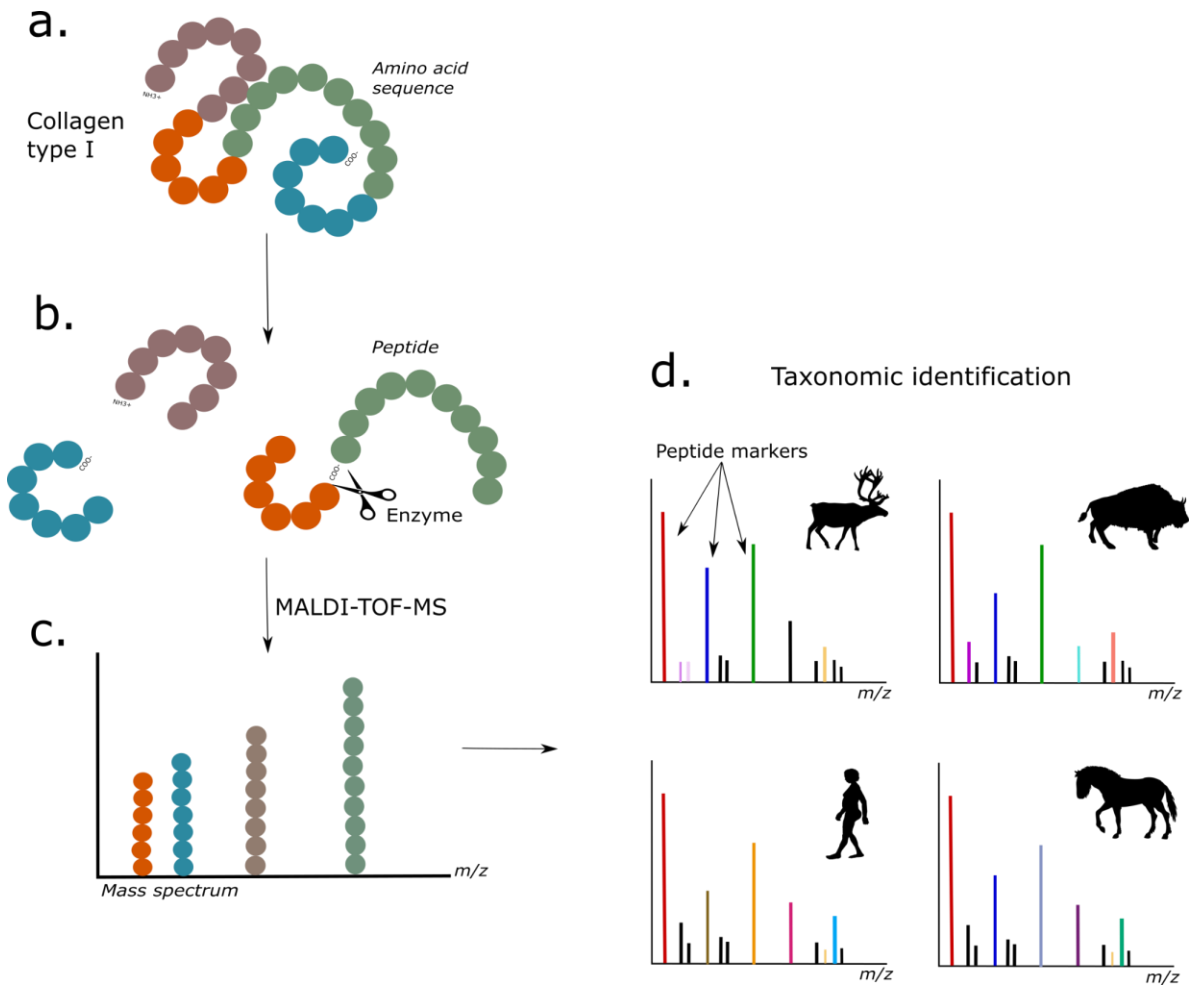


**Figure 4:** Schematic overview of ZooMS analysis of bone fragments. a. Denaturation of the amino acid sequence of collagen type I. b. Digestion with an enzyme to cleave the amino acid sequence into peptides. c. MALDI-TOF MS analysis generating a mass spectrum. d. Taxonomic determination of the sample through the identification of a combination of specific peptide markers. Animal silhouettes are from phylopic.org.

The AmBic approach has been preferentially used for the analysis of the large datasets allowing for a more rapid peptide extraction, and providing glutamine deamidation ratios (van Doorn et al., 2012; Welker et al., 2017; Wilson et al., 2012). The procedure of glutamine deamidation involves the transformation of glutamine into glutamic acid, resulting in an addition of 1 Da to the peptide. Glutamine (Gln) deamidation ratios are measured for the peptide COL1α1 508–519 following existing protocols (Wilson et al., 2012), to assess protein

degradation and collagen preservation for the detection of outliers, thus permitting the identification of possibly intrusive material or differential bone preservation (van Doorn et al., 2012; Welker et al., 2017), although with varying success (Brown et al., 2021). The deamidation ratio ranges from %Gln = 1.0, indicating no deamidation from glutamine to glutamic acid, to %Gln = 0.0 indicating complete deamidation of glutamine to glutamic acid.

Because of the low sample input requirement, the recent development of non-invasive collagen extraction techniques for ZooMS analysis opens up the possibility for the analysis of rare and fragile specimens. Some of these non-invasive strategies are called electrostatic ZooMS (eZooMS), and rely on static electricity generated either by friction or by contact with a plastic polymer, in order to capture proteins from the surface of an organic tissue. The friction generates a triboelectric charge between the organic surface and the plastic polymer, releasing proteins from the specimen surface that binds to the plastic polymer (Richter et al., 2022; Welker et al., 2017). First developed in biocodicology (Fiddyment et al., 2015, 2021; Teasdale et al., 2017) by rubbing a soft polymer eraser on the surface of a parchment, alternative non-destructive sampling protocols have employed this technique to extract proteins from sample plastic bags or membrane boxes which had contained archaeological specimens (Martisius et al., 2020; McGrath et al., 2019). While these methods are increasingly raising interest and applied to various archaeological materials including worked bones (Brandt et al., 2018; Coutu et al., 2021; McGrath et al., 2019), the impact on bone surfaces has not been addressed. The application of the eraser sampling technique generates a friction on the bone surfaces, with the potential to remove, modify or even generate ancient features comparable to use-wear traces, thus bringing caution on its potential abrasiveness. Recently, new methods have been developed using polishing films with grit (Kirby et al., 2020), ethylene vinyl acetate films studded with strong cation and anion exchanges and C8/C18 resins (Zilberstein et al., 2020), enzyme functionalized films (Cicatiello et al., 2018) or hydrogels (Calvano et al., 2020). The rapid methodological development of minimally destructive extraction techniques opens the possibilities of non-invasive analyses while preserving the integrity of rare and culturally significant specimens, e.g. worked bones (Dekker et al., 2021; Martisius et al., 2020; McGrath et al., 2019).

## 3.3. ZooMS applications: an overview

Since 2018, the field has experienced a rapid growth of the application of ZooMS (Brown et al., 2021; Richter et al., 2022). It has provided taxonomic identification of collagenous material such as bone/ivory/antler including worked bone, bone points, arrowheads, daggers, rings and combs (Ashby et al., 2015; Bradfield et al., 2019; Dekker et al., 2021; Desmond et al., 2018; Martisius et al., 2020; McGrath et al., 2019; Pétillon et al., 2019;

Tomasso et al., 2018; von Holstein et al., 2014), but also parchment and soft tissues (Fiddyment et al., 2015; Kirby et al., 2013; Ruffini-Ronzani et al., 2021; Teasdale et al., 2017; Vnouček et al., 2020), leather (Ebsen et al., 2019), and bone fragments contained within dog palaeofaeces (Runge et al., 2021). The technique has been applied to a wide range of fragmentary and/or morphologically similar taxa from various archaeological and palaeontological assemblages.

In these cases, ZooMS is commonly performed in a targeted manner on individual bone specimens, for example to identify bone tools (as discussed previously), for radiocarbon or isotopic studies (Fewlass et al., 2020; McCormack et al., 2022), or to verify a taxonomic identity. However, ZooMS has been developed as a screening tool to distinguish closely related species that are difficult to identify morphologically (Buckley et al., 2011; Buckley & Kansa, 2011; Evans et al., 2016) and to identify species of interest among unidentifiable remains such as hominins or extinct megafauna (Brown et al., 2016; Buckley et al., 2017; Charlton et al., 2016; Devièse et al., 2017; Evans et al., 2016; Welker et al., 2016; Welker et al., 2017). When applied to faunal bone assemblages, ZooMS studies have often focused on ecological purposes to improve the faunal spectrum of a bone assemblage or to address domestic herd management, choices relating to secondary product use, exploitation of wild species, and appearance of commensal species (Eda et al., 2020; von Holstein et al., 2014). However, the assessment of the fragmented and morphologically unidentifiable component of bone assemblages through ZooMS may provide a clearer picture of assemblage composition and inform about specific hominin strategies in relation to faunal carcass processing and selection, and remain to be investigated. Indeed, the correlation of patterns of bone fragmentation revealed by bone surface modification analysis with taxonomic identification of morphologically unidentifiable faunal specimens may provide access to previously unavailable information on past hominin behaviour.

# 4. Doctoral project aims and objectives

The overall objective of the research presented in this thesis is to integrate two complementary fields: palaeoproteomics, specifically ZooMS, and traditional zooarchaeology, by combining the analyses of bone surface modifications with biomolecular analysis, and to show the potential of the inclusion of ancient protein analysis within the current framework of zooarchaeological analysis at Palaeolithic sites. This project provides an integrative zooarchaeological and ZooMS workflow in order to assess the morphologically unidentifiable portion of Late Pleistocene bone assemblages and to yield complementary

and comprehensive data on taxonomic abundance, site formation, human subsistence and site use. Because of high bone fragmentation and an often low proportion of identifiable bone remains based solely on morphology, this work focuses on the transition from the Middle to Upper Palaeolithic seeking to contribute to the understanding of human behavioural response to changing conditions (environmental, biological and technological changes). The ZooMS-identified and morphologically-identified bone specimens constitute two artificial components defined based on identifiability or size-cut-off, but in reality represent subsamples of the same death assemblage. The initial hypothesis previously presented expects that both components show similar taxonomic composition and abundances for the dominant taxa (Welker, 2017). Several ZooMS screening studies have shown that this hypothesis could be verified (Berto et al., 2021; Buckley et al., 2017; Welker et al., 2016; Welker et al., 2015). However, the increased taxonomic richness noted by Welker et al., 2015 within the ZooMS component of the faunal assemblage from Les Cottés but not among the assemblages of Quinçay (Welker et al., 2017) and Grotte du Renne (Welker et al., 2016) raises questions about the representativity of the morphologically identified component over the complete faunal assemblage.

Several objectives will be addressed throughout three projects:

- Demonstration that the morphologically unidentifiable bone components do not necessarily reflect the taxonomic abundance of the morphologically identified component of Palaeolithic faunal assemblages (Chapters 2 and 3)
- Further, through ZooMS identification, these previously unidentified bone components can provide new and complementary data about specific human subsistence behaviours and bone assemblage formation (Chapters 2 and 3)
- Demonstration that ZooMS analysis of the morphologically unidentified bone component allows a better understanding of potential biases, such as differential identification rates affecting the bone assemblage (Chapter 3)
- Development of a controlled sampling experiment in order to explore the effects of eraser sampling for eZooMS analysis on Palaeolithic bone surface microtopography (Chapter 4)

Chapters 2 and 3 focus on the integration of complementary datasets from zooarchaeological and ZooMS-based analysis and its contribution to the understanding of subsistence behaviour and hunting strategies during the MUPT. Chapter 4 emphasises the importance of testing the effect on bone surfaces and bone surface modifications of so-called “non-destructive” collagen extraction techniques such as the eraser sampling for ZooMS analysis.

## 4.1. Chapter Two - Project 1: Combining ZooMS and zooarchaeology at Fumane Cave (Italy)

This project presents, for the first time, the integration of complementary data sets from zooarchaeological and ZooMS-based analyses at Fumane Cave (Italy). This study aims to provide a more accurate picture of species proportions and explores the synthesis and analysis of comparable data for both the identifiable and morphologically unidentifiable portions of faunal assemblages from Final Mousterian and Uluzzian layers. In order to address these objectives, this work will:

- Provide taxonomic identifications of almost 700 bone morphologically unidentifiable specimens through ZooMS analysis, within a highly fragmented bone assemblage
- Explore the causes of a compositional difference between the ZooMS and morphology component, highlighted by frequency differences in the presence of a particular taxon within the same archaeological unit
- Investigate the involvement of human groups in the formation of the morphologically unidentifiable bone component from the Late Pleistocene bone assemblage of Fumane
- Examine the categorisation of taxonomically unidentifiable bone specimens into body size classes

This work has been published in Scientific Reports: **Sinet-Mathiot, V**., Smith, G. M., Romandini, M., Wilcke, A., Peresani, M., Hublin, J.-J., & Welker, F. (2019). Combining ZooMS and zooarchaeology to study Late Pleistocene hominin behaviour at Fumane (Italy). *Scientific Reports*, 9: 12350. https://doi.org/10.1038/s41598-019-48706-z

## 4.2. Chapter Three - Project 2: Contribution of ZooMS to the understanding of subsistence strategies during MUPT

Following the results obtained for Project 1, this second project aims to explore the implications of incorporating the analysis of the morphologically unidentifiable bone components into the interpretation of several faunal assemblages covering the MUPT, both in terms of overall bone accumulation and, more specifically, human subsistence strategies. This project seeks to address methodological limits commonly faced during the morphological assessment of faunal assemblages and to demonstrate how the addition of biomolecular methods such as untargeted ZooMS screening can complement our understanding of

subsistence behaviour by providing a clearer picture of prey selection and site occupation. In order to assess these aims, this project will:

- Integrate taxonomic identifications for more than 2,600 morphologically unidentifiable bone specimens from three key Late Pleistocene sites covering the MUPT (Bacho Kiro Cave (Bulgaria), Les Cottés and La Ferrassie (France)) into the zooarchaeological assessment of the faunal assemblages
- Investigate differences in taxonomic abundance of the dominant taxa between the ZooMS and morphology components
- Contribute to our understanding of prey selection, bone assemblage formation and subsistence strategies during the MUPT
- Address methodological limits commonly faced during the zooarchaeological analysis of faunal assemblages with the addition of ZooMS

This research is under review at Archaeological and Anthropological Sciences: **Sinet-Mathiot, V**., Rendu, W., Steele, T.E., Spasov R., Madelaine, S., Renou, S., Soulier, M.-C., Martisius, N.L., Aldeias, V., Endarova, E., Goldberg, P., McPherron, S.J.P., Rezek, Z., Sandgathe, D., Sirakov, N., Sirakova, S., Soressi, M., Tsanova, T., Turq, A., Hublin, J.-J., Welker, F., Smith, G.M. Identifying the unidentified fauna enhances insights into hominin subsistence strategies during the Middle to Upper Palaeolithic transition. *Archaeological and Anthropological Sciences.*

## 4.3. Chapter Four – Project 3: Testing the effect of a non-destructive collagen extraction method on Palaeolithic bone surfaces

With the expansion of ZooMS applications and the development of non-destructive collagen extraction techniques, this third project aims to address the impact of the eraser extraction method on ancient bone surfaces, and alert the community on the potential invasiveness of this sampling method when applied on Palaeolithic bone surfaces. In order to approach these objectives, this project will:

- Design a controlled sampling experiment measuring the force applied and the rate of the eraser movements
- Assess the macro- and micro-structure of the bone surfaces at multiple scales prior to and after eraser sampling using both qualitative and quantitative methods
- Discuss the implication of potential bone modifications for future analysis of the bone artefact

This project was published in Scientific Reports: **Sinet-Mathiot, V.,** Martisius, N.L., Schulz-Kornas, E., Van Casteren A., Tsanova T., Sirakov N., Spasov R., Welker F., Smith G. M., Hublin J.-J. (2021). The effect of eraser sampling for proteomic analysis on Palaeolithic bone surface microtopography. *Scientific Reports* 11: 23611. https://doi.org/10.1038/s41598-021-02823-w

## 4.4. Chapter Five: Discussion and Conclusion

The final section of this thesis will synthesise Chapters two to four by providing a brief conclusion of the outcomes of these projects. Next, it will give an overview for outstanding challenges and opportunities in the field of zooarchaeological research through palaeoproteomic methods, particularly in the context of Late Pleistocene hunter-gatherer subsistence strategies.

Chapter Two

# Combining ZooMS and zooarchaeology to study Late Pleistocene hominin behaviour at Fumane (Italy)

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## Abstract

Collagen type I fingerprinting (ZooMS) has recently been used to provide either palaeoenvironmental data or to identify additional hominin specimens in pleistocene contexts, where faunal assemblages are normally highly fragmented. However, its potential to elucidate hominin subsistence behaviour has been unexplored. Here, ZooMS and zooarchaeology have been employed in a complementary approach to investigate bone assemblages from final Mousterian and Uluzzian contexts at fumane cave (Italy). Both approaches produced analogous species composition, but differ significantly in species abundance, particularly highlighted by a six fold-increase in the quantity of *Bos/Bison* remains in the molecularly identified component. Traditional zooarchaeological methods would therefore underestimate the proportion of *Bos*/*Bison* in these levels to a considerable extent. We suggest that this difference is potentially due to percussion-based carcass fragmentation of large *Bos/Bison* bone diaphyses. Finally, our data demonstrates high variability in species assignment to body size classes based on bone cortical thickness and fragment size. Thus, combining biomolecular and traditional zooarchaeological methods allows us to refine our understanding of bone assemblage composition associated with hominin occupation at Fumane.

## Introduction

Zooarchaeological analyses use faunal remains to address archaeological questions. This provides a wealth of information on local and regional palaeoenvironments, the timing of hominin occupation, and interactions with other species[1–5]. Most specifically, such studies have been used to reconstruct hominin diet and subsistence patterns. However, faunal remains are often highly fragmented by taphonomic, including anthropogenic processes, precluding any type of taxonomic identification for most specimens. The non-identifiable component of Pleistocene bone assemblages frequently incorporates 60–70% of the excavated assemblage[6,7]. This leads to an extensive taxonomically uninformative proportion of bone assemblages, which could represent a source of bias in zooarchaeological studies of hominin subsistence behaviour.

Bone fragmentation can also provide a wealth of detail about site formation and depositional processes, but also more specifically about butchery practices and subsistence patterns. The species body part representation and the occurrence and location of cut-marks, percussion traces and bone breakage patterns can illustrate specific transport decisions by human groups[8–10]. However, large portions of bone assemblages remain taxonomically unidentifiable, and in the best cases can only be attributed to body size classes. Patterns of human subsistence behaviour are therefore often reliant on a relatively small proportion of morphologically identifiable remains. To provide a more comprehensive picture of human subsistence behaviour at a site requires the synthesis and analysis of comparable taxonomic

and taphonomic data from both identifiable and unidentifiable fraction of Pleistocene faunal assemblages.

With the advancement of biomolecular studies in the past 20 years, different methods have been developed in order to aid the identification and the analysis of biological markers preserved in unidentifiable bone fragments. First, ancient DNA metabarcoding of bone samples has been employed to study the taxonomic composition of hundreds or thousands of bone samples simultaneously[11–13]. Second, various approaches involving ancient DNA sequencing have allowed the identification of vertebrate DNA directly from Pleistocene soil and sediment samples[14–17]. Both approaches provide qualitative insights into species composition but,currently, little resolution in terms of quantitative aspects[11]. In addition, all genetic and genomic approaches rely on ancient DNA survival, a biomolecule prone to fragmentation in comparison to other biomolecules, such as proteins[18–20]. Therefore, proteomic approaches, in particular collagen type I peptide mass fingerprinting through Zooarchaeology by Mass Spectrometry analysis (ZooMS[21]), have been suggested as a biomolecular alternative to study the taxonomic composition of the unidentifiable component of Pleistocene bone assemblages. Proteins such as collagen type I are phylogenetically informative, easily accessible, and survive beyond the temporal range of ancient DNA[22–24].

ZooMS is a proteomic approach that allows taxonomic identification based on protein amino acid sequence variation through peptide mass fingerprinting[21]. This method is commonly performed on individual bone specimens in a targeted manner (for example on bone tools, particular taxonomic groups, or for radiocarbon or isotopic studies[25–28]) and thereby provides quantitative datasets potentially comparable with traditional zooarchaeological studies. ZooMS can add additional information on hominin behaviour in relation to faunal carcass processing and selection[29,30], but this potential has not been explored. Nevertheless, previous studies have demonstrated that ZooMS is a robust tool that provides a high identification success rate (>95%) in the European Late Pleistocene. Initial taxonomic identifications through ZooMS have allowed the recovery of additional hominin specimens[30–33]. Bone specimens individually identified through ZooMS can be utilised in subsequent ancient DNA, isotopic, and radiocarbon dating analysis[34–37]. Finally, peptide mass fingerprints of collagen type I provide specimen-specific information of molecular diagenesis, allowing insights into spatial and temporal biomolecular preservation within a site[38–40].

In previous studies, ZooMS- and morphologically-identified components from the same layers are comparable in terms of species composition and abundance (Fig. 1). On some sites, the application of this method has allowed for the identification of species previously unconfirmed through traditional morphological analysis[29,30,38,41] However, no ZooMS studies have investigated the relationship between faunal composition and bone fragmentation and, in turn, whether this is related to specific hominin behaviour at a site. In this study, 684 bone

specimens across the Middle to Upper Palaeolithic transition (MUPT) corresponding to layers A6 to A2 at Fumane (Italy), have been analysed, with a focus on the Final Mousterian layer A4 (previously attributed to the Uluzzian: see ref.[42]) and the Uluzzian layer A3[43–46].

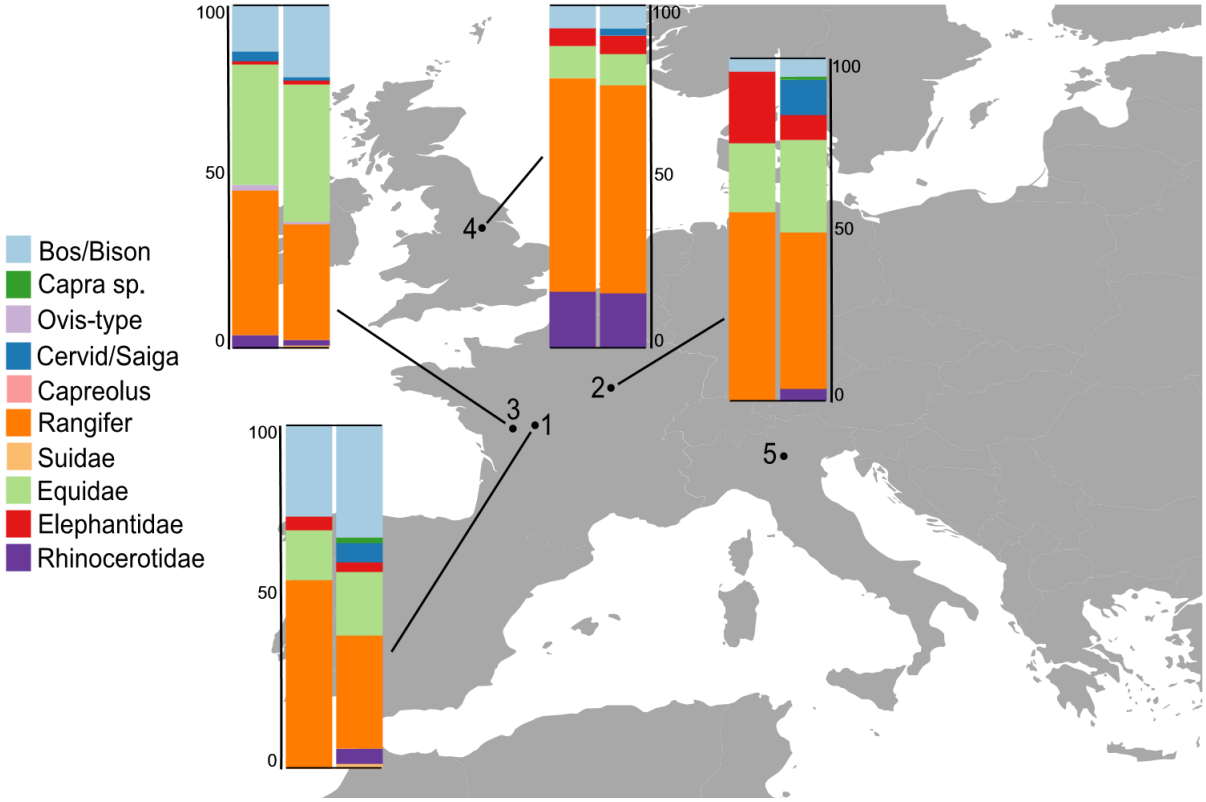


**Figure 1.** Site location of Fumane and other published, non-targeted ZooMS studies with zooarchaeological data available for the same archaeological layers. For each site the barplot indicates the percentage of number of identified specimens (%NISP) of herbivores for the morphologically identified (left) and the ZooMScomponent (right). 1: Les Cottés (France)[29] (ZooMS: N = 70, Morph: N = 75), 2: Grotte du Renne (France)[30] (ZooMS: N = 108, Morph: N = 100), 3: Quinçay (France)[38] (ZooMS: N = 412, Morph: N = 213), 4: Pin Hole Cave (UK)[41] (ZooMS: N = 72, Morph: N = 78), 5: Fumane (Italy; this study see Fig. 3). Further details are provided in Supplementary Table S1.

# Methods

## Fumane

Fumane cave is located at the bottom of the Venetian Pre-Alps within the Western Monti Lessini in North of Italy (Fig. 1). The site has been known since the late 19th century, and was first excavated in 1964 by the Natural History Museum of Verona. The current excavations are led by a team from the University of Ferrara, and the faunal assemblage from these excavations were sampled and analysed to form the basis of this study.

The cave is part of a karst system composed of several cavities which has permitted the accumulation of a sedimentary sequence including Mousterian, Uluzzian and Aurignacian

cultural complexes[45–49]. Human occupation at Fumane is attested by numerous faunal remains, lithics artefacts and combustion features. The site also offers unusual finds such as ornamental objects, painted stones, and evidence for the intentional removal of feathers from birds[50–53]. Various studies have presented radiocarbon dates, Uranium-Thorium dates, and electron spin resonance (ESR) combined dates, that provide a clear chronological framework for the entire stratigraphy[47,54], in addition to palaeoecological contexts[55]. Within this framework, layers A4 and A3 date between 41.3 and 39.1 ka (Table 1)[56,57].

The bone assemblages from Fumane are highly fragmented across the stratigraphy[43,58–60]. For example, for layers A3 and A4 about 3% of the assemblage (1,188 out of 36,944 bone remains including dental remains) can be securely identified based on morphological characteristics. For these 2 layers, the faunal spectrum based on the morphologically identifiable bones includes various ungulates, carnivores and birds, which together indicate a closed wooded environment indicative of temperate to cool climatic conditions[43,59]. The differences in faunal composition between layers A3 and A4 are relatively minor, and they occur in the abundance of the dominant species (Table 1).

| Layer | Cultural attribution | Approximate age | Dominant faunal components (%NISP) |
|---|---|---|---|
| D3 | Aurignacian | | Ibex (*Capra ibex*, 43.0%) |
| D6 | Aurignacian | | Ibex (*Capra ibex*, 35.5%) |
| A1 | Protoaurignacian | | Ibex (*Capra ibex*, 43.9%), red deer (*Cervus elaphus*, 18.4%) |
| A2-A2R | Protoaurignacian | 41–38 ka cal BP | Ibex (*Capra ibex*, 49.5%), red deer (*Cervus elaphus*, 18.8%) |
| A3 | Uluzzian | 44–42 ka cal BP | Red deer (*Cervus elaphus*, 29.5%), ibex (*Capra ibex*, 20.3%) |
| A4 | Final Mousterian (Levallois) | 44–42 ka cal BP | Red deer (*Cervus elaphus*, 39.3%), ibex (*Capra ibex*, 20.3%) |
| A5-A6 | Mousterian (Levallois) | 45–44 ka cal BP | Red deer (*Cervus elaphus*, 70.3%), roe deer (*Capreolus capreolus*, 11.7%) |
| A7 | (-) | | No human presence |
| A9 | Mousterian (discoidal) | >47.6 ka cal BP | Red deer (*Cervus elaphus*, 39.3%), roe deer (*Capreolus capreolus*, 22.3%) |
| A10 | Mousterian (Levallois/discoidal) | >47.6 ka cal BP | Roe deer (*Capreolus capreolus*, 43.8%), red deer (*Cervus elaphus*, 29.5%) |
| A11 | Mousterian (Levallois) | >47.6 ka cal BP | Roe deer (*Capreolus capreolus*, 39.5%), red deer (*Cervus elaphus*, 32.3%) |

**Table 1.** Fumane stratigraphy, chronological age, and faunal composition based on morphologically identifiable bone specimens. Reference data on chronology taken from[44,47,56]. Reference data for zooarchaeological analysis taken from[43,51,58–60,115]. Note that layer A4 is now attributed to the Final Mousterian. See discussion in[42].

## Zooarchaeological analysis

In the zooarchaeological analysis of the bone assemblages from Fumane, all the remains have been counted and grouped by size (0–1 cm, 1–2 cm, 2–3 cm, 3–4 cm, 4–5 cm, >5 cm). Burned and calcined bones were separated from the unburned specimens. All bone specimens were also grouped by body size class (large, medium-large, medium, medium-small, and small) based on bone cortical thickness and fragment size.

Taxonomic and skeletal identification was based on two reference collections. The first is stored at Lazio Museum Pole at the National Prehistoric Ethnographic Museum "Luigi Pigorini" in the Bioarchaeology Section in Rome, while the second is in the Prehistoric and Anthropological Sciences Section at the University of Ferrara. Microscopic analyses of the bone surfaces were carried out using portable low-magnification lenses (10–20X) and Leica S6D Green Ough stereomicroscopes with 0.75–70X magnification range. In specific cases, observation was also carried out using scanning electron microscopy (SEM).

In order to determine the nature of surface bone alterations, and to distinguish hominin from animal traces, trampling abrasion, and modern mechanical modifications produced by excavation tools, reference was made to the well-established taphonomic literature[61–68]. The degree of combustion was evaluated employing the methodology developed by Stiner *et al*.[69]. All faunal specimens were analysed, regardless of their taxonomic identifiability by one of the authors (M.R.) using traditional morphological observation. For our study, species abundance was assessed using the number of identified specimens (NISP)[70], as minimum number of skeletal element (MNE) and minimum number of individuals (MNI) cannot be compared quantitatively with ZooMS data, which is inherently a NISP count. The percentage of the number of identified specimens have been calculated based on the taxonomically identified faunal specimens. Finally, bone fragmentation indices were calculated to evaluate the skeletal representation of the different animals and the skeletal survival rate[61,62,67].

## ZooMS

684 morphologically unidentifiable bone and dental (dentine) specimens have been randomly sampled across levels A6 to A2 excavated in the same squares in the western area of the cave entrance (Supplementary Table S2). The majority of these bone specimens (73%) derive from the two layers A3 and A4. All selected specimens were recorded as individual specimens during excavation. For bone specimens, selection was based on the presence of cortical bone surface and a length of at least 2 cm. Dental specimens comprise a minor proportion of the analyzed samples (n = 8, 1.2%) and were excluded from surface modification analysis. For layers A3 and A4, our sampling covered the same spatial distribution (Supplementary Table S2). The maximum length of the bone specimens was measured individually with a digital calliper. ZooMS-identified bone specimens had previously been

analysed morphologically and various taphonomic attributes recorded, allowing for the comparison of surface modification frequencies related to taphonomic and anthropogenic processes present in both components of the bone assemblages.

ZooMS extraction methods followed protocols outlined in detail elsewhere30. In short, soluble collagen is first extracted from small bone samples (<20 mg) by incubation in 100 µL 50 mM ammonium-bicarbonate buffer at 65 °C for 1 hour. Half of this is digested using trypsin (0.5 µg/µL, Promega) overnight, acidified to pH < 1 using TFA (10% TFA), and cleaned on C18 ZipTips (Thermo Scientific). Hereafter, this is referred to as the "AmBic" extraction method40. Digested peptides are subsequently spotted in triplicate on a MALDI Bruker plate (MTP AnchorChip 384) with the addition of α-Cyano-4-hydroxycinnamic acid (CHCA) matrix. MALDI-TOF MS analysis was conducted at the University of York on an Ultraflex mass spectrometer (Bruker) in the mass-to-charge range 900–4000 m/z. MALDI-TOF stands for Matrix-assisted Laser-Desorption/Ionization, a method to ionize molecules, and is based on the co-crystallization of the matrix and an analyte, i.e. the substance to be analysed, in this case a bone proteome digested with trypsin. Analyte molecules are incorporated into the matrix while crystallization takes place. Subsequent laser impulses result in the detachment of crystalline particles into the vacuum of the mass spectrometer. Based on their time-of-flight (TOF) to the spectrometer´s detector, the molecular mass(es) of the analyte can be determined. Triplicates were merged for each sample, and taxonomic identification proceeded through peptide marker mass identification in comparison to a published database containing peptide marker series for all medium-to larger sized mammalian genera in existence in Europe during the Pleistocene[21,30].

For 24 samples, the AmBic taxonomic identity based on soluble collagen was verified through subsequent demineralization of the sample in 0.6 M HCl, neutralization to pH 6–7, and protein solubilization again in 0.6 M ammonium-bicarbonate (hereafter the "HCl" extraction method)[21]. All subsequent steps for these 24 specimens were identical to the "AmBic" extraction method except that they were analysed at the MALDI-TOF MS facility at the Fraunhofer IZI in Leipzig, Germany, using an autoflex speed LRF MALDI-TOF (Bruker) in reflector mode, positive polarity, matrix suppression of 590 Da, and collected in the mass-to-charge range 800–4000 m/z.

Soluble collagen deamidation was calculated for selected peptides frequently observed in peptide fingerprints of collagen type I through published protocols[71,72]. Glutamine deamidation has been suggested as an indicator of collagen preservation variability[38,72]. Only slow-deamidating peptides have been observed to be frequently present in the Fumane spectra, and we hence limit our analyses to these peptides (P1105 and P1706). Deamidation ratios are presented on a scale from 0 (complete deamidation, all glutamines converted into glutamic acid) to 1 (no deamidation, all glutamines unmodified).

All analyses were conducted in R[73], and figures were produced using the package ggplot2[74].

## Results

Our analysis resulted in successful ZooMS identifications for 97.8% of a total of 684 bone specimens, with nearly identical success rates across all sampled levels (Supplementary Table S2). Deamidation values for all bone specimens indicate a temporal cline towards more extensive diagenetic modification for older layers (Supplementary Fig. S1). Extraction blanks to monitor protein contamination in the lab were empty of collagen type I. Furthermore, HCl demineralization and MALDI-TOF-MS analysis of a randomly selected set of 24 *Bos/ Bison* specimens after AmBic analysis resulted in identical taxonomic identifications for both AmBic and HCl extraction methods (Supplementary Fig. S2). Our results are therefore difficult to explain by (laboratory) protein contamination.

Species presence in A3 and A4 is consistent between the ZooMS- and morphology-components of both levels (Supplementary Table S3). Exceptions are the addition of *Elephantidae* and *Rhinocerotidae* through ZooMS analysis for layer A4 and the presence of several carnivore species in the morphology-component[43]. This observation is similar to those made for previous untargeted ZooMS studies[29,30,38]. There are no herbivore species identified morphologically that are not represented in the ZooMS-component.

In zooarchaeology, bone specimens are frequently categorised in body size classes when species identification is not possible based on morphological criteria. At Fumane, bone fragment size and cortical thickness has been used as a proxy for body size class assignments. ZooMS analysis of bone specimens with body size class (BSC) assignments reveals that such categorizations are highly variable. For example, we note the presence of *Caprinae* within the large body size class and bone fragments identified as *Elephantidae* and *Bos/Bison* assigned to the medium body size class (Fig. 2). Thus, using the Fumane dataset, we illustrate that attributing taxonomically unidentifiable components to body size class categories (large, medium-large, medium) remains a useful, but problematic, qualitative tool. Moreover, these attributions are not taxonomically reliable as bone fragment size and cortical thickness are dependent on numerous overlapping and interrelated biological and taphonomic factors. As such, these body size class categories may not accurately reflect overall species composition at a site.

In contrast to previous studies (Fig. 1), a large difference in the quantitative composition of the ZooMS-component and the morphologically-identified component have been observed for layers A3 and A4 (Fig. 3). As ZooMS analysis cannot be performed on burned bone it was important to have comparable datasets for both the morphological and ZooMS component. Therefore, we assessed the proportion of burned and unburned specimens by taxon in the

morphological component from A3 and A4 (Fig. 3). The species representation is similar for both burned and unburned portions.

Overall, species representation among layers A3 and A4 is driven by an almost 6-fold increase in the number of *Bos/Bison* specimens in the ZooMS-component (36%) compared to the morphology-component (6%) (Fig. 3), and counterbalanced by a relative decrease in the number of specimens attributed to *Capra* sp. Such a frequency difference in the presence of a particular species between the ZooMS- and morphology-components of the same archaeological layer has never been observed until now (Fig. 1). The remainder of this paper aims to explore potential causes of this compositional difference by focusing on the three main taxonomic components (*Capra* sp., Cervid/Saiga, and *Bos/Bison*) of the layers A3 and A4.

For these three species groups, the spatial distribution of the bone specimens is more restricted in the ZooMS component (Fig. 4). The studied bone fragments have nearly identical distributions of specimen length (Fig. 5a). Whilst *Bos/Bison* specimens (41.7 ± 16.9 mm) are longer than *Capra* sp. specimens (37.1 ± 16.0 mm) and Cervid/

Saiga specimens (39.6 ± 15.0 mm), there is no significant difference in the overall distributions (Cervid/Saiga versus *Capra* sp.: t-test(0.7), df = 20, $p = 0.48$; Cervid/Saiga versus *Bos/Bison*: t-test(−1.1), df = 178, $p = 0.27$; *Capra* sp. versus *Bos/Bison*: t-test(−1.2), df = 22, $p = 0.23$). However, considering that bone specimens of over 2 cm in length have been selected for this study, the distribution might not be similar for the smallest, unstudied, size range (0–2 cm). Finally, there is no apparent difference in the spatial distribution of bone fragment size (Fig. 4d). Altogether, we therefore conclude that, assuming *Bos*/*Bison* individuals are generally larger than *Capra* sp. and Cervid/Saiga individuals, *Bos*/*Bison* bone elements have been subjected to a larger amount of fragmentation.

Bone length and P1105 deamidation, an indicator of molecular collagen type I preservation, display no significant relationship for any of the three species groups (Fig. 5b; Spearman rank correlation, $R_s = 0.09$ $p = 0.19$). All specimens from layers A3 and A4 display an identical distribution of P1105 deamidation (t-test(1.5), df = 420, $p = 0.14$) (Fig. 5c) and show no spatial differences in the amount of average deamidation per square in the area analyzed (Fig. 4c). However, Cervid/Saiga specimens have a deamidation distribution significantly different from that observed for *Capra* sp. and *Bos/Bison* specimens (Cervid/Saiga versus *Capra* sp.: t-test(−5.9), df = 21, $p = 6.4*10^{-06}$; Cervid/Saiga versus *Bos/Bison*: t-test(−6.8), df = 171, $p = 1.4*10^{-10}$), while *Capra* sp. and *Bos/Bison* specimens have similar distributions (t-test(0.9), df = 25, $p = 0.39$). This reveals that Cervid/Saiga specimens have undergone a different extent of molecular diagenesis, but not fragmentation, compared to bone specimens from the other ZooMS-identified species.

The frequency of bone surface modifications due to non-anthropogenic taphonomic processes (e.g., weathering, concretion, corrosion, mineral staining and root etching) is broadly similar

for all three species groups in both the ZooMS and morphology component, as is the presence of carnivore and/or rodent marks (Supplementary Tables S4, S5). Thus, non-anthropogenic bone surface modifications appear to have affected all three species groups to a similar extent. Similarly, increased levels of molecular damage for Cervid/Saiga specimens can only be explained by a mechanism unrelated to bone fragmentation processes, but this cannot explain the increase in *Bos/Bison* specimens in the ZooMS component.

Likewise, bone surface modifications resulting from anthropogenic processes are present to a similar extent in the ZooMS- and morphologically-identified components. In general, such bone surface modifications are recorded in comparable frequencies, but the frequencies for cut marks and impact points (or loading point)[67] are more distinct (Supplementary Table S4). It should be noted that frequencies differ between species to some extent, but generally not between the morphology- and ZooMS-components within the same species. For example, there seem to be fewer anthropogenic modifications of *Capra sp*. specimens compared to both Cervid/Saiga and *Bos/ Bison* specimens (Supplementary Table S4). We note, however, high frequencies of percussion marks for *Bos/ Bison* specimens in the ZooMS-component of both A3 (30%) and A4 (11%; Supplementary Table S4 and Fig. 6). Such marks are absent for *Bos/Bison* in the same layers (0% and 0%, respectively) in the morphology-component, mostly represented by bone epiphysis, but also by carpals, tarsals, and distal limb bones. This is in contrast to the ZooMS assemblages, which are mainly composed of long bone fragments (diaphysis) and ribs. Indeed, percussion marks on *Bos/Bison* specimens are exclusively present on long bone diaphyses in our ZooMS-identified sample set. These are bone elements subjected to more intense processing during butchering and bone marrow extraction[4,8,75]. Moreover, these specific traces appear to occur at much lower frequencies in the Cervid/Saiga (2–4%) and *Capra* sp. (0–0%) specimen groups.

All these observations related to bone surface modifications are replicated when the analysed specimens are restricted to the same set of squares for both the ZooMS and morphology components of the faunal assemblage (Supplementary Table S2). Alongside the absence of spatial patterns in bone fragmentation (Fig. 4d) and molecular degradation (Fig. 4c), there is therefore also no apparent spatial patterning in occurrence and frequency of bone surface modifications.

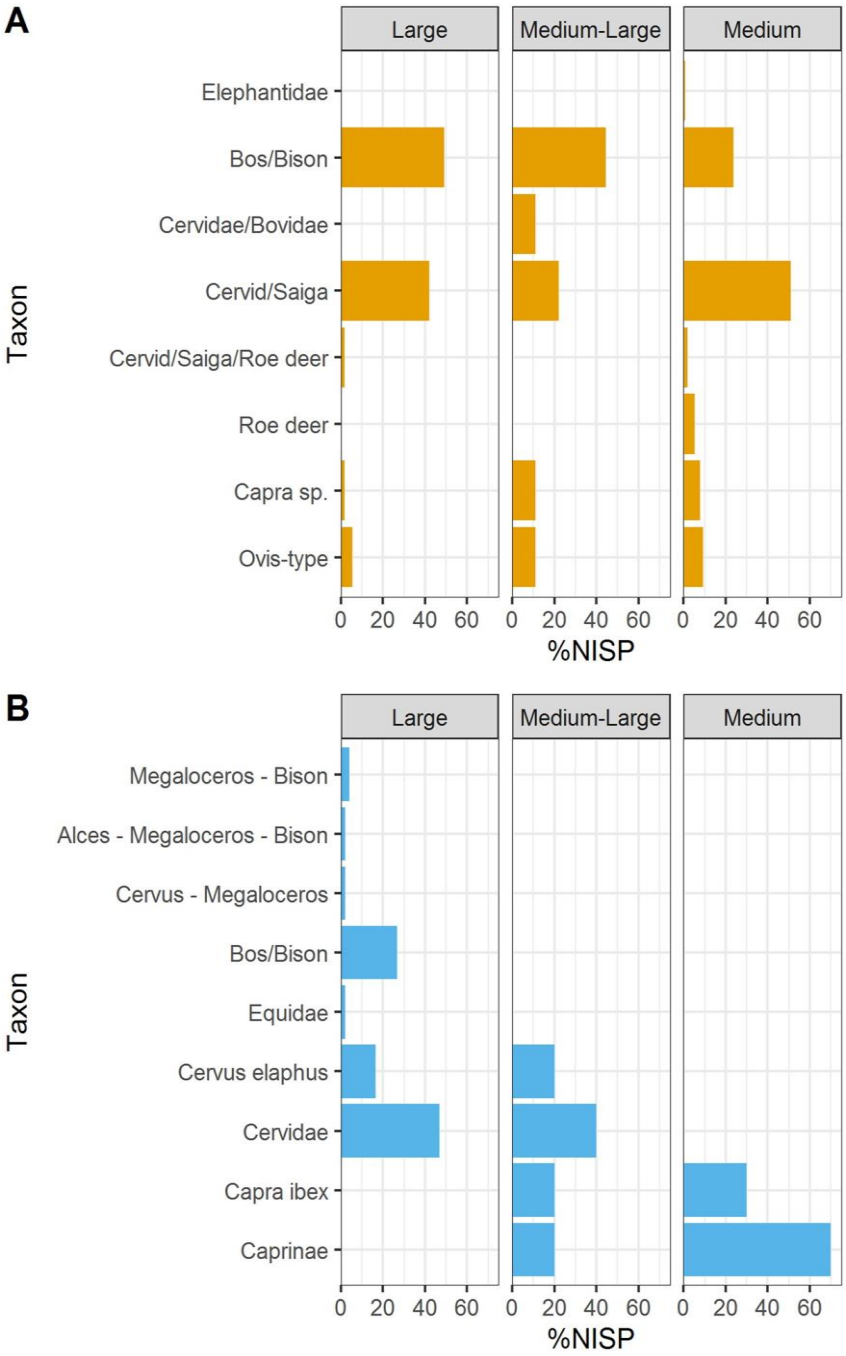


**Figure 2.** Barplot illustrating relative frequency (%NISP) for taxa identified using ZooMS (**A**) and morphology (**B**) in relation to their body size class attribution.

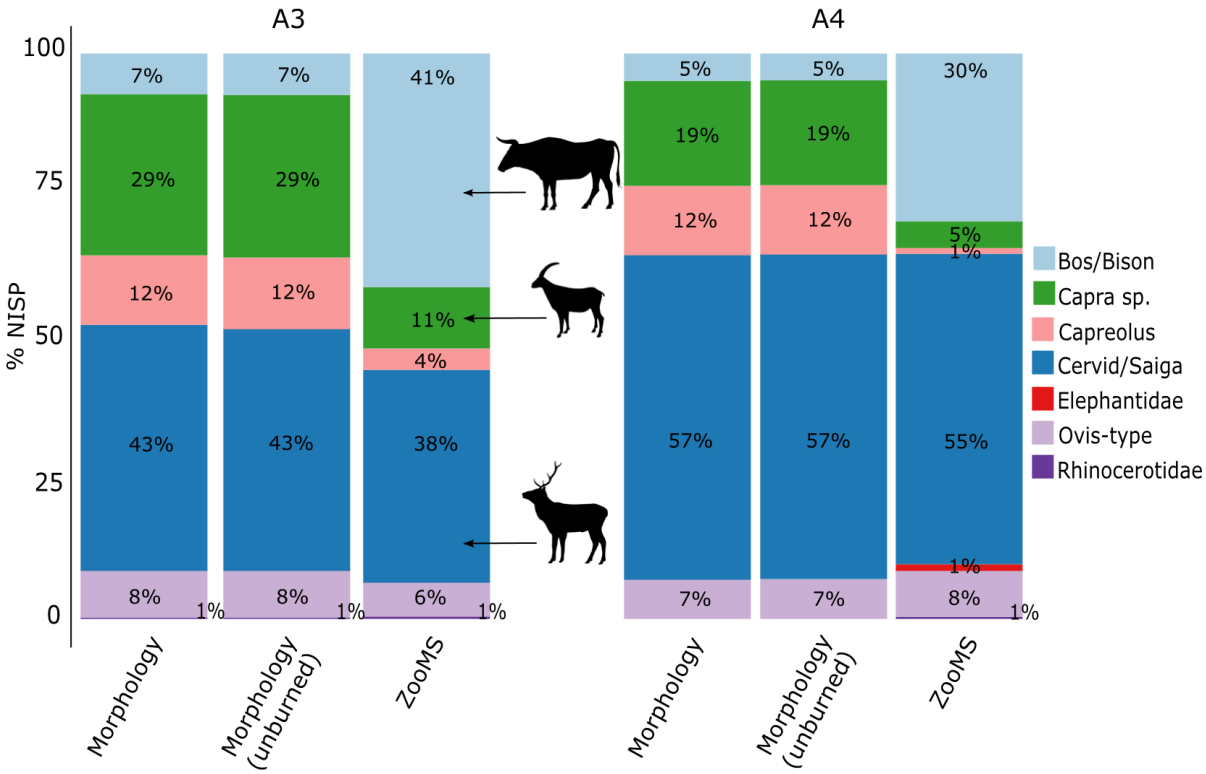


**Figure 3.** Barplot of %NISP of identified herbivores at Fumane. Morphology: this includes all specimens identified morphologically. Morphology (unburned): this includes specimens identified morphologically but excludes burned fragments (A3: 0.11% of burned specimens out of the morphology-identified assemblage (N = 453); (A4) 0.16% of burned bone fragments out of the morphological faunal assemblage (N = 681)). ZooMS: all specimens identified through ZooMS analysis (does not include burned fragments; see text for details). Colours are similar to Fig. 1. Data for the morphology-component derives from Tagliacozzo *et al*.[43]. Animal silhouettes are not to scale and derive from phylopic.org.

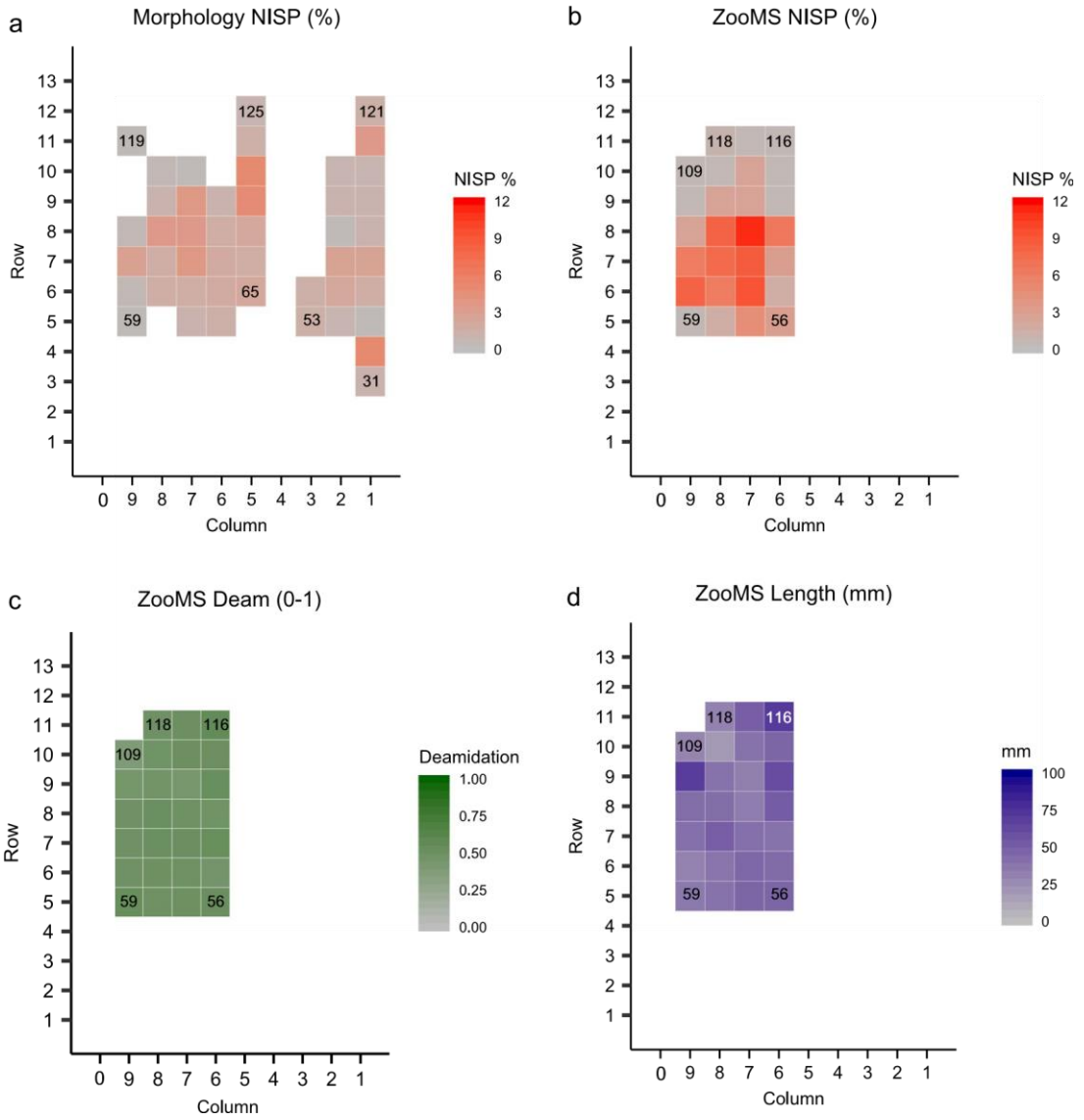


**Figure 4.** Spatial distribution maps of all bone specimens from the species groups Cervid/Saiga, *Capra* sp. and *Bos/Bison* from layers A3 and A4 at Fumane cave. (**a**) Distribution of %NISP of the three identified species for the morphology-component, over the sampled squares. (**b**) Distribution of %NISP of the three identified species for the ZooMS-component, over the sampled squares. (**c**) Average deamidation per square for the ZooMS component. (**d**) Average length (mm) per square for the ZooMS component. Squares are 1 × 1 meter, and the corresponding excavation numbers for each square can be obtained by joining the y-axis number and the x-axis number (for a detailed excavation plan, see[59]). The numbers within the squares represent the square numbers from the excavation grid. For **a** and **b**, a %NISP of 12% would indicate that 12% of the NISP of the combined total of *Capra* sp., *Bos*/*Bison*, and Cervid/Saiga is derived from that square.

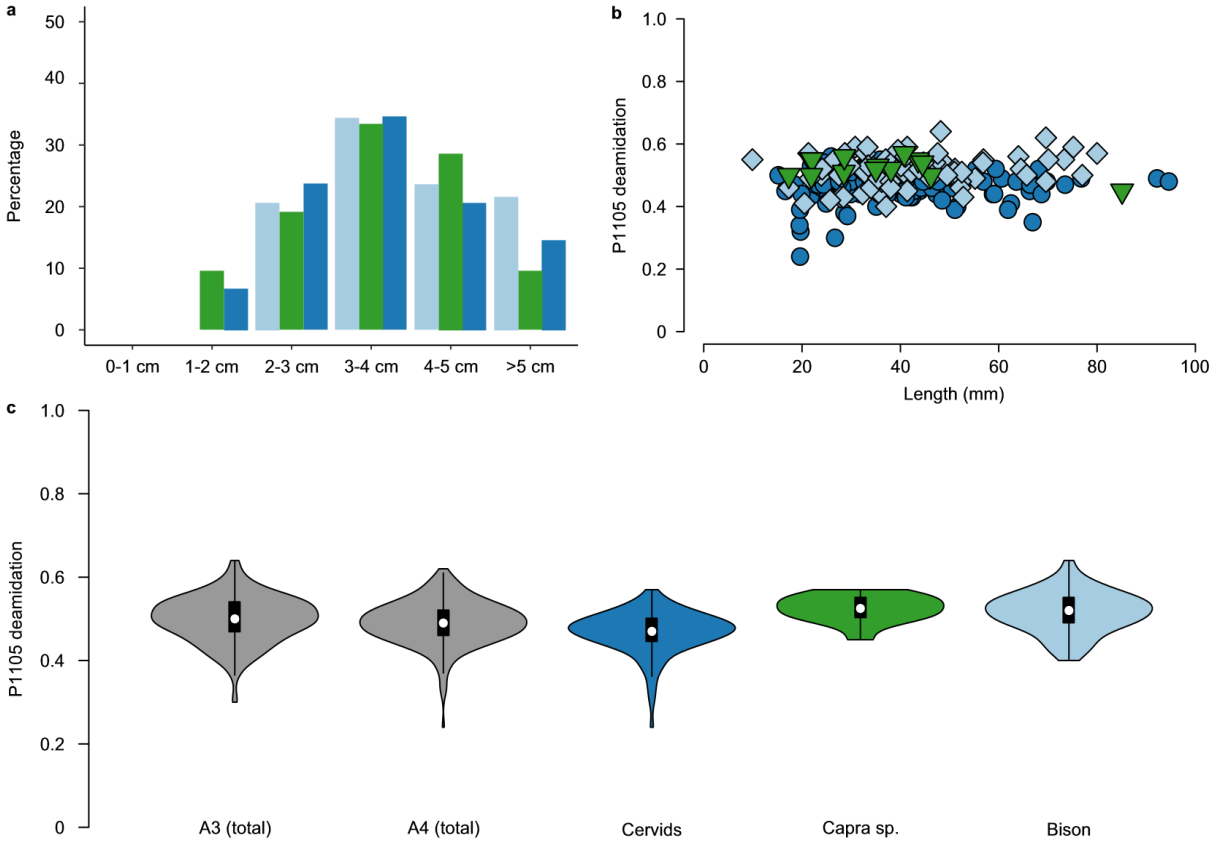


**Figure 5.** Taphonomic and molecular preservation of Cervid/Saiga, *Capra* sp., and *Bos/Bison* specimens. (**a)** Bone length distribution in mm. (**b)** Absence of a relationship between bone length (mm) and molecular diagenesis (P1105 deamidation). (**c**) Violin plots of P1105 deamidation. A3 and A4 include all specimens identified through ZooMS for these levels. Note that Cervid/Saiga is the dominant species group for both A3 and A4, significantly influencing the total violin plots for both levels displayed on the left. Only data for A3 and A4 are included for each panel. Colour legend is identical across panels as well as Figs. 1 and 3 (Cervid/Saiga: dark blue, *Capra* sp.: green and *Bos/Bison*: light blue).

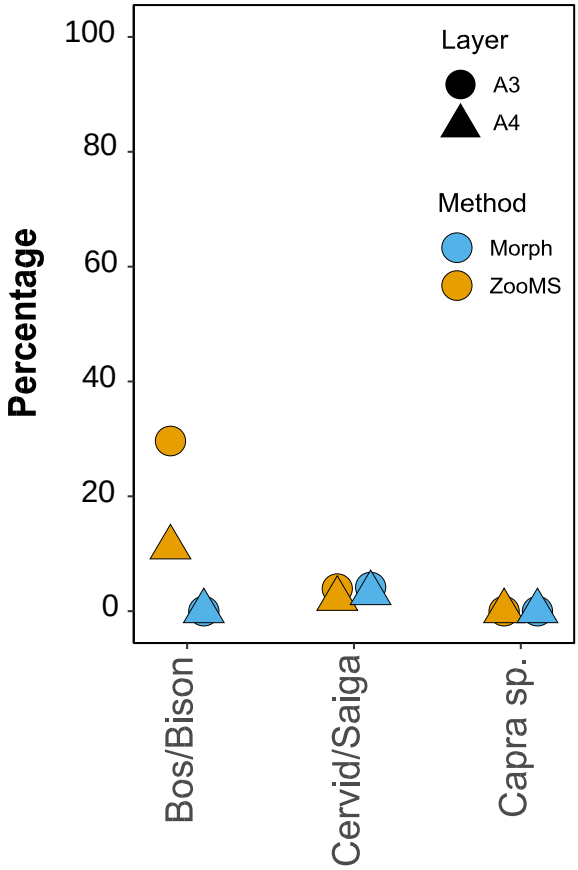


**Figure 6.** Percussion marks frequencies for the three main species groups within the morphology and the ZooMS-identified component in layers A3 and A4. Y-axis gives the percentage of occurrence (0–100) of percussion marks per specimen for the three major species groups (x-axis). Different point shapes indicate different layers (circle: A3; triangle: A4) whilst colour illustrates different identification method (blue: morphologically identified; gold: ZooMS). See Supplementary Table S4 for associated NISP numbers.

## Discussion

Palaeoproteomics, including ZooMS, is a recent addition to the molecular toolkit available to explore past faunal communities[21,29], the phylogenetic relationships between those species[20,22,76,77], and hominin interactions with their immediate environment[78]. ZooMS in particular has been adopted to survey the unidentifiable bone component of Palaeolithic sites in order to identify additional hominin remains[30–33] and to explore the qualitative aspects of faunal assemblages. Archaeological complexes like the Uluzzian in Italy have been attributed to the transitional phase between the Middle and the Upper Palaeolithic marked by the diffusion of populations of anatomically and genetically modern hominins and the local extinction of Neanderthals[46,79–82]. However, few hominin remains are directly attributable to the Uluzzian. Those that are available are associated with complicated debates on their taphonomic history[83–85], or cannot be reliably assigned to Neanderthals or modern hominins based on morphological characteristics because of their elevated degree of fragmentation or

the uncertain stratigraphic position[86,87]. Although no additional hominin specimen attributable to the Uluzzian has been identified here, our dataset adds to a growing understanding of hominin interactions with the environment around the MUPT[88–92].

Alongside similar methods based on ancient DNA sequencing, ZooMS has the ability to provide quantitative data on the abundance of particular species. This quantitative aspect has not been explored up to now, however, partly because previous studies indicated little quantitative difference between morphology-identified and ZooMS-identified components of the same assemblage (Fig. 1). Here we have encountered an assemblage where the morphology- and ZooMS-identified components are relatively similar in terms of species composition but markedly different in quantitative aspects for two distinct archaeological layers at the same site. In particular, a 6-fold increase in *Bos/Bison* specimens in the ZooMS-component. This is counterbalanced by a 3-fold decrease in *Capra* sp. (Fig. 3). We observe no apparent spatial differences in bone fragmentation (Fig. 4d) or molecular diagenesis (Fig. 4c). However, we note that Cervid specimens are more deamidated than other bone specimens at the site. It is therefore possible that the enhanced deamidation of Cervids in A3 and A4 is the result of hominin behaviour, although we are unable, at present, to precisely define which kind of anthropogenic process might be responsible. Possibilities include boiling, low-temperature roasting, or fermentation, but a precise assessment requires the development of further molecular methods to identify and distinguish these different anthropogenic processes. Furthermore, slightly higher rates of Cervid collagen deamidation cannot explain the higher incidence of *Bos/Bison* specimens.

Bones fractured deliberately to extract marrow have been previously noted in both layers at Fumane[43]. Compared to the morphologically identified assemblage, high frequencies of percussion marks on ZooMS-identified *Bos/Bison* specimens have been observed (Fig. 6). Therefore, the larger size of *Bos*/*Bison* elements and higher frequency of long bone diaphysis fragments and marrow fractures in the ZooMS-identified assemblage might explain the higher incidence of this species. Consequently, without the addition of the ZooMS dataset our interpretation of assemblage composition and human subsistence behaviour at Fumane would have been incomplete. The complementary ZooMS and zooarchaeological datasets from Fumane have provided a more comprehensive picture of assemblage composition and highlighted variation in the intensity and treatment of different prey sizes. This is exemplified by the increased fragmentation of *Bos/Bison* remains.

Palaeolithic faunal assemblages are often characterized by a high degree of fragmentation. This phenomenon can result from a number of natural taphonomic agents and processes[67,93–102] but also due to intensive hominin carcass processing. Indeed, such patterns appear similar whether in the Lower[98,101,103–106], Middle[2,107–111] or Upper Palaeolithic[88,112]. Often, long bones and rib fragments represent, by far, the largest proportion of the unidentified component of

faunal assemblages[109,113]. Similarly, these body regions represent high utility in terms of available resources (e.g., meat, marrow) and are thus frequently fragmented[6–8,114]. This, undoubtedly, leads to a loss of taxonomic identification and hominin behavioural information, with behavioural interpretations based on a relatively small proportion of identifiable remains. The novel application of ZooMS to taxonomically unidentifiable specimens has the potential to provide a clearer picture of overall species composition at a site and can help to reduce analyst error, especially when faced with a large proportion of one species within the morphologically identified component.

Comparisons of the relative proportions of species within the morphological and ZooMS components provides complementary data about species abundance and environmental context at sites though these datasets have not, to date, been used to address broader zooarchaeological questions related to site use, assemblage formation, or hominin subsistence behaviour. The current study presents a first attempt to integrate complementary data sets from zooarchaeological and ZooMS-based analyses. Whilst the morphologically-identified assemblage may be dominated by a small number of species, sometimes a single species, this may not necessarily reflect true assemblage abundance. Body size class based on bone cortical thickness can provide a qualitative assessment of assemblage fragmentation. Comparative analysis at Fumane illustrates considerable variation between the ZooMS and morphological datasets when assigning bone fragments to specific body size classes based on fragment size and cortical bone thickness. Subsequent ZooMS analysis illustrates a scattering of species across and within these categories (e.g. *Elephantidae* in the medium size class, *Capra* sp. in the large size class) (Fig. 2). Body size class attributions should therefore be used with caution. Instead, molecular approaches like ZooMS can provide a more secure assignment of taxonomic identity and gives a more informative picture of species proportions, and associated bone surface modifications, within an assemblage.

## Conclusion

Faunal remains from archaeological sites allow us to reconstruct how hominin populations adapted to changing climates and environments through the detailed study of patterns of hominin subsistence. Faunal analysis provides ecological information and also illustrates hominin behaviour associated with prey choice and carcass exploitation. High bone fragmentation rates, due to both natural and anthropogenic processes, result in low proportions of morphologically identifiable remains for many Palaeolithic faunal assemblages. Previous studies have relied solely on morphologically identifiable fauna, which can potentially exclude vast quantities of specimens and archaeologically valuable data. Through the biomolecular analysis of a large number of unidentifiable bone fragments, we have observed a significant quantitative difference in the ZooMS faunal spectrum compared to the

morphologically identifiable portion within the same assemblage. This is most evident as a 6-fold increase in the number of *Bos/Bison* specimens in the morphologically unidentifiable fragments; this is possibly due to the size of *Bos*/*Bison* bone elements, their processing during food procurement, and differences in bone elements identified through molecular and morphological methods of taxonomic identification. We also demonstrate that assigning bone fragments to body size classes based on bone cortical thickness and fragment size is an unreliable predictor of taxonomic identity, and these categorizations should therefore be used cautiously in behavioural interpretations of assemblage formation. We have thereby demonstrated that combining molecular and traditional zooarchaeological analysis can provide additional complementary insights into Pleistocene faunal assemblages and hominin subsistence behaviour.

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## Author contributions

V.S.M., F.W. and G.M.S. designed the research. V.S.M. and F.W. performed the proteomic analysis. M.R. and M.P. performed zooarchaeological analysis. J.J.-H. and A.W. provided technical support. V.S.M., F.W. and G.M.S. wrote the manuscript with contributions of all authors.*

## Additional information

**Supplementary information** accompanies this paper at https://doi.org/10.1038/s41598-019-48706-z.

# SUPPLEMENTARY INFORMATION TO:

# Combining ZooMS and zooarchaeology to study Late Pleistocene hominin behaviour at Fumane (Italy).

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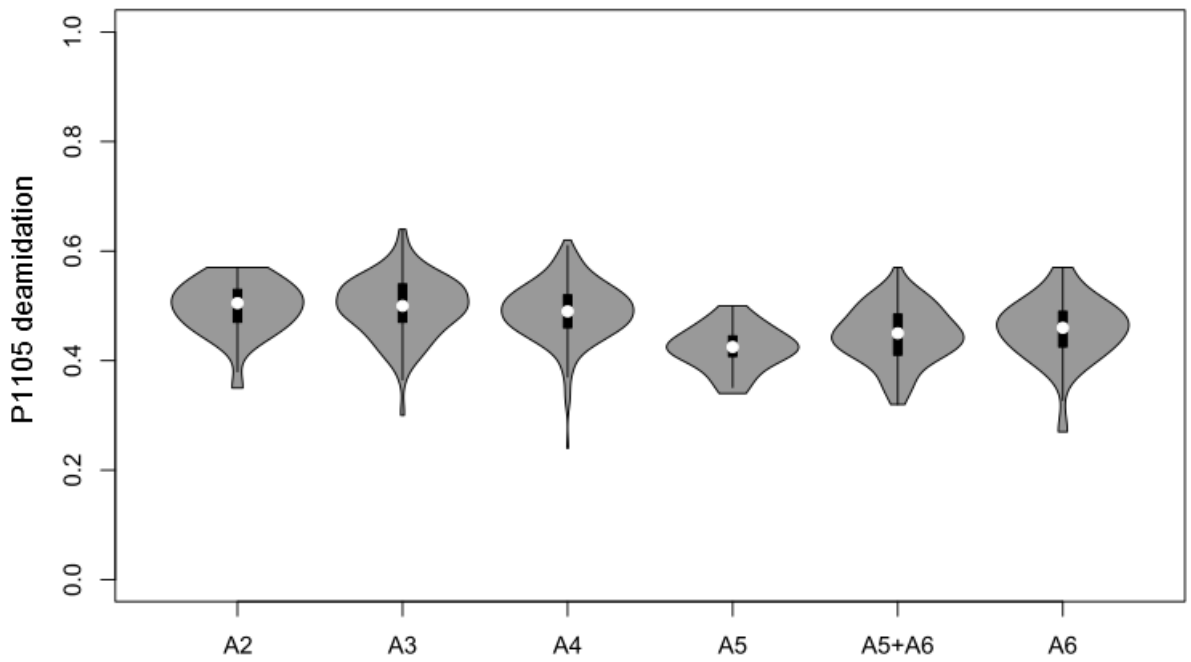
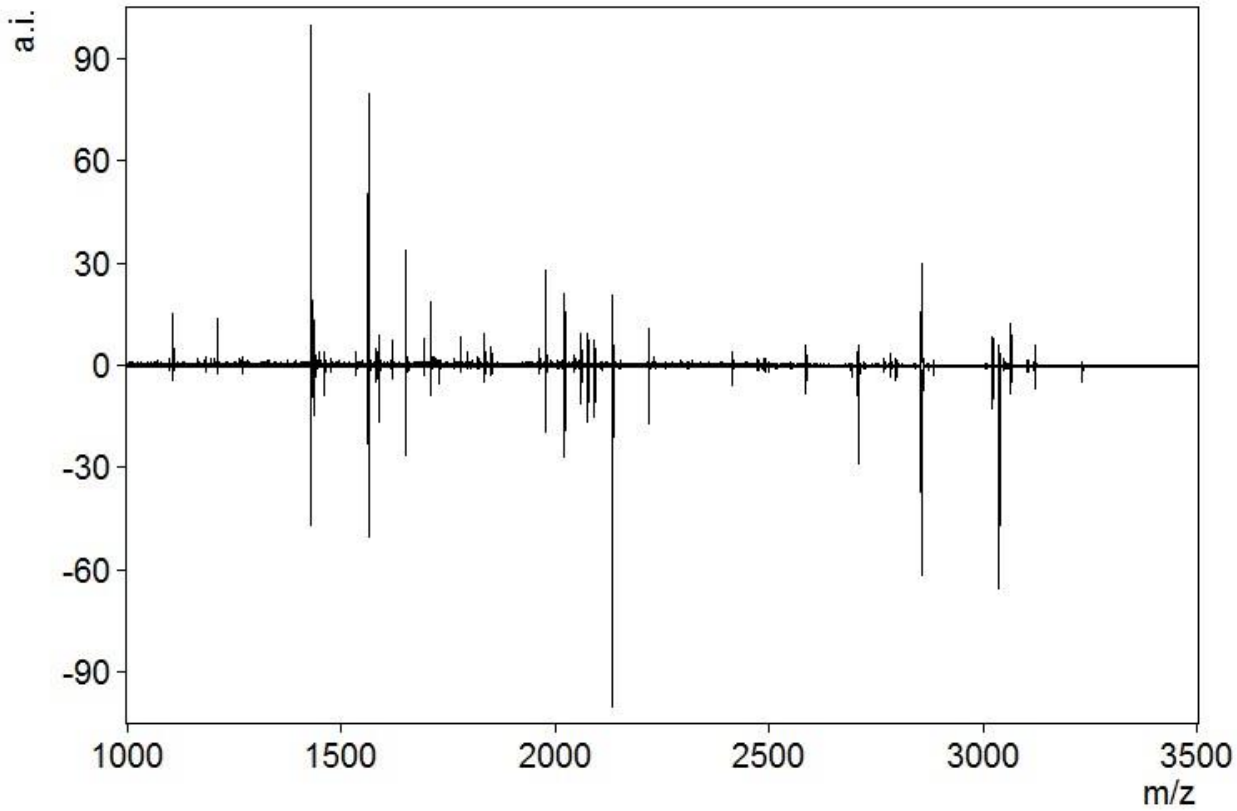



**Supplementary Figure S1. Temporal cline of bone P1105 deamidation values across the Fumane stratigraphy.** Chronologically younger layers (from A2) are less deamidated than chronologically older layers (to A6). P1105 deamidation distributions between different archaeological layers are significantly different at $p<0.05$ for all comparisons except between the layers A3 (Uluzzian) and A4 (Final Mousterian; see main text).




**Supplementary Figure S2. Comparison of an AmBic (top) and Acid (bottom) extract analysed in York and Leipzig, respectively.** Spectra are displayed in the m/z range of 1000-3500, and concern the same morphologically unidentifiable bone specimen (F-258; Bos/Bison). Y-axis (top) and inverted y-axis (bottom) shows relative intensity to the highest peak in either spectrum.

**Supplementary Table S1. Percentage of number of identified specimens (%NISP) of herbivores for the morphologically identified (Morph.) and the ZooMS-component (ZooMS) from Les Cottés (France), Quinçay (France), Pin Hole Cave (UK), and Grotte du Renne (France)**. Note that the values for Pin Hole are estimates as the original publication does not provide numeric information, only graphical.

| Species | Les Cottés ZooMS | Les Cottés Morph . | Quincay ZooMS | Quincay Morph. | Pin Hole ZooMS | Pin Hole Morph. | Grotte du Renne ZooMS | Grotte du Renne Morph. |
|---|---|---|---|---|---|---|---|---|
| *Bos/Bison* | 32.9 | 26.7 | 21.1 | 13.6 | 7.0 | 6.8 | 5.6 | 4.0 |
| *Capra* sp. | 1.4 | 0 | 0 | 0 | 0 | 0 | 0.9 | 0 |
| Ovis-type | 0 | 0 | 0.7 | 1.4 | 0 | 0 | 0 | 0 |
| Cervid/Saiga | 5.7 | 0 | 1.0 | 2.8 | 2.1 | 0 | 10.2 | 0 |
| *Capreolus capreolus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Rangifer tarandus* | 32.9 | 54.7 | 33.7 | 42.2 | 60.5 | 62.0 | 45.4 | 55.0 |
| Suidae | 1.4 | 0 | 1.0 | 0 | 0 | 0 | 0 | 0 |
| Equidae | 18.6 | 14.7 | 39.8 | 35.2 | 9.2 | 9.3 | 26.9 | 20.0 |
| Elephantidae | 2.9 | 4.0 | 1.2 | 0.9 | 5.2 | 5.3 | 7.4 | 21.0 |
| Rhinocerotidae | 4.3 | 0 | 1.5 | 3.8 | 16.0 | 16.5 | 3.7 | 0 |

**Supplementary Table S2. Squares, sublayers and layers studied with ZooMS.** For further information see Peresani et al. [44,81,92].

| Layers | Sublayers | Squares |
|---|---|---|
| A2 | A2R; A2+A2R | 116; 117; 127; 128 |
| A3 | A3; A3I; A3II; A3III; A3IV; A3-A4II; A3 tetto; | 56; 57; 58; 59; 66; 67; 68; 69; 76; 77; 78; 79; 86; 87; 88; 89; 97; 98 |
| A4 | A4; A4II; A4II/A5-A6; A4IV; A4V; A4V/A5-A6; A4V/A5; A4VI; A4VI/A5A6; A4-A5 | 56; 57; 58; 59; 66; 67; 68; 69; 76; 77; 78; 79; 86; 87; 88; 89; 96; 97; 98; 99; 106; 107; 108; 109; 116; 117; 118 |
| A5 | A5 | 41; 53; 61; 62; 71; 72 |
| A5+A6 | A5+A6; A5+A6-A6 | 60; 65; 70; 75; 81; 90; 91; 95; 100; 101; 105; 106; 107;<br>108; 111; 116; 117; 118 |
| A6 | A6 | 51; 61; 80; 117; 118 |

**Supplementary Table S3. ZooMS species IDs for all studied levels.** Percentages in the bottom row indicate the identification success rate per level. In the context of Fumane, Cervid/Saiga can be attributed to either *Cervus elaphus* (red deer), *Megaloceros giganteus* (giant deer) or *Alces alces* (elk), as an attribution to *Saiga* sp. or *Dama* sp. can be excluded based on our knowledge of the fauna in this region for this period. Ovis-type includes non-*Capra* sp. members of the Caprinae. In the context of Fumane an attribution to *Rupicapra rupicapra* (chamois) is possible. Canidae includes members of *Canis* sp. and *Vulpes alopex* (arctic fox), both of which are known morphologically at Fumane, but based on the ZooMS marker series an attribution to *Vulpes vulpes* can be excluded. Felinae is represented morphologically by *Lynx lynx* at the site for the stratigraphic portion considered in this study, and is the most likely species candidate for this taxonomic group in the ZooMS component.

| **Species** | **A2** | **A3** | **A4** | **A5** | **A5+A6** | **A6** |
|---|---|---|---|---|---|---|
| Canidae (not red fox) | 0 | 1 | 4 | 0 | 0 | 0 |
| Red fox | 0 | 1 | 3 | 0 | 0 | 0 |
| Felinae | 0 | 2 | 1 | 0 | 0 | 0 |
| Ursidae | 0 | 0 | 7 | 0 | 5 | 1 |
| *Bos/Bison* | 7 | 88 | 76 | 1 | 7 | 1 |
| *Capra* sp. | 5 | 23 | 12 | 0 | 8 | 1 |
| Ovis-type | 1 | 13 | 21 | 2 | 3 | 1 |
| Cervid/Saiga | 11 | 80 | 141 | 15 | 86 | 22 |
| *Capreolus* sp. | 0 | 8 | 3 | 1 | 2 | 0 |
| Rhinocerotidae | 0 | 1 | 1 | 0 | 0 | 0 |
| Elephantidae | 0 | 0 | 3 | 0 | 0 | 0 |
| | | | | | | |
| Pantherinae/Hyaenidae | 0 | 1 | 0 | 0 | 0 | 0 |
| Cervidae/Bovidae | 0 | 1 | 0 | 0 | 0 | 0 |
| Cervid/Saiga/Roe deer | 0 | 3 | 3 | 1 | 0 | 0 |
| | | | | | | |
| Indeterminate | 0 | 0 | 2 | 0 | 4 | 0 |
| | | | | | | |
| **Total** | **24** (100%) | **222** (98%) | **277** (98%) | **20** (95%) | **115** (97%**)** | **26** (100%) |

**Supplementary Table S4. A3 and A4 surface modification frequencies for the three main species groups per identification method.** All agents of surface modification presented in the table below relate to the frequency of presence for each of these attributes. Data for all studied squares for both ZooMS and morphological analysis are included. Dental remains and burned specimens are not included in the calculation of the shown percentages. See Supplementary Table S5 for similar frequencies derived from a restricted set of squares. Numbers listed are percentages of occurrence and those in parentheses are number of specimens. ZooMS: bone component identified through ZooMS analysis. Morph.: bone component identified through morphology.

| | | **Cervid/Saiga** | | ***Capra* sp.** | | ***Bos/Bison*** | |
|---|---|---|---|---|---|---|---|
| | | **ZooMS** | **Morph.** | **ZooMS** | **Morph.** | **ZooMS** | **Morph.** |
| **Weathering** | A3 | 27.4 (14) | 15.6 (25) | 21.4 (3) | 15.6 (13) | 20.7 (11) | 40.0 (8) |
| | A4 | 43.0 (40) | 21.9 (40) | 25.0 (1) | 24.0 (12) | 15.5 (7) | 52.9 (9) |
| **Concretion** | A3 | 33.3 (17) | 26.8 (43) | 14.2 (2) | 32.5 (27) | 24.5 (13) | 60.0 (12) |
| | A4 | 20.4 (19) | 39.0 (71) | 50.0 (2) | 22.0 (11) | 17.7 (8) | 52.9 (9) |
| **Corrosion** | A3 | 15.6 (8) | 5.6 (9) | 42.8 (6) | 24.0 (20) | 20.7 (11) | 30.0 (6) |
| | A4 | 10.7 (10) | 9.9 (18) | 50.0 (2) | 18.0 (9) | 20.0 (9) | 17.6 (3) |
| **Exfoliation** | A3 | 5.9 (3) | 25.6 (41) | 0.0 (0) | 15.6 (13) | 16.9 (9) | 35.0 (7) |
| | A4 | 10.7 (10) | 22.5 (41) | 0.0 (0) | 18.0 (9) | 11.1 (5) | 23.5 (4) |
| **Mineral staining** | A3 | 29.4 (15) | 16.8 (27) | 21.4 (3) | 16.8 (14) | 9.4 (5) | 15.0 (3) |
| | A4 | 25.8 (24) | 32.4 (59) | 25.0 (1) | 34.0 (17) | 13.3 (6) | 5.9 (1) |
| **Root etching** | A3 | 64.7 (33) | 59.3 (95) | 50.0 (7) | 44.5 (37) | 49.0 (26) | 60.0 (12) |
| | A4 | 69.8 (65) | 53.2 (97) | 75.0 (3) | 46.0 (23) | 57.7 (26) | 47.0 (8) |
| **Carnivore and/or Rodent marks** | A3 | 5.9 (3) | 1.9 (3) | 14.2 (2) | 15.6 (13) | 1.9 (1) | 25.0 (5) |
| | A4 | 3.2 (3) | 3.8 (7) | 0.0 (0) | 4.0 (2) | 8.9 (4) | 0.0 (0) |
| **Cut marks** | A3 | 58.8 (30) | 36.8 (59) | 35.7 (5) | 24.0 (20) | 32.0 (17) | 50.0 (10) |
| | A4 | 44.0 (41) | 48.9 (89) | 25.0 (1) | 16.0 (8) | 13.3 (6) | 41.1 (7) |
| **Impact points** | A3 | 7.8 (4) | 21.8 (35) | 7.14 (1) | 4.81 (4) | 15.0 (8) | 10.0 (2) |
| | A4 | 13.9 (13) | 19.7 (36) | 0.0 (0) | 6.0 (3) | 24.4 (11) | 35.2 (6) |
| **Percussion Marks** | A3 | 3.9 (2) | 4.4 (7) | 0.0 (0) | 0.0 (0) | 30.1 (16) | 0.0 (0) |
| | A4 | 2.2 (2) | 3.3 (6) | 0.0 (0) | 0.0 (0) | 11.1 (5) | 0.0 (0) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Total NISP** | A3 | 51 | 160 | 14 | 83 | 53 | 20 |
| | A4 | 93 | 182 | 4 | 50 | 45 | 17 |

**Supplementary Table S5. A3 and A4 surface modification frequencies for the three main species groups per identification method, derived from the squares also covered by ZooMS sampling.** All agents of surface modification presented in the table below relate to the frequency of presence for each of these attributes. Data for all studied squares for both ZooMS and morphological analysis are included. Dental remains and burned specimens are not included in the calculation of the shown percentages. Numbers listed are percentages of occurrence and those in parentheses are number of specimens. ZooMS: bone component identified through ZooMS analysis. Morph.: bone component identified through morphology.

| | | **Cervid/Saiga** | | ***Capra* sp.** | | ***Bos*/*Bison*** | |
|---|---|---|---|---|---|---|---|
| | | **ZooMS** | **Morph.** | **ZooMS** | **Morph.** | **ZooMS** | **Morph.** |
| **Weathering** | A3 | 27.4 (14) | 35.0 (7) | 21.4 (3) | 23.0 (3) | 20.7 (11) | 50.0 (3) |
| | A4 | 43.0 (40) | 37.2 (19) | 25.0 (1) | 63.6 (7) | 15.5 (7) | 85.7 (6) |
| **Concretion** | A3 | 33.3 (17) | 15.0 (3) | 14.2 (2) | 15.3 (2) | 24.5 (13) | 50.0 (3) |
| | A4 | 20.4 (19) | 21.5 (11) | 50.0 (2) | 9.1 (1) | 17.7 (8) | 57.1 (4) |
| **Corrosion** | A3 | 15.6 (8) | 20.0 (4) | 42.8 (6) | 38.4 (5) | 20.7 (11) | 33.3 (2) |
| | A4 | 10.7 (10) | 11.7 (6) | 50.0 (2) | 27.2 (3) | 20.0 (9) | 14.2 (1) |
| **Exfoliation** | A3 | 5.88 (3) | 15.0 (3) | 0.0 (0) | 7.7 (1) | 16.9 (9) | 16.6 (1) |
| | A4 | 10.7 (10) | 25.4 (13) | 0.0 (0) | 9.1 (1) | 11.1 (5) | 28.5 (2) |
| **Mineral staining** | A3 | 29.4 (15) | 15.0 (3) | 21.4 (3) | 7.7 (1) | 9.4 (5) | 0.0 (0) |
| | A4 | 25.8 (24) | 47.0 (24) | 25.0 (1) | 27.2 (3) | 13.3 (6) | 0.0 (0) |
| **Root etching** | A3 | 64.7 (33) | 65 (13) | 50.0 (7) | 61.5 (8) | 49.0 (26) | 50.0 (3) |
| | A4 | 69.8 (65) | 43.1 (22) | 75.0 (3) | 45.4 (5) | 57.7 (26) | 57.1 (4) |
| **Carnivore and/or rodent marks** | A3 | 5.88 (3) | 10.0 (2) | 14.2 (2) | 15.3 (2) | 1.88 (1) | 16.6 (1) |
| | A4 | 3.22 (3) | 1.96 (1) | 0.0 (0) | 0.0 (0) | 8.9 (4) | 0.0 (0) |
| | A3 | 58.8 (30) | 55.0 (11) | 35.7 (5) | 15.3 (2) | 32.0 (17) | 33.3 (2) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Cut marks** | A4 | 44.0 (41) | 52.9 (27) | 25.0 (1) | 18.1 (2) | 13.3 (6) | 71.4 (5) |
| **Impact points** | A3 | 7.8 (4) | 15.0 (3) | 7.1 (1) | 7.7 (1) | 15.0 (8) | 33.3 (2) |
| | A4 | 13.9 (13) | 19.6 (10) | 0.0 (0) | 27.2 (3) | 24.4 (11) | 57.1 (4) |
| **Percussion Marks** | A3 | 3.9 (2) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 30.1 (16) | 0.0 (0) |
| | A4 | 2.2 (2) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 11.1 (5) | 0.0 (0) |
| **Total NISP** | A3 | 51 | 20 | 14 | 13 | 53 | 6 |
| | A4 | 93 | 51 | 4 | 11 | 45 | 7 |

Chapter Three

# Identifying the unidentified enhances insights into hominin subsistence strategies during the Middle to Upper Palaeolithic transition

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## Abstract

Understanding Palaeolithic hominin subsistence strategies requires the comprehensive taxonomic identification of faunal remains. The high fragmentation of Late Pleistocene faunal assemblages often prevents proper taxonomic identification based on bone morphology. It has been assumed that the morphologically unidentifiable component of the faunal assemblage would reflect the taxonomic abundances of the morphologically identified portion. In this study, we analyse three faunal datasets covering the Middle to Upper Palaeolithic transition (MUPT) at Bacho Kiro Cave (Bulgaria), and Les Cottés and La Ferrassie (France) with the application of collagen type I peptide mass fingerprinting (ZooMS). Our results emphasise that the fragmented component of Palaeolithic bone assemblages can differ significantly from the morphologically identifiable component. We obtain contrasting identification rates between taxa resulting in an overrepresentation of morphologically identified reindeer (*Rangifer tarandus*) and an underrepresentation of aurochs/bison (*Bos/Bison)* and horses (Equus) at Les Cottés and La Ferrassie. Together with an increase in the relative diversity of the faunal composition, these results have implications for the interpretation of subsistence strategies during a period of possible interaction between Neanderthals and anatomically modern humans in Europe. Furthermore, shifts in faunal community composition and in carnivore activity suggest a change in the interaction between humans and carnivores across the MUPT, and indicate a possible difference in site use between Neanderthals and anatomically modern humans. The combined use of traditional and biomolecular methods allows (zoo)archaeologists to tackle some of the methodological limits commonly faced during the morphological assessment of Palaeolithic bone assemblages.

## Introduction

The investigation of behavioural shifts in prey selection and hunting strategies during phases of major changes in the material record is key to understanding the relationship between human behavioural evolution, cultural variation, and population dynamics (Delagnes & Rendu, 2011; Discamps et al., 2011; Niven et al., 2012; Rendu et al., 2012; Steele, 2004). Traditionally, such behavioural patterns have been approached through the analysis of the stone tools and faunal remains recovered from excavations at Palaeolithic sites. In particular, faunal specimens provide the opportunity to identify and document behaviour developed by human populations for the exploitation of their environment (Gaudzinski & Roebroeks, 2000; Gaudzinski-Windheuser et al., 2014; Morin, 2012; Pederzani et al., 2021; Smith et al., 2021; Stiner, 1993). However, studying ancient fauna not only provides paleoenvironmental information, but when combined with the analysis of bone surface modifications related to

human activity, it can fine-tune the timing of human occupations and helps to reconstruct human diet and interactions with other groups or even species (Steele, 2015). Indeed, faunal exploitation is related to a wide range of behaviours and cognitive aspects entwined with mobility, social organisation, technological development and subsistence capacities (Marean & Assefa, 1999).

However, Late Pleistocene bone assemblages are often highly fragmented, preventing proper taxonomic identification and anatomical attribution of many specimens based on morphology alone (Lyman, 2002; Morin et al., 2017a). Several processes affect faunal remains, starting from decomposition, selective destruction in the sediment, post mortem transport and burial, to preserved bone specimens that are potentially altered during excavation, cleaning treatment, and storage (Lyman & Lyman, 1994; Marean, 1991). All these factors, geological, biological, and cultural, can lead to variability in faunal identification. Together with differential preservation, they can create a potential source of bias for the interpretation and quantification of relative abundances of taxa (Dirrigl, 2002; Marean, 1991; Marean & Kim, 1998; Morin, 2004; Morin et al., 2017a, 2017b; Pickering et al., 2006). Indeed, combined with the impact of human and carnivore activities at the site, these factors contribute to reduced proportions of taxonomically diagnostic bones resulting in a lower number of identifiable specimens. Such processes generate the potential to seriously distort various archaeological and ecological inferences (Faith, 2007; Morin et al., 2017a).

Recent developments of biomolecular methods allow us to exploit the collagen preserved in these bone fragments to taxonomically identify faunal specimens (Buckley et al., 2009). The inclusion of the analysis of highly fragmented bone through proteomic screening using zooarchaeology by mass spectrometry (ZooMS) for the taxonomic assessment of Palaeolithic faunal assemblages has already demonstrated its great potential (Berto et al., 2021; Brown, Wang, et al., 2021; Buckley et al., 2017; Pothier Bouchard et al., 2020; Ruebens et al., 2022; Sinet-Mathiot et al., 2019; Welker et al., 2015) and highlighted the necessity to use a multi-methodological approach in studying human subsistence. Taxonomic identities from both the morphologically identified and the ZooMS identified components can thus be correlated with bone surface modification analysis to address specimen surface preservation and bone accumulation agents through the reconstruction of taphonomic history. The analysis of collagen protein type I provides a taxonomic identity based on the variation in protein amino acid sequence and allows the taxonomic identification of bone assemblages to be extended to the morphologically-unidentifiable component. The previous application of ZooMS as a screening tool for faunal assemblages has provided variable results in terms of the comparability of the two components. Taxonomic abundances of the morphologically unidentifiable component of a faunal assemblage may not generally differ from the morphologically identifiable component (Berto et al., 2021; Buckley et al., 2017; Welker et al.,

2016, 2017), but that does not necessarily indicate a pattern (Ruebens et al., 2022; Sinet-Mathiot et al., 2019). Moreover, such differences could reflect a specific human behavioural signature related to bone fragmentation and intensity of carcass processing (Sinet-Mathiot et al., 2019). A better understanding of the source(s) of variability will help in anticipating the potential differences that may occur within certain bone assemblages.

The zooarchaeological literature frequently contains body size class attributions of bone specimens that cannot be reliably assigned to a particular taxon or clade. It is generally assumed that these body size class attributions are reliable and reflect or contain taxonomic information about the bone assemblage as a whole. However, previous ZooMS research has highlighted that this is a potentially unreliable approach (Sinet-Mathiot et al., 2019). Here we test the fragmentary component of bone assemblages of three Late Pleistocene sites: Bacho Kiro Cave (Bulgaria), Les Cottés and La Ferrassie (France). They all show rich and well-preserved stratigraphic sequences spanning the Middle to Upper Palaeolithic transition (MUPT). These sites offer the opportunity to discuss diachronic changes in subsistence strategies during the period of possible interaction between Neanderthal and Late Pleistocene *Homo sapiens* populations (Hajdinjak et al., 2021; Higham et al., 2014; Hublin, 2015; Hublin et al., 2020; Prüfer et al., 2021). This work explores the implications of incorporating the analysis of the morphologically unidentifiable bone component into the description of faunal assemblages in terms of both overall bone accumulation and aims to advance our interpretation of human subsistence strategies during the MUPT. We address methodological limits commonly faced during the morphological assessment of faunal assemblages and demonstrate how the addition of biomolecular methods, such as untargeted ZooMS screening, can complement our understanding of subsistence behaviour by providing a clearer picture of prey selection and site occupation. By including assemblages that span the MUPT in Europe, we are thereby able to demonstrate that the assessment of the fragmented component of bone assemblages through ZooMS can provide different patterns of species frequencies than previously interpreted based solely on the morphologically identifiable record.

## Material and Methods

### Sample selection

This study includes the ZooMS analysis of bone material from three Late Pleistocene sites (Bacho Kiro Cave, Les Cottés and La Ferrassie; SI Figure 1, SI Table 1). All the material taxonomically identified through bone morphology by zooarchaeologists will be referred to as the morphology component. Similarly, all fragmentary specimens morphologically unidentifiable and taxonomically identified through ZooMS will be referred to as the ZooMS component. All three sites were recently excavated and have provided large, well-contextualised and highly fragmented bone assemblages of individually piece-provenienced

faunal remains. Bone surface analyses of both the morphologically identified and the fragmentary unidentifiable bone assemblages were assessed using comparable zooarchaeological methods and protocols. All faunal data were derived from recent excavation campaigns, and specimens from both the morphology and ZooMS components show similar spatial distributions over the excavated areas. Fragmentary and morphologically unidentifiable piece-provenienced specimens, generally >2 cm in length, were selected for proteomic analysis. Bone material resulting from sediment sieving during the excavation of the archaeological sites are not included in this study. All morphologically unidentifiable piece-provenienced specimens from the La Ferrassie layer 6 faunal assemblage were selected for ZooMS analysis. In the case of Bacho Kiro Cave and Les Cottés, specimens were randomly selected from among the unidentifiable components of the bone assemblages defined by the zooarchaeologists.

## Bacho Kiro Cave

Bacho Kiro Cave (Dryanovo, Bulgaria) is located on the northern slope of the Balkan mountain range (Stara Planina) and about 70 km south of the Danube River. Previously investigated during the 20th century (Garrod et al., 1939; Kozłowski & Ginter, 1982), the site was reopened for excavation in 2015 by the National Archaeological Institute with Museum from the Bulgarian Academy of Sciences (Sofia, Bulgaria) and the Max Planck Institute for Evolutionary Anthropology (Leipzig, Germany). The archaeological sequence spans the Middle Palaeolithic (MP) through to the Upper Palaeolithic (UP). The archaeological material recovered from two sectors (Main Sector and Niche 1) from Layers I and J was recognized as part of the Initial Upper Palaeolithic marked by the earliest occurrence of Late Pleistocene *Homo sapiens* in Europe (Hublin et al., 2020). This starts around 45,990 cal BP in the upper part of Layer J and considerably intensifies in Layer I which is dated to 45,040-43,280 cal BP (Fewlass et al., 2020; Pederzani et al., 2021). This material comprises the earliest and largest number of *Homo sapiens* bone tool and ornament assemblages in Europe, partly taxonomically identified through ZooMS (Martisius et al., 2022). The assemblage recovered from Layer K was technologically associated with the MP and was deposited between 61 ± 6,000yr and 51,000 yr BP (Fewlass et al., 2020; Pederzani et al., 2021). We investigated 1,595 faunal remains through ZooMS from Layer I (814 specimens), Layer J (438 specimens) and Layer K (343 specimens) from both the Niche 1 and the Main Sector (Hublin et al., 2020). Zooarchaeological analysis was performed on 7,013 faunal remains from Layers I, J and K from both sectors following previously described methodology (Smith et al., 2021) and including 1,453 specimens assigned to a taxonomic group (1,077 from Layer I, 232 from Layer J and 143 from Layer K).

## Les Cottés

Les Cottés (Vienne, France) is a cave located in the corridor between the Parisian basin and the Poitou in west-central France. The site was discovered in 1878 and was explored through several excavation campaigns (Bastin et al., 1976; Lévêque, 1997; Pradel, 1967), but the material included in this study derive from an excavation initiated in 2006 by M. Soressi with support of the French Ministry of Culture and Communication and the Max Planck Institute for Evolutionary Anthropology (Soressi et al., 2010). Through ZooMS, we analysed 523 morphologically unidentifiable faunal specimens, which, together with the 152 presented in Welker et al. 2015 (137 undiagnostic fragments, and 15 specimens analysed in a ZooMS blind test), means 675 specimens from Les Cottés were analysed with ZooMS. Of these, 220 are from the Mousterian (US08, dated between 46,051 to 42,034 cal BP using radiocarbon and between 55 and 48 ka according to the OSL measurements (Jacobs et al., 2015)), 217 are from the Châtelperronian (US06, dated between 42,961 to 40,344 cal BP), 168 are from the Protoaurignacian (US04 lower) and 70 are from the Early Aurignacian (US04 upper). The dates for the Aurignacian layers extend from 40,372 to 36,697 cal BP (Talamo et al., 2012) in radiocarbon years, or from 43 to 36 ka in OSL years (Jacobs et al., 2015). Interpretations coming from US04 upper are considered with caution due to the low number of specimens in comparison to the other layers. Bone surface analysis was standardised over the assemblage and was previously described elsewhere (Rendu et al., 2019). Of a total of 5,169 bone remains assessed through traditional zooarchaeology, 1,922 bone and dental specimens were morphologically identified in the range of subfamily to species (397 specimens from US08, 166 from US06, 715 from US04 lower and 629 from US04 upper).

## La Ferrassie

The Grand Abri of La Ferrassie (Savignac-de-Miremont, France) is in the Dordogne region of south-western France in a tributary valley to the Vézère River and was first excavated during the 20th century by Capitan and Peyrony and then by Delporte (Delporte & Delibrias, 1984; Peyrony, 1934). An excavation conducted from 2010 to 2015 by Turq and colleagues further refine the stratigraphic sequence spanning the MUPT (Guérin et al., 2015; Turq et al., 2012). The Châtelperronian layer (Layer 6) was dated to between 45,100 and 39,520 cal BP (Talamo et al., 2020) marking the earliest appearance of this lithic industry in the region. The faunal material from this layer that was morphologically identifiable to taxon is limited to 17.5% of the bone assemblage (142 specimens) and is dominated by reindeer (*Rangifer tarandus*). All piece-plotted, morphologically indeterminate specimens were processed through ZooMS (527 specimens).

## ZooMS methodology

ZooMS extraction protocols employed for this study were partially described previously (Buckley et al., 2009; van Doorn et al., 2011; Welker et al., 2016). All 2,645 specimens were sampled (10-30 mg) using pliers and placed into 96-well plates. Soluble collagen was extracted through incubation in 100µl of 50mM ammonium-bicarbonate (AmBic) buffer at 65°C for one hour. In order to improve and verify the taxonomic identity obtained from soluble collagen, 440 specimens (70 for La Ferrassie, 369 for Les Cottés and 1 for Bacho Kiro Cave) (SI Table 2) were demineralised in 130µl 0.6M HCl at 4°C for 18-20 hours, neutralised to pH 7, and solubilised again in AmBic. Then 50µl of the resulting supernatant was digested using trypsin (0.5µg/µl, Promega) overnight at 37°C, acidified using trifluoroacetic acid (20% TFA), and then cleaned on Hypersep C18 96-well plates (Thermo Scientific) using a vacuum manifold. In short, a 96-well deepwell plate (Eppendorf) is placed beneath the Hypersep plate to collect the solutions. C18 filter tips from the Hypersep plate were conditioned with 200µL of 0.1% TFA in 50:50 acetonitrile and UHQ water (conditioning solution) and washed with 200µL of 0.1% TFA and UHQ water (washing solution). Peptide extracts were then vacuumed through the filters slowly to ensure optimal binding efficiency. The obtained waste solution was discarded. Filters were then washed again with 200µL of washing solution and peptides were extracted in 100µL of conditioning solution and transferred to a 96-well plate. Digested peptides were spotted in triplicate on a MALDI Bruker plate with the addition of α-cyano-4-hydroxycinnamic acid (CHCA, Sigma) matrix, using a multichannel pipette (Thermo Fisher).
MALDI-TOF-MS analysis was conducted at the Fraunhofer IZI in Leipzig (Germany), using an autoflex speed LRF MALDI-TOF (Bruker) in reflector mode, positive polarity, matrix suppression up to 590 Da and collected in the mass-to-charge range 700-3500 m/z. Triplicates were merged for each sample in R version 4.0.5 (R Core Team, 2021, n.d.) and MALDIquant v. 1.21 (Gibb & Strimmer, 2012). First, we smooth the intensity using a moving average and remove the baseline using the TopHat approach. Then, for each sample we align the replicate spectra using SuperSmoother and a signal to noise ratio of 3, sum the three replicates to obtain a single spectrum, and remove the baseline once more, again using TopHat. Spectra were exported as .msd files. Taxonomic identifications were made using mMass (Strohalm et al., 2010) through manual peptide marker mass identification in comparison to a database of peptide marker series for all European Pleistocene medium- to large-sized mammals (Welker et al., 2016). To assess any potential contamination by non-endogenous peptides, we performed laboratory blanks alongside the samples. These remained empty of collagenous peptides, excluding the possibility of modern laboratory or storage contamination.
Peptide marker series can be similar for some closely related species, which is the case for the species belonging to the following taxonomic groups: *Bos/Bison*, Cervid/Saiga, Equidae and Ursidae. Cervid/Saiga can be attributed to either *Cervus elaphus* (red deer), *Megaloceros*

*giganteus* (giant deer), *Alces alces* (elk) or *Dama* sp. (fallow deer). Equidae and Ursidae include, respectively, species from the genera *Equus* and *Ursus*, most likely *Equus ferus* and *Equus hydruntinus* or *Ursus spelaeus* and *Ursus arctos*. In order to facilitate the comparison between ZooMS and morphology components, the most common species and taxa were grouped into broader categories of: *Bos/Bison* (*Bos primigenius*, *Bison priscus* and *Bos/Bison* sp.), Cervid/Saiga (*C. elaphus*, *D. dama*, *M. giganteus*), Ursidae (*U. arctos*, *U. spelaeus* and *Ursus* sp.), *Capra* sp. (*C. ibex* and *Capra* sp.), and Equidae (*E. ferus*, *E. hydruntinus* and Equid sp.). At Bacho Kiro Cave, due to the high proportion of this taxonomic group, cervid specimens from the morphology component were also included into the broader group Cervid/Saiga alongside the few specimens identified as *Rangifer tarandus* and *Capreolus capreolus*. Within the ZooMS component, the few specimens identified as Cervid/Saiga/*Capreolus capreolus* were included in the broader taxonomic group Cervid/Saiga, in order to allow the comparability of both components.

Suggested as an indicator of collagen preservation (Welker et al., 2017; Wilson et al., 2012), glutamine (Gln) deamidation ratios were calculated on all samples for peptide COL1α1 508-519 (Brown, Douka, et al., 2021), which is frequently observed in peptide fingerprints of collagen type I, following published protocols (van Doorn et al., 2012; Wilson et al., 2012). The deamidation ratio ranges from %Gln=1.0 with non-deamidated glutamines to %Gln=0.0 indicating a full deamidation of the glutamines. The Gln deamidation ratios obtained during routine ZooMS screening have been previously suggested to assess bone assemblage homogeneity (spatial and temporal variability within a site), to detect stratigraphic outliers (intrusive material or differential bone preservation), to inform on the preservational quality of specific peptides and specimens, or to look at the taxonomic distribution from a biomolecular perspective (Sinet-Mathiot et al., 2019; Welker et al., 2017; Wilson et al., 2012), although with varying success (Brown, Wang, et al., 2021).

## Zooarchaeological methodology

All taphonomic modifications were recorded on the morphology- and ZooMS-identified specimens by the respective zooarchaeologists, consistently within and between studied sites. Bone surfaces for both the morphologically identified and unidentified components were assessed through visual inspection, using magnification when needed (up to 20x magnification) (Blumenschine et al., 1996). The maximum length of the bone specimens was measured individually with digital calipers.

Although traces of burning were recorded during taphonomic analyses using the scale proposed by Stiner and colleagues (Stiner & Kuhn, 1995) (0: Unburnt to 6: Completely calcined), these burnt remains were excluded from subsequent ZooMS analyses due to poor collagen preservation. Weathering stages were recorded for all bones and provide a qualitative scale for understanding the exposure (short/long duration) of the bones prior to

burial (Behrensmeyer, 1978). A slightly modified scheme was used on Les Cottés bone assemblages where specific modifications were recorded related to weathering (see Rendu et al., 2019). Specifically, weathering was recorded according to three variables: exfoliation (the peeling of bone surface), cracking (the emergence of longitudinal cracks on bone surface), and disintegration (the complete destruction of the bone). In addition, other recorded modifications included root etching and abrasion (expressed as a percentage of bone surface affected). The schemes range from 0% (no visible modification observed) through 100% (the whole bone surface covered; (Smith et al., 2021; Behrensmeyer, 1978; Blumenschine et al., 1996; Domínguez-Rodrigo et al., 2017; Fisher, 1995; Lee Lyman, 1994; Olsen & Shipman, 1988; Soulier & Costamagno, 2017).

For all three bone assemblages, human modifications included traces related to butchery and carcass processing (cut marks, scraping marks, chop marks, marrow bone breakage), and carnivore modifications included tooth marks, gnawing traces and damage from bone breakage and digestion as well as rodent tooth traces. The number of identified specimens (NISP) represents the number of specimens assigned to a taxon.

When it was not possible to morphologically assign fragmentary bone specimens to a specific taxon, these were assigned to a specific body size class based on previous assignments (SI Table 3; Morin, 2012). The separation of specific taxa into different body size classes was normally done on the basis of both body and skeletal size (following (Morin, 2012; Rendu et al., 2019; Smith et al., 2021)).

The combination of the ZooMS and morphology component allows for the assessment of skeletal element distributions and the possible identification of previously unrecognised skeletal elements, which has implications for our understanding of hunting strategies and carcass transport. As skeletal elements were identified, when possible, on taxonomically unidentifiable specimens, we aimed to correlate the skeletal part identifications with the ZooMS taxonomic identities in order to assess skeletal representation among both the morphology- and ZooMS-identified components. To assess skeletal element representation for the dominant taxa within each component, bone elements were categorised into body parts for each method of identification (cranial: cranium, mandible; axial: vertebrae, pelvis, rib; forelimb: humerus, radius, ulna; hindlimb: femur, tibia; distal limbs: carpals, metacarpal, tarsals, metatarsal, phalanges; LBN: long bone fragments, FBN: flat bone fragments) (based on Stiner, 1991a, 1991b). Within all three datasets, teeth and antler were categorised separately from the cranial body part, as their inclusion might bias the comparison between components. Indeed, antlers and horn cores tend to be rare and are more easily identified morphologically, reducing their representation in the ZooMS component. Anatomically unidentifiable specimens (NID) were excluded from the assessment of skeletal element representation as they did not provide substantial information.

Ecological diversity indices were calculated in order to investigate the effect of the addition of the ZooMS-identified specimens on the diversity of the faunal community of each layer and site. We used the Shannon-Wiener Index (H') (Shannon, 1948) (R package *vegan* v. *2.6-2*, Oksanen et al., 2019) to quantify the taxonomic diversity of our three faunal assemblages among each component, taking into account the taxonomic richness and the distribution of their abundance. As the Shannon-Wiener index is sensitive to sample size, values should be considered with caution when the sample size is small. Along with species richness, Pielou's evenness (J') measures taxonomic diversity by giving the count of individuals of each taxonomic group among each component and reflecting the evenness of the distributed abundances between taxa. The index value ranges from 0 (no evenness) to 1 (complete evenness).

# Results

## ZooMS analysis

ZooMS analysis of all three datasets shows well-preserved collagen type I with a high success rate of taxonomic identification, up to the range of subfamily or genus, between 90% and 97% (SI Table 1). For 82% of the samples, the semi-destructive extraction protocol (AmBic) is sufficient to obtain a ZooMS identification. At Bacho Kiro Cave, collagen preservation is excellent (also noted by Fewlass et al., 2020) resulting in a high proportion of distinct taxonomic identities. All ZooMS samples could be extracted using only the AmBic protocol, while we extracted one specimen through acid demineralization as well to verify its taxonomic identity (*Castor fiber*). At both Les Cottés and La Ferrassie, samples were processed using both AmBic and acid demineralisation protocols to improve and optimise taxonomic identifications (SI Table 1).

<u>Deamidation between stratigraphic units and taxa:</u>

Glutamine deamidation ratios are calculated in order to detect potential intrusive material between archaeological layers or differential collagen preservation between taxa. Because the data is not normally distributed (Shapiro-Wilk normality test, p-value < 0.05), we used Wilcoxon-Mann-Whitney tests to compare the glutamine deamidation ratios between taxa and layers. At Bacho Kiro Cave, we observe that older samples from Layer K show elevated levels of deamidation with values significantly different between layers (SI Table 4, SI Figure 2). In contrast, we note overlapping deamidation values between layers at Les Cottés (SI Figure 3) with the exception of US06 which showed values significantly different from US04 Lower and US08 (SI Table 4). Glutamine deamidation ratios seem to overlap between dominant taxa which would suggest that they have undergone similar molecular diagenetic processes within each site (SI Figure 4). However, a few exceptions could be

identified. At Bacho Kiro Cave, deamidation ratios show similarities between taxa, particularly within layer K, but ursid specimens tend to have glutamine deamidation values significantly different from other taxa in layers I and J, notably in comparison with *Bos/Bison*, *Capra* sp. and Equidae (SI Table 5). At Les Cottés, all taxonomic groups show similar deamidation ratios within each layer, except for a few *Rangifer tarandus* specimens (n = 6) showing deamidation values significantly different from Equidae in US04 Lower (Wilcoxon-Mann-Whitney tests, statistic = 64, p-values = 0.013, SI Table 5). At La Ferrassie, *Rangifer tarandus* and Cervid/Saiga specimens show deamidation values significantly different from *Bos/Bison* (SI Table 5). The statistical differences observed between some of the taxonomic groups and layers could be driven by discrepancies in sample sizes, taphonomic history and site formation or butchery practices. However, further exploration is required in order to interpret these differences.

## Taxonomic representation

Species representation among both ZooMS and morphology components are generally consistent within each site, but the addition of ZooMS permits the identification of taxa that were unrecognisable through morphology. At Les Cottés, ZooMS identified Felidae and Ursidae in the faunal community obtained from US06, but also resulted in the addition of Cervid/Saiga in US04 Lower (SI Table 6). At Bacho Kiro Cave, the ZooMS analysis allowed for the identification of Elephantidae in Layer J (SI Table 7). At La Ferrassie, the use of ZooMS results in a 4-fold increase of the number of taxonomically identified specimens. Consequently, the taxonomic diversity for this layer was broadened, with the addition of *Capra* sp., Rhinocerotidae, Ursidae and several carnivores (SI Table 8).

Shannon-Wiener index calculations show that the diversity of the faunal community identified on a site can significantly change with the addition of ZooMS. More specifically, we observe an increase in the faunal diversity of the combined ZooMS and morphology components in the layers under study here at La Ferrassie, at Les Cottés and at Bacho Kiro Cave (Figure 1, SI Table 9). In contrast, the lower values of the Shannon-Wiener index, after the addition of ZooMS identities to the Layer I faunal assemblage, at Bacho Kiro Cave indicate a lower taxonomic diversity. Such a pattern possibly emphasises a better identification rate within the morphology component related to a larger sample size, or highlights a higher evenness of the ZooMS component due to the repeated identification of taxa showing a low abundance among the morphology component.

The occurrence of the dominant taxa, i.e. the taxa showing the highest proportions, among both components are consistent within each site (Bacho Kiro Cave: Ursidae, Equidae, Cervid/Saiga, *Capra* sp. and *Bos/Bison*; Les Cottés: *Bos/Bison*, Equidae and *Rangifer tarandus*; La Ferrassie: *Bos/Bison*, Cervid/Saiga, Equidae and *Rangifer tarandus*), but we

observe differences in their relative contributions to the overall bone assemblage (Figure 2). At Les Cottés and La Ferrassie, the ZooMS component indicates lower proportions of reindeer, offset by higher proportions of *Bos/Bison* and Equidae (SI Table 6, 8 and 10). We note a 9-fold increase in the proportion of *Bos/Bison* at La Ferrassie. At Les Cottés, we observe an on average 2-fold increase of *Bos/Bison* and Equidae with the addition of ZooMS to the analysis of the faunal assemblage. At Bacho Kiro Cave, and similar to Les Cottés and La Ferrassie, *Bos/Bison* remains are slightly more abundant within the ZooMS component, particularly in Layers I and J. Conversely, Ursidae show a similar pattern as reindeer at La Ferrassie and Les Cottés with slightly lower proportions notably in Layers J and K. We note a large difference between methods of identification for Cervid/Saiga in Layer I, but these differences are not consistent throughout the other layers. When comparing the faunal composition between layers to assess any changes or shifts in the NISP of different taxa across the MUPT, we note at Les Cottés a progressive decrease in the proportions of *Bos/Bison* offset by an increase of Equidae from US08 to US04, which is particularly clear through the use of ZooMS. Despite the low number of specimens analysed through ZooMS from the Early Aurignacian layer (US04 upper) of Les Cottés, the results obtained show a continuous pattern with those from the layers below in terms of taxonomic abundances between the dominant taxa.

While the categorisation of morphologically unidentifiable specimens into body size classes remains a useful tool when no other alternative is available for the interpretation of this component of the assemblage, the correlation between taxonomic identifications provided by ZooMS with the body size classes indicates inconsistencies. Therefore, the observations made previously at Fumane Cave therefore do not seem to be an exception, but rather the norm (Martisius et al., 2020; Sinet-Mathiot et al., 2019). We observe inconsistencies between body size class attributions, which are largely based on bone size and cortical thickness, and ZooMS taxonomic assignments (SI Table 3). For example, Ursidae specimens are present in most carnivore and ungulate body size class units (Figure 3), several equid specimens are categorised among the large carnivore class, and Caprinae and *Capra* sp. among the large ungulate class (Figure 3). Although many zooarchaeologists are already using alternative nomenclatures (i.e. *mammal classes* or *unknown* instead of *ungulate* or *carnivore classes (Castel, 2011))*, or standardisation tools (Discamps, 2021), these results simply confirm that body size class attributions should be used with caution, especially when translating these classes to more specific taxonomic units and/or assessment of hominin subsistence strategies. When assigning bone specimens to generalised family attributions, one should cautiously avoid “taxonomic blindness” based on presumed abundance of cladistic assignments that are based on the thickness of the cortical bone.

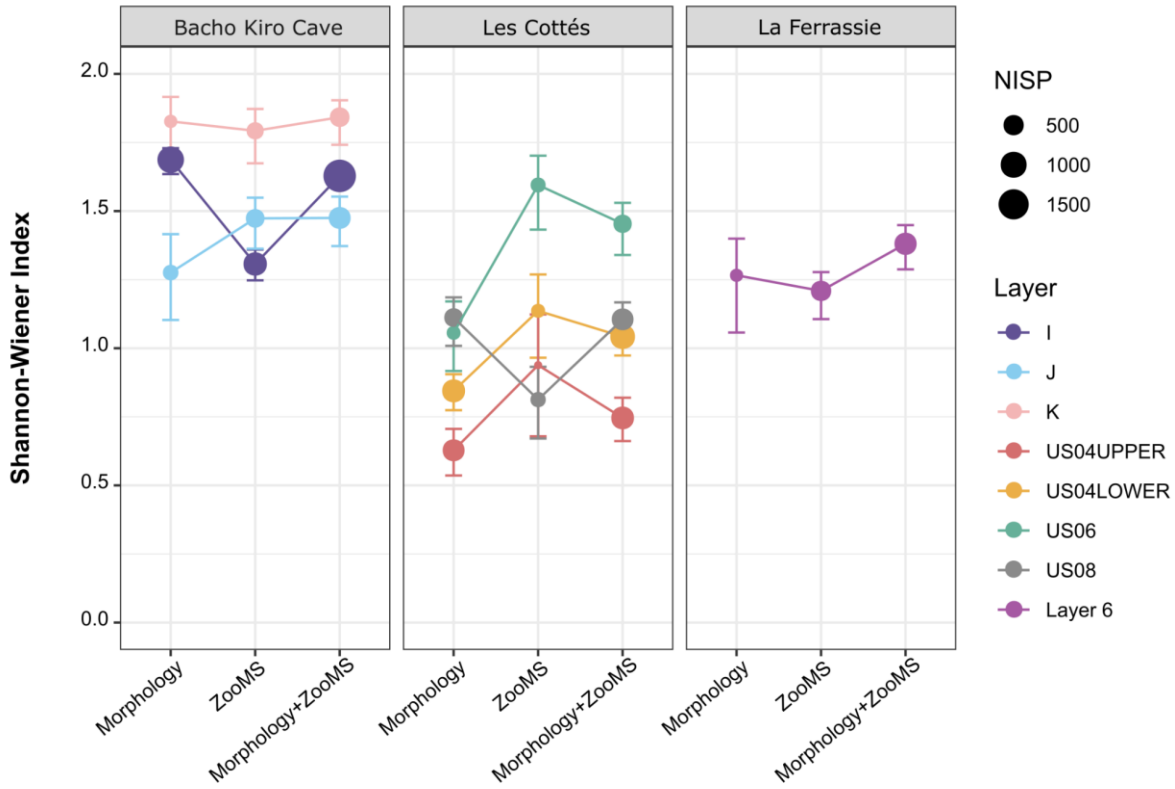


**Figure 1:** Shannon-Wiener Index for each studied layer of Bacho Kiro Cave, Les Cottés and La Ferrassie compared between methods of taxonomic identification (see SI Table 9 for details). Confidence intervals (2.5%-97.5%) are given for each value.

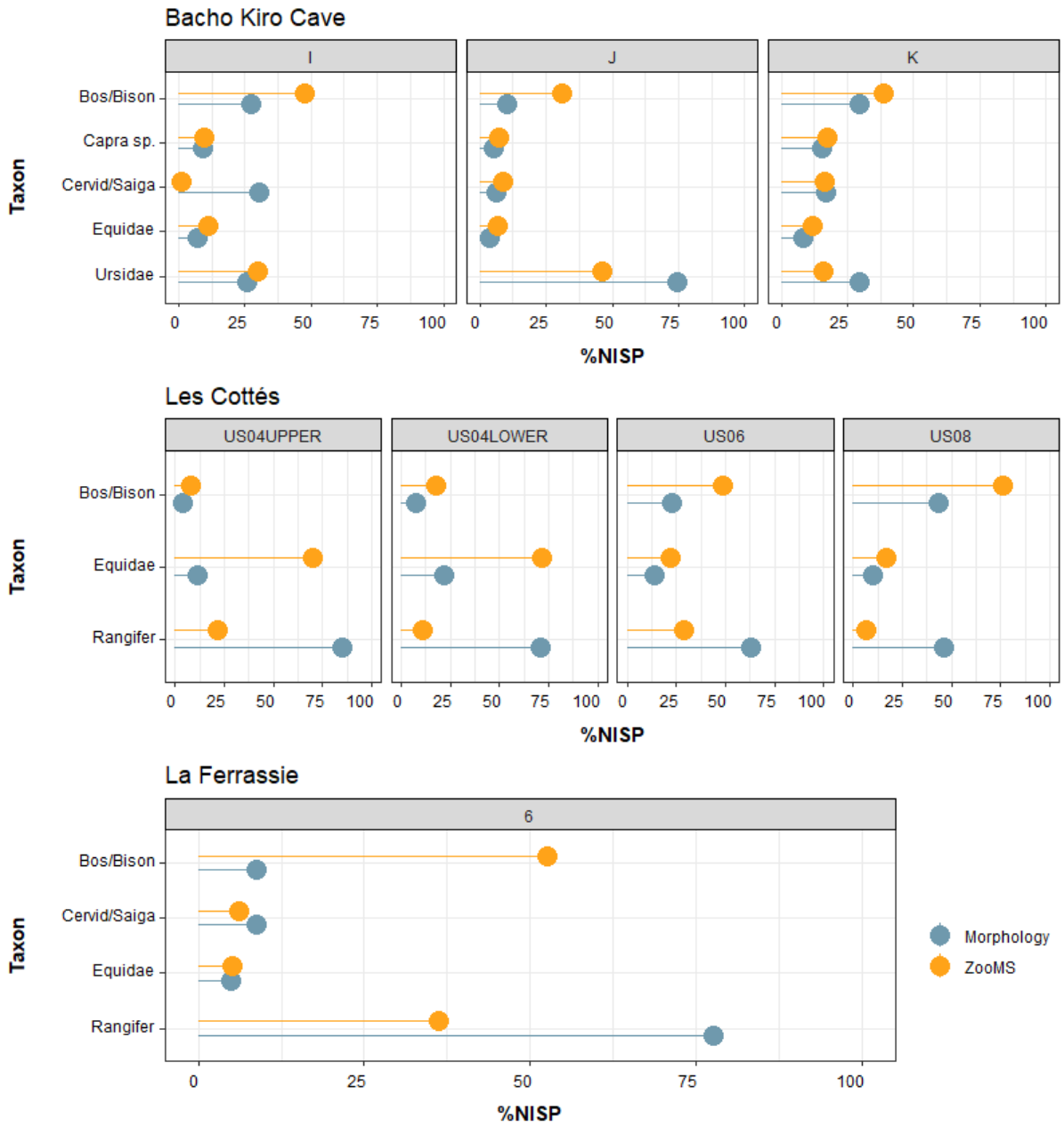


**Figure 2:** Percentage of the dominant taxa among both ZooMS and morphology components at Bacho Kiro Cave, Les Cottés and La Ferrassie.

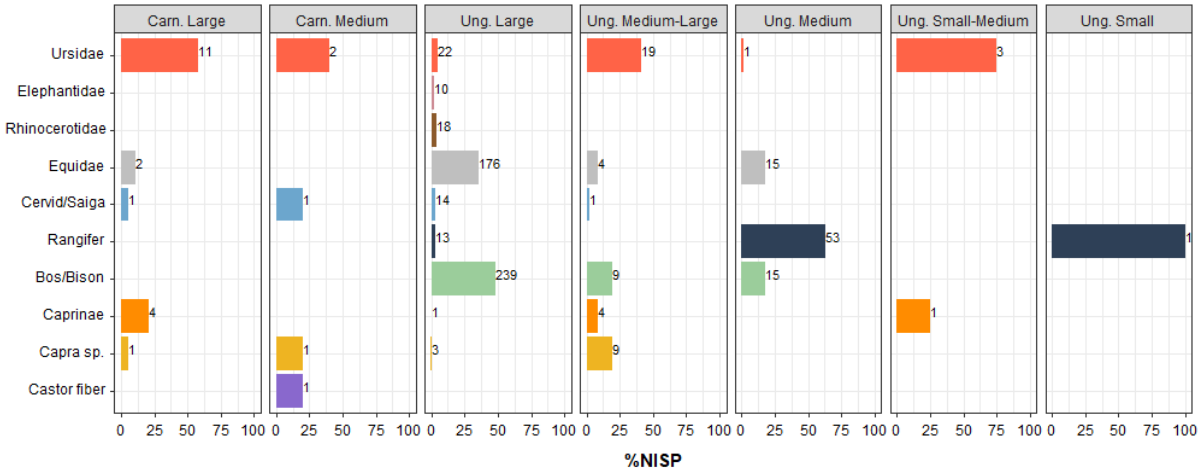


**Figure 3:** Combined %NISP from Bacho Kiro Cave, Les Cottés and La Ferrassie of taxa identified using ZooMS (rows) and morphology (column headings) in relation to their body size class attribution. Carn=Carnivore and Ung=Ungulate. Each taxon is assigned a colour to help the visualisation of the graph. Numbers on the bars are the NISP per category.

## Bone length distribution

As expected, larger bone fragments are generally more identifiable through comparative morphology as they often preserve more morphologically distinctive features. Smaller fragments tend to be identifiable only through ZooMS (Figure 4). This pattern is particularly noticeable at Bacho Kiro Cave in Layers I and K (SI Figure 5). However, this is not the case for all taxa. We note a different specimen length distribution between both ZooMS and morphology components among dominant taxa. At Bacho Kiro Cave, *Bos/Bison*, Cervid/Saiga and Equidae specimens show an opposite bipolar distribution of their specimen length whereas the two distributions are more similar for *Capra* sp. and Ursidae. Because the data is not normally distributed (Shapiro-Wilk normality test, p-value < 0.05), we used Wilcoxon-Mann-Whitney tests to compare the bone length distribution between taxa, layers and method of identification. Bone specimens identified as *Capra* sp. and Ursidae through ZooMS show a fragment length distribution significantly different from other taxonomic groups particularly in layer I (SI Table 11). Likewise, observations of the bone assemblage from Les Cottés indicate a similar trend with *Bos/Bison* and Equidae most often exhibiting opposite distributions, compared to the similar distributions of both ZooMS and morphology components for the reindeer specimens (SI Figure 6). At Les Cottés, the bone length distribution of specimens identified morphologically as reindeer are significantly different from the *Bos/Bison* and of Equidae distributions in US04 and US08 (SI Table 12), but no differences are observed among the ZooMS component. When comparing the distribution between methods of identification, we also note significant differences for *Bos/Bison* and equid specimens in US04 and US08 (SI Table 13). The absence of metric measurements on the morphologically identified component from La Ferrassie prevents comparisons of bone length

distribution between the ZooMS and morphological components. However, the ZooMS component represents 82.5% of the faunal assemblage, so a comparison of specimen length between dominant taxa for the ZooMS component is possible. Although specimens from the dominant taxa generally show similar length distributions, with a large proportion within the 2-3 cm range, equid bones tend to have fewer large fragments illustrated by a higher proportion of specimens within the smaller size classes (SI Figure 7). Equid fragments identified through ZooMS present a length distribution significantly different from *Bos/Bison*, Cervid/Saiga and reindeer (SI Table 14), most likely due to an over-representation of equid fragments of 2-3 cm counterbalanced by an under-representation of specimens of 3-4 cm. Nevertheless, it should be noted that Equidae is the taxa with the smallest sample size, which might influence these results.

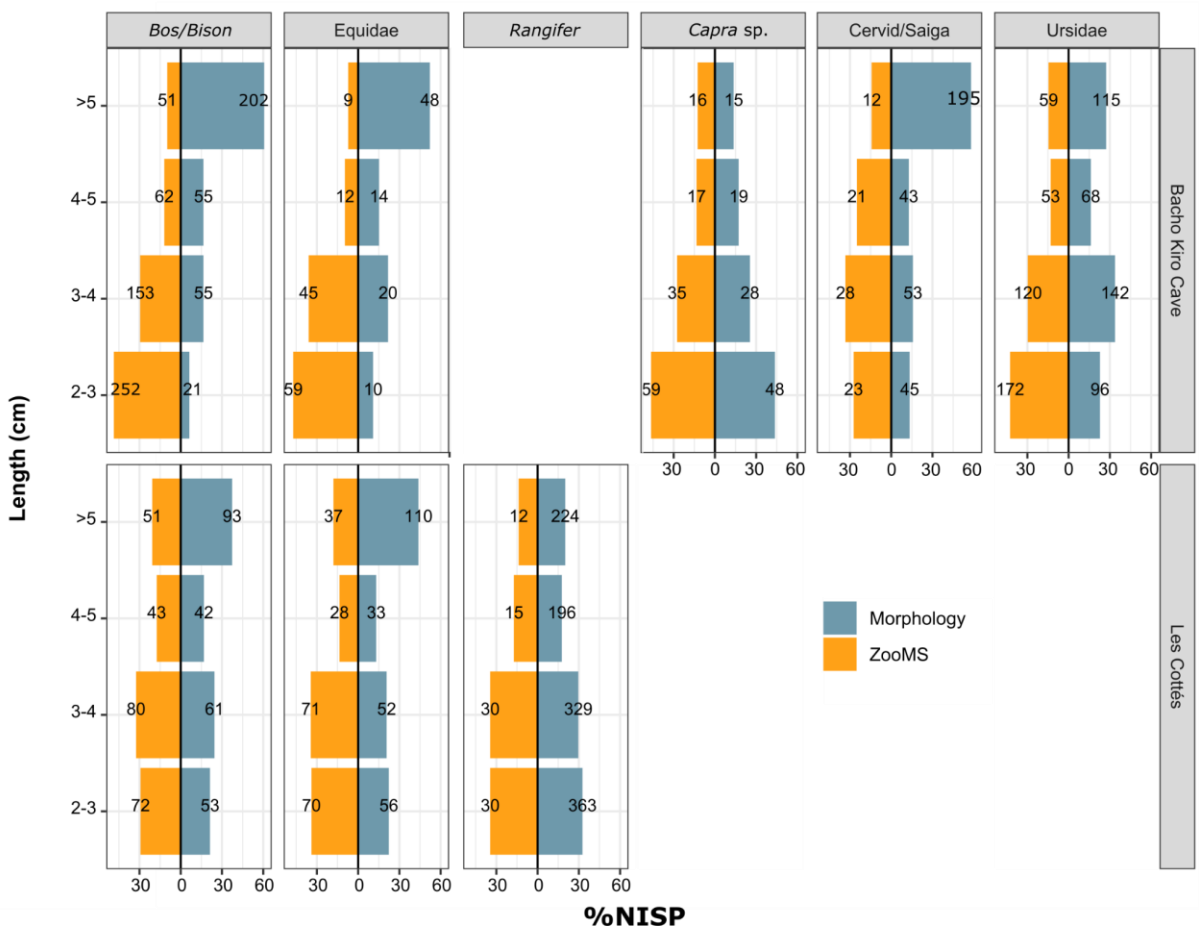


**Figure 4:** Bone length distribution of the dominant taxa within the ZooMS (orange) and the morphology (blue) component for all studied layers at the sites of Bacho Kiro Cave and Les Cottés. Numbers on the bars are the NISP for each size class.

## Bone surface modification analysis

*Bone surface preservation*:

We investigated readability of the bone surfaces to rule out bone fragmentation related to environmental taphonomic factors. We find that, at Bacho Kiro Cave and La Ferrassie, the bone surfaces of specimens taxonomically identified both through ZooMS and morphology are only affected by low degrees of surface weathering, which cannot explain the differences in fragmentation between taxa and/or layers (SI Table 15 and 16).

Due to high stages of weathering at Les Cottés, many bone surfaces from the ZooMS component exhibit natural fractures. In particular, a large percentage of *Bos/Bison* fragments, from US06 and US08 and equid specimens in US04 indicate multiple types of surface damage (SI Figure 8). These patterns are also recorded on reindeer at a high percentage (>50% for US04 and US06) within the morphology component. The readability of the surfaces, which reflects how bones were affected by weathering or other factors possibly leading to fragmentation, is generally better for the reindeer specimens compared to bones from *Bos/Bison* and equids (SI Figure 9).

*Bone assemblage accumulator:*

We investigated bone modifications associated with carnivore and human activity to identify the accumulation agents of the bone assemblage. We find that, within all three sites, ZooMS analysis allows for improved association of taxonomic identity with taphonomic data, which in several cases provides additional behavioural information. Overall, the inclusion of ZooMS identifications within zooarchaeological analyses highlights a diverse range of taxa exhibiting bone modifications from carnivore and human activity (SI Figures 10 and 11). These results are particularly informative for Layers J and K at Bacho Kiro Cave and at La Ferrassie with the addition of three to four taxa previously unassociated with the modifying agents (carnivores and humans).

With the addition of ZooMS, carnivore modifications were identified at Bacho Kiro Cave on Cervid/Saiga (Layer J: 3% NISP, Layer K: 23% NISP) and *Capra* sp. (Layer J: 11% NISP, Layer K: 11% NISP), and on *Bos/Bison* (31% NISP) and Equidae (31% NISP) in Layer K. Carnivore modifications within the ZooMS component of Layer K affected 21% of the remains from the dominant taxa, a considerably higher percentage than previously obtained through morphology (SI Figure 12). At La Ferrassie, the proportion of carnivore activity within Layer 6 appears relatively low compared to human activity as carnivore modifications were identified on only two *Bos/Bison* specimens within the ZooMS component (SI Figure 12). , affecting all dominant taxa.

In addition to evidence of carnivore activity, anthropogenic modifications are also present on most taxa within all studied layers. Human modifications were recognised on equids (20%

NISP) and *Capra* sp. (22% NISP) in Layer J at Bacho Kiro Cave (Figure 5) and we noted a relatively high proportion of percussion marks on Cervid/Saiga specimens from Layer J (22% NISP) (SI Figure 11 and SI Figure 15). At La Ferrassie, human activity is identified on Cervid/Saiga (4% NISP) and *Bos/Bison* (6% NISP) but not on equid specimens, and percussion traces occur on a higher proportion of reindeer remains (9% NISP; Figure 5 and SI Figure 13). At Les Cottés, human modifications range between 10 and 20% among dominant taxa over all studied layers and occur at higher proportions on reindeer specimens (particularly in US04 and US06), mainly represented by cut marks and percussion traces (Figure 5 and SI Figure 14). At Bacho Kiro Cave and Les Cottés, we note a progressive reduction of carnivore activity from the Late Middle Palaeolithic to the Upper Palaeolithic alongside an increase of human modifications at Bacho Kiro Cave, reinforcing patterns previously described (Rendu et al., 2019; Smith et al., 2021). In addition, we note the recurrent occurrence of anthropogenic modifications on carnivore remains (n = 93) from various taxa such as canids (*Canis lupus*, *Vulpes vulpes*), felids (*Panthera leo spelaea*, *Panthera pardus*), cave hyaenas (*Crocuta crocuta spelaea*), and ursids within layers I and J at Bacho Kiro Cave while layer K exhibits only two carnivores remains with human modifications (SI Table 17). At Les Cottés, only two canid specimens show human modifications, and no human modifications were observed on carnivore remains within layer 6 at La Ferrassie (SI Table 17).

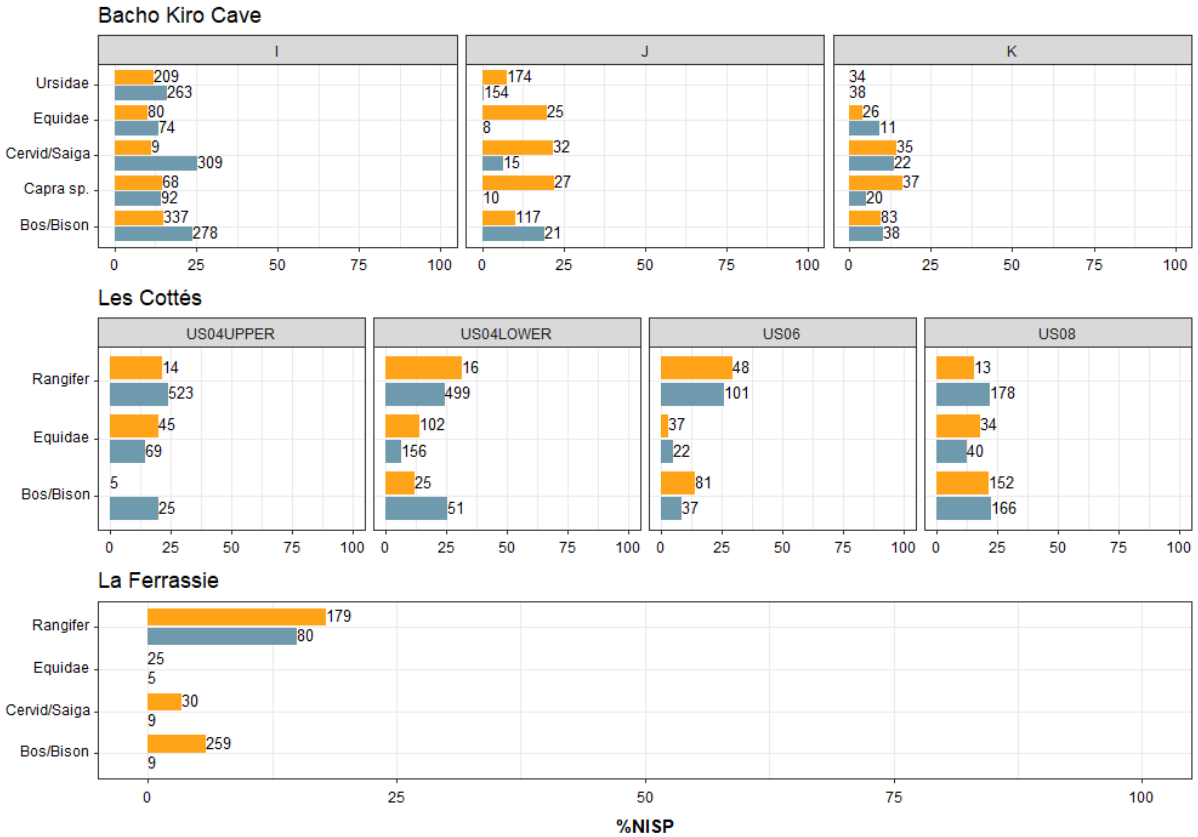


**Figure 5:** Percentages of anthropogenic modifications within the ZooMS (orange) and morphology (blue) components on the dominant taxa at the sites of Bacho Kiro Cave, Les Cottés and La Ferrassie. Numbers on the bars are the total NISP of specimens identified for the taxa.

## Skeletal representation

Due to their morphological specificities and as they are affected differently by taphonomic processes, teeth are largely represented in the morphology component and show the highest proportions among skeletal elements, particularly illustrated by the material from Bacho Kiro Cave and Les Cottés (Figure 6 & SI Figure 16). At Bacho Kiro Cave, the skeletal composition of the ZooMS component is mostly represented by long bones (LBN), cranial and axial remains, with a higher proportion of axial elements within the ZooMS component explained by an overrepresentation of ribs (SI Table 18, Figure 6). Rib elements are difficult to taxonomically identify as they do not retain many specific morphological features relative to their size and proportion in a skeleton. Long bone fragments (LBN) correspond to unidentified bone fragments from forelimbs, hindlimbs and distal limbs (metacarpals and metatarsals). Bone specimens categorised as LBN within the ZooMS component are predominantly represented by diaphysis fragments (either from the mid-shaft or near the epiphysis of the bones) but rarely from the epiphysis, as illustrated by the example on the material of Bacho Kiro Cave (SI Figure 17). Within the morphology component at Les Cottés, we observe relatively similar proportions of limb remains between the taxa, with the exception of the absence of hindlimb and distal limb remains recorded for *Bos/Bison* in US06 of both

components, but higher proportions of cranial specimens from *Bos/Bison* and Equidae (SI Figure 16). At La Ferrassie, the elemental representation of the ZooMS component only contributes to a small extent to the skeletal representation of the morphology component as most of the remains were unidentifiable and had not been assigned to a body part (SI Figure 18).

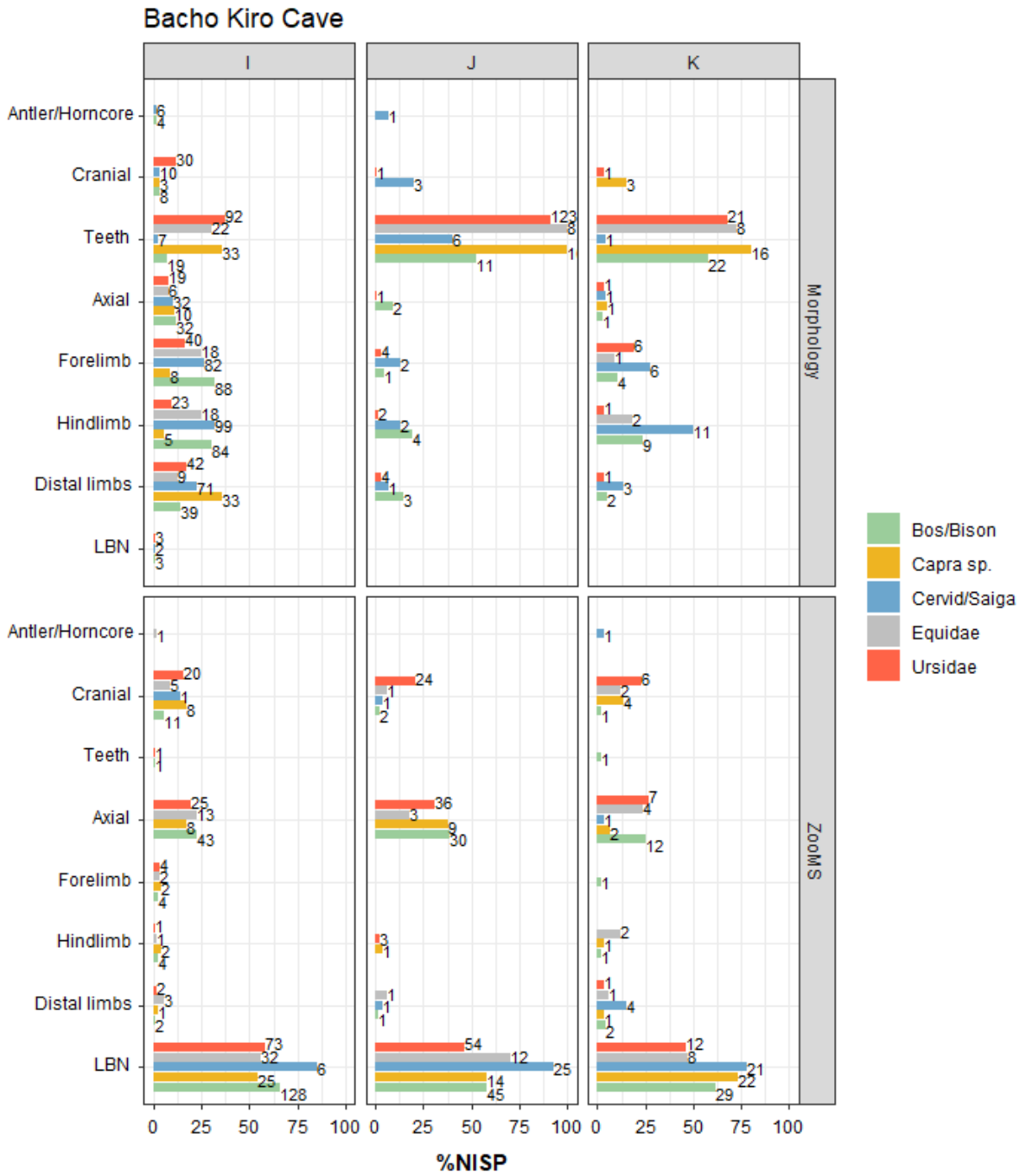


**Figure 6:** Skeletal distribution of the bone specimens identified through morphology (top) and ZooMS (bottom) from the dominant taxa at Bacho Kiro Cave. Numbers on the bars give the total NISP for each body part, layers and ID-method. Unidentified body parts (NID) were excluded from the plot. LBN: Long Bone fragment.

## Discussion

This study represents the first combined palaeoproteomic and zooarchaeological analysis of faunal material from three datasets covering the Middle to Upper Palaeolithic transition. It aims to overcome methodological limits in taxonomic identification resulting from bone fragmentation and to address human subsistence and fauna processing behaviour during a period of possible interaction between Neanderthals and Late Pleistocene *Homo sapiens* groups in Europe. Together with a high success rate of taxonomic identification, the inclusion of ZooMS analysis of the fragmented, unidentifiable component of bone assemblages can identify species previously unrecognised through traditional morphological analysis and, furthermore, be integrated and correlated with traditional zooarchaeological, taphonomic and ecological data (Berto et al., 2021; Sinet-Mathiot et al., 2019; Welker et al., 2015). In the case of highly fragmented bone assemblages, this addition can provide highly valuable information for the interpretation of human subsistence. This is notably exemplified in our study at La Ferrassie Layer 6 with a 4-fold increase in taxonomic identification through ZooMS compared to the morphologically identified component ($NISP_{Morph}$ = 142, $NISP_{ZooMS}$ = 518).

### *Prey selection and sampling bias.*

In the absence of alternative methods to address the fragmented component of Palaeolithic bone assemblages, previous studies of past human behaviour related to subsistence strategies have relied solely on morphologically identifiable fauna, excluding a vast majority of the available bone specimens. However, the fragmented component of Palaeolithic bone assemblages can differ significantly from the morphologically identifiable component, highlighted by differences in proportions of the dominant taxonomic groups between morphologically identified and ZooMS components. Our study does not reflect the pattern observed in several other ZooMS screening studies which found a similar taxonomic composition of dominant species between both components. (Berto et al., 2021; Buckley et al., 2017; Welker et al., 2016, 2017). In this study, discrepancies in taxonomic abundances between both components are seen through an overrepresentation of reindeer and an underrepresentation of *Bos/Bison* and equids at the sites of Les Cottés and La Ferrassie. These differences seem to be related to differential identification rates between taxa, possibly creating a reporting bias in the representation of the dominant taxa depending on their ease of identification. Thus, taxa such as reindeer or Ursidae will be overrepresented in the morphologically identified component as they are easy to differentiate even when fragmented. On the contrary, *Bos/Bison* and Equidae are more difficult to distinguish when fragmented and are often categorised as unidentifiable remains.

***Assemblage composition and identification rates*.**

The uniformity in low weathering patterns on the bone material from Bacho Kiro Cave and La Ferrassie sites indicate that, throughout the stratigraphy, natural factors played a limited role in bone fragmentation. Overall, bone material was relatively quickly buried and suffered minimal re-exposure at these two sites. At Les Cottés, the degree of weathering was comparable among the dominant taxa, although reindeer showed slightly better bone surface readability. Further study is required to understand if this pattern could be explained through bone morphology or specific depositional conditions of the reindeer specimens (shorter exposition of the specimens prior to burying), especially knowing that glutamine deamidation ratios do not indicate a clear differential molecular preservation. Further, our analysis of collagen deamidation at each site does not provide a molecular diagenetic explanation for the differences in taxonomic proportion between the two bone components of each assemblage. When incorporating ZooMS identifications into the zooarchaeological analysis we should keep in mind that both components, by definition, commonly show different bone length distributions, as larger fragments tend to be more easily identifiable morphologically. However, when comparing taxa, we note that this is not the case for all taxonomic groups (Pickering et al., 2006). Certain taxa, such as reindeer at Les Cottés and *Capra* sp. at Bacho Kiro Cave, can show a bone length distribution significantly different from other taxonomic groups (*Bos/Bison* and Equidae), possibly resulting from the size of the bone fragments most likely produced during marrow extraction and a different identification rate between these taxa. Indeed, because of the low cortical thickness relative to bone diameter and their smaller body size compared to *Bos/Bison* and equids, reindeer fragments will cover more of the reindeer bone proportionally, which would give it a better chance of preserving identifiable features. On the other hand, breaking open the bones of larger animals such as *Bos/Bison* or equids will produce larger fragments on average. Fragments of bovine bone specimens are often difficult to distinguish from homologous parts of Equidae or red deer as the skeletal elements of these taxa tend to overlap in size and morphology (Morin, 2012). However, since reindeer are more easily identifiable, this results in increasing representation of this species within the morphological component alongside a limited proportion of identified *Bos/Bison* and equid specimens (Gobalet, 2001).

The assessment of prey skeletal part distribution is often closely related to the taxonomic identification of the bone specimens. Small long bone shaft fragments tend to be difficult to identify due to a lack of diagnostic features on the bone diaphyses in combination with their high fragmentation rate due to marrow extraction (Morin et al., 2017a). Thus, the morphologically unidentifiable component analysed through ZooMS often contains a high proportion of long bone, particularly diaphysis portions, and rib fragments challenging the evaluation of skeletal distributions. Epiphysis portions tend to retain more specific

morphological criteria facilitating the taxonomic identification of the remains. However, their representation within the long bone fraction of the ZooMS component do not strongly differ from the morphology component at Bacho Kiro Cave. Thus, an underrepresentation of epiphyses can also result from selective destruction due to various factors such as differential preservation and bone density, carnivore activity, specific butchering practices like extraction of bone grease, and post-depositional or sampling bias during the archaeological excavation (Binford, 1981; Grayson & Delpech, 2008; Morin, 2010, 2020; Yravedra & Domínguez-Rodrigo, 2009). Behavioural inferences such as carcass processing and the selected transport of different body parts are often made based on skeletal part representation and abundance (Bartram et al., 1999; Binford, 1981; Klein et al., 1999; Marean & Assefa, 1999). The integration of skeletal representation with the taxonomic identification obtained through ZooMS has the potential to add elements to the inventory of the faunal record, contributing to our understanding of the transport of articulated remains to the site.

### ***Subsistence during the Middle to Upper Palaeolithic transition*.**

The addition, through peptide mass fingerprinting, of taxonomically identified bone specimens to faunal assemblages spanning a transitional phase during human evolution, contribute to our understanding of patterns of shifts observed during the MUPT. Our results contribute further detail to the general picture that, over this period, the hominin diet was dominated by a range of medium and large herbivores (Discamps et al., 2011; Gaudzinski-Windheuser & Niven, 2009; Gaudzinski-Windheuser & Roebroeks, 2011; Jaouen et al., 2019; Niven et al., 2012; Rendu et al., 2019; Richards et al., 2008; Smith, 2015). Our work highlights the exploitation of a more diverse range of taxa by both hominins and carnivores, permitting the correlation of certain taxa with particular agents that were contributing to the bone accumulation on site, notably at Bacho Kiro Cave. Across dominant taxa, human modifications mainly consist of cut marks, with a low occurrence of percussion traces from marrow extraction, thus providing no suitable explanation for the difference in proportions between the components. The ZooMS analysis emphasises and refines shifts of proportions of taxa throughout the stratigraphy at Les Cottés, particularly between equids and *Bos/Bison* specimens (Rendu et al., 2019). These shifts in the faunal composition could represent either a slow change in the prey availability in the environment around the site or human selection strategies paralleling the expansion of Late Pleistocene *Homo sapiens* over Europe. Nonetheless, while the morphologically identified fauna suggests a more specialised focus on hunting reindeer (Rendu et al., 2019), our results suggest this underestimates the exploitation of other species; in particular, Equidae. These results are particularly of interest within the framework of the debate about reindeer hunting specialisation (Grayson & Delpech, 2002; Mellars, 2004). Although the progressive increase of reindeer remains through the MUPT

transition correlates with a progressive climatic degradation during MIS3 and can be explained by an adaptation of the human groups to environmental fluctuations (Banks et al., 2013; Discamps et al., 2011), the role of large ungulates in the human diet throughout the MUPT might have been under-represented due to differential identification rates.

The incidence of carnivore modifications during late Neanderthal occupation of the sites suggests a context where both humans and carnivores were important in faunal accumulation and modification, still indicating frequent human occupation of the cave and sporadic carnivore visits, but the latter possibly more frequent than previously considered (Straus, 1982). The progressive decrease of carnivore activity highlighted by the reduction of carnivore modifications from the MP to the UP at Bacho Kiro Cave and Les Cottés fits with the pattern previously detected in some other sites in Europe from this time period (Discamps, 2014; Discamps et al., 2019; Rendu et al., 2019; Smith et al., 2021; Stiner & Kuhn, 2006). This possible change of relationship of carnivores to humans, from competitor to prey or source of raw material is emphasised by the appearance of human modifications on Ursidae remains during the IUP at Bacho Kiro Cave (alongside modifications on other carnivore species at Bacho Kiro Cave; (Smith et al., 2021)). *Homo sapiens* started to exploit carnivore remains more intensively as a raw material, notably illustrated by the increase in bone artefacts made from cave bear bones and teeth at Bacho Kiro Cave and other sites in southeast Europe and southwest Asia during the IUP (Bosch et al., 2019; Guadelli et al., 2011; Kuhn et al., 2009; Martisius et al., 2022; Stiner et al., 2013). Such specific needs in raw material can be investigated through skeletal part representation and carcass processing (Rendu et al., 2019). Furthermore, the higher percentage of carnivore traces in the Middle Palaeolithic layers at Bacho Kiro Cave and at Les Cottés attest to their repetitive use of the site correlated with possible short duration of human occupation (Hublin et al., 2020; Smith et al., 2021). The interaction between human groups and large carnivores seems to change during the MUPT and might indicate an increasing predatory pressure of human groups on their environment (Stiner & Kuhn, 2006) and/or a shorter duration of site occupation by Neanderthals compared to Late Pleistocene *Homo sapiens*.

ZooMS screening of fragmentary components of Palaeolithic bone assemblages should be systematically undertaken alongside the taphonomic analysis of the taxonomically unidentifiable specimens (see for example (Discamps, 2021)). In addition, the integration of the faunal data obtained from aDNA retrieved from the sediment of an archaeological site with the zooarchaeological and ZooMS analysis of palaeolithic faunal assemblages has the potential to provide a better understanding of the various episodes of occupation of a site or inform about the potential origin of the DNA preserved in the sediment.

## Conclusion

The analysis of the morphologically unidentifiable component of Pleistocene bone assemblages offers an exciting new avenue for research. Our work on faunal assemblages from sites with occupational sequence that span the MUPT has highlighted inter- and intra-site differences between assemblages, taxa, layers and identification methods. We emphasise that the morphologically unidentifiable component of faunal assemblages does not necessarily reflect the morphologically identified component. Certain taxa are more readily identifiable based on morphology compared to others. Their bone elements show particular features allowing for their recognition even when fragmented (Morin et al., 2017a). This results in a discrepancy in the identification rate of differing taxa during the analysis of bone material. Taxonomic abundances are influenced by these methodological limits and any interpretation related to past human subsistence behaviour and hunting strategies can potentially be biased. Similar patterns might be expected in other monospecific faunal assemblages, and the assessment of morphologically unidentifiable bone fractions through ZooMS can reveal conditions that influence the variability of the results.

The integration of fragmentary bone components, identified through ZooMS or other biomolecular methods (Rüther et al., 2022), within a coherent zooarchaeological framework allows for a more exhaustive evaluation of the preserved bone assemblage, unlocking behavioural information based on skeletal part profiles, bone surface modifications and ecological indices. Our large-scale, non-targeted ZooMS studies across the MUPT at Bacho Kiro Cave, Les Cottés and La Ferrassie indicate an underestimated exploitation of the large ungulates such as *Bos/Bison* and Equidae, a progressive shift in prey selection from *Bos/Bison* to equids, a reduction in the frequency of site occupation by carnivores and an increase in their exploitation by Upper Palaeolithic *Homo sapiens* over the course of their progressive dispersal across Europe. This approach provides complementary data for assessing preserved bone remains, contributes to our understanding of bone assemblage formation, and represents a future path for Palaeolithic zooarchaeology.

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## Authors' contributions

V.S.M., G.M.S. and F.W. designed the research. V.S.M. and F.W. performed the proteomic analysis. W.R., T.E.S., R.S., S.M., S.R., M.-C.S., and N.L.M. performed zooarchaeological analysis. V.A., E.E., P.G., S.J.P.M., Z.R., D.S., N.S., S.S., M.S., T.T., A.T. and J.-J.H. provided samples and archaeological context. V.S.M., G.M.S. and F.W. wrote the manuscript with contributions of all authors.

# Supplementary information to:

## Identifying the unidentified enhances insights into hominin subsistence strategies during the Middle to Upper Palaeolithic transition

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**SI Table 1:** Count of specimens identified up to subfamily or genus through both methods of identification, and the total amount of faunal specimens analysed by zooarchaeologists (including teeth) for each layer and its cultural attribution at Bacho Kiro Cave, Les Cottés and La Ferrassie.

| | Layer | Cultural attribution | ZooMS | Morphology | Total fauna (including teeth) |
|---|---|---|---|---|---|
| Bacho Kiro | I | Initial Upper Palaeolithic | 776 | 1077 | 5631 |
| | J | | 433 | 232 | 776 |
| | K | Middle Palaeolithic | 337 | 143 | 606 |
| Les Cottés | US04upper | Aurignacian | 70 | 630 | 1609 |
| | US04lower | | 168 | 715 | 2003 |
| | US06 | Châtelperronian | 217 | 166 | 477 |
| | US08 | Mousterian | 220 | 397 | 1080 |
| La Ferrassie | 6 | Châtelperronian | 527 | 142 | 809 |

**SI Table 2:** Count of ZooMS samples analysed for this study from Bacho Kiro Cave, Les Cottés and La Ferrassie per extraction protocols, and total of taxonomically unidentified specimens through ZooMS. Les Cottés specimens from Welker et al., 2015 are not included in the table.

| | Bacho Kiro Cave | Les Cottés | La Ferrassie |
|---|---|---|---|
| Total ZooMS samples | 1,595 | 523 | 527 |
| AmBic protocol | 1,595 | 523 | 527 |
| Acid demineralisation | 1 (0.06%) | 369 (58.11%) | 70 (13.28%) |
| Total unidentified | 53 (3.32%) | 16 (2.52%) | 9 (1.71%) |

**SI Table 3:** Body size classes adopted at Bacho Kiro Cave, La Ferrassie and Les Cottés for ungulates and carnivores (modified from Morin 2012). Birds, leporids, fishes and rodents are excluded from this table. For ungulates, Size 1 = Small, Size 2 = Small/Medium, Size 3 = Medium-Large, Size 4 = Large, Size 5 = megafauna For carnivores Size 1 = small, Size 2 and 3 = medium, Size 3 = Large.

| Size classes | | | | |
|---|---|---|---|---|
| **Size 1** | **Size 2** | **Size 3** | **Size 4** | **Size 5** |
| wolf (Canis lupus) | hyena (Crocuta spelaea) | cave lion (Panthera leo spelaea) | rhinoceros (Stephanorhinus hemeiotechus) | mammoth (Mammuthus primigenius) |
| red fox (Vulpes vulpes) | reindeer (Rangifer tarandus) | leopard (Panthera pardus) | giant deer (Megaloceros giganteus) | |
| dhole (Cuon alpinus) | wild ass (Equus hydruntinus) | bear (Ursus spelaeus, Ursus | Bos/Bison (Bison priscus, Bos primigenius), | |
| roe deer (Capreolus capreolus) | | horse (Equus ferus) | | |
| goat (Caprinae sp.) | | red deer (Cervus elaphus) | | |
| fallow deer (Dama dama) | | Cervidae sp. | | |
| ibex (Capra ibex) | | | | |
| pig (Sus scrofa) | | | | |

**SI Table 4:** Wilcoxon-Mann-Whitney tests of the comparison of glutamine deamidation of the peptide COL1α1 508-519 between layers from Les Cottés and Bacho Kiro Cave. The adjusted p value (P.adj.signif) is the smallest familywise significance level at which a particular comparison will be declared statistically significant as part of the multiple comparison testing, and was considered to address here the statistical comparison. The adjusted p-value significance symbols correspond to the following cutpoints: <1e-04: "****", <0.001: "***", <0.01: "**", <0.05: "*", >0.05: "ns".

| Site | group1 | group2 | statistic | p | p.adj | p.adj.signif |
|---|---|---|---|---|---|---|
| Les Cottés | US04LOWER | US04UPPER | 819 | 0.51 | 0.51 | ns |
| Les Cottés | US04LOWER | US06 | 3383 | 0.000762 | 0.004 | ** |
| Les Cottés | US04LOWER | US08 | 5041 | 0.049 | 0.197 | ns |
| Les Cottés | US04UPPER | US06 | 1277.5 | 0.23 | 0.46 | ns |
| Les Cottés | US04UPPER | US08 | 1689 | 0.085 | 0.256 | ns |
| Les Cottés | US06 | US08 | 10287 | 1.80E-08 | 1.08E-07 | **** |
| Bacho Kiro Cave | I | J | 122911 | 8.19E-11 | 8.19E-11 | **** |
| Bacho Kiro Cave | I | K | 115535 | 1.05E-81 | 3.15E-81 | **** |
| Bacho Kiro Cave | J | K | 70140.5 | 3.18E-63 | 6.36E-63 | **** |

**SI Table 5:** Wilcoxon-Mann-Whitney tests of the comparison of glutamine deamidation of the peptide COL1α1 508-519 between taxa and layers from Les Cottés and Bacho Kiro Cave. The adjusted p value (P.adj.signif) is the smallest familywise significance level at which a particular comparison will be declared statistically significant as part of the multiple comparison testing, and was considered to address here the statistical comparison. The adjusted p-value significance symbols correspond to the following cutpoints: <1e-04: "****", <0.001: "***", <0.01: "**", <0.05: "*", >0.05: "ns".

| Measurement | Site | Layer | group1 | group2 | statistic | p | p.adj | p.adj.signif |
|---|---|---|---|---|---|---|---|---|
| Ambic_P1105 | Les Cottés | US04UPPER | Bos/Bison | Equidae | 13 | 0.641 | 0.641 | ns |
| Ambic_P1105 | Les Cottés | US04UPPER | Bos/Bison | Rangifer | 1.5 | 0.241 | 0.482 | ns |
| Ambic_P1105 | Les Cottés | US04UPPER | Equidae | Rangifer | 23.5 | 0.147 | 0.441 | ns |
| Ambic_P1105 | Les Cottés | US04LOWER | Bos/Bison | Equidae | 434 | 0.147 | 0.294 | ns |
| Ambic_P1105 | Les Cottés | US04LOWER | Bos/Bison | Rangifer | 22 | 0.205 | 0.294 | ns |
| Ambic_P1105 | Les Cottés | US04LOWER | Equidae | Rangifer | 64 | 0.013 | 0.038 | * |
| Ambic_P1105 | Les Cottés | US06 | Bos/Bison | Equidae | 883.5 | 0.481 | 0.84 | ns |
| Ambic_P1105 | Les Cottés | US06 | Bos/Bison | Rangifer | 1025.5 | 0.28 | 0.84 | ns |
| Ambic_P1105 | Les Cottés | US06 | Equidae | Rangifer | 414.5 | 0.28 | 0.84 | ns |
| Ambic_P1105 | Les Cottés | US08 | Bos/Bison | Equidae | 813.5 | 0.236 | 0.507 | ns |
| Ambic_P1105 | Les Cottés | US08 | Bos/Bison | Rangifer | 350 | 0.477 | 0.507 | ns |
| Ambic_P1105 | Les Cottés | US08 | Equidae | Rangifer | 44 | 0.169 | 0.507 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Bos/Bison | Capra sp. | 7067.5 | 0.175 | 0.875 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Bos/Bison | Cervid/Saiga | 1438 | 0.349 | 0.956 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Bos/Bison | Equidae | 8839 | 0.078 | 0.465 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Bos/Bison | Ursidae | 32807 | 3.70E-16 | 3.70E-15 | **** |
| Ambic_P1105 | Bacho Kiro Cave | I | Capra sp. | Cervid/Saiga | 331 | 0.239 | 0.956 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Capra sp. | Equidae | 2104.5 | 0.02 | 0.136 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Capra sp. | Ursidae | 7641 | 1.56E-10 | 1.40E-09 | **** |
| Ambic_P1105 | Bacho Kiro Cave | I | Cervid/Saiga | Equidae | 241.5 | 0.786 | 0.956 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Cervid/Saiga | Ursidae | 911 | 0.268 | 0.956 | ns |
| Ambic_P1105 | Bacho Kiro Cave | I | Equidae | Ursidae | 6099.5 | 0.001 | 0.009 | ** |
| Ambic_P1105 | Bacho Kiro Cave | J | Bos/Bison | Capra sp. | 1695.5 | 0.164 | 0.656 | ns |
| Ambic_P1105 | Bacho Kiro Cave | J | Bos/Bison | Cervid/Saiga | 2696 | 4.76E-06 | 4.28E-05 | **** |
| Ambic_P1105 | Bacho Kiro Cave | J | Bos/Bison | Equidae | 1665.5 | 0.002 | 0.018 | * |
| Ambic_P1105 | Bacho Kiro Cave | J | Bos/Bison | Ursidae | 12377 | 6.29E-10 | 6.29E-09 | **** |
| Ambic_P1105 | Bacho Kiro Cave | J | Capra sp. | Cervid/Saiga | 625.5 | 0.007 | 0.052 | ns |
| Ambic_P1105 | Bacho Kiro Cave | J | Capra sp. | Equidae | 375.5 | 0.116 | 0.58 | ns |
| Ambic_P1105 | Bacho Kiro Cave | J | Capra sp. | Ursidae | 2850 | 0.008 | 0.052 | ns |
| Ambic_P1105 | Bacho Kiro Cave | J | Cervid/Saiga | Equidae | 286.5 | 0.19 | 0.656 | ns |
| Ambic_P1105 | Bacho Kiro Cave | J | Cervid/Saiga | Ursidae | 2422.5 | 0.457 | 0.732 | ns |
| Ambic_P1105 | Bacho Kiro Cave | J | Equidae | Ursidae | 1969.5 | 0.366 | 0.732 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Bos/Bison | Capra sp. | 1630.5 | 0.59 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Bos/Bison | Cervid/Saiga | 1727.5 | 0.55 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Bos/Bison | Equidae | 1380.5 | 0.032 | 0.318 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Bos/Bison | Ursidae | 1531.5 | 0.47 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Capra sp. | Cervid/Saiga | 750 | 0.771 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Capra sp. | Equidae | 569 | 0.219 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Capra sp. | Ursidae | 644 | 0.867 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Cervid/Saiga | Equidae | 597.5 | 0.227 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Cervid/Saiga | Ursidae | 671 | 0.934 | 1 | ns |
| Ambic_P1105 | Bacho Kiro Cave | K | Equidae | Ursidae | 360 | 0.223 | 1 | ns |
| Ambic_P1106 | La Ferrassie | 6 | Bos/Bison | Cervid/Saiga | 4380.5 | 0.008 | 0.042 | * |
| Ambic_P1107 | La Ferrassie | 6 | Bos/Bison | Equidae | 2831.5 | 0.574 | 1 | ns |
| Ambic_P1108 | La Ferrassie | 6 | Bos/Bison | Rangifer | 25867.5 | 3.66E-08 | 2.20E-07 | **** |
| Ambic_P1109 | La Ferrassie | 6 | Cervid/Saiga | Equidae | 234.5 | 0.152 | 0.456 | ns |
| Ambic_P1110 | La Ferrassie | 6 | Cervid/Saiga | Rangifer | 2339.5 | 0.833 | 1 | ns |
| Ambic_P1111 | La Ferrassie | 6 | Equidae | Rangifer | 2264 | 0.046 | 0.182 | ns |

**SI Table 6:** Faunal spectrum of the ZooMS and morphology component from US04 (Upper and Lower), US06 and US08 of Les Cottés.

| Les Cottés ZooMS | US04 UPPER | | US04 LOWER | | US06 | | US08 | |
|---|---|---|---|---|---|---|---|---|
| **Taxon** | NISP | %NISP | NISP | %NISP | NISP | %NISP | NISP | %NISP |
| Canidae | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 0 | 0.00 |
| Felidae | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 0 | 0.00 |
| Ursidae | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 0 | 0.00 |
| Elephantidae | 1 | 1.43 | 9 | 5.36 | 7 | 3.23 | 0 | 0.00 |
| Rhinocerotidae | 2 | 2.86 | 5 | 2.98 | 13 | 5.99 | 1 | 0.45 |
| Equidae | 46 | 65.71 | 106 | 63.10 | 40 | 18.42 | 37 | 16.82 |
| Cervid/Saiga | 0 | 0.00 | 2 | 1.19 | 9 | 4.17 | 3 | 1.36 |
| *Rangifer tarandus* | 15 | 21.43 | 16 | 9.52 | 48 | 22.12 | 15 | 6.82 |
| *Bos/Bison* | 5 | 7.14 | 30 | 17.86 | 86 | 39.63 | 163 | 74.09 |
| *Capra* sp. | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 1 | 0.45 |
| Caprinae (not *capra* sp.) | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 0 | 0.00 |
| Suidae | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | | |
| Bovidae | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 0 | 0.00 |
| Bovidae/Cervidae | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 0 | 0.00 |
| *Capra* sp./Rangifer | 1 | 1.43 | 0 | 0.00 | 5 | 2.30 | 0 | 0.00 |
| Caprinae | 0 | 0.00 | 0 | 0.00 | 1 | 0.46 | 0 | 0.00 |
| Total | **70** | **100.00** | **168** | **100.00** | **217** | **100.00** | **220** | **100.00** |

| Les Cottés Morphology | US04 UPPER | | US04 LOWER | | US06 | | US08 | |
|---|---|---|---|---|---|---|---|---|
| **Taxon** | NISP | %NISP | NISP | %NISP | NISP | %NISP | NISP | %NISP |
| Lagomorpha | 2 | 0.32 | 2 | 0.28 | 0 | 0.00 | 0 | 0.00 |
| Canidae | 2 | 0.32 | 4 | 0.56 | 0 | 0.00 | 4 | 1.01 |
| Canidae (not *Vulpes vulpes*) | 3 | 0.48 | 1 | 0.14 | 0 | 0.00 | 0 | 0.00 |
| Elephantidae | 4 | 0.64 | 0 | 0.00 | 4 | 2.41 | 4 | 1.01 |
| Rhinocerotidae | 0 | 0.00 | 1 | 0.14 | 0 | 0.00 | 2 | 0.50 |
| Equidae | 69 | 10.95 | 156 | 21.82 | 22 | 13.25 | 40 | 10.07 |
| Cervid/Saiga | 0 | 0.00 | 0 | 0.00 | 1 | 0.60 | 3 | 0.75 |
| *Rangifer tarandus* | 524 | 83.17 | 499 | 69.79 | 101 | 60.85 | 178 | 44.83 |
| *Bos/Bison* | 25 | 3.97 | 51 | 7.13 | 37 | 22.29 | 166 | 41.83 |
| *Capra* sp. | 1 | 0.16 | 0 | 0.00 | 1 | 0.60 | 0 | 0.00 |
| Suidae | 0 | 0.00 | 1 | 0.14 | 0 | 0.00 | 0 | 0.00 |
| Total | **630** | **100.00** | **715** | **100.00** | **166** | **100.00** | **397** | **100.00** |

**SI Table 7:** Faunal spectrum of the ZooMS and morphology component from Layers I, J and K of Bacho Kiro Cave.

| Bacho Kiro Cave ZooMS | I | | J | | K | |
|---|---|---|---|---|---|---|
| **Taxon** | NISP | %NISP | NISP | %NISP | NISP | %NISP |
| Canidae | 0 | 0.00 | 2 | 0.46 | 0 | 0.00 |
| Felinae | 0 | 0.00 | 3 | 0.69 | 1 | 0.30 |
| *Vulpes vulpes* | 0 | 0.00 | 0 | 0.00 | 3 | 0.89 |
| Ursidae | 223 | 28.74 | 174 | 40.12 | 46 | 13.69 |
| Elephantidae | 0 | 0.00 | 2 | 0.46 | 7 | 2.08 |
| Rhinocerotidae | 0 | 0.00 | 2 | 0.46 | 6 | 1.79 |
| Equidae | 84 | 10.82 | 25 | 5.76 | 31 | 9.23 |
| Cervid/Saiga | 12 | 1.55 | 37 | 8.63 | 50 | 14.88 |
| *Bos/Bison* | 357 | 46.01 | 117 | 26.96 | 111 | 33.04 |
| *Capra* sp. | 69 | 8.89 | 27 | 6.32 | 46 | 13.69 |
| Caprinae (not *Capra* sp.) | 2 | 0.26 | 3 | 0.69 | 1 | 0.30 |
| Caprinae | 2 | 0.26 | 6 | 1.38 | 13 | 3.87 |
| *Castor fiber* | 0 | 0.00 | 0 | 0.00 | 1 | 0.30 |
| Felinae/Ursidae | 4 | 0.52 | 26 | 5.99 | 6 | 1.69 |
| Hyaenidae/Pantherinae/Mustelidae | 8 | 1.03 | 0 | 0.00 | 5 | 1.38 |
| Cervid/*Saiga*/Caprinae/*Capreolus capreolus* | 0 | 0.00 | 1 | 0.23 | 2 | 0.60 |
| Bovidae/Cervidae | 1 | 0.13 | 0 | 0.00 | 0 | 0.00 |
| Bovidae/*Rangifer* | 3 | 0.39 | 0 | 0.00 | 0 | 0.00 |
| Caprinae/*Rangifer* | 0 | 0.00 | 5 | 1.15 | 6 | 1.69 |
| *Capra* sp./*Rangifer* | 11 | 1.42 | 3 | 0.69 | 2 | 0.60 |
| Total | **776** | **100.00** | **433** | **100.00** | **337** | **100.00** |

| Bacho Kiro Cave Morphology | I | | J | | K | |
|---|---|---|---|---|---|---|
| **Taxon** | NISP | %NISP | NISP | %NISP | NISP | %NISP |
| *Canis* sp. | 5 | 0.46 | 0 | 0.00 | 0 | 0.00 |
| *Canis lupus* | 12 | 1.11 | 4 | 1.72 | 4 | 2.80 |
| *Crocuta spelaea* | 21 | 1.95 | 7 | 3.02 | 1 | 0.70 |
| *Cuon alpinus* | 0 | 0.00 | 1 | 0.43 | 0 | 0.00 |
| Felidae | 0 | 0.00 | 1 | 0.43 | 0 | 0.00 |
| *Gulo gulo* | 1 | 0.09 | 1 | 0.43 | 0 | 0.00 |
| *Hyaena* sp. | 2 | 0.19 | 4 | 1.72 | 0 | 0.00 |
| *Panthera leo spelaeus* | 5 | 0.46 | 0 | 0.00 | 0 | 0.00 |
| *Panthera pardus* | 1 | 0.09 | 1 | 0.43 | 5 | 3.50 |
| Ursidae | 263 | 24.42 | 154 | 66.38 | 38 | 26.57 |
| *Vulpes vulpes* | 4 | 0.37 | 2 | 0.86 | 1 | 0.70 |
| *Mammuthus Primigenius* | 0 | 0.00 | 0 | 0.00 | 1 | 0.70 |
| *Stephanorhinus hemiotechus* | 0 | 0.00 | 2 | 0.86 | 0 | 0.00 |
| *Rhinoceros* sp. | 2 | 0.19 | 0 | 0.00 | 0 | 0.00 |
| Equidae | 74 | 6.87 | 8 | 3.45 | 11 | 7.69 |
| Cervid/Saiga | 309 | 28.69 | 15 | 6.465517 | 22 | 15.38 |
| *Bos/Bison* | 278 | 25.81 | 21 | 9.05 | 38 | 26.57 |
| *Capra* sp. | 92 | 8.54 | 10 | 4.31 | 20 | 13.99 |
| *Rupicapra rupicapra* | 1 | 0.09 | 1 | 0.43 | 0 | 0.00 |
| Leporidae | 5 | 0.46 | 0 | 0.00 | 0 | 0.00 |
| *Sus scrofa* | 2 | 0.19 | 0 | 0.00 | 2 | 1.40 |
| Total | **1077** | **100.00** | **232** | **100.00** | **143** | **100.00** |

**SI Table 8:** Faunal spectrum of the ZooMS and morphology component from layer 6 at La Ferrassie.

| La Ferrassie | ZooMS | | Morphology | |
|---|---|---|---|---|
| **Taxon** | NISP | %NISP | NISP | %NISP |
| Canidae | 1 | 0.19 | 0 | 0.00 |
| Mustelidae/Hyaenidae/Pantherinae | 1 | 0.19 | 0 | 0.00 |
| Ursidae | 3 | 0.58 | 0 | 0.00 |
| Elephantidae | 1 | 0.19 | 0 | 0.00 |
| Rhinocerotidae | 6 | 1.16 | 0 | 0.00 |
| Equidae | 25 | 4.83 | 5 | 3.52 |
| Cervid/*Saiga* | 30 | 5.79 | 9 | 6.34 |
| *Capreolus capreolus* | 1 | 0.19 | 2 | 1.41 |
| *Rangifer tarandus* | 179 | 34.56 | 80 | 56.34 |
| *Bos/Bison* | 259 | 50.00 | 9 | 6.34 |
| *Capra* sp. | 3 | 0.58 | 0 | 0.00 |
| Leporidae | 0 | 0.00 | 1 | 0.70 |
| Suidae | 2 | 0.39 | 1 | 0.70 |
| Caprinae/Muskox/*Rangifer* | 1 | 0.19 | 0 | 0.00 |
| Caprinae/*Rangifer* | 6 | 1.16 | 0 | 0.00 |
| Cervus/*Rangifer* | 0 | 0.00 | 35 | 24.65 |
| Total | **518** | **100.00** | **142** | **100.00** |

**SI Table 9:** Ecological diversity indices for each component (morphology and ZooMS) and the combination of both (morphology + ZooMS) of La Ferrassie (LF), Bacho Kiro Cave (BK) and Les Cottés (CTS). The table includes values of the Shannon-Wiener Index (H), Pielou's Evenness (J), NISP (number of identified specimens), NTAXA (number of taxa) and confidence intervals for the Shannon's index.

| Site | Layer | Method of Identification | Shannon-Wiener Index | Evenness | NISP | NTAXA | Shannon Index 2.5% | Shannon Index 97.5% |
|---|---|---|---|---|---|---|---|---|
| BK | I | Morphology | 1.687 | 0.679 | 1077 | 15 | 1.635 | 1.729 |
| BK | I | ZooMS | 1.307 | 0.672 | 755 | 10 | 1.248 | 1.36 |
| BK | I | Morphology+ZooMS | 1.628 | 0.655 | 1832 | 15 | 1.589 | 1.66 |
| BK | J | Morphology | 1.276 | 0.554 | 232 | 13 | 1.103 | 1.416 |
| BK | J | ZooMS | 1.473 | 0.614 | 393 | 14 | 1.363 | 1.549 |
| BK | J | Morphology+ZooMS | 1.475 | 0.575 | 625 | 16 | 1.372 | 1.553 |
| BK | K | Morphology | 1.827 | 0.793 | 143 | 13 | 1.662 | 1.916 |
| BK | K | ZooMS | 1.793 | 0.721 | 308 | 15 | 1.674 | 1.872 |
| BK | K | Morphology+ZooMS | 1.842 | 0.698 | 451 | 17 | 1.742 | 1.904 |
| LF | Layer 6 | Morphology | 1.266 | 0.609 | 142 | 11 | 1.057 | 1.399 |
| LF | Layer 6 | ZooMS | 1.209 | 0.487 | 511 | 15 | 1.106 | 1.278 |
| LF | Layer 6 | Morphology+ZooMS | 1.381 | 0.523 | 653 | 17 | 1.288 | 1.449 |
| CTS | US04UPPER | Morphology | 0.628 | 0.302 | 630 | 11 | 0.536 | 0.706 |
| CTS | US04UPPER | ZooMS | 0.938 | 0.583 | 67 | 8 | 0.679 | 1.123 |
| CTS | US04UPPER | Morphology+ZooMS | 0.746 | 0.339 | 697 | 12 | 0.662 | 0.82 |
| CTS | US04LOWER | Morphology | 0.845 | 0.406 | 715 | 11 | 0.774 | 0.905 |
| CTS | US04LOWER | ZooMS | 1.136 | 0.634 | 168 | 9 | 0.965 | 1.269 |
| CTS | US04LOWER | Morphology+ZooMS | 1.042 | 0.453 | 883 | 13 | 0.973 | 1.092 |
| CTS | US06 | Morphology | 1.056 | 0.589 | 166 | 9 | 0.917 | 1.171 |
| CTS | US06 | ZooMS | 1.595 | 0.642 | 209 | 15 | 1.432 | 1.702 |
| CTS | US06 | Morphology+ZooMS | 1.454 | 0.585 | 375 | 15 | 1.34 | 1.53 |
| CTS | US08 | Morphology | 1.112 | 0.571 | 397 | 10 | 1.009 | 1.185 |
| CTS | US08 | ZooMS | 0.813 | 0.454 | 220 | 9 | 0.671 | 0.932 |
| CTS | US08 | Morphology+ZooMS | 1.105 | 0.532 | 617 | 11 | 1.031 | 1.167 |

**SI Table 10:** Proportions (%NISP) of the dominant taxa within the ZooMS and morphology components per layers of Les Cottés and La Ferrassie. Numbers in brackets indicate the NISP for each category.

| **La Ferrassie** | | **ZooMS** | **Morph** |
|---|---|---|---|
| *Bos/Bison* | | 53% (259) | 9% (9) |
| *Rangifer tarandus* | | 36% (179) | 78% (80) |
| | | | |
| **Les Cottés** | | | |
| *Bos/Bison* | US04 UPPER | 5% (3) | 4% (25) |
| | US04 LOWER | 17% (25) | 7% (51) |
| | US06 | 49% (81) | 23% (37) |
| | US08 | 76% (152) | 43% (166) |
| *Rangifer tarandus* | US04 UPPER | 23% (14) | 85% (524) |
| | US04 LOWER | 11% (16) | 71% (499) |
| | US06 | 29% (48) | 63% (101) |
| | US08 | 7% (13) | 46% (178) |
| Equidae | US04 UPPER | 72% (43) | 11% (69) |
| | US04 LOWER | 71% (102) | 22% (156) |
| | US06 | 22% (37) | 14% (22) |
| | US08 | 17% (34) | 10% (40) |

**SI Table 11:** Wilcoxon-Mann-Whitney tests of the comparison of bone length distribution between taxa and layers at Bacho Kiro Cave. The adjusted p-value significance symbols correspond to the following cutpoints: <1e-04: "****", <0.001: "***", <0.01: "**", <0.05: "*", >0.05: ns".

| Method of Identification | Layer | Measurement | group1 | group2 | statistic | p | p.adj | p.adj.signif |
|---|---|---|---|---|---|---|---|---|
| Morphology | I | BONE_LENGTH | *Bos/Bison* | *Capra sp.* | 21838.5 | 2.43E-23 | 2.43E-22 | **** |
| Morphology | I | BONE_LENGTH | *Bos/Bison* | Cervid/Saiga | 47846 | 0.009 | 0.027 | * |
| Morphology | I | BONE_LENGTH | *Bos/Bison* | Equidae | 11694.5 | 0.07 | 0.141 | ns |
| Morphology | I | BONE_LENGTH | *Bos/Bison* | Ursidae | 49553.5 | 8.58E-13 | 6.86E-12 | **** |
| Morphology | I | BONE_LENGTH | *Capra sp.* | Cervid/Saiga | 5853.5 | 8.05E-18 | 7.24E-17 | **** |
| Morphology | I | BONE_LENGTH | *Capra sp.* | Equidae | 1435.5 | 1.05E-10 | 7.35E-10 | **** |
| Morphology | I | BONE_LENGTH | *Capra sp.* | Ursidae | 7946 | 5.14E-07 | 3.08E-06 | **** |
| Morphology | I | BONE_LENGTH | Cervid/Saiga | Equidae | 11468.5 | 0.863 | 0.863 | ns |
| Morphology | I | BONE_LENGTH | Cervid/Saiga | Ursidae | 49698.5 | 1.31E-06 | 6.55E-06 | **** |
| Morphology | I | BONE_LENGTH | Equidae | Ursidae | 11929.5 | 0.003 | 0.012 | * |
| ZooMS | I | BONE_LENGTH | *Bos/Bison* | *Capra sp.* | 12239 | 0.293 | 1 | ns |
| ZooMS | I | BONE_LENGTH | *Bos/Bison* | Cervid/Saiga | 1341.5 | 0.593 | 1 | ns |
| ZooMS | I | BONE_LENGTH | *Bos/Bison* | Equidae | 14313.5 | 0.3 | 1 | ns |
| ZooMS | I | BONE_LENGTH | *Bos/Bison* | Ursidae | 33623 | 0.508 | 1 | ns |
| ZooMS | I | BONE_LENGTH | *Capra sp.* | Cervid/Saiga | 248 | 0.362 | 1 | ns |
| ZooMS | I | BONE_LENGTH | *Capra sp.* | Equidae | 2701 | 0.943 | 1 | ns |
| ZooMS | I | BONE_LENGTH | *Capra sp.* | Ursidae | 6257.5 | 0.139 | 1 | ns |
| ZooMS | I | BONE_LENGTH | Cervid/Saiga | Equidae | 431 | 0.337 | 1 | ns |
| ZooMS | I | BONE_LENGTH | Cervid/Saiga | Ursidae | 1028.5 | 0.637 | 1 | ns |
| ZooMS | I | BONE_LENGTH | Equidae | Ursidae | 7420 | 0.139 | 1 | ns |
| Morphology | J | BONE_LENGTH | *Bos/Bison* | *Capra sp.* | 201 | 0.000346 | 0.003 | ** |
| Morphology | J | BONE_LENGTH | *Bos/Bison* | Cervid/Saiga | 184 | 0.096 | 0.77 | ns |
| Morphology | J | BONE_LENGTH | *Bos/Bison* | Equidae | 107 | 0.279 | 1 | ns |
| Morphology | J | BONE_LENGTH | *Bos/Bison* | Ursidae | 2570.5 | 1.21E-05 | 0.000121 | *** |
| Morphology | J | BONE_LENGTH | *Capra sp.* | Cervid/Saiga | 60 | 0.531 | 1 | ns |
| Morphology | J | BONE_LENGTH | *Capra sp.* | Equidae | 24 | 0.109 | 0.77 | ns |
| Morphology | J | BONE_LENGTH | *Capra sp.* | Ursidae | 635 | 0.167 | 1 | ns |
| Morphology | J | BONE_LENGTH | Cervid/Saiga | Equidae | 42 | 0.5 | 1 | ns |
| Morphology | J | BONE_LENGTH | Cervid/Saiga | Ursidae | 1000 | 0.998 | 1 | ns |
| Morphology | J | BONE_LENGTH | Equidae | Ursidae | 751 | 0.298 | 1 | ns |
| ZooMS | J | BONE_LENGTH | *Bos/Bison* | *Capra sp.* | 1152.5 | 0.029 | 0.261 | ns |
| ZooMS | J | BONE_LENGTH | *Bos/Bison* | Cervid/Saiga | 1666.5 | 0.343 | 1 | ns |
| ZooMS | J | BONE_LENGTH | *Bos/Bison* | Equidae | 1546 | 0.657 | 1 | ns |
| ZooMS | J | BONE_LENGTH | *Bos/Bison* | Ursidae | 8932 | 0.077 | 0.613 | ns |
| ZooMS | J | BONE_LENGTH | *Capra sp.* | Cervid/Saiga | 506 | 0.266 | 1 | ns |
| ZooMS | J | BONE_LENGTH | *Capra sp.* | Equidae | 466 | 0.019 | 0.191 | ns |
| ZooMS | J | BONE_LENGTH | *Capra sp.* | Ursidae | 2681 | 0.238 | 1 | ns |
| ZooMS | J | BONE_LENGTH | Cervid/Saiga | Equidae | 473.5 | 0.24 | 1 | ns |
| ZooMS | J | BONE_LENGTH | Cervid/Saiga | Ursidae | 2754 | 0.924 | 1 | ns |
| ZooMS | J | BONE_LENGTH | Equidae | Ursidae | 1734.5 | 0.102 | 0.714 | ns |
| Morphology | K | BONE_LENGTH | *Bos/Bison* | *Capra sp.* | 510 | 0.033 | 0.266 | ns |
| Morphology | K | BONE_LENGTH | *Bos/Bison* | Cervid/Saiga | 365 | 0.424 | 1 | ns |
| Morphology | K | BONE_LENGTH | *Bos/Bison* | Equidae | 161 | 0.259 | 1 | ns |
| Morphology | K | BONE_LENGTH | *Bos/Bison* | Ursidae | 843 | 0.212 | 1 | ns |
| Morphology | K | BONE_LENGTH | *Capra sp.* | Cervid/Saiga | 120 | 0.011 | 0.1 | ns |
| Morphology | K | BONE_LENGTH | *Capra sp.* | Equidae | 39 | 0.003 | 0.025 | * |
| Morphology | K | BONE_LENGTH | *Capra sp.* | Ursidae | 333.5 | 0.452 | 1 | ns |
| Morphology | K | BONE_LENGTH | Cervid/Saiga | Equidae | 110 | 0.693 | 1 | ns |
| Morphology | K | BONE_LENGTH | Cervid/Saiga | Ursidae | 541 | 0.06 | 0.419 | ns |
| Morphology | K | BONE_LENGTH | Equidae | Ursidae | 276 | 0.112 | 0.672 | ns |
| ZooMS | K | BONE_LENGTH | *Bos/Bison* | *Capra sp.* | 1520.5 | 0.934 | 1 | ns |
| ZooMS | K | BONE_LENGTH | *Bos/Bison* | Cervid/Saiga | 1276 | 0.3 | 1 | ns |
| ZooMS | K | BONE_LENGTH | *Bos/Bison* | Equidae | 1025.5 | 0.706 | 1 | ns |
| ZooMS | K | BONE_LENGTH | *Bos/Bison* | Ursidae | 1488 | 0.646 | 1 | ns |
| ZooMS | K | BONE_LENGTH | *Capra sp.* | Cervid/Saiga | 564 | 0.352 | 1 | ns |
| ZooMS | K | BONE_LENGTH | *Capra sp.* | Equidae | 465 | 0.83 | 1 | ns |
| ZooMS | K | BONE_LENGTH | *Capra sp.* | Ursidae | 657 | 0.753 | 1 | ns |
| ZooMS | K | BONE_LENGTH | Cervid/Saiga | Equidae | 486 | 0.656 | 1 | ns |
| ZooMS | K | BONE_LENGTH | Cervid/Saiga | Ursidae | 697 | 0.225 | 1 | ns |
| ZooMS | K | BONE_LENGTH | Equidae | Ursidae | 483 | 0.548 | 1 | ns |

**SI Table 12:** Wilcoxon-Mann-Whitney tests of the comparison of bone length distribution between taxa and layers at Les Cottés. The adjusted p-value significance symbols correspond to the following cutpoints: <1e-04: "****", <0.001: "***", <0.01: "**", <0.05: "*", >0.05: "ns".

| Method of Identification | Layer | Measurement | group1 | group2 | statistic | p | p.adj | p.adj.signif |
|---|---|---|---|---|---|---|---|---|
| Morphology | US04UPPER | Bone length | *Bos/Bison* | Equidae | 982.5 | 0.306 | 0.306 | ns |
| Morphology | US04UPPER | Bone length | *Bos/Bison* | *Rangifer* | 8961 | 0.002 | 0.004 | ** |
| Morphology | US04UPPER | Bone length | Equidae | *Rangifer* | 23096 | 0.000176 | 0.000528 | *** |
| Morphology | US04LOWER | Bone length | *Bos/Bison* | Equidae | 3611.5 | 0.294 | 0.588 | ns |
| Morphology | US04LOWER | Bone length | *Bos/Bison* | *Rangifer* | 13259.5 | 0.638 | 0.638 | ns |
| Morphology | US04LOWER | Bone length | Equidae | *Rangifer* | 44674 | 0.009 | 0.027 | * |
| Morphology | US06 | Bone length | *Bos/Bison* | Equidae | 339.5 | 0.193 | 0.386 | ns |
| Morphology | US06 | Bone length | *Bos/Bison* | *Rangifer* | 1959.5 | 0.731 | 0.731 | ns |
| Morphology | US06 | Bone length | Equidae | *Rangifer* | 1473 | 0.056 | 0.169 | ns |
| Morphology | US08 | Bone length | *Bos/Bison* | Equidae | 3055.5 | 0.25 | 0.25 | ns |
| Morphology | US08 | Bone length | *Bos/Bison* | *Rangifer* | 21367.5 | 2.94E-09 | 8.82E-09 | **** |
| Morphology | US08 | Bone length | Equidae | *Rangifer* | 5333.5 | 2.83E-06 | 5.66E-06 | **** |
| ZooMS | US04UPPER | Bone length | *Bos/Bison* | Equidae | 43 | 0.057 | 0.17 | ns |
| ZooMS | US04UPPER | Bone length | *Bos/Bison* | *Rangifer* | 10 | 0.429 | 0.67 | ns |
| ZooMS | US04UPPER | Bone length | Equidae | *Rangifer* | 139 | 0.335 | 0.67 | ns |
| ZooMS | US04LOWER | Bone length | *Bos/Bison* | Equidae | 1228.5 | 0.169 | 0.507 | ns |
| ZooMS | US04LOWER | Bone length | *Bos/Bison* | *Rangifer* | 119.5 | 0.222 | 0.507 | ns |
| ZooMS | US04LOWER | Bone length | Equidae | *Rangifer* | 390 | 0.702 | 0.702 | ns |
| ZooMS | US06 | Bone length | *Bos/Bison* | Equidae | 694 | 0.622 | 1 | ns |
| ZooMS | US06 | Bone length | *Bos/Bison* | *Rangifer* | 569.5 | 0.981 | 1 | ns |
| ZooMS | US06 | Bone length | Equidae | *Rangifer* | 242 | 0.829 | 1 | ns |
| ZooMS | US08 | Bone length | *Bos/Bison* | Equidae | 2031 | 0.725 | 1 | ns |
| ZooMS | US08 | Bone length | *Bos/Bison* | *Rangifer* | 681.5 | 0.411 | 1 | ns |
| ZooMS | US08 | Bone length | Equidae | *Rangifer* | 137.5 | 0.947 | 1 | ns |

**SI Table 13:** Wilcoxon-Mann-Whitney tests of the comparison of bone length distribution between method of identification, taxa and layers at Les Cottés. The adjusted p-value significance symbols correspond to the following cutpoints: <1e-04: "****", <0.001: "***", <0.01: "**", <0.05: "*", >0.05: "ns".

| Layer | Taxon | Measurement | group1 | group2 | statistic | p | p.signif |
|---|---|---|---|---|---|---|---|
| US04LOWER | Bos/Bison | Bone length | Morphology | ZooMS | 534 | 0.543 | ns |
| US04LOWER | Equidae | Bone length | Morphology | ZooMS | 8049.5 | 0.0686 | ns |
| US04LOWER | Rangifer | Bone length | Morphology | ZooMS | 2159 | 0.7 | ns |
| US04UPPER | Bos/Bison | Bone length | Morphology | ZooMS | 101.5 | 0.032 | * |
| US04UPPER | Equidae | Bone length | Morphology | ZooMS | 1564.5 | 0.0566 | ns |
| US04UPPER | Rangifer | Bone length | Morphology | ZooMS | 1815.5 | 0.515 | ns |
| US06 | Bos/Bison | Bone length | Morphology | ZooMS | 919.5 | 0.523 | ns |
| US06 | Equidae | Bone length | Morphology | ZooMS | 333 | 0.229 | ns |
| US06 | Rangifer | Bone length | Morphology | ZooMS | 975.5 | 0.523 | ns |
| US08 | Bos/Bison | Bone length | Morphology | ZooMS | 13575 | 0.00202 | ** |
| US08 | Equidae | Bone length | Morphology | ZooMS | 838.5 | 0.00471 | ** |
| US08 | Rangifer | Bone length | Morphology | ZooMS | 715 | 0.538 | ns |

**SI Table 14:** Wilcoxon-Mann-Whitney tests of the comparison of bone length distribution between taxa at La Ferrassie. The adjusted p-value significance symbols correspond to the following cutpoints: <1e-04: "****", <0.001: "***", <0.01: "**", <0.05: "*", >0.05: "ns".

| Method of Identification | Measurement | group1 | group2 | statistic | p | p.adj | p.adj.signif |
|---|---|---|---|---|---|---|---|
| ZooMS | BONE_LENGTH | *Bos/Bison* | Cervid/Saiga | 2412 | 0.127 | 0.381 | ns |
| ZooMS | BONE_LENGTH | *Bos/Bison* | Equidae | 3529.5 | 0.02 | 0.098 | ns |
| ZooMS | BONE_LENGTH | *Bos/Bison* | *Rangifer* | 22524 | 0.92 | 0.92 | ns |
| ZooMS | BONE_LENGTH | Cervid/Saiga | Equidae | 415 | 0.008 | 0.047 | * |
| ZooMS | BONE_LENGTH | Cervid/Saiga | *Rangifer* | 2803.5 | 0.13 | 0.381 | ns |
| ZooMS | BONE_LENGTH | Equidae | *Rangifer* | 1577.5 | 0.032 | 0.128 | ns |

**SI Table 15:** Percentage of specimens displaying low weathering (stage 0-2) or high weathering (3-5) based on the study by Behrensmeyer (1978) for the dominant taxa in layers I, J and K at Bacho Kiro Cave.

| Bacho Kiro Cave | | Low weathering | | | High weathering | | |
|---|---|---|---|---|---|---|---|
| | | I | J | K | I | J | K |
| *Bos/Bison* | ZooMS | 100 (333) | 97.43 (114) | 100 (83) | 0 (0) | 2.56 (3) | 0 (0) |
| | Morphology | 98.92 (275) | 95.24 (20) | 100 (38) | 1.08 (3) | 4.76 (1) | 0 (0) |
| *Capra* sp. | ZooMS | 100 (68) | 100 (27) | 100 (37) | 0 (0) | 0 (0) | 0 (0) |
| | Morphology | 100 (93) | 100 (11) | 100 (20) | 0 (0) | 0 (0) | 0 (0) |
| Cervid/Saiga | ZooMS | 100 (9) | 100 (32) | 100 (35) | 0 (0) | 0 (0) | 0 (0) |
| | Morphology | 99.35 (304) | 100 (13) | 100 (22) | 0.65 (2) | 0 (0) | 0 (0) |
| Equidae | ZooMS | 100 (80) | 96.00 (24) | 100 (26) | 0 (0) | 4.00 (1) | 0 (0) |
| | Morphology | 100 (74) | 100 (8) | 100 (11) | 0 (0) | 0 (0) | 0 (0) |
| Ursidae | ZooMS | 100 (209) | 98.28 (171) | 100 (34) | 0 (0) | 1.72 (3) | 0 (0) |
| | Morphology | 99.24 (261) | 100 (154) | 100 (38) | 0.76 (2) | 0 (0) | 0 (0) |

**SI Table 16:** Percentage of specimens displaying low weathering (stage 0-2) or high weathering (3-5) based on the study by Behrensmeyer (1978) for the dominant taxa at La Ferrassie.

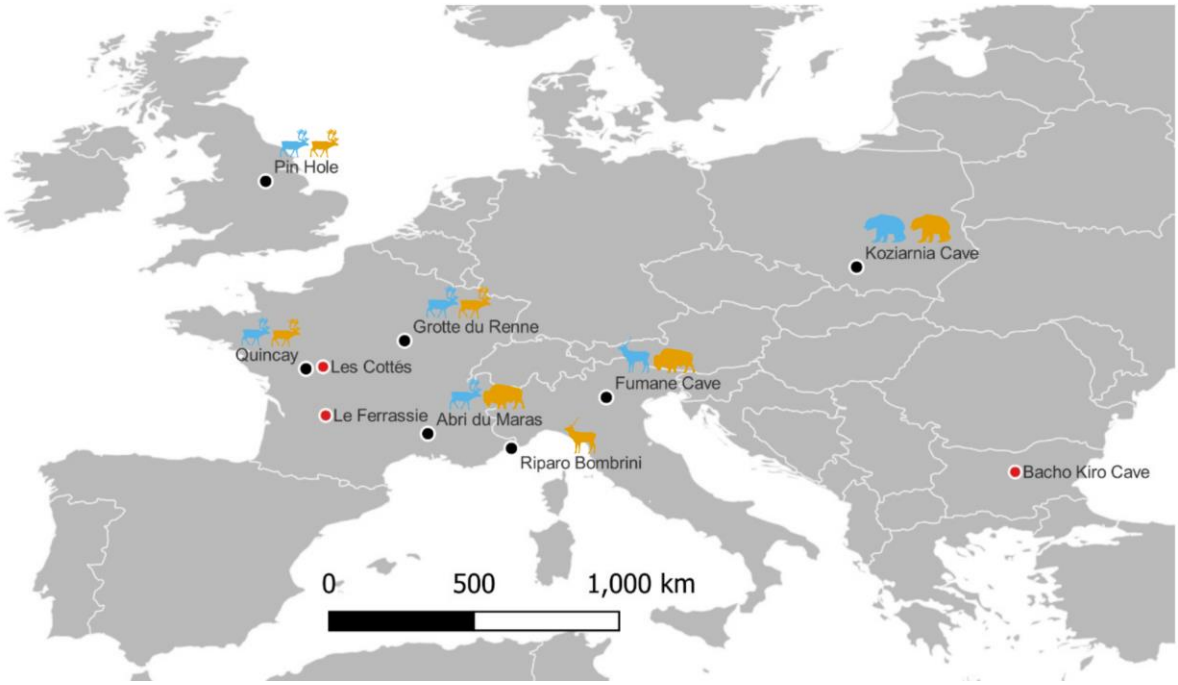
| La Ferrassie | | Low weathering | High weathering |
|---|---|---|---|
| *Bos/Bison* | ZooMS | 98.23% (222) | 1.77% (4) |
| | Morphology | 100% (4) | 0 (0) |
| Cervid/*Saiga* | ZooMS | 94.74% (18) | 5.26% (1) |
| | Morphology | 100% (3) | 0 (0) |
| Equidae | ZooMS | 100% (23) | 0 (0) |
| | Morphology | 100% (2) | 0 (0) |
| Rangifer | ZooMS | 99.30% (141) | 0.70% (1) |
| | Morphology | 100% (46) | 0 (0) |

**SI Table 17:** Number of specimens identified as carnivore (ZooMS or Morphology) showing anthropogenic bone surface modifications at Bacho Kiro Cave and Les Cottés.

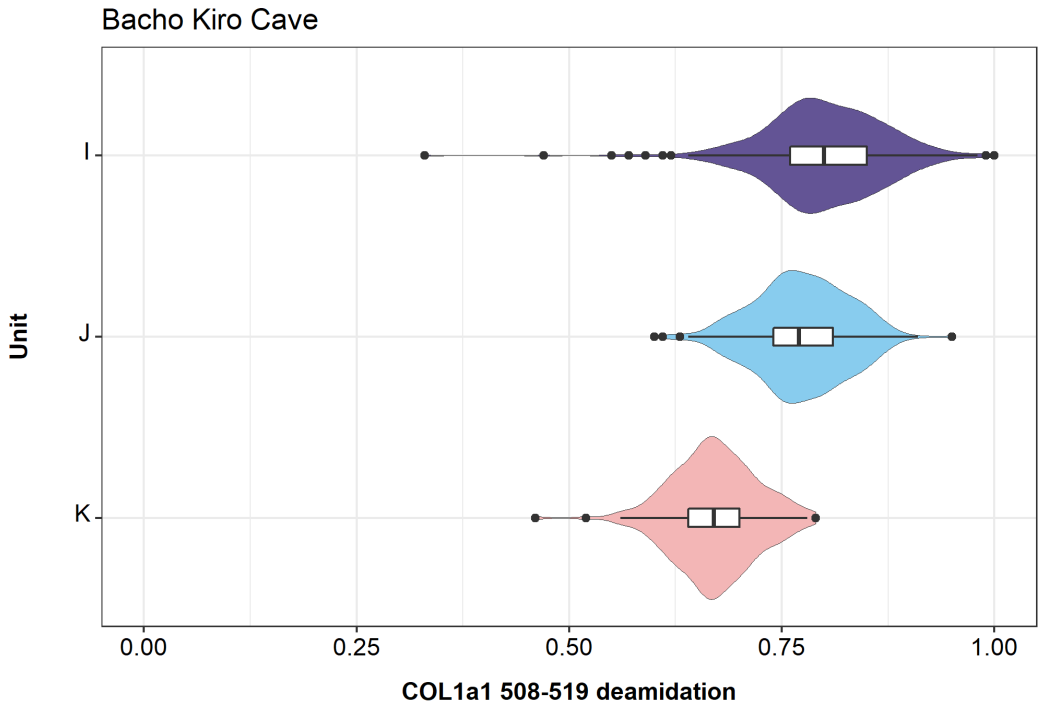
| Site | Method of Identification | Layer | Taxon | NISP |
|---|---|---|---|---|
| Bacho Kiro Cave | Morphology | I | *Canis lupus* | 3 |
| Bacho Kiro Cave | Morphology | I | *Crocuta spelaea* | 3 |
| Bacho Kiro Cave | Morphology | I | *Panthera (leo) spelaea* | 1 |
| Bacho Kiro Cave | Morphology | I | Ursidae | 42 |
| Bacho Kiro Cave | Morphology | I | *Vulpes vulpes* | 1 |
| Bacho Kiro Cave | Morphology | J | *Crocuta spelaea* | 1 |
| Bacho Kiro Cave | Morphology | J | Ursidae | 1 |
| Bacho Kiro Cave | Morphology | K | *Panthera pardus* | 2 |
| Bacho Kiro Cave | ZooMS | I | Hyaenidae/Pantherinae/Mustelidae | 1 |
| Bacho Kiro Cave | ZooMS | I | Ursidae | 25 |
| Bacho Kiro Cave | ZooMS | J | Felinae/Ursidae | 2 |
| Bacho Kiro Cave | ZooMS | J | Ursidae | 13 |
| Les Cottés | Morphology | US04UPPER | Canidae | 1 |
| Les Cottés | Morphology | US08 | Canidae | 1 |

**SI Table 18:** Percentages of the identified axial bone elements among methods of identification at Bacho Kiro Cave (morphology and ZooMS).

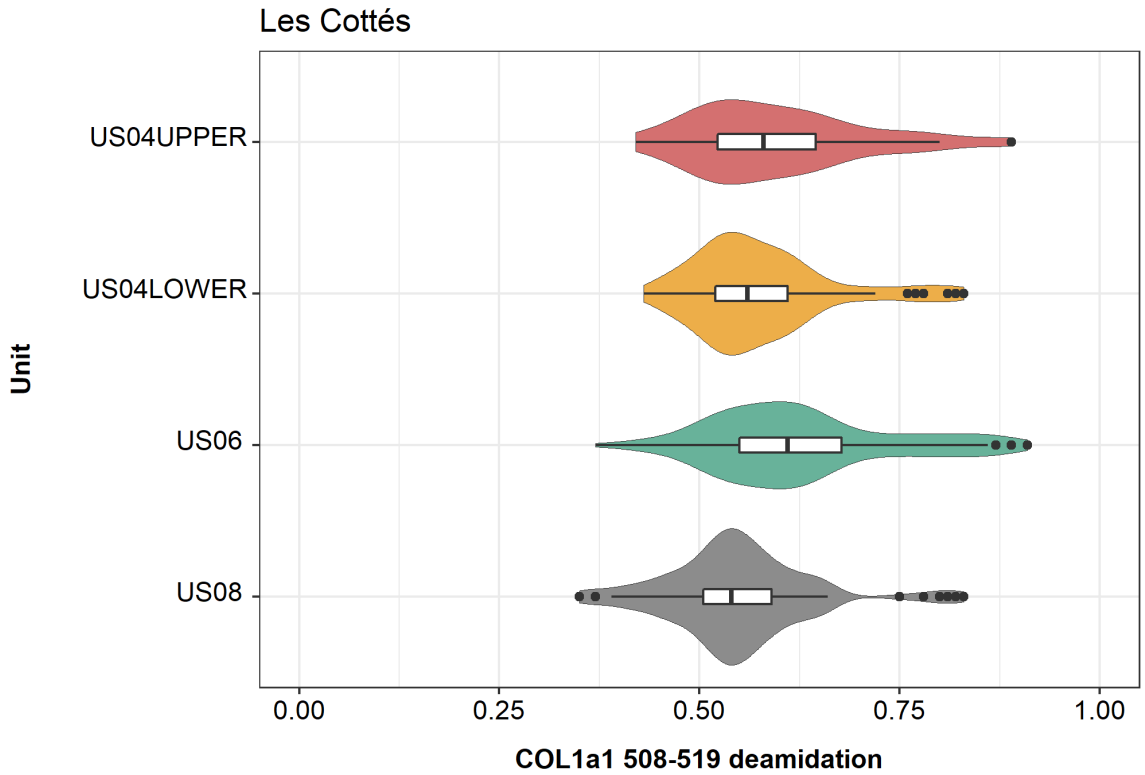
| Skeletal Elements | Method of Identification | NISP | %NISP |
|---|---|---|---|
| Acetabulum | Morphology | 6 | 100 |
| Atlas | Morphology | 3 | 100 |
| Axis | Morphology | 4 | 80 |
| Ischium | Morphology | 4 | 100 |
| Rib | Morphology | 36 | 18.85 |
| Scapula | Morphology | 9 | 75 |
| Vertebrae Caudal | Morphology | 1 | 100 |
| Vertebrae Cervical | Morphology | 8 | 72.73 |
| Vertebrae Lumbar | Morphology | 3 | 75 |
| Vertebrae Thoracic | Morphology | 10 | 100 |
| Axis | ZooMS | 1 | 20 |
| Ilium | ZooMS | 1 | 100 |
| Rib | ZooMS | 155 | 81.15 |
| Scapula | ZooMS | 3 | 25 |
| Sternabrae | ZooMS | 1 | 100 |
| Vertebrae Cervical | ZooMS | 3 | 27.27 |
| Vertebrae Indeterminate | ZooMS | 28 | 100 |
| Vertebrae Lumbar | ZooMS | 1 | 25 |



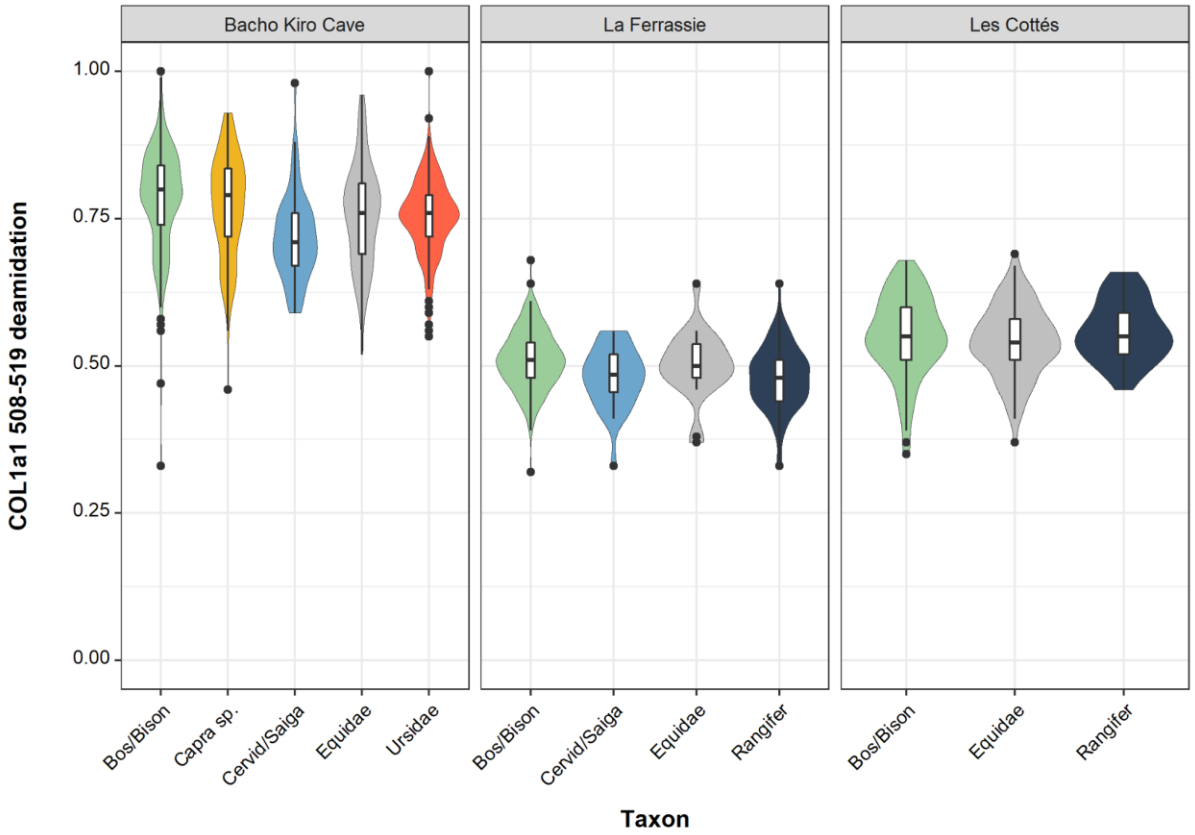

**SI Figure 1:** Site location of Bacho Kiro Cave, Les Cottés and La Ferrassie (red dots) and other published european non-targeted ZooMS studies with zooarchaeological data available for the same archaeological layers. For each site (Pin Hole Cave (UK)(Buckley et al., 2017), Quinçay (France)(Welker et al., 2017), Grotte du Renne (France)(Welker et al., 2016), Abri du Maras (France)(Ruebens et al., 2022), Fumane Cave (Italy)(Sinet-Mathiot et al., 2019), Riparo Bombrini(Pothier Bouchard et al., 2020) and Koziarnia Cave (Poland)(Berto et al., 2021)), animal silhouettes (phylopic.org) indicate the dominant taxa in each component (ZooMS: orange and morphology: blue), although the complete faunal spectrum of each sites includes various taxa. The morphology component from Riparo Bombrini is not illustrated on the map as it is represented by a low NISP (<20 morphologically identified specimens).



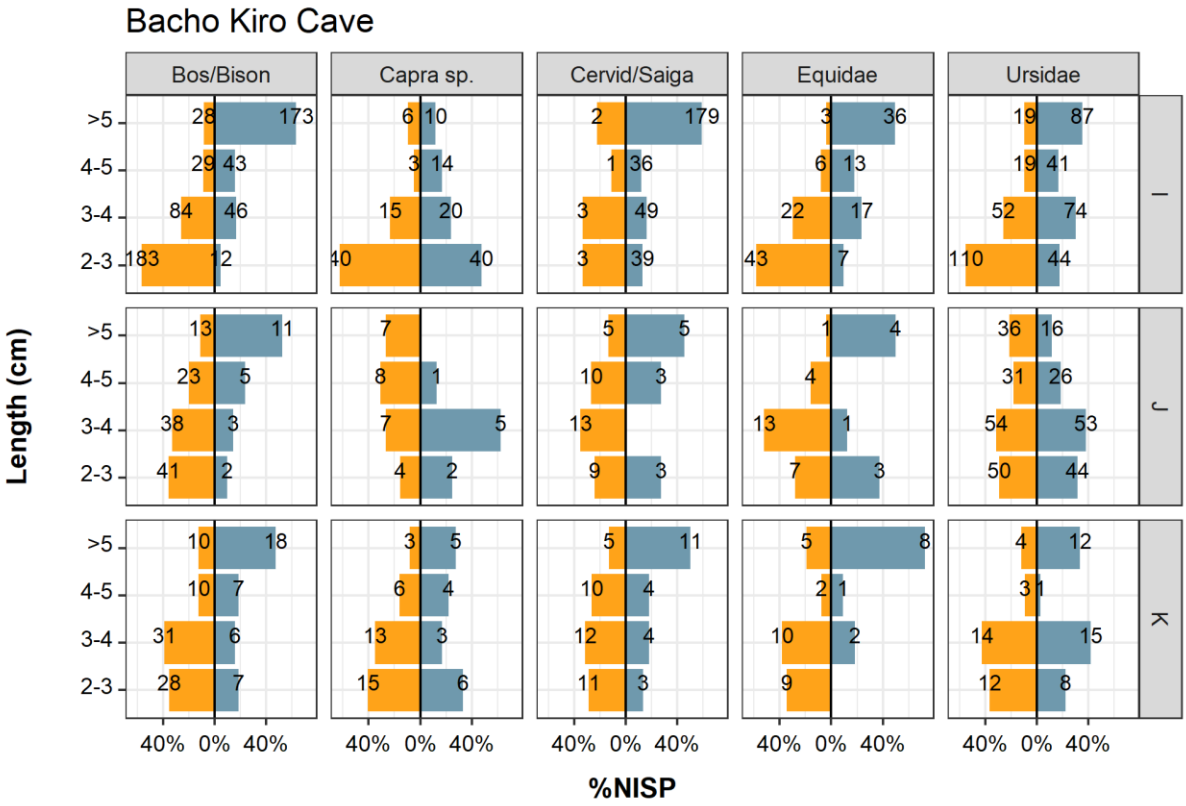

**SI Figure 2:** Peptide deamidation (all taxa combined) obtained for layers I, J and K at Bacho Kiro Cave. Sample sizes for each layer are K (n = 219), J (n = 349) and I (n = 561).



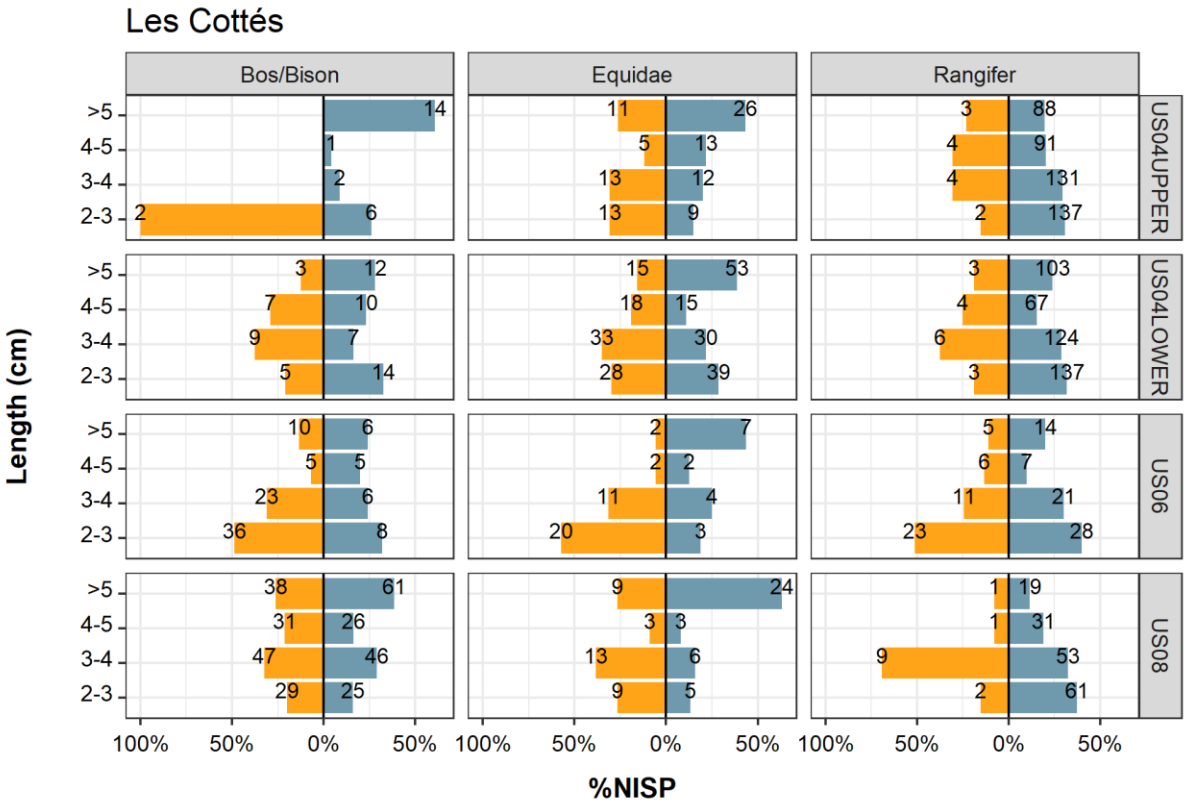

**SI Figure 3:** Peptide deamidation (all taxa combined) obtained for US04 Upper, US04 Lower, US06 and US08 at Les Cottés. Sample sizes for each layer are US08 (n = 115), US06 (n = 126), US04LOWER (n = 75) and US04UPPER (n = 24).



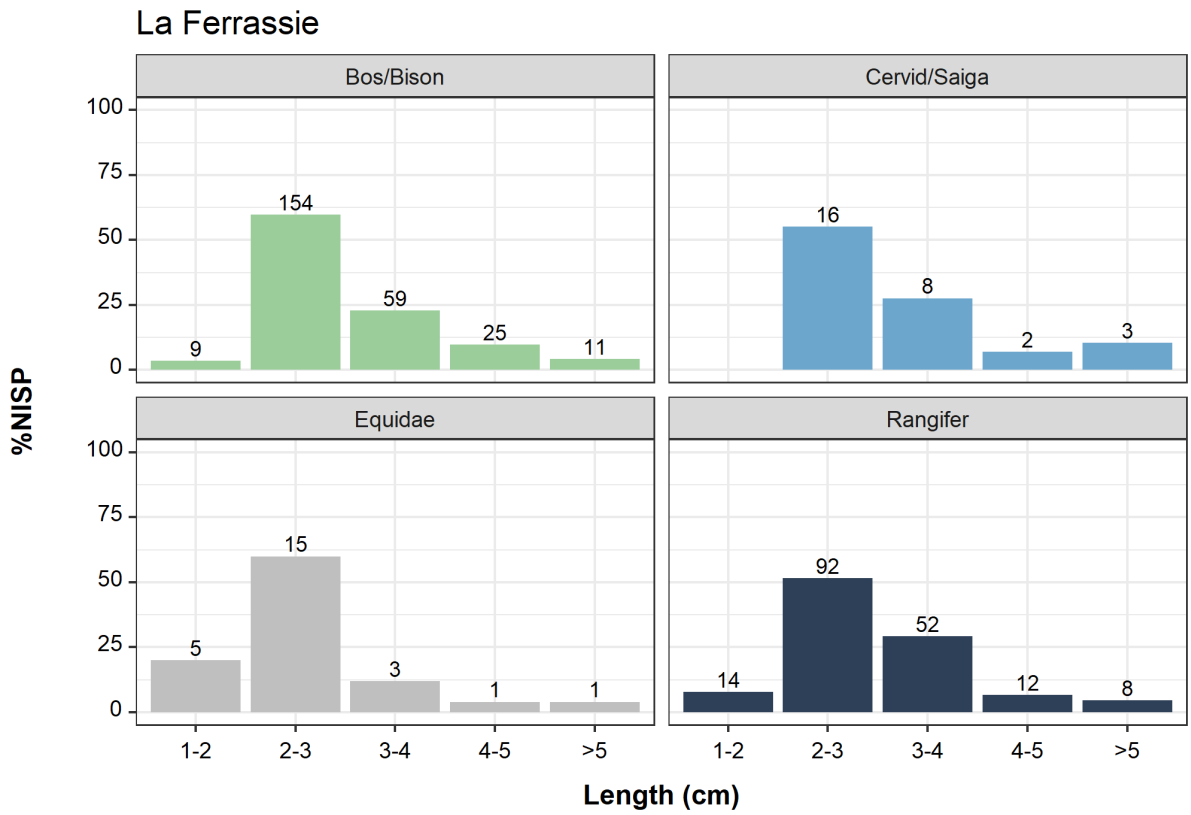

**SI Figure 4:** Peptide deamidation of the dominant taxa for all studied layers at Bacho Kiro Cave, Les Cottés and La Ferrassie.



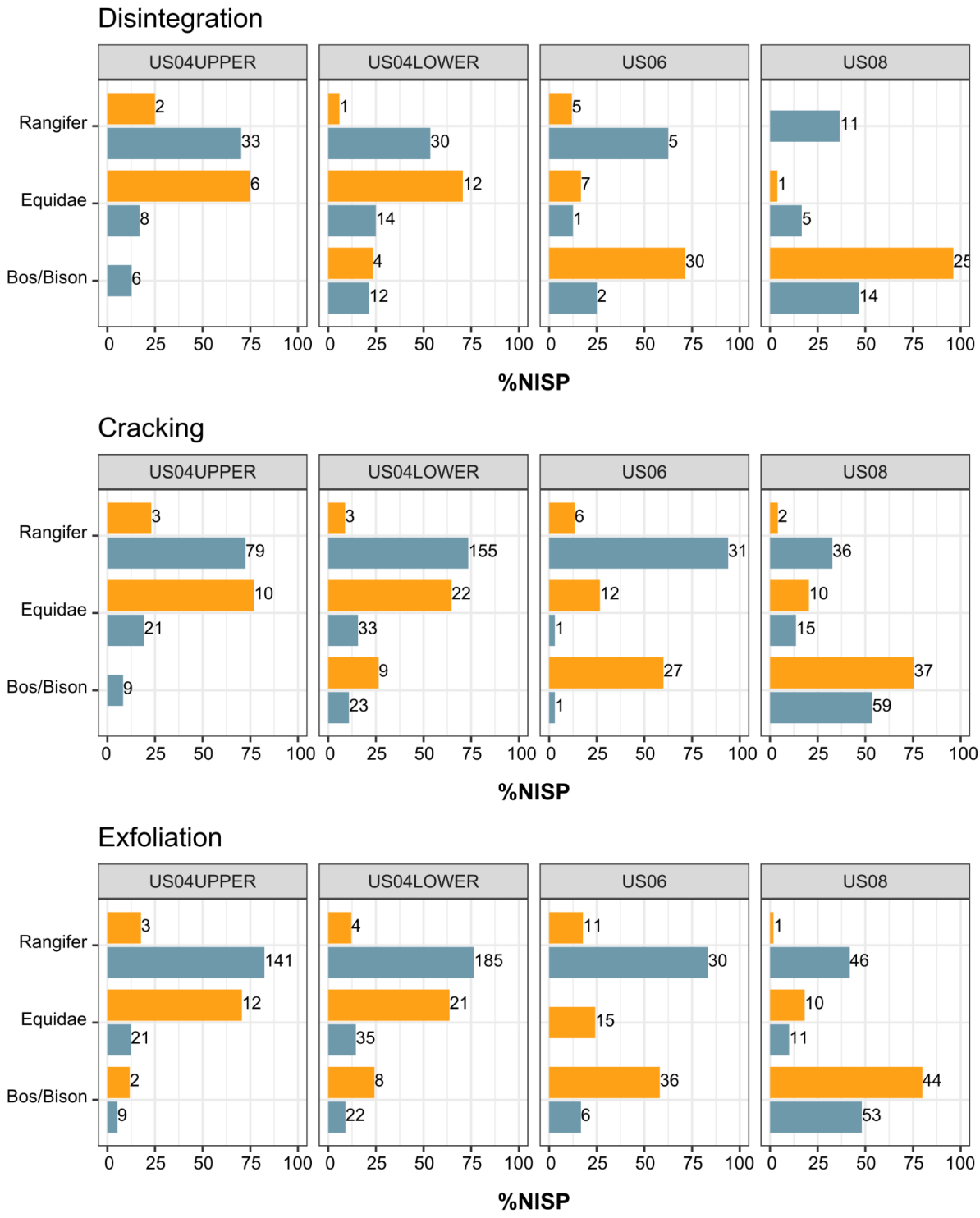

**SI Figure 5:** Bone length distribution of the dominant taxa within the ZooMS (orange) and the Morphology (blue) dataset for Layers I, J and K at Bacho Kiro Cave. Numbers on the bars are the NISP for each size class.



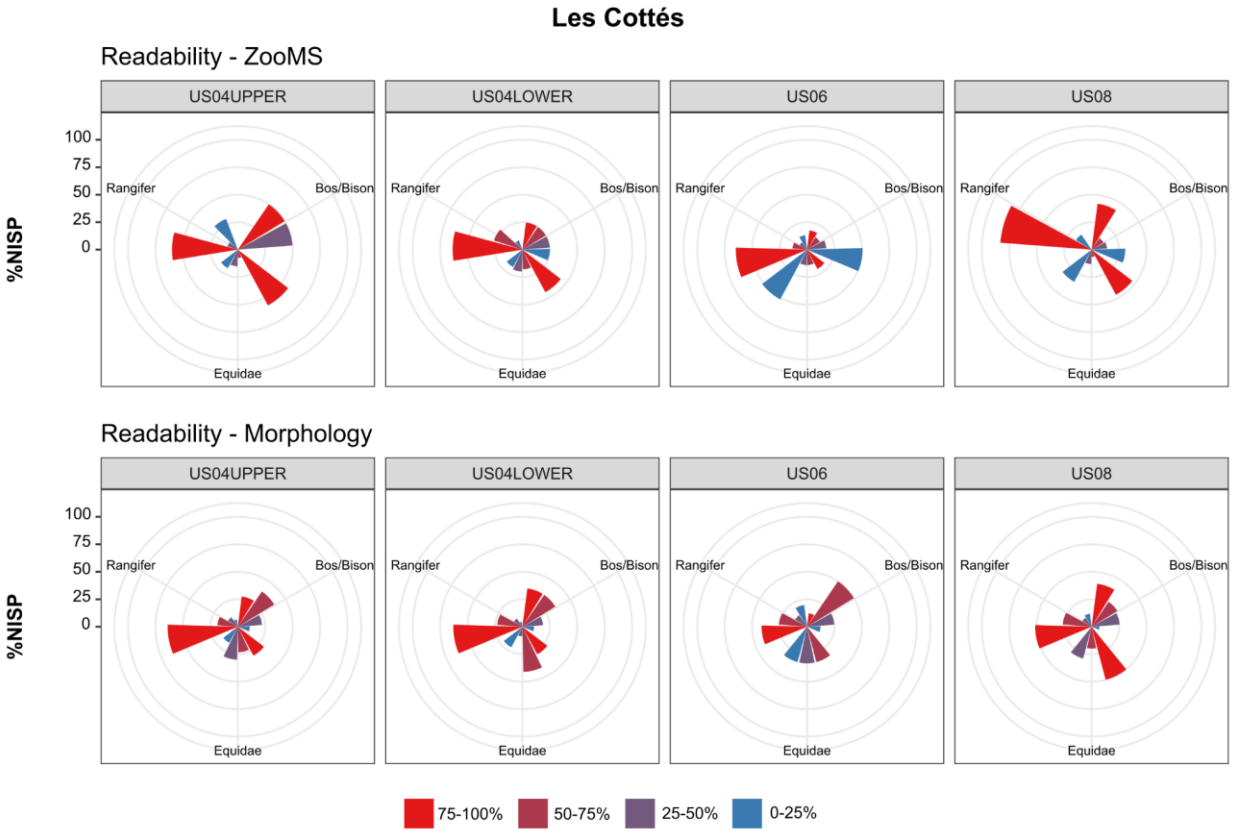

**SI Figure 6:** Bone length distribution of the dominant taxa within the ZooMS (orange) and the Morphology (blue) dataset for US04 (Upper and Lower), US06 and US08 at Les Cottés. Numbers on the bars are the NISP for each size class.



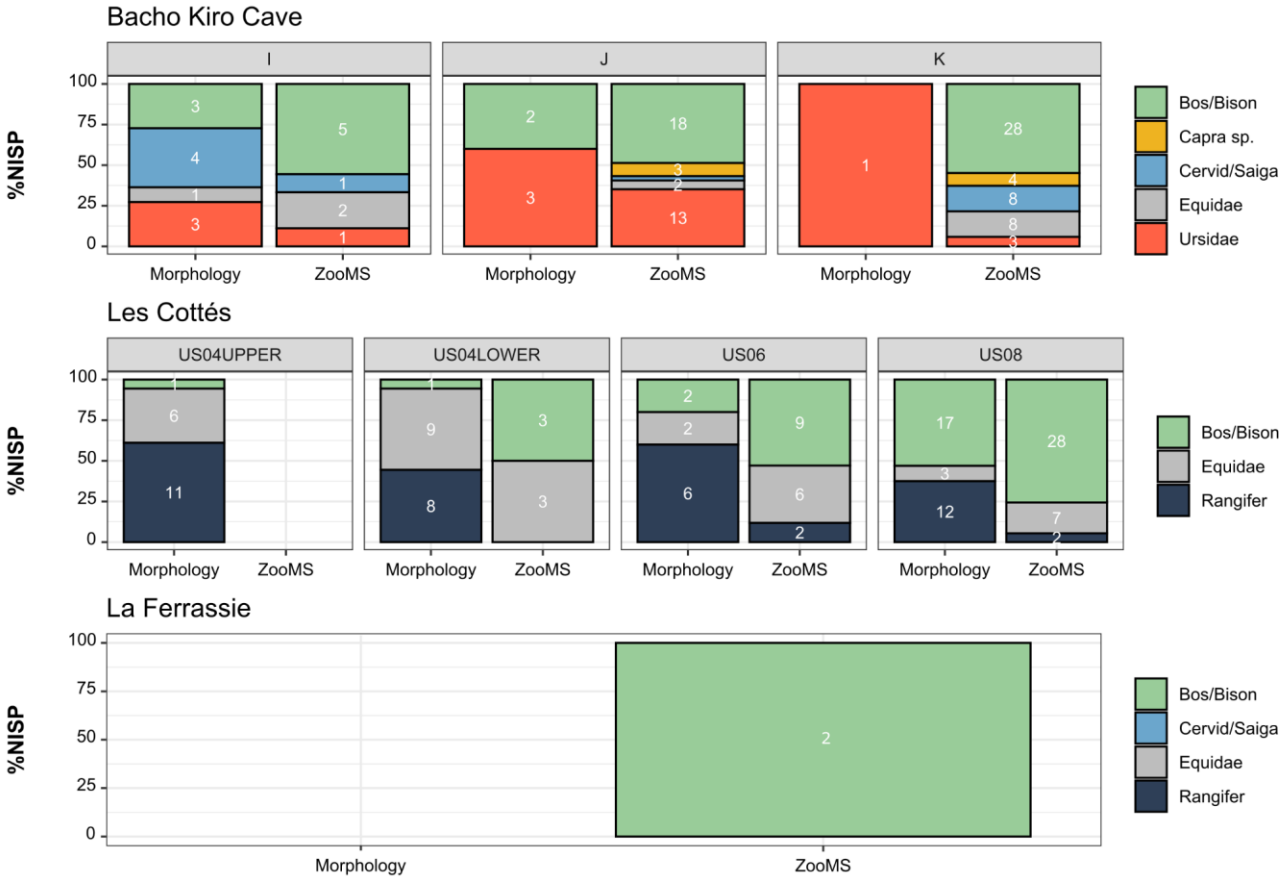

**SI Figure 7:** Bone length distribution of the dominant taxa within the ZooMS dataset at La Ferrassie. Numbers on the bars are the NISP for each size class.



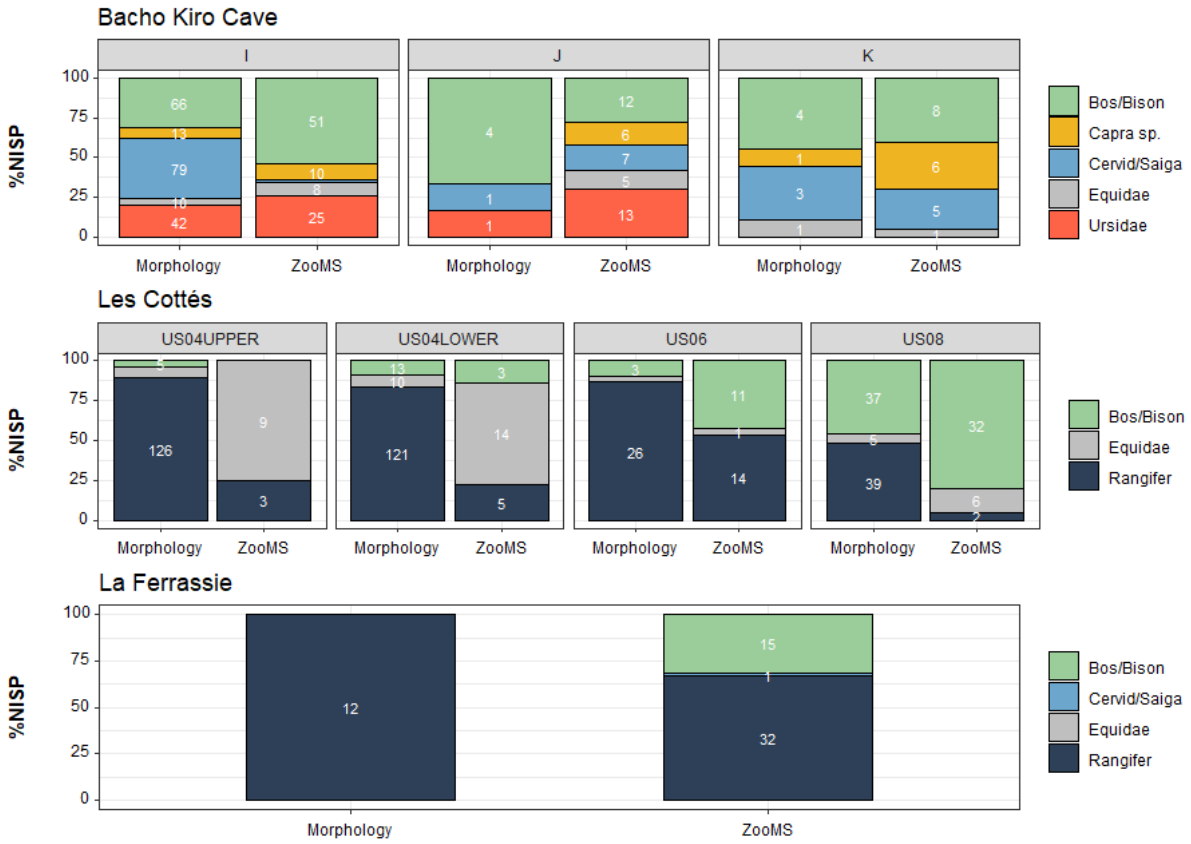

**SI Figure 8:** Percentage of disintegration, cracking and exfoliation on dominant taxa specimens in ZooMS (orange) and Morphology (blue) datasets from US04, US06 and US08 at Les Cottés. Numbers on the bars are the NISP.



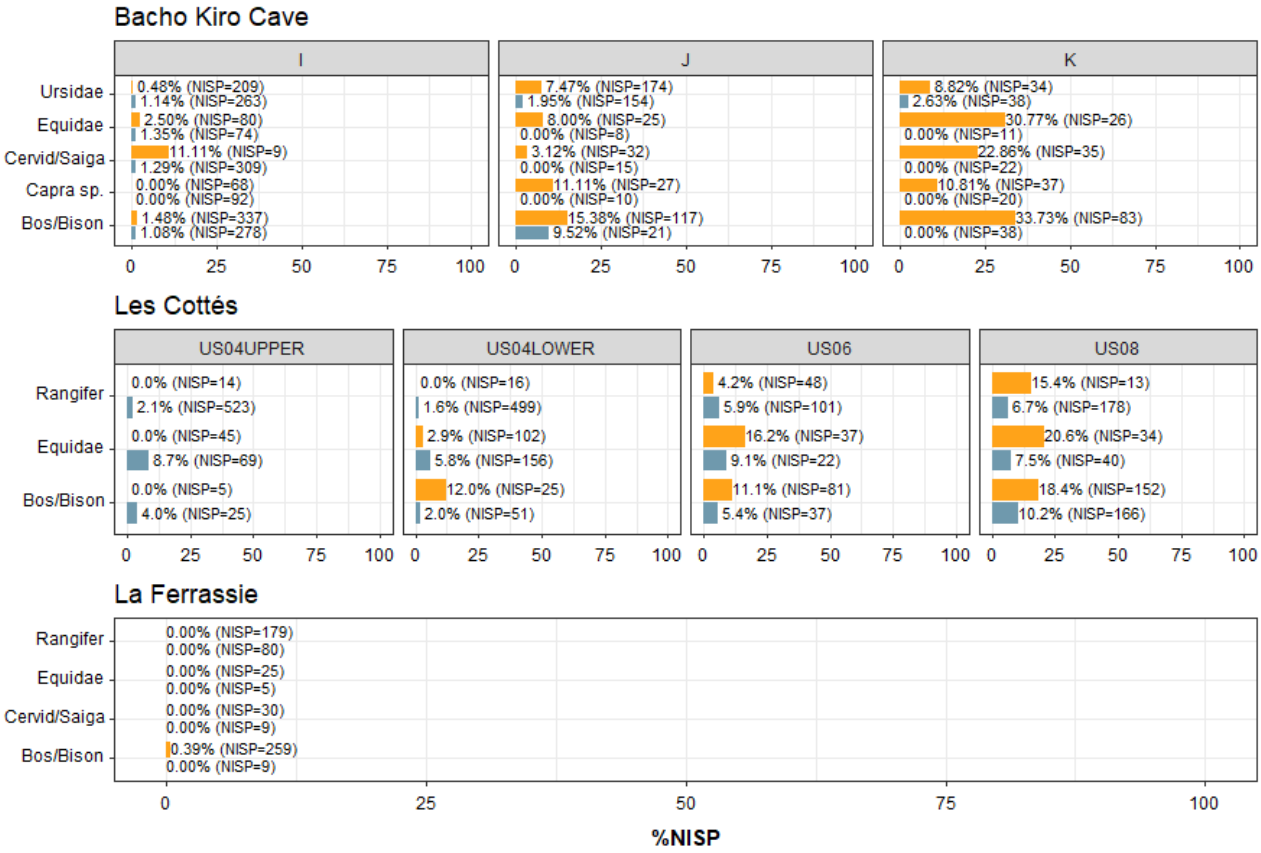

**SI Figure 9:** Percentage of readable bone surfaces (75-100% meaning 75-100% of the bone surface of the specimen is readable) for each dominant taxa from ZooMS and Morphology datasets from US04, US06 and US08 at Les Cottés.



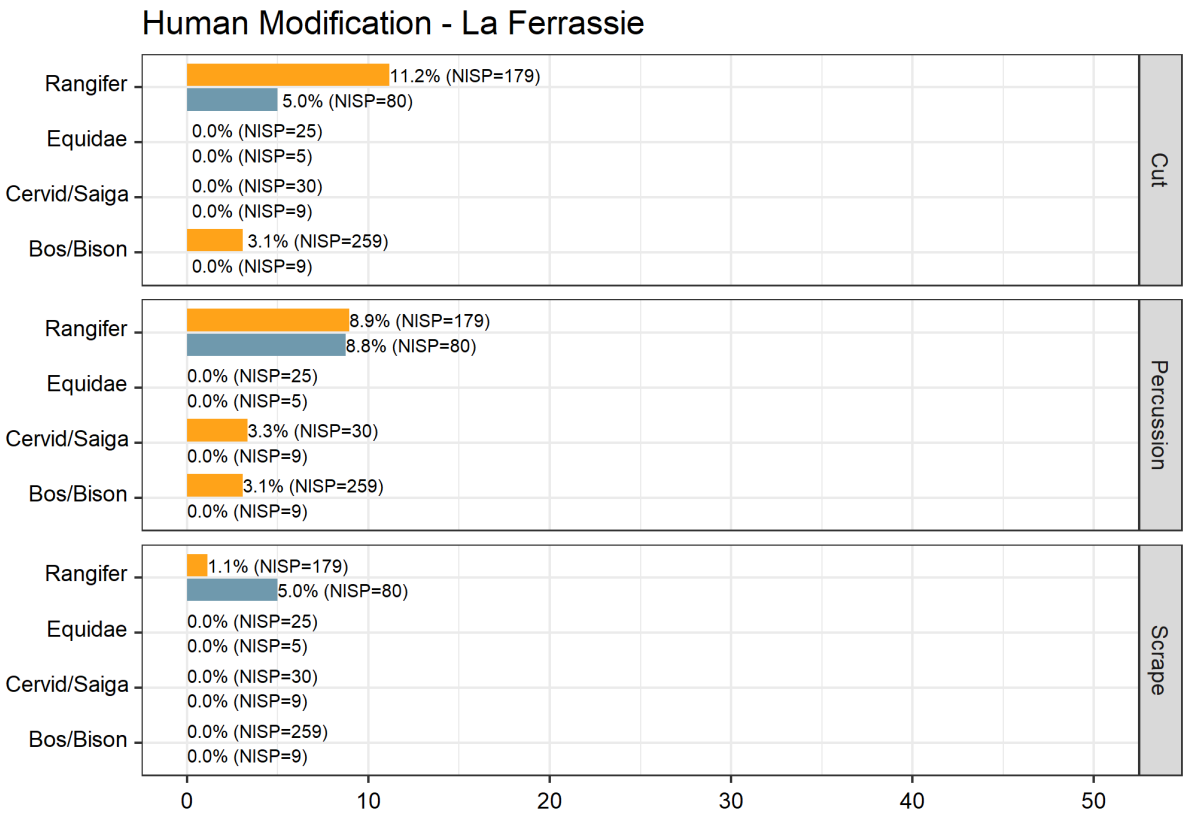

**SI Figure 10:** Percentages of carnivore modifications on the dominant taxa within each method of identification and layers at Bacho Kiro Cave, Les Cottés and La Ferrassie. Numbers on the bars are the NISP of specimens showing carnivore modifications.



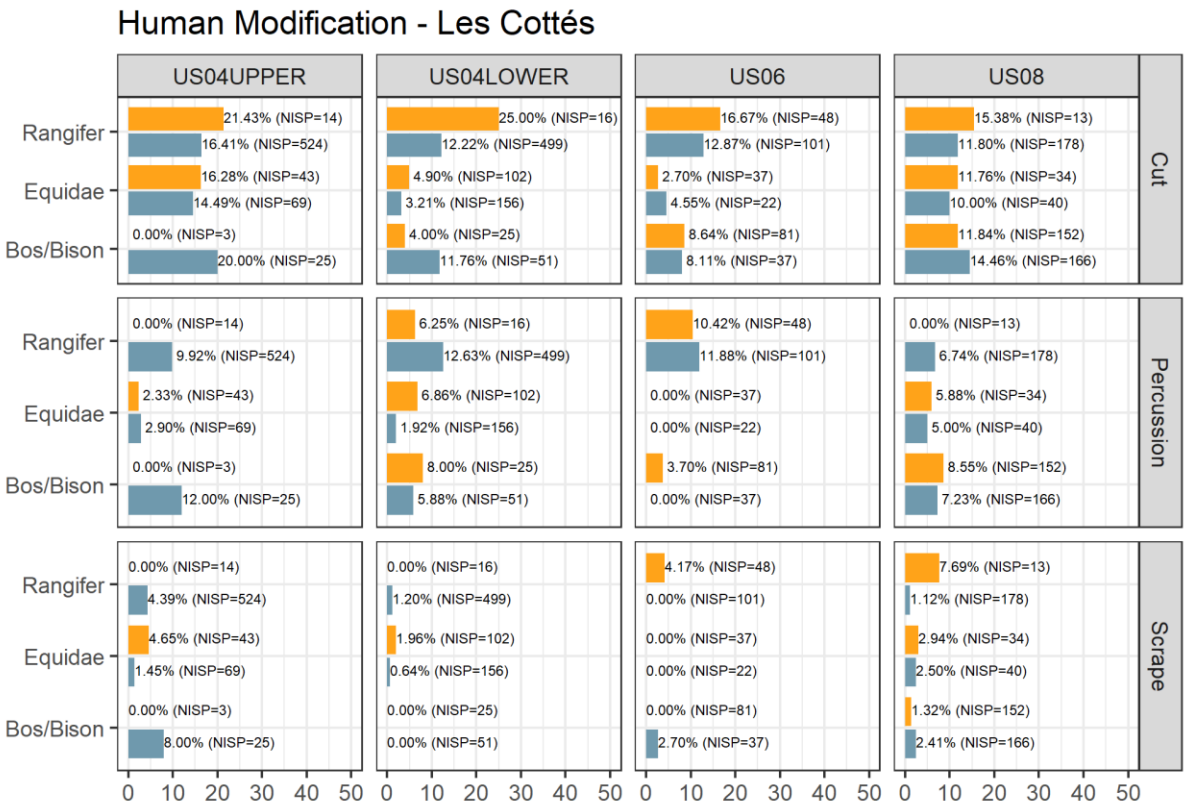

**SI Figure 11:** Percentages of anthropogenic modifications on the dominant taxa within each method of identification and layers at Bacho Kiro Cave, Les Cottés and La Ferrassie. Numbers on the bars are the NISP of specimens showing carnivore modifications.



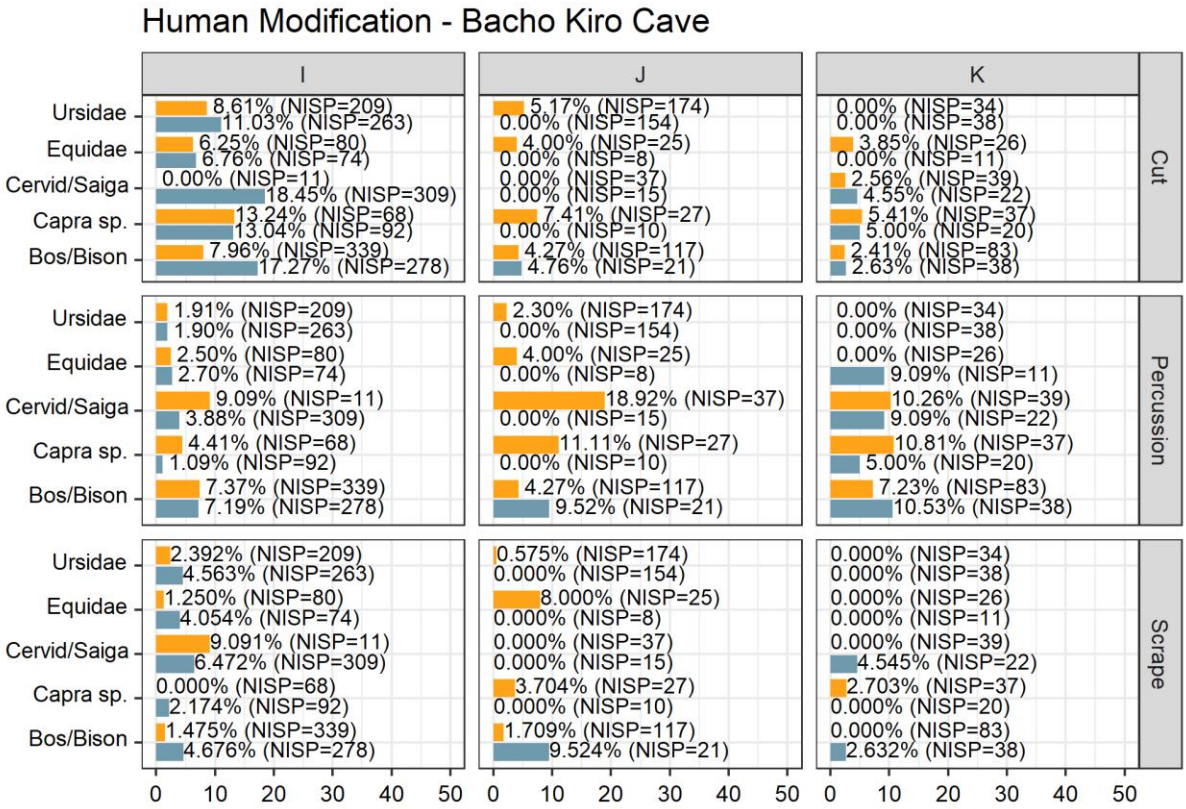

**SI Figure 12:** Percentages of carnivore modifications within the ZooMS (orange) and morphology (blue) datasets on the dominant taxa at Bacho Kiro Cave, Les Cottés and La Ferrassie. Numbers on the bars are the %NISP and total NISP of specimens identified for the taxon.



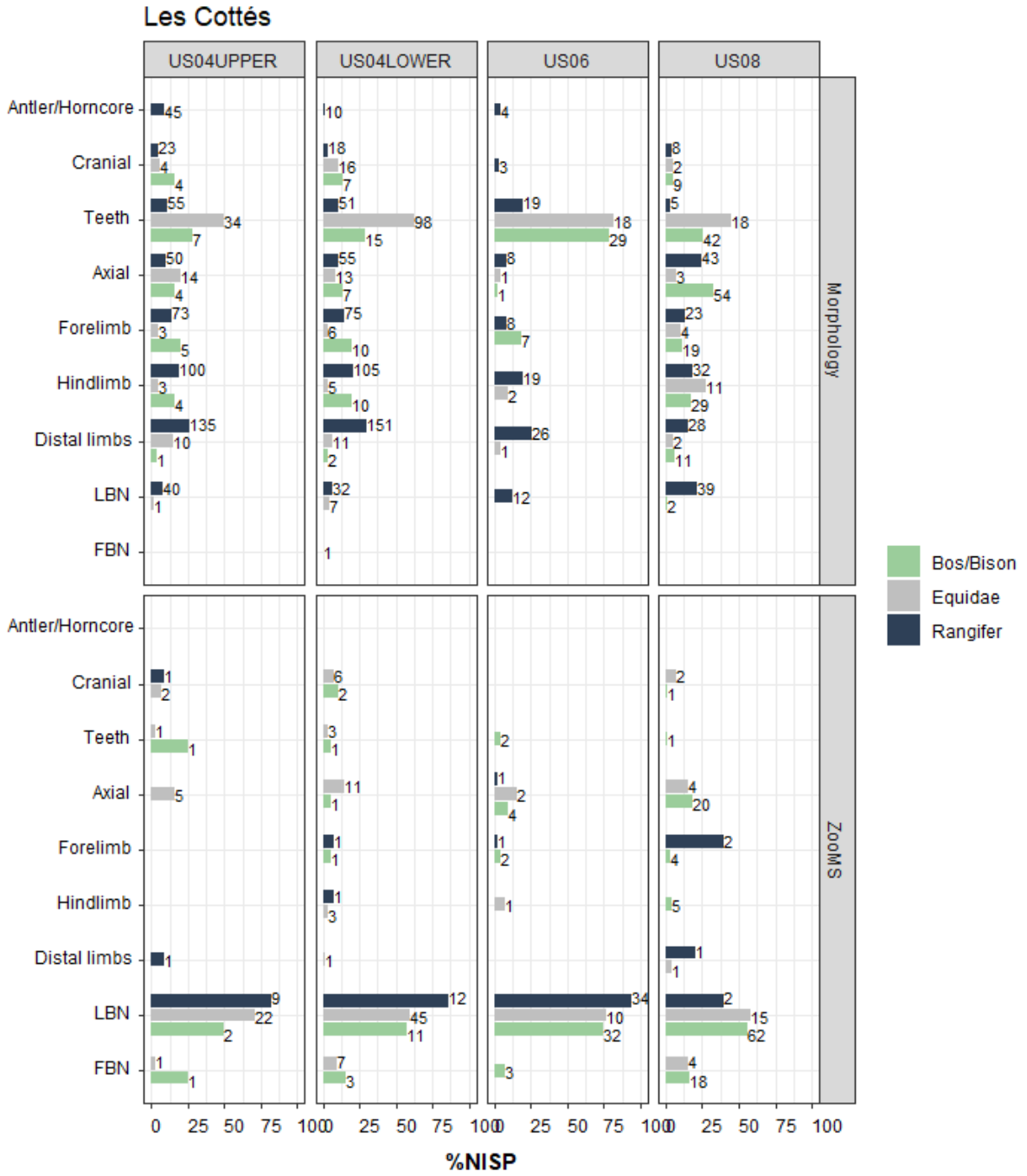

**SI Figure 13:** Percentage of specimens with human bone surface modifications (cut marks, percussion and scraping traces) across dominant taxa within ZooMS (orange) and Morphology (blue) datasets at La Ferrassie. Numbers on the bars are the %NISP and total NISP of specimens identified for the taxon.



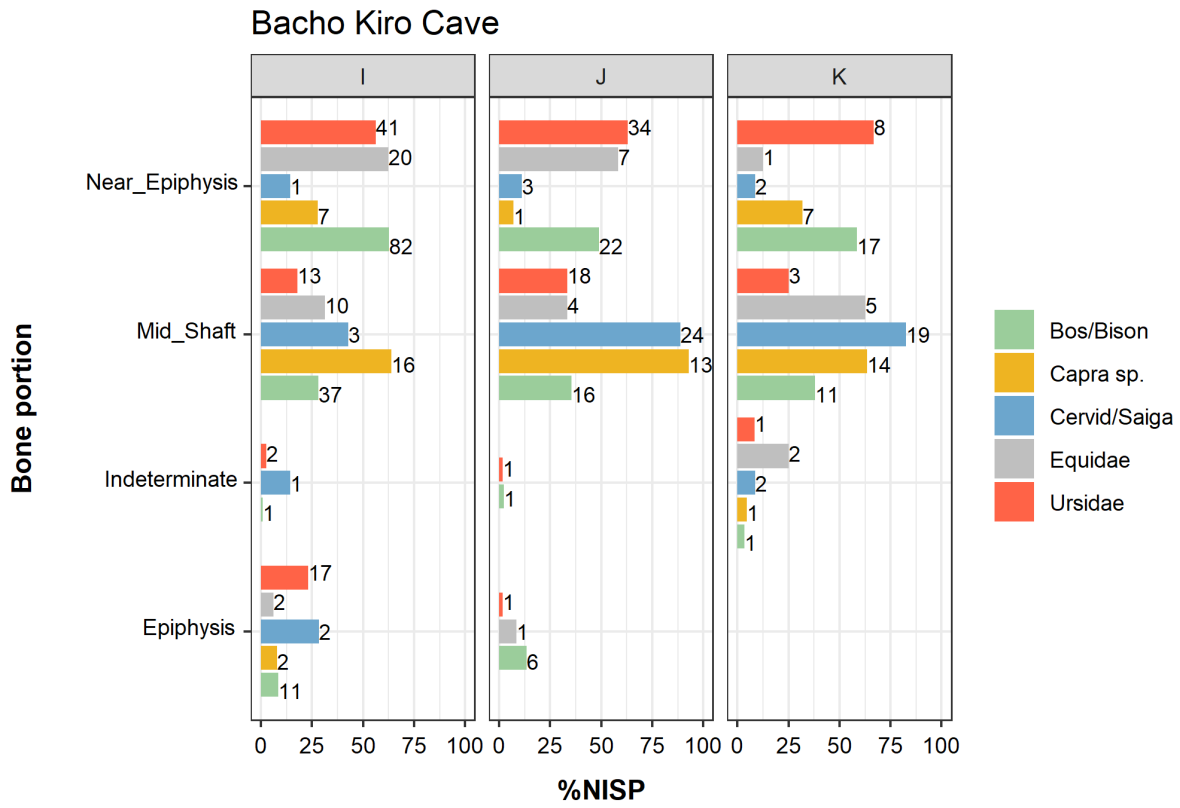

**SI Figure 14**: Percentage of specimens with human bone surface modifications (cut marks, percussion and scraping traces) across dominant taxa within ZooMS (orange) and Morphology (blue) datasets from US04 Lower, US06 and US08 at Les Cottés. Numbers on the bars are the %NISP and total NISP of specimens identified for the taxon.



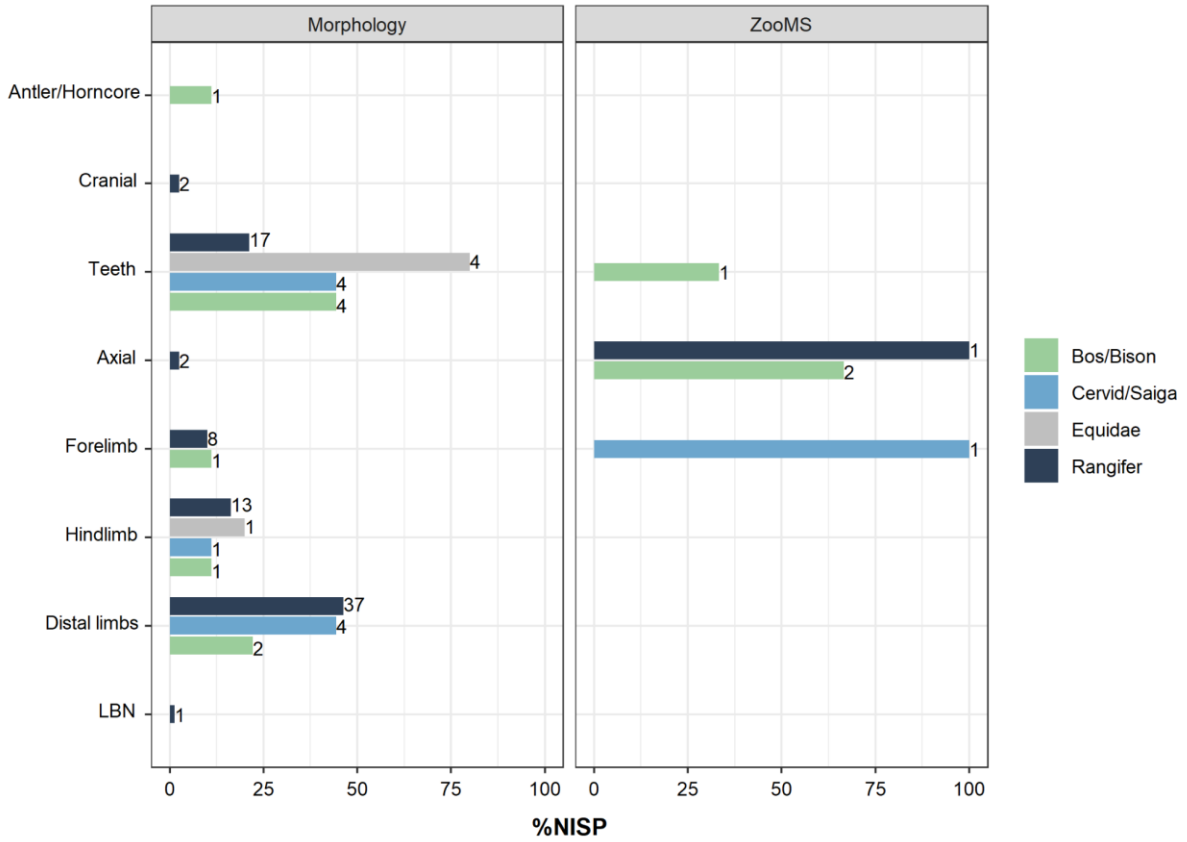

**SI Figure 15:** Percentage of specimens with human bone surface modifications (cut marks, percussion and anvil marks, and scraping traces) across dominant taxa among ZooMS (orange) and Morphology (blue) datasets from Layer I, J and K at Bacho Kiro Cave. Numbers on the bars are the %NISP and total NISP of specimens identified for the taxon.




**SI Figure 16**: Skeletal distribution of the bone specimens identified through morphology (top) and ZooMS (bottom) from the dominant taxa at Les Cottés. Numbers on the bars give the total NISP for each body part, layers and ID-method. Unidentified body parts (NID) were excluded from the plot. LBN: Long Bone fragment, FBN: Flat Bone fragment.




**SI Figure 17**: Distribution of the bone portions (Epiphysis, Near Epiphysis, Mid Shaft or Indeterminate) among the long bones (LBN) from the ZooMS component, between taxa and layers at Bacho Kiro Cave. Numbers on the bars of the graph correspond to the NISP for each category.




**SI Figure 18**: Skeletal distribution of the bone specimens identified through morphology (left) and ZooMS (right) from the dominant taxa at the site of La Ferrassie. Numbers within the bars give the total NISP for each body part and ID-method. Unidentified body parts (NID) were excluded from the plot. LBN: Long Bone fragment.

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Chapter Four

# The effect of eraser sampling for proteomic analysis on Palaeolithic bone surface microtopography

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## Abstract

Bone surface modifications are crucial for understanding human subsistence and dietary behaviour, and can inform about the techniques employed in the production and use of bone tools. Permission to destructively sample such unique artefacts is not always granted. The recent development of non-destructive proteomic extraction techniques has provided some alternatives for the analysis of rare and culturally significant artefacts, including bone tools and personal ornaments. The Eraser Extraction Method (EEM), first developed for ZooMS analysis of parchment, has recently been applied to bone and ivory specimens. To test the potential impact of the EEM on ancient bone surfaces, we analyse six anthropogenically modified Palaeolithic bone specimens from Bacho Kiro Cave (Bulgaria) through a controlled sampling experiment using qualitative and 3D quantitative microscopy. Although the overall bone topography is generally preserved, our findings demonstrate a slight flattening of the microtopography alongside the formation of micro-striations associated with the use of the eraser for all bone specimens. Such modifications are similar to ancient use-wear traces. We therefore consider the EEM a destructive sampling approach for Palaeolithic bone surfaces. Together with low ZooMS success rates in some of the reported studies, the EEM might not be a suitable approach to taxonomically identify Pleistocene bone specimens.

## Introduction

Bone is one of the most common archaeological remains recovered from Palaeolithic sites. Analysis of these remains can provide insights into human subsistence behaviour, dietary practices and site formation processes. Bone surface modifications are informative in this regard, particularly to retrace the taphonomic history of a bone fragment or to understand the manufacturing process and the potential use of a worked b one[1,2]. The taxonomic identification of these specimens through morphological assessments then becomes c rucial[3], in particular related to raw material selection at the species or skeletal element level. Worked bones such as bone tools represent technological innovation during human e volution[4–8], and the selection of raw material reflects behavioural choices and, potentially, also the function of the t ool[9]. Tool production can be driven by opportunistic bone selection made among the available faunal assemblage on-site resulting from food consumption[10,11]. In such a case, species composition and skeletal representation of a bone artefact assemblage could reflect species (and element) composition at the archaeological site or the faunal community present on the landscape. Alternatively, the raw material choice of a specific taxa and/or bone element can be based on the biomechanical properties required by the function of the tool, and knowledge thereof[12–14], or can be driven by behavioural choices related to cultural and/or symbolic meanings associated with a specific taxa (and/or element)[15,16]. Therefore, species

determination is greatly informative for our understanding of their production and the culturally-mediated behaviours associated with technological choices.

The vast majority of bone material, including bone tools, found on Palaeolithic sites are highly fragmented due to various taphonomic processes and prevent the taxonomic assessment of these bone specimens based on morphology. Moreover, taxonomic assignments of bone artefacts based on visual inspection of the external appearance are rendered difficult by the removal of morphological features during the fabrication of the tools or, subsequently, during tool use[13,16,17,18]. Often, bone artefacts are analyzed without knowing species identity, or lack specific taxonomic assignment; e.g. are assigned to broad taxonomic groups such as large- or medium size classes based on bone thickness, or even in relation to the most frequent species within the morphologically identifiable portion of the assemblage. Such assignments are not necessarily c orrect[19], and lack taxonomic precision.

To overcome these obstacles, researchers are using biomolecular approaches like palaeoproteomics, in particular Zooarchaeology by Mass Spectrometry (ZooMS)[20,21], ancient DNA analysis, and high-resolution CT scanning of bone histological thin sections to assess raw material selection and behavioural aspects associated with the artefact[22,23]. In particular proteomic peptide mass fingerprinting such as using ZooMS, has been applied frequently to the study of archaeological bone artefacts[18,24–29] and provides a precise taxonomic identification based on the analysis of the bone protein collagen type I[20]. Collagen type I survives beyond the temporal range of ancient D NA[30] and provides specimen-specific information about molecular d iagenesis[31,32]. However, despite the small sample size required in its traditional version, such destructive sampling is problematic for the analysis of rare, culturally significant and highly valuable archaeological artefacts.

With the expansion of ZooMS applications, recent studies have focused on developing non-destructive collagen extraction techniques, which began in 2015 using an eraser method initially applied to thirteenth century parchments[33]. This significant advance in biocodicology has unlocked the development of biomolecular analysis of parchments[34,35], and has been replicated for the extraction of DNA from herbarium specimens[36]. The Polyvinyl Chloride (PVC) eraser method consists of rubbing a soft polymer eraser on the surface of an organic t issue[33]. It is generally believed that the friction caused by the eraser rubbing generates a triboelectric charge between the organic surface and the eraser, releasing protein from the bone surface that binds to the eraser w aste[32]. Alternative non-destructive sampling protocols also employ static electricity to extract proteins from sample surfaces[13,37]. PVC erasers are widely used by conservators as a conventional conservation treatment for cleaning parchment and paper s urfaces[38]. This method does not require specialized equipment and the protein extract can be obtained on-site without transporting the specimen. The Eraser Extraction Method (EEM) is one of the non-invasive strategies called “eZooMS” (electrostatic ZooMS)

and has recently been extended to various archeological materials such as bone and ivory[25,37,39].

Using EEM to assess taxonomic identification allows for the possibility of non-invasive analyses while preserving the integrity of these specimens[37]. However some specialists have also shown that undertaking surface cleaning treatment using an eraser on paper can be abrasive to the specimen surface, particularly through the removal of fi bers[40–42]. Likewise, due to its potentially abrasive nature, we hypothesize that the use of PVC erasers on bone surfaces might modify macro- and microscopic features of the surface topography. We assume that it could remove large-scale macroscopic traces, such as the cross sectional shape of cut marks at mm-scale. In addition, it could potentially alter the surface roughness of the bone microtopography and produce small-scale microscopic use-wear traces such as micro-striations at µm-scale. This would result in unintentionally modified surfaces of the archaeological material, similar to natural processes that can produce pseudotools[43], but also may cover or overprint ancient traces. Such aspects have recently received increased attention, as they can be informative on taphonomic processes and human behaviour[44–47].

We note that although the advantages of the EEM for biomolecular analysis are clear, its impact on bone surfaces and bone surface modifications has not been assessed. To address this caveat, we characterize any potential modifications to archaeological bone surfaces resulting from EEM sampling. In addition, we assess the implications of such modifications relating to subsequent archaeological analyses like use-wear or bone surface modification analysis. To describe the modifications, first, we measure downward forces applied during EEM, and second, analyze bone surfaces with and without cut marks before and after EEM at different scales using qualitative (digital microscope, 2D) and quantitative microscopy (confocal disc-scanning microscope, 3D)[13,48].

## Methods

### Bone selection and sampling location.

We selected six archeological bone specimens from Bacho Kiro Cave (Dryanovo, Bulgaria), which were directly dated to approx. 45,000 c alBP[49]. These large long bone fragments have previously been taxonomically identified as *Bos/Bison* through ZooMS using destructive sampling. We determined by visual inspection that they show good surface preservation with clear anthropogenic traces including cut marks, marrow fractures or damage from reshaping lithic tools[49,50,51]. We defined two regions of interest (ROI) on each bone surface, represented by two squares of 1 × 1 cm each. One ROI was located on top of a butchery trace with cut marks (cut area), and another ROI was located on an unmodified surface (control area) (Fig. 1).

**Eraser sampling protocol.**

The bone specimens were placed into a styrofoam block that fit the shape of the bone fragment so that a standardised position of the bone specimen was maintained throughout the sampling process. The eraser sampling was done by one individual (VSM) to avoid any potential inter-individual variability and followed details provided by[33]. All samples were obtained through the rubbing of a sterile PVC eraser on a bone surface. An eraser holder and eraser sticks (Staedtler Mars plastic 52855) were used. The erasers were wiped with ethanol (Roth, 99.98%) and wrapped separately in aluminium foil before sampling. The holder was cleaned with ethanol between each sampling event. The eraser piece was replaced after each sampling event. Sampling was done with unidirectional movements[33]. Eraser movements were orientated perpendicular to the cut mark, limited to a duration of 2 min in order to standardize the experiment for all specimens. This duration is, in our experience, generally sufficient to generate the recommended amount of eraser scrubbings, equivalent to 20 µl[52]. The eraser wastes were collected in aluminium foil placed directly underneath the bone specimen and transferred into labeled LoBind tubes (Eppendorf).

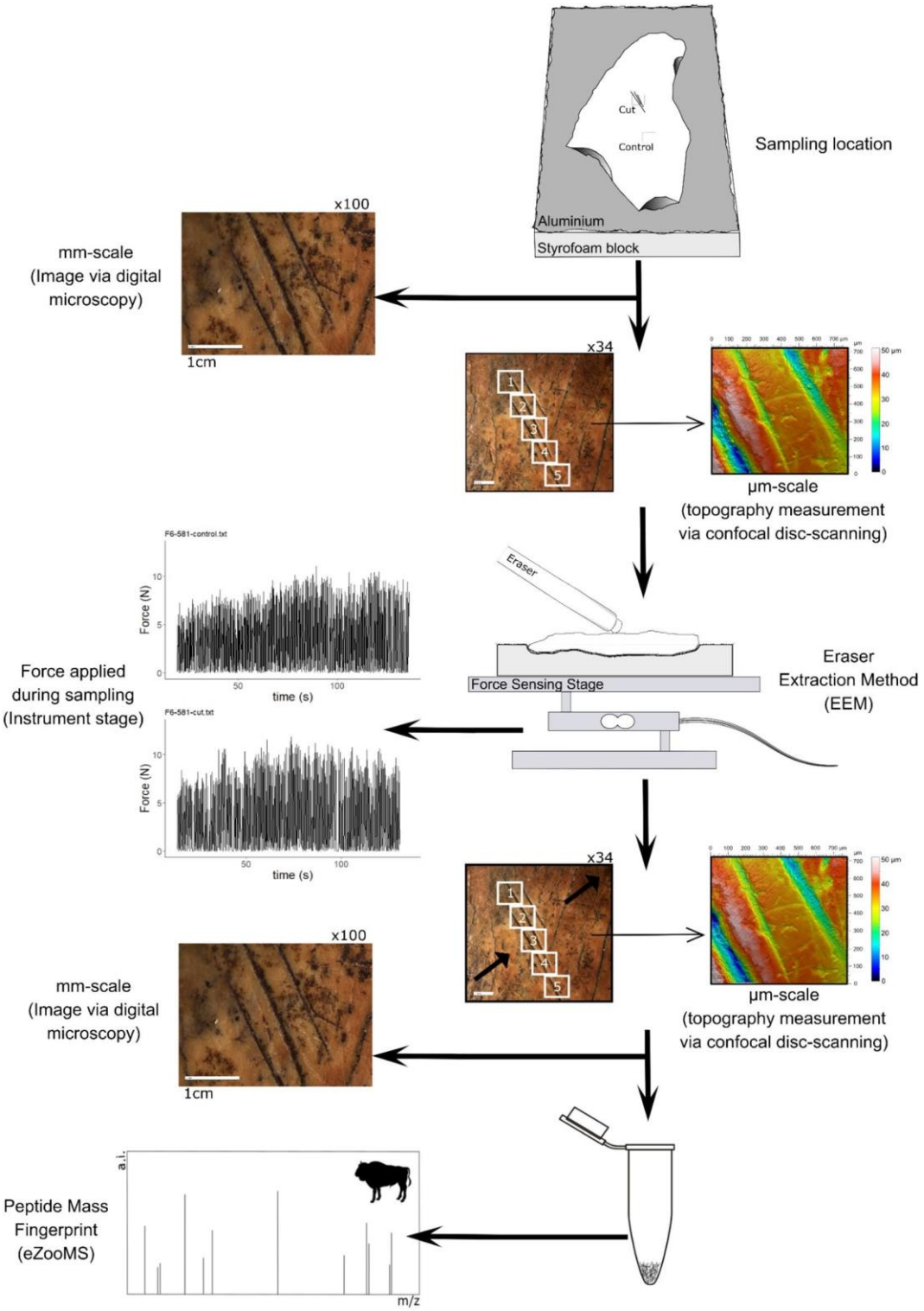


**Figure 1.** Description of the experimental workflow. The location of ROIs (cut and control) was defined on each bone specimen included in the study. The macro- and microscopic surface topography of the bone surface of each area was visually inspected using photos by digital microscopy (ZEISS, Smartzoom 5) and measurements by confocal disc-scanning microscopy (µsurf mobile, Nanofocus AG) before and after EEM. Cut and control areas were sampled using EEM while the downward force applied during sampling was measured via an instrumented stage. Each sample collected was analysed through peptide mass fingerprinting (n = 12). Animal silhouette is not to scale and derives from http:// phylo pic. org.

## ZooMS analysis.

The eraser waste obtained during sampling was analyzed through previously described ZooMS protocols[20,33,53]. In short, eraser waste was centrifuged for 1 min and incubated with 100 µL of 50 mM ammonium bicarbonate solution ($NH_3CO_3$) for 1 h at 65 °C. 50 µL of the supernatant was transferred into a new Lo-Bind tube (Eppendorf). 1 µL of trypsin (0.5 µg/µL, Promega) was added to the gelatin extract and incubated at 37 °C for 18 h. After digestion, each sample was acidified using 1 µL of 10% TFA and cleaned on C18 ZipTips (Thermo Scientific). Subsequently, the eluted peptides were spotted in triplicate on a MALDI Bruker plate with the addition of matrix solution (CHCA). MALDI-TOF MS analysis was conducted at the IZI Fraunhofer in Leipzig and spectra were identified in comparison to a database containing peptide marker masses for all medium to larger sized mammalian genera in existence in Europe during the Pleistocene[53]. To compare spectral quality between sample extracts, we calculated signal-to-noise ratios (S/N) for a selected set of peptides using MALDIquant, including baseline removal and peak picking[54].

In order to assess any potential contamination by non-endogenous peptides, we performed a set of blank extractions, consisting of empty tubes, alongside the rest of the samples in order to exclude any potential protein contamination during laboratory extraction. The MALDI-TOF MS spectra obtained show no collageneous peptides, demonstrating that the taxonomic identification does not derive from laboratory contamination. Secondly, all spectra were checked against known contaminant peptide masses such as human keratin and any matching peaks were excluded from further analysis.

## Measuring the force applied using a force sensing stage.

The intensity of force and the rate of erasing movements during the experiment were recorded by a self-made instrumented stage that reads the force applied to bones. The stage is composed of a load cell (5 kg CZL635, Tinkerforge, Stukenbrock, Germany) mounted between two 1 cm thick aluminium plates. The load cell has a precision of 0.05% across its full range of 49.04 N (equating to 5 kg). The load cell output is amplified by a HX711 load cell amplifier (SparkFun, Colorado, USA), which is then connected to an Arduino Nano microcontroller (Arduino, Ivrea, Italy). The stage interfaces with a laptop computer through two custom programs: one program to read the forces and one to calibrate the load cell. Force data is recorded at a rate of 10 Hz. The force sensing stage was secured to the laboratory bench using two screw clamps to limit the movement or vibrational noise. Before each sampling session the load cell calibration was checked using a series of known weights (Supplementary Fig. S1). In order to provide a reference point for comparison, the force applied during the erasing of pencil traces from paper was measured. Peak forces, above a threshold of 0.5 N, for each contact event between eraser and bone surface were identified using the SciPy library in Python 3.9 and the “find_peaks” function. This allowed the

identification of the frequency of contact events and the peak force associated with each event (Fig. 2).

## Qualitative microscopy (digital microscopy).

Cut and control areas on each bone specimen were assessed using a digital microscope (Smartzoom 5 with lens PlanApoD 1.6/0.1FWD 36 mm, Zeiss) and photo images were taken using the Smartzoom 5 software version 1.4 (Zeiss), in a standardised manner, before and after EEM using a digital zoom of × 34/100 (Supplementary Fig. S2). The magnification was standardised for all bone specimens and ROIs, and the position and orientation of the bone specimens were normalized by their inclusion into their styrofoam block. The intensity, orientation and inclination of the light source were kept consistent across samples. The focus was adjusted manually and the segmented ringlight was used on different fixed positions for all images (full, top, bottom, right and left ringlight). In order to obtain the extended depth of field, the lower and higher focal plane were assigned manually. Each ROIs was assessed at two magnifications (× 34 and × 100) before and after eraser sampling. Visual inspection was conducted by one individual (VSM), and photo images were qualitatively described using the following terminology: the general appearance and rugosity of the surface were compared before and after EEM, the location and morphology of the cut marks were reported, as well as the presence of residues and their distribution on the surface. Surface reflectivity was addressed through a visual comparison of the intensity and localisation of the most reflective area. Removal/ creation of traces appearing after the use of eraser was described including the indication of their orientation.

## Quantitative microscopy (confocal disc-scanning microscopy).

In order to assess the surface texture of the bone microtopography, each bone specimen was scanned with a confocal disc-scanning microscope (μsurf mobile, Nanofocus AG, Oberhausen, Germany), using a 20 × lens (numerical aperture = 0.4, field of view = 0.8 $mm^2$). The confocal disc-scanning was done by one individual (ESK) to avoid any potential interindividual variability and the measuring procedure followed details provided by[48,55]. For the two ROIs on each specimen, we ensured the same scanned position before and after EEM, through the creation of two reference points on a piece of tape placed on the surface of the bone (an incised X and a drawn point). Five scans within both ROIs were taken in sequence along the longitudinal axis of each cut mark (cut area). The area without cut marks (control area) was scanned in a similar manner along the same axis for each bone specimen. Scans were reviewed for quality and accepted for further study if 95% or more of the surface points were measured. Those with lesser accuracy were re-measured by altering exposure, brightness, gain, or pitch values until 95% of the surface was captured.

Within MountainsMap Premium v. 8.1.9369 Analysis software by Digital Surf (Besançon, France), meshed axiomatic 3D models of each scan were constructed using the following procedures: extract layers (topography layer), leveling (LS-plane), outlier removal (isolated outlier removal, with normal strength, and fill in non-measured points) following the pre-processing algorithms as described previously[48,55–57]. To compare the same scan area before and after eraser sampling, the following three pre-processing operators were included in the workflow: build series of surfaces (settings: copy after, use X/Y-offset, T-axis spacing 2), shift surface (settings: fixed reference studiable, offset settings manually defined, intersection set as kept area), and extract surfaces (settings: all surfaces of the series). One set of the paired before and after scans (second measurement location in the control area of specimen CC7-379) was removed at this point due to minimal overlap in surface portions. ISO 25178 parameters were then calculated from the S-L (roughness surface) using a filter set of an S-filter (Robust Gaussian polynomial of second order, 0.8 µm) and an L-filter (Robust Gaussian polynomial of second order, 0.008 mm).

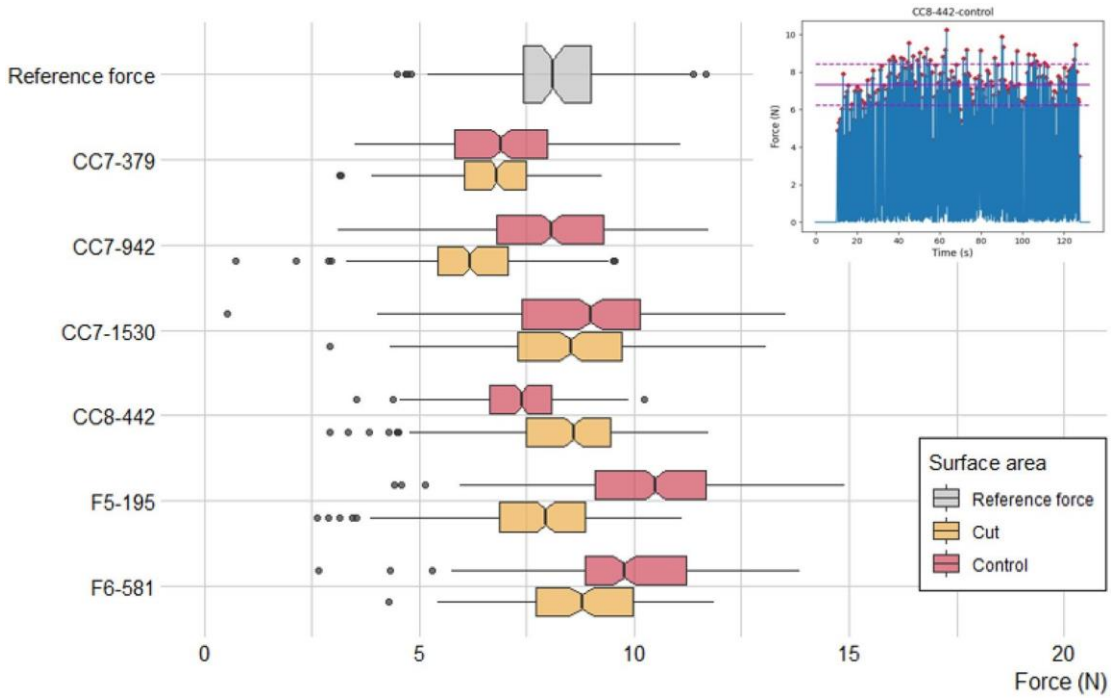


**Figure 2.** Distribution of the peak forces applied during the EEM of each sampling cut area (in yellow) and control area (in red) for each bone specimen. The reference peak force measurements (eraser on paper) is shown in grey. The insert in the top right is an example of how peak forces were acquired from force data. The mean peak force (red line) for each surface area consists of the maximum force values (red markers) for each eraser and bone contact during the 2 min of the EEM event. Dashed lines equal to + 1 and − 1 SD.

We selected four ISO 25178 p arameters[58] for quantitative analysis based on previous experience assessing diagnostic alterations to bone microtopography: arithmetic peak height (*Sa*), arithmetic mean peak curvature (*Spc*), closed hill area (*Sha*), and upper material ratio

(*Smrk1*)[48,57] (Supplementary Fig. S3). *Sa* is a height parameter that represents the distribution of heights along the z-axis of the surface in comparison to the arithmetic mean value of the surface. Thus, higher values indicate a surface with greater variation or roughness along the z-axis. *Spc* is a feature parameter that represents the arithmetic mean curvature of the peaks of the surface. Higher values indicate more pointed peak forms. Another feature parameter, *Sha,* represents the average hill area that is not connected to the boundary edge at a given height of the material ratio. Higher values indicate a surface with large cross sections in the upper portion of the surface. *Smrk1* is a functional parameter calculated from the material ratio curve of the distribution of surface depths and represents the uppermost portion of the surface. Higher values indicate a plateaued surface.

### Statistical analysis of the ISO 25178 surface texture parameters.

We employed Bayesian modeling following previous protocols[48,57]. To stabilize the variances and distributions, the ISO 25178 parameters were log transformed (Table 1, Supplementary Table S1). The statistical model for the observations Y, a matrix of p = 4 columns (log-transformed ISO 25178 parameters) and n = 59 rows (3D models), is a multivariate mixed model of the form $Y=XB+ZU+E$ ; where XB represents the fixed effects, ZU the random effects, and E the residual error. ZU captures idiosyncratic bone *specimens* and *measurement location* effects. U is a 65 × 4 matrix of random intercepts; each column of U contains 6 unique *specimen* effects and 59 unique *measurement location* effects. The four ISO 25178 parameters are represented by a column of U. Z is a 118 × 65 matrix of zeros and ones, which indicates the *specimen* and *measurement location* of each scan. The dimensions of X and B depend on the number of fixed effects. We fit two models of increasing complexity with different effects. M0 includes one fixed effect *area*, while M1 includes the additional effect *erasing*. We compared leave-one-out cross-validation (LOO) scores [59], which found design (M1) to generate model predictions best (Supplementary Table S2). This indicates that there is statistical support for change in at least one ISO 25178 parameter related to eraser use on the bone surfaces. For M1, B is a 3 × 4 matrix of fixed effect *area* (control, cut) and *erasing* (after) and an intercept for each surface texture parameter. Design matrix X is a 118 × 3 matrix of zeros and ones. E is a 118 × 4 residual matrix. Therefore, M1 is a multilevel, multivariate Bayesian model with fixed effects (*area* and *erasing*), random effects (*specimen* and *measurement location*), and *error* (Supplementary Table S2).

We applied a goodness of fit check to ensure that *specimen* and *measurement location* random effects were adequately modeled using multivariate Gaussian distributions. For additional model details see Martisius et al.[48]. We estimated effects by a Hamiltonian Markov-chain Monte Carlo method, using the library rstan version 2.21.2[60] of the statistical computing language R version 4.1.0[61]. We allowed a 2000-iteration warm-up for four chains generating

1000 parameter samples per chain resulting in 4000 posterior samples for inference. We examined scaled and squared Mahalanobis distances between observations and predicted values to check for goodness of fit, and compared these distances to theoretical quantiles of the F-distribution[62] using a quantile–quantile plot (Supplementary Fig. S4).

| ISO 25178 parameter | Descriptive statistics (raw values) | | Model ratios (based on log-transformed values) | | |
|---|---|---|---|---|---|
| | BEFORE Mean (SD) [Unit] | AFTER Mean (SD) [Unit] | 2.5% Quantile | Median | 97.5% Quantile |
| *Sa* | 0.60 (0.32) [µm] | 0.59 (0.32) [µm] | 0.97 | 0.99 | 1.01 |
| *Spc* | 2.71 (1.29) [1/µm] | 2.69 (1.37) [1/µm] | 0.96 | 0.98 | 1.01 |
| *Sha* | 123.50 (59.73) [µm$^2$] | 127.85 (67.71) [µm$^2$] | 0.95 | 1.00 | 1.04 |
| *Smrk1* | 19.87 (0.95) [%] | 20.24 (0.82) [%] | 1.01 | 1.02 | 1.03 |

**Table 1.** Descriptive statistics of the raw ISO 25178 data and model ratios based on posterior effects of each fixed effect in the model after and before EEM for each surface texture parameter including 95% credibility intervals. Intervals that include 1.00 indicate that the parameter ratio values are not well distinguishable prior to and after EEM.

# Results

## ZooMS analysis.

eZooMS analysis shows preserved collagen type I in each sample and, at a minimum, provides a MALDI-TOF MS spectrum containing two peptide markers (COL1α1 508–519 and COL1α2 978–990). In our spectra, we observe a systematic absence of peptides of higher molecular weight, in particular markers COL1α2 454–483, COL1α1 586–618 and COL1α2 757–789, which are absent in all eZooMS spectra, and COL1α2 793–816 present in one spectra (Supplementary Table S3 + Supplementary Fig. S5). This is in contrast to the previously obtained spectra from the same specimens, which were generated using a destructive sampling approach, and where such heavier peptide markers are present. The general absence of heavy m/z peptides within non-destructively extracted samples has also been observed in previous eZooMS s tudies[13,37]. The assessment of the peak intensity between comparable samples illustrates a signal considerably lower for the eraser samples than for the bone fragments of the same specimens analysed through ZooMS. The signal-to-noise ratio (S/N) of the three dominant peptide markers (COL1α1 508–519, COL1α2 978–990, and COL1α2 484–498) of the eZooMS samples show, in the case of COL1α1 508–519, COL1α2 978–990, lower to similar values compared to the bone samples with the exception of specimen CC7-1530. The S/N values for the peptide marker COL1α2 484–498 are consistently higher for the bone samples compared to the values obtained from eZooMS (Supplementary Fig. S6).

Each spectrum obtained with the EEM produced a taxonomic identification in agreement with those previously made using a destructive sample of the same bone specimens[50]. However, eZooMS identifications are broader due to the absence of the higher mass peptides, resulting in identifications as *Bos/Bison/Ovibos* instead of *Bos/Bison*. The mass 1208 m/z corresponds to the peptide COL1α2 978–990 present in *Bos* sp*., Bison* sp*.,* and *Ovibos* sp. This peptide was systematically identified within each MALDI-TOF MS spectra and, despite the absence of higher mass peptides, permitted such a specific taxonomic attribution. The genera *Bos* sp. and *Bison* sp. cannot be separated from each other based on standard ZooMS peptide markers, while in our case the peptide COL1α1 586–618 is absent and also prevents the eZooMS identifications to exclude *Ovibos* sp. as a possibility.

## Force sensing.

We analysed the peak forces applied during each EEM event (n = 12) and compared these to a reference set of peak forces obtained when erasing a pencil mark from paper (Fig. 2). All forces remain low at less than 15 N of force, the mean peak force applied to the bones during the EEM (8.12 N, ± 1.21 N) is comparable to the mean peak forces applied during the test on paper (mean = 8.15 N, ± 1.44 N). The peak forces applied to the bones were significantly different between specimens and sampled areas (Kruskal Wallis test, p-value < 0.05), but without a clear pattern or direction. However, these significant differences amount to only very small deviations in the mean peak force between conditions, all occurring within less than 5 N of each other. With this in mind we assert that the peak forces recorded throughout this experiment are representative of real-world erasing events and are broadly comparable in each experimental condition.

Sample weights consisting of the eraser wastes collected during the experiment ranged from 0.5 to 15.6 mg and were consistently higher for the control area compared to the cut area (Supplementary Table S3). However we observe no significant difference of the mean peak force applied between cut and control areas ($t(10) = 0.41$, $p = 0.7$) and also no significant difference between the mean number of eraser movements applied to cut area compared to control area ($t(10) = 0.58$, $p = 0.6$). There is no correlation between generated sample weight and generated force during the EEM procedure either, nor between generated force and the signal-to-noise ratio (S/N) of three low-weight peptide markers (COL1α1 508–519, COL1α2 978–990, and COL1α2 484–498) across samples (Supplementary Fig. S7). We therefore conclude that the force applied in these experiments has had no measurable influence on our eZooMS spectral quality.

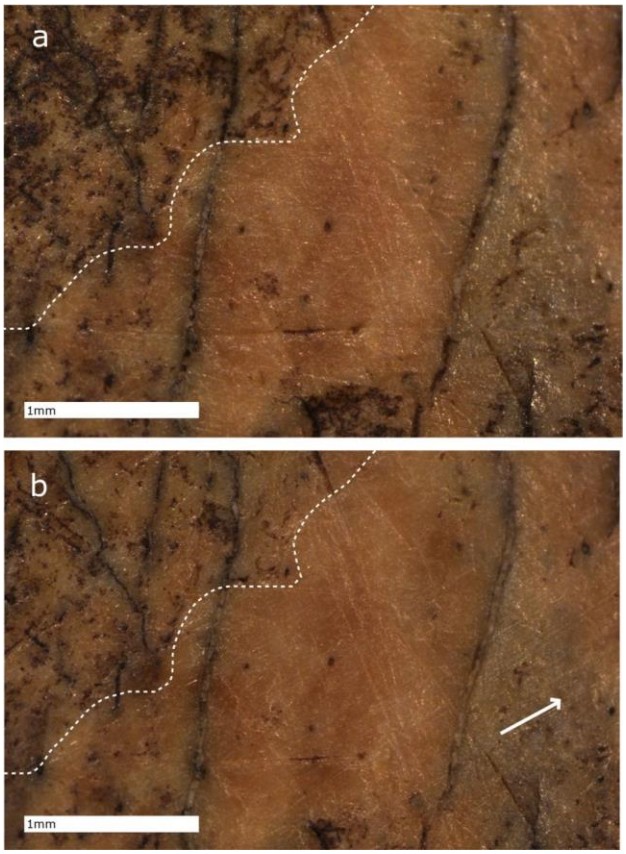


**Figure 3.** Micrographs of the control area of CC7-379 using automated digital microscopy (ZEISS, Smartzoom 5), (**a**) before the use of EEM, (**b**) after the use of EEM. The white arrow highlights the orientation of the microstriations which follow the orientation of the erasing movement. In addition, we note the removal of surface residues, visible in particular in the top-left corner as the removal of dark-stained regions (white dashed line).

## Qualitative microscopy.

The qualitative assessment of the bone specimens through digital microscopy reveals several types of modifications to the bone surfaces after eraser use. First, the friction caused by the repetitive movement of the eraser during EEM results in the removal of residues and particles from the highest portion of the topography of the bone surfaces of 9 out of 12 ROIs (Fig. 3, Supplementary Fig. S2, Supplementary Table S3), which, in the case of 3 specimens, is also observable at the macroscopic scale. In contrast, the particles trapped within the lower portion of the topography do not seem to be removed during the limited duration of the experiment. We note that the removed residues will most likely get trapped within the eraser wastes generated during sampling. They therefore represent a potential source of contamination during the proteomic extraction of the organic matter present on the surface of a bone, in case these residues are proteinaceous in nature. Secondly the movement of dust, sediment particles and/or bone residues on the surface associated with the pressure applied during EEM sampling results in the formation of multiple linear features. Although we do not observe modification of the gross features of the bone surfaces after EEM through digital

microscopy, we do observe the generation of parallel and regular micro-striations in the direction of the eraser movement on the control area of CC7-379 (Fig. 3). These modern striations are comparable to ancient use-wear traces[63,64] but are unrelated to use of the bone. No other specimens exhibit micro-striations at the scale observed through digital microscopy.

## Quantitative microscopy.

Model predictions for the four ISO 25178 surface texture parameters indicate no distinguishable differences in the bone microtopography after EEM for three of the parameters (*Sa*, *Spc*, and *Sha*) (Table 1). Ratios of the estimated after-to-before parameter values that are close to 1 indicate that the before and after state are very similar (Fig. 4; Table 1). It should be noted that a small amount of variation in the parameters is expected due to the difficulty of repositioning the surfaces on the micrometer scale required. Even so, the estimated before and after EEM values for *Sa* and *Spc* are most often very similar and indicate that the surfaces retain their overall roughness and curvature of the surface peaks after use with the eraser. Though model predictions for *Sa* and *Spc* are overall statistically indistinguishable, there appears to be a modest trend for lower *Sa* and *Spc* values after EEM, which is associated with a greater number of eraser movements (Supplementary Fig. S8). We postulate that if sampling were to occur over a longer duration, this would result in a more substantial decrease in both *Sa* and *Spc*. While the overall predicted values for *Sha* also appear to be similar after EEM, the ratio of the estimated differences for this parameter has the largest credibility interval of the four tested ISO 25178 parameters (Table 1). Further, empirical observations of the matched before and after pairwise scatterplots indicate some degree of variation, including a number of outliers (Fig. 4). Because this variation scatters relatively equally around the 45-degree line and not to one side of it, these differences are either unrelated to eraser sampling or are the result of unpredictable and irregular surface alterations. If the latter, hill area as calculated by standard default settings used for the calculation of *Sha* may not be an appropriate settings for assessing microscopic bone alterations and need to be re-adjusted.

In contrast to *Sa*, *Spc* and *Sha*, model predictions for *Smrk1* indicate that there is an increase in values after EEM resulting in a larger portion of material in the peaks of the surfaces (Table 1). This shift in predicted values demonstrates that eraser use uniformly wears the highest areas, or hills, of the surface, causing a general flattening or plateauing of the uppermost part of the surface microtopography (Fig. 4). A comparison of the observed before and after differences with both the number of eraser movements and the amount of force exerted during EEM, shows a slight trend for greater before and after differences in association with both variables (Supplementary Figs. S8 and S9). However, we observe an absence of correlation between sample weights and the after to before differences of the four ISO 25178 parameters indicating that the mass of the samples collected (consisting of eraser wastes) had no influence on surface texture measurements (Supplementary Fig. S10). Though the increase

in *Smrk1* is generally observed for most of the surfaces, the differences are more pronounced for those in the control area (Fig. 4). Because this parameter is a proportion of the surface material, the surfaces with less material [i.e., those with less surface roughness (< *Sa*)] are altered at a greater relative rate.

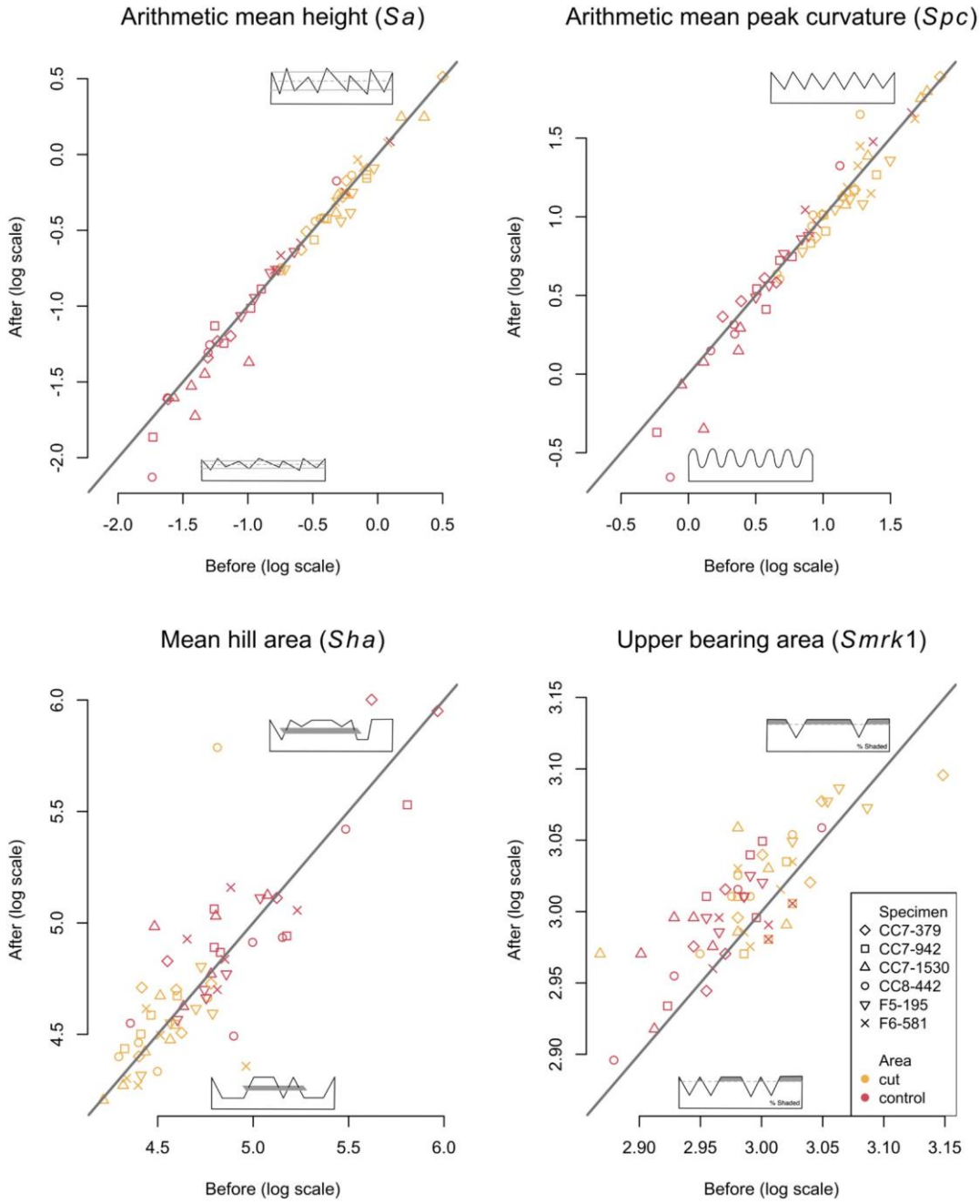


**Figure 4.** Matched before and after EEM pairwise scatterplot for each ISO 25178 surface texture parameter measured in this study (*Sa, Spc, Sha* and *Smrk1*). Lines represent equivalent parameter values after and before EEM. Each specimen is represented by different symbols, cut areas are in yellow and control areas are in red. 2D depictions of low and high values for each surface texture parameter are indicated on each plot.

**Comparison of quantitative and qualitative microscopy.**

A qualitative assessment of the bone surfaces through confocal microscopy supports the observations made using digital microscopy. At this higher magnification, we observe both "surface cleaning" and subparallel micro-striations oriented in the direction of eraser movement. Whereas digital microscopy reveals striations on one surface area of specimen CC7-379, visual inspection of the 2D intensity micrographs produced through confocal microscopy demonstrates that these surface modifications are present for every bone specimen, though not within each scanning location (Fig. 5; Supplementary Fig. S11). When visually comparing the same surfaces in both two and three dimensions (2D, 3D), these micro-striations or furrows appear to be superficial and do not alter the overall features of the bone surfaces (Fig. 5). The combined qualitative observations at different scales along with the quantitative increase of *Smrk1* indicates that EEM on bone creates friction that cleans the bone surface, while flattening the microtopography and creating fine micro-striations or furrows, which also causes the bone surface to appear polished at a macroscopic scale. The combination of multiple techniques and the assessment of surfaces at different scales is then crucial in order to obtain a comprehensive picture of the bone surface alterations after EEM.

## Discussion

Bone surface modifications provide the opportunity to associate an artefact retrieved from a Palaeolithic site with human occupation and activity, and potentially subsequent taphonomic processes at such archaeological sites. Therefore, the preservation of these modifications is crucial for the future analysis of such a specimen. With the development of non-destructive proteomic methods allowing for species determination, it is important to characterise the potential effects on bone surfaces. The Eraser Extraction Method constitutes one of the so-called non-destructive sampling techniques, and is based on the electrostatic extraction of proteinaceous molecules through repetitive movement of a soft Polyvinyl Chloride eraser directly on a bone surface[13,33,37].

Our analysis of bone surfaces after EEM shows, overall, neither major modifications of the bone topography nor the removal of features, such as cut marks, at a macroscopic level. This is supported by the similar before and after values obtained for three of the four tested ISO 25178 parameters (*Sa*, *Spc*, and *Sha*) through quantitative microscopy. The relatively short duration of EEM appears to have little to no effect on these variables at the scale studied in this experiment. However, a similar analysis at higher magnification or with a larger selection of ISO parameters may have provided different results. Even so, the repetitive movement of the eraser on these bone surfaces generated several permanent modifications at the µm-scale that should be acknowledged prior to the use of this sampling technique on any archaeological bone.

A measurable increase in one of the ISO 25178 parameters (*Smrk1*) provides compelling evidence that EEM on bone flattens the surface. Given the relationship between the shift in *Smrk1* values and both the number of eraser movements and the force applied, it is likely that eraser sampling for a duration longer than 2 min, or with greater pressure, would further alter the bone surfaces. A previous study on experimentally worked bone surfaces found a similar pattern associated with duration of use when bones were worn against fresh animal skin, a supple, sticky material that readily incorporates external particles adding to its a brasiveness[48]. Another experimental study testing the effects of cleaning procedures on stone tools also found an increase in this surface texture parameter after rubbing dirt off of a flint flake for about 1 min[65]. As with fresh skin and sediment particles, this increase in *Smrk1* indicates that eraser use causes alterations to the highest portion of bone surfaces resulting in the plateauing of the microtopography at the µm-scale. This pattern is most likely the result of friction generated during EEM combined with microscopic particles such as dust or calcite crystals wearing the surface when pressed against the bone and dragged by eraser movement. Further, this mechanical action likely led to the formation of the micro-striations or furrows observed on the bone specimens at multiple scales, which has also been observed on lithic a rtifacts[66]. Therefore, the effect of the EEM on bone surface microtopography could be related to the abrasiveness and size of the particles present on the bone surfaces during sampling.

We interpret the increase in *Smrk1* after EEM as an explanation for the qualitative observation that eraser use appears to clean and remove residues preserved on bone surfaces. This observation in both 2D and 3D microscopy represents an irreversible pattern. This implies that any potential traces of substances, such as adhesives, pigments, organic residues or residue traces, can potentially be removed from the surface, which in turn could prevent any subsequent residue analysis seeking to address the function of the worked p iece[67].

Although the presence of multiple micro-striations are only observed on a single specimen (CC7-379) through visual inspection using digital microscopy, they are measurable on each studied specimen at higher magnification using confocal disc-scanning microscopy. When looking at potential variables that could have influenced the creation of these traces and their appearance at different magnifications, we note the similarity in the force exerted and the number of eraser movements applied to the cut and control areas of the specimen CC7-379. At lower magnification using digital microscopy, the control area exhibits micro-striations after eraser use, while the cut area does not. This discrepancy cannot be explained by differences in force applied or eraser movements. However, it should be considered that the standardised parameters used in this experiment may have not permitted the complete visualisation of these surface alterations through digital microscopy, and variation in bone inclination along with differing oblique light orientations might have provided a clearer shadow effect and better assessment of the bone surfaces at that s cale[68]. Nonetheless, the standardised protocol

presented in this study allowed for the identification of various surface alterations caused by the use of EEM on palaeolithic bone specimens.

These observed micro-striations are comparable to surface modifications produced either during the use of a bone as a tool or during other taphonomic processes, and are observed at different magnifications using distinct microscopic methods[46,48,63,64]. While the observations made on the 2D intensity micrographs show clear and welldefined striations, they appear more superficial within the 3D surface texture. If such a bone was subsequently studied without a detailed sampling record, a functional analysis could lead to misinterpretation of such t races[69]. Worse, this sampling method applied to a bone tool could overprint any ancient use-wear traces indicative of the tool's function, obscuring interpretation. Thus, if one chooses to use EEM on bone, it would be important to incorporate this method into a phased approach, one in which EEM should be conducted subsequent to any functional analyses. The application of EEM on other mineralized and non-mineralized tissue surfaces might generate similar modifications, which is something that should be investigated prior to future applications of this technique. Indeed, our results emphasize the importance of maintaining a detailed record associated with this extraction technique, similar to any protocol for destructive sampling. This is especially important for future analyses that have not been anticipated. Moreover, the exclusive capture of low-molecular weight peptides and the low signal-to-noise ratio of the three dominant peptide markers limits the opportunity to obtain a discriminant species assignment for taxonomic groups not separable based on low-mass peptide markers. As a result, it can be expected that EEM, and other eZooMS approaches, result in a potentially low success rate when applied to Palaeolithic bone s pecimens[13,37]. Thus, the bone surface alterations and the potential low success rate of the eZooMS analysis using EEM highlighted in this paper should bring caution to the use of this extraction method on Palaeolithic faunal assemblages, and especially worked bones such as bone tools. The creation of modern alterations to the surfaces of archaeological specimens unrelated to their fabrication or use should be avoided to prevent subsequent misinterpretations.

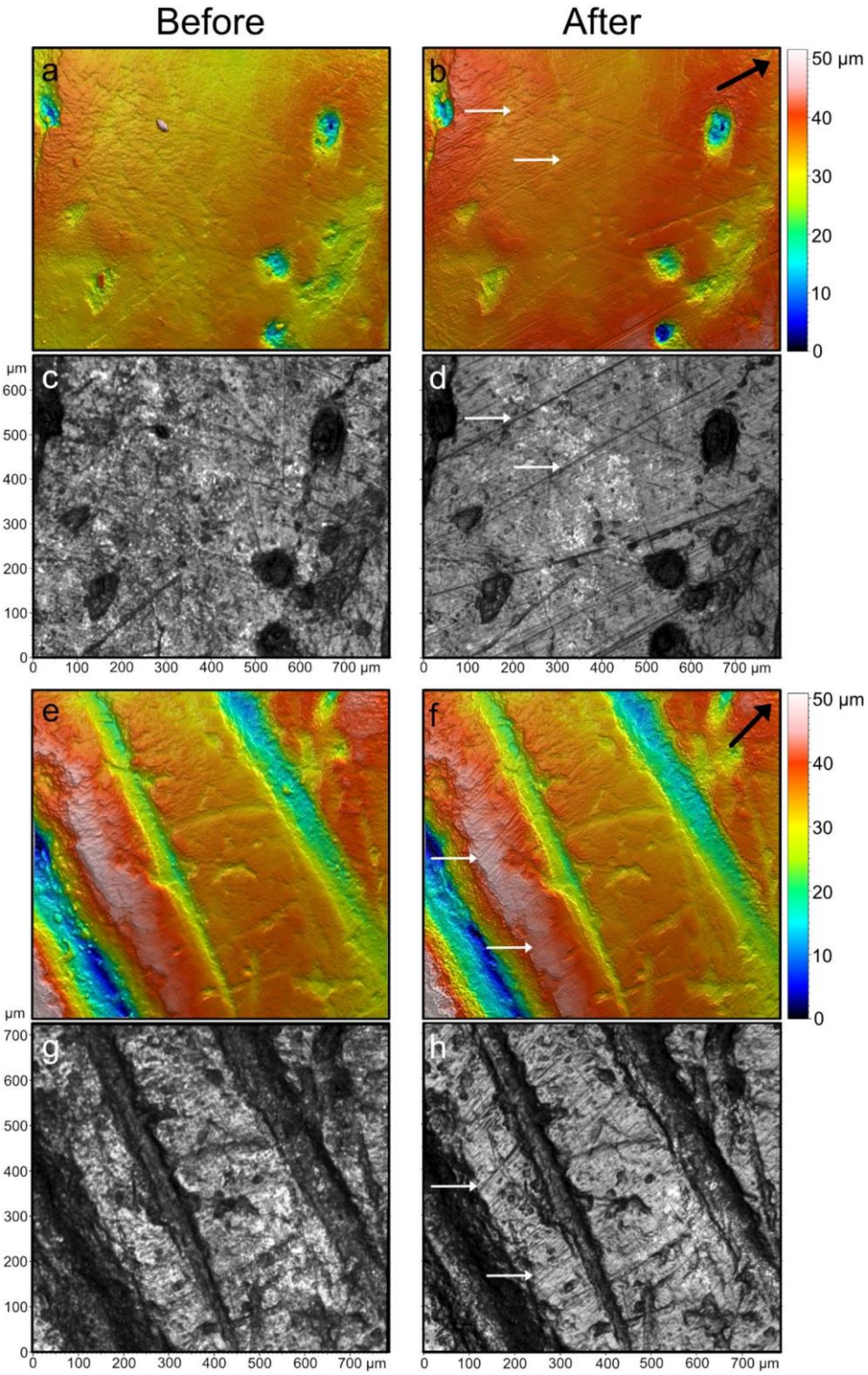


**Figure 5.** Bone surface microtopography of the specimen CC7-379, before (left) and after (right) EEM, using confocal disc-scanning microscopy. 3D surface models (**a**,**b**,**e**,**f**) and 2D intensity micrographs (**c**,**d**,**g**,**h**) of control (**a**–**d**) and cut areas (**e**–**h**). Orientation of eraser movements are indicated by the black arrows. Depth of the bone microtopography is color-coded with blue indicating the lowest valleys and white the highest peaks. We note the generation of microstriations after the use of EEM with some examples indicated by white arrows. We note the presence of a residue in the middle of the bone surface (**a**) which has been removed with the use of EEM (**b**) and is potentially related to the formation of the deeper traces located near its initial position.

## Conclusion

The taxonomic assessment of fragmented or heavily modified bone artefact specimens represents an ongoing problem, especially in Palaeolithic archaeology. The recent development of non-destructive extraction techniques has opened up the possibility to contribute to the understanding of hominin behaviour related to the manufacture and use of such objects. To understand the impact of such biomolecular sampling methods on Palaeolithic bones, we performed a controlled sampling experiment measuring applied force in addition to qualitative and 3D quantitative microscopy prior to and after the use of the EEM. Overall, while the EEM can be used on Palaeolithic bone objects, it provides low-quality MALDI-TOF MS spectra and modifies bone surfaces. These modifications include aspects mimicking use-wear traces, and involve striations on all bone surfaces. Although the gross features of the bone microtopography remain, the quantitative differences shown by one of the four tested ISO 25178 surface texture parameters (*Smrk1*) indicates a general flattening of the bone surface. Based on our results, we conclude that the EEM should not be considered as a non-destructive sampling method when applied on Palaeolithic bone surfaces. Further work is therefore required to overcome sampling limitations for the analysis of worked bones. We recommend that such development should be done in a manner that takes into account the analysis of bone surfaces via other methods, including functional analyses aimed at interpreting use-wear traces and residues.

## Data availability

All original, unfiltered surface texture scans, surface texture templates (MountainsMap) and raw data for bayesian modeling (R and stan) used in this study are stored at the Edmond database of the Max Planck Society (MPS, Munich, Germany); and can be accessed via Edmond: https:// doi. org/ 10. 17617/3. 6z.

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## Author contributions

V.S.-M., N.L.M., E.S.-K., and G.M.S. designed the research. A.V.C. designed and performed force transducer measurements. V.S.M. and F.W. performed the proteomic analysis. V.S.M., N.L.M., and E.S.-K. performed photography, microscopy, and microtopography analyses. T.T., N.S., R.S. and J.J.-H. provided samples and archaeological context. V.S.-M., N.L.M., F.W. and G.M.S. wrote the manuscript with contributions of all authors.

## Additional information

**Supplementary Information** The online version contains supplementary material available at https:// doi. org/ 10. 1038/ s41598- 021- 02823-w.

# Supplementary Information to:

## The effect of eraser sampling for proteomic analysis on Palaeolithic bone surface morphology

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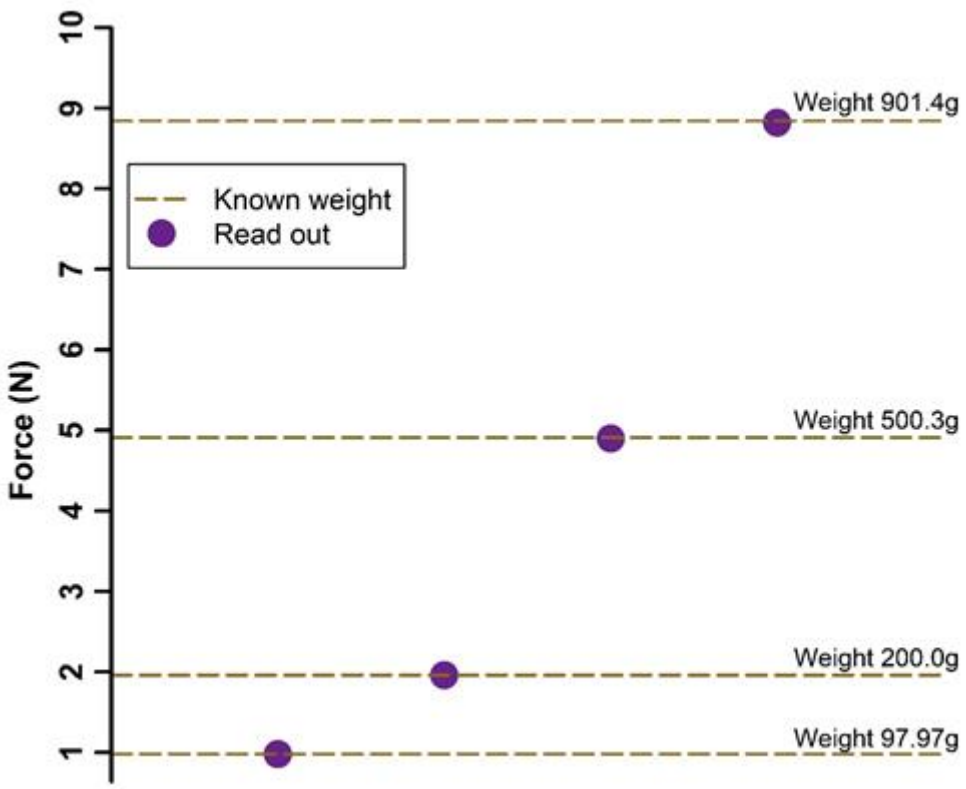
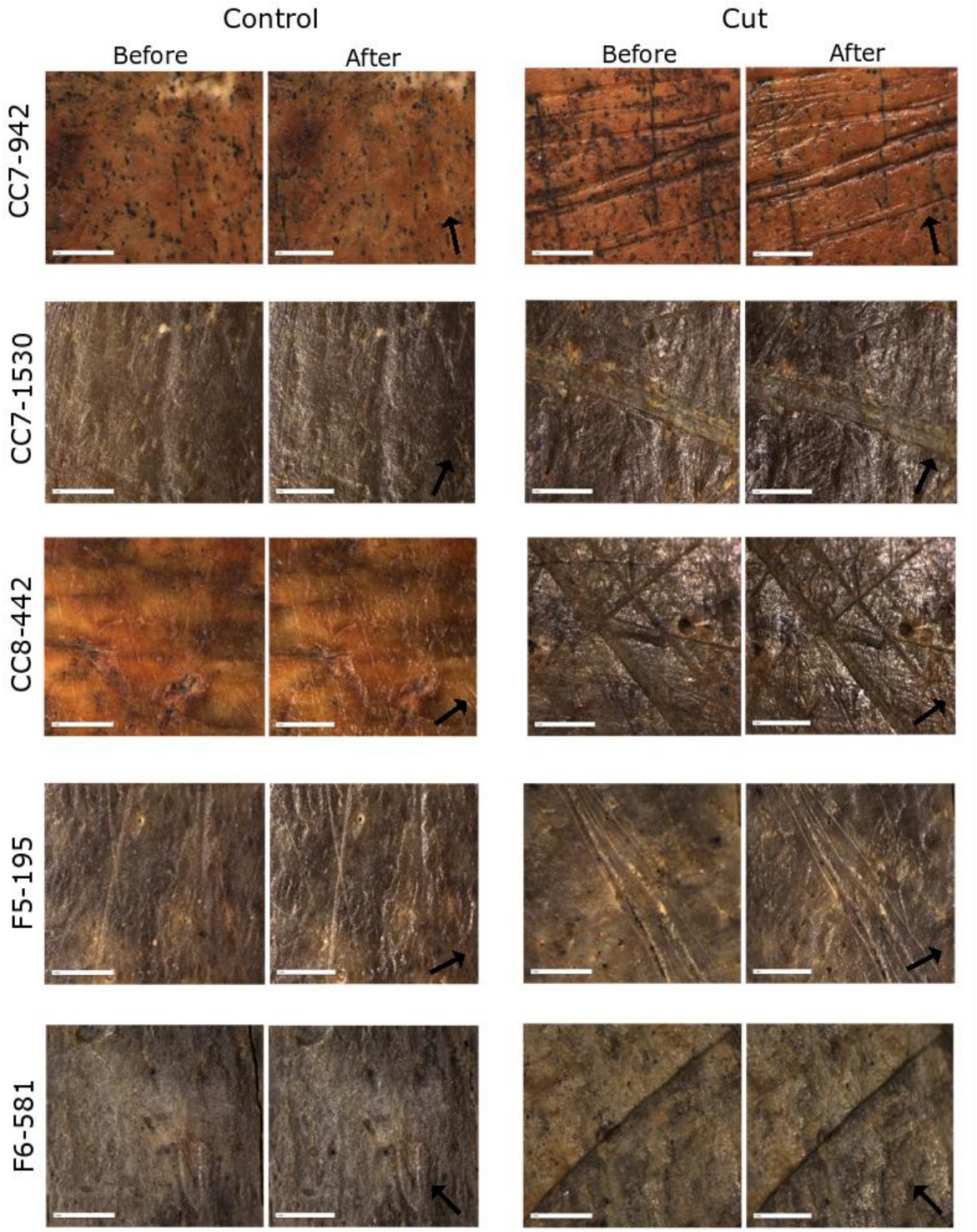
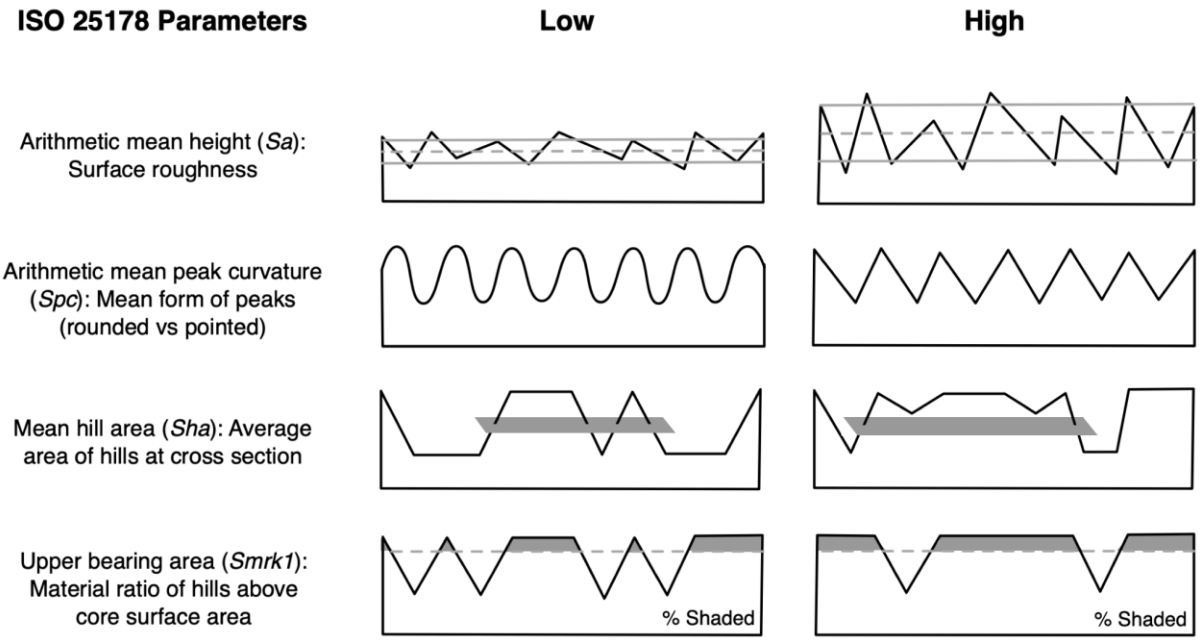


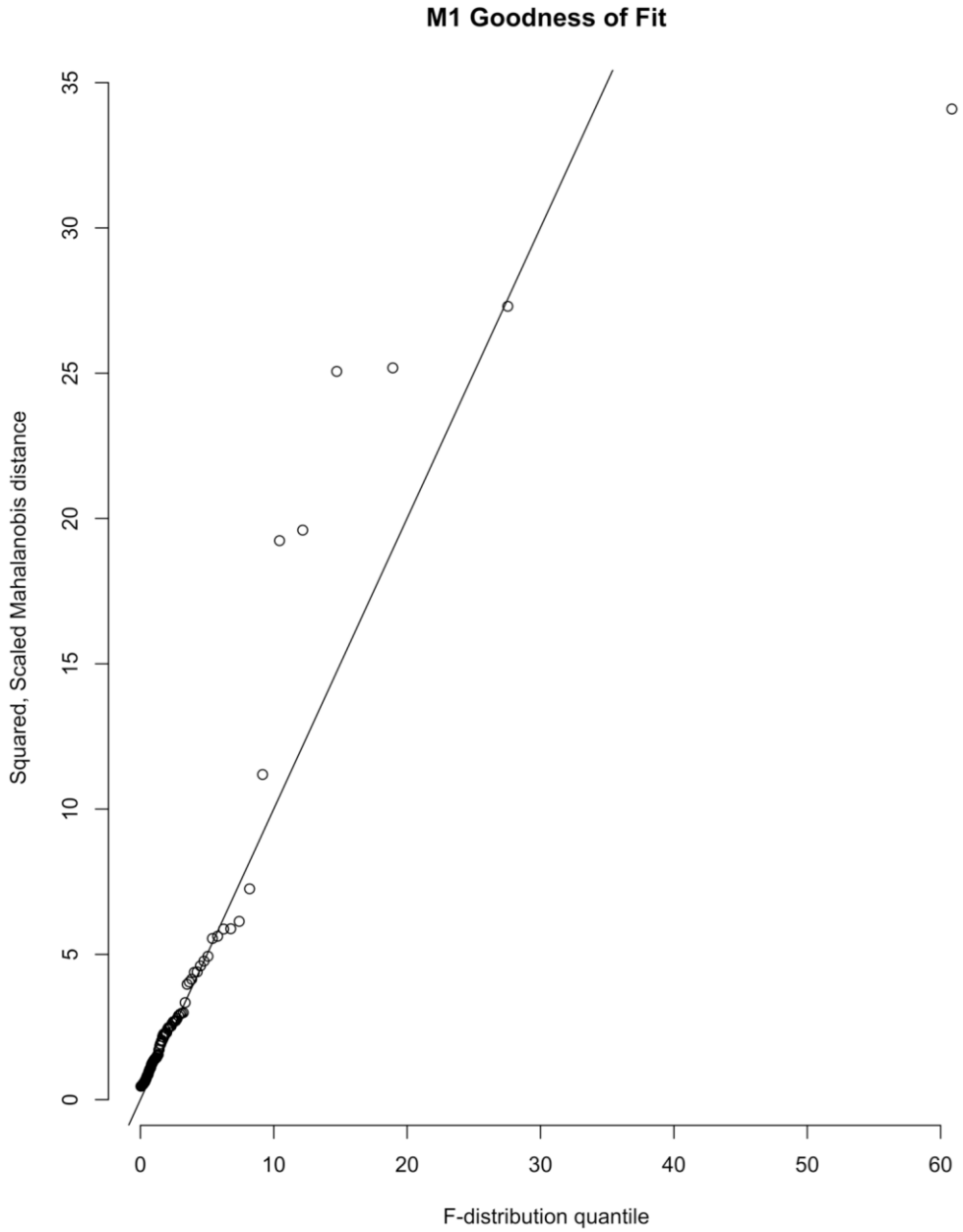

Supplementary Figure S1: The load cell was calibrated using a series of known weights. A weight was placed on the instrumented stage and the readout recorded for 15 seconds. If the readout matched (within the 0.05% precision range) what was expected for the known weight in Newtons, then the load cell was considered calibrated correctly. If they differed, then recalibration was required, and the calibration factor adjusted until the readout aligned with what was expected for the known weight.



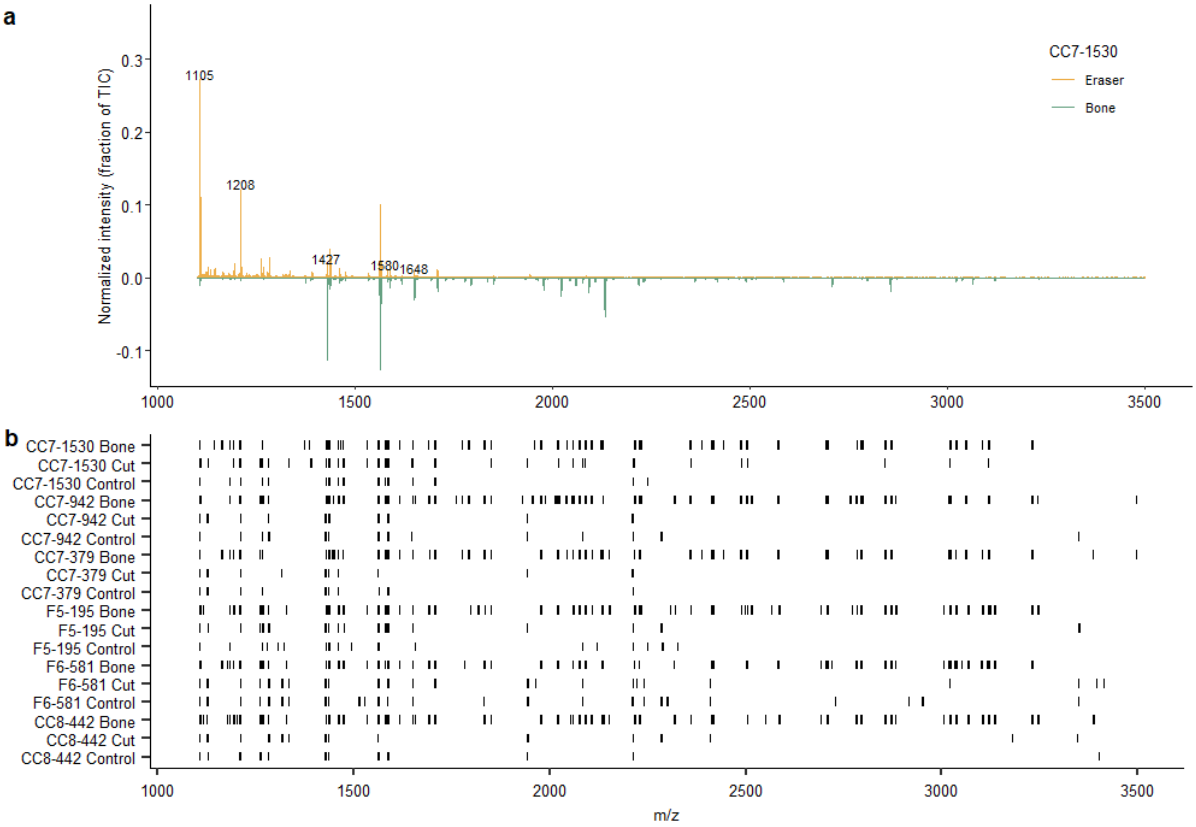

Supplementary Figure S2: Micrographs of the control and cut areas of all studied specimens using automated digital microscopy (ZEISS, Smartzoom 5, magnification x106), before (left) and after (right) EEM. The scale represents 1cm on each image. Black arrows indicate the orientation of the eraser movements during the experiment. We note an increase of the bone surface shininess in the case of 9 ROIs out of 12, generally located on the highest areas or hills of the surface topography after the use of EEM, resulting from the repetitive movement of the soft eraser rubbing the bone surface. This reflectivity mimics the visual pattern of polished surfaces which one would expect to observe on a bone that has been used or handled repetitively.



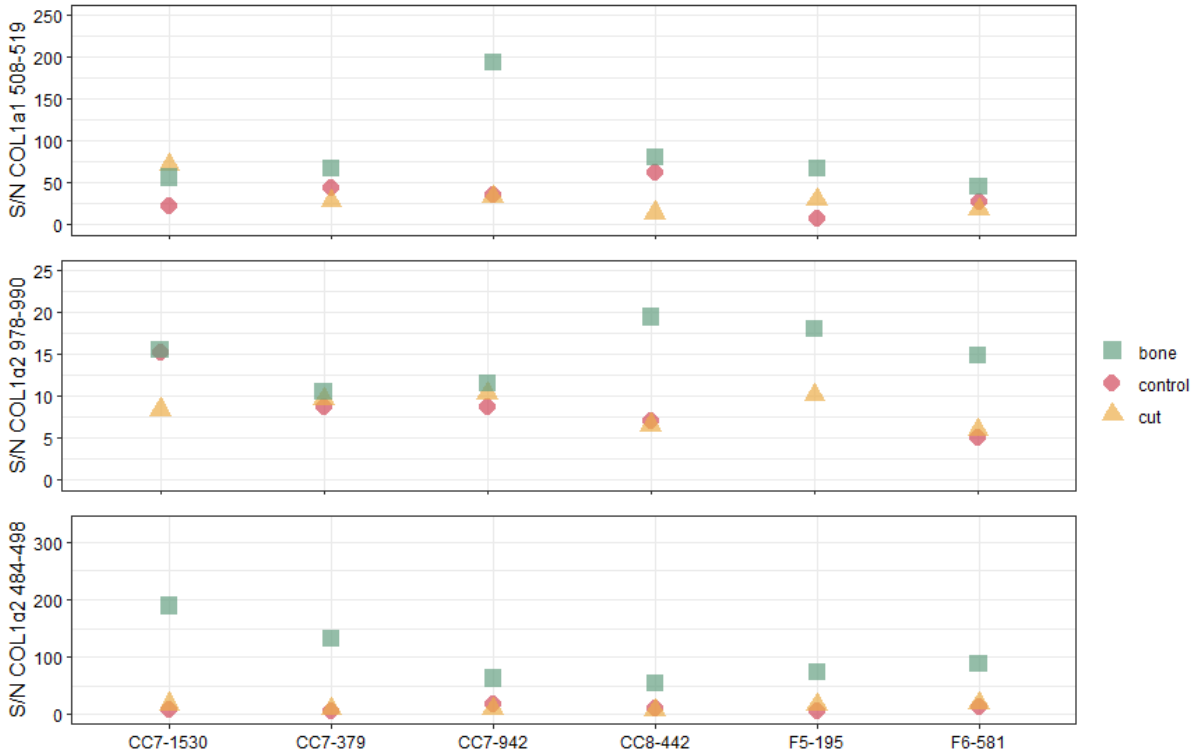

Supplementary Figure S3: 2D depictions of surface texture parameters (ISO 25178) (*Sa, Spc, Sha and Smrk1*) indicating low and high values (adapted/modified from [1–3]).



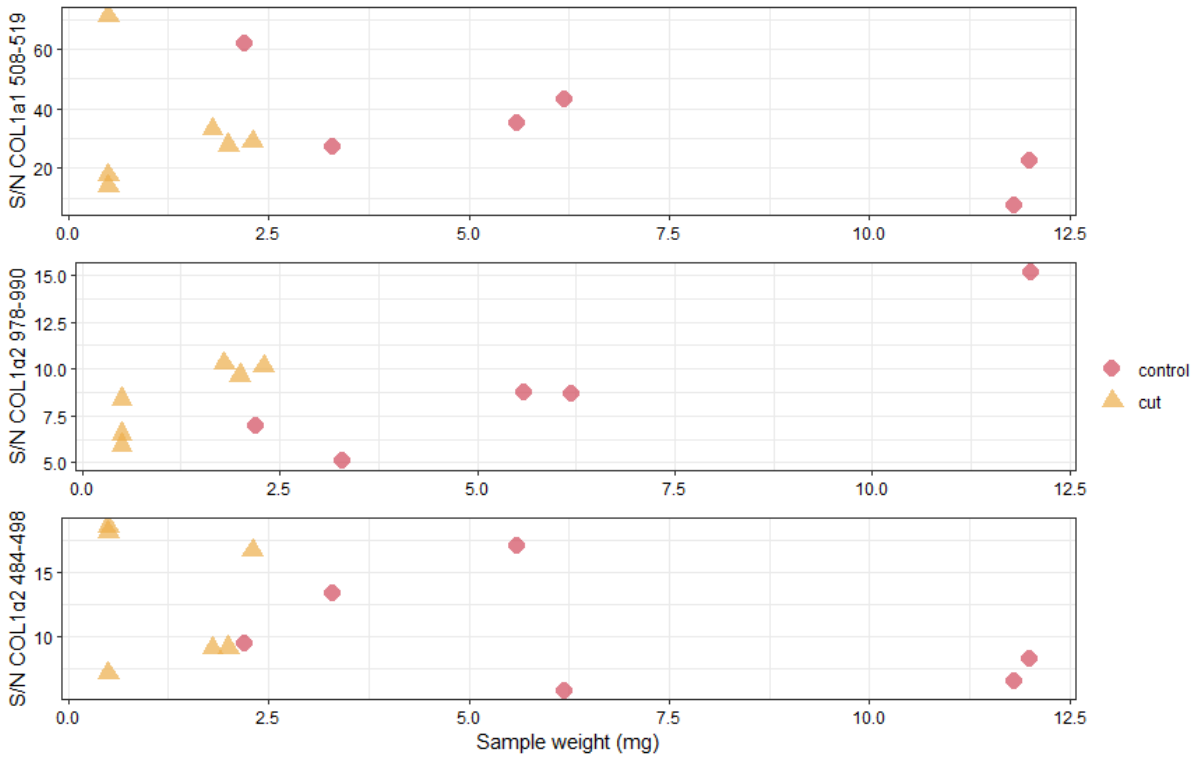

Supplementary Figure S4: Quantile-quantile plot of scaled and squared Mahalanobis distances (between observations and their predicted values) versus theoretical quantiles of the F- distribution [4]. Most observations follow the theoretical quantiles well.



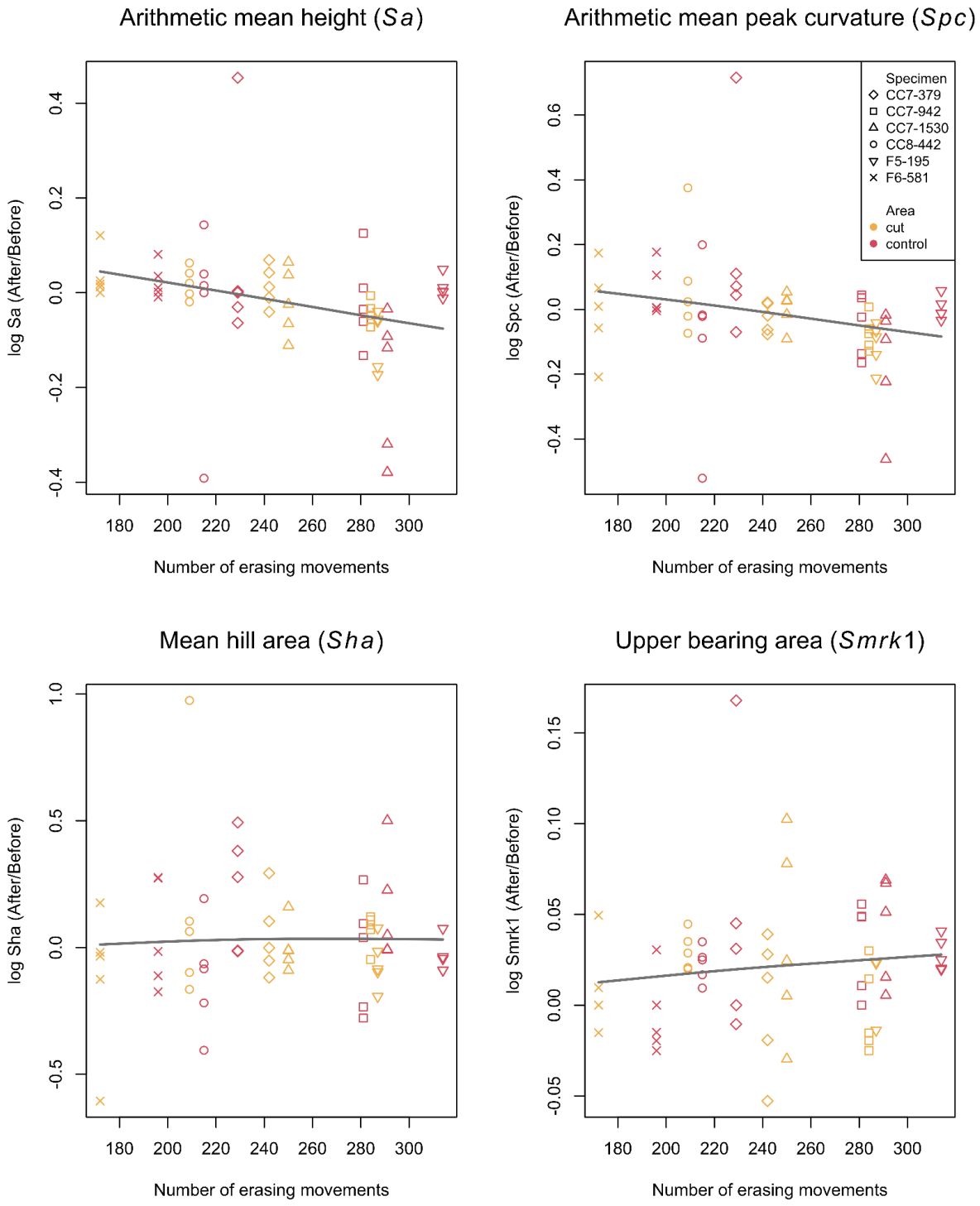

Supplementary Figure S5: a) Example of MALDI-TOF mass spectra obtained from the eraser extraction of a bone surface (in orange) and from the buffer extraction of a bone sample (in green) of CC7-1530. The intensity is normalised as a fraction of the TIC for better comparison. b) Gel view representation of the spectra obtained for each specimen from the buffer extraction of a bone sample (Bone) and the eraser extraction of the Control and Cut area. Each bar represents a peak with a S/N ratio of 5 or higher. This representation highlights the systematic absence of high-molecular weight peptide markers within the spectra obtained from the eraser extraction of bone surfaces.



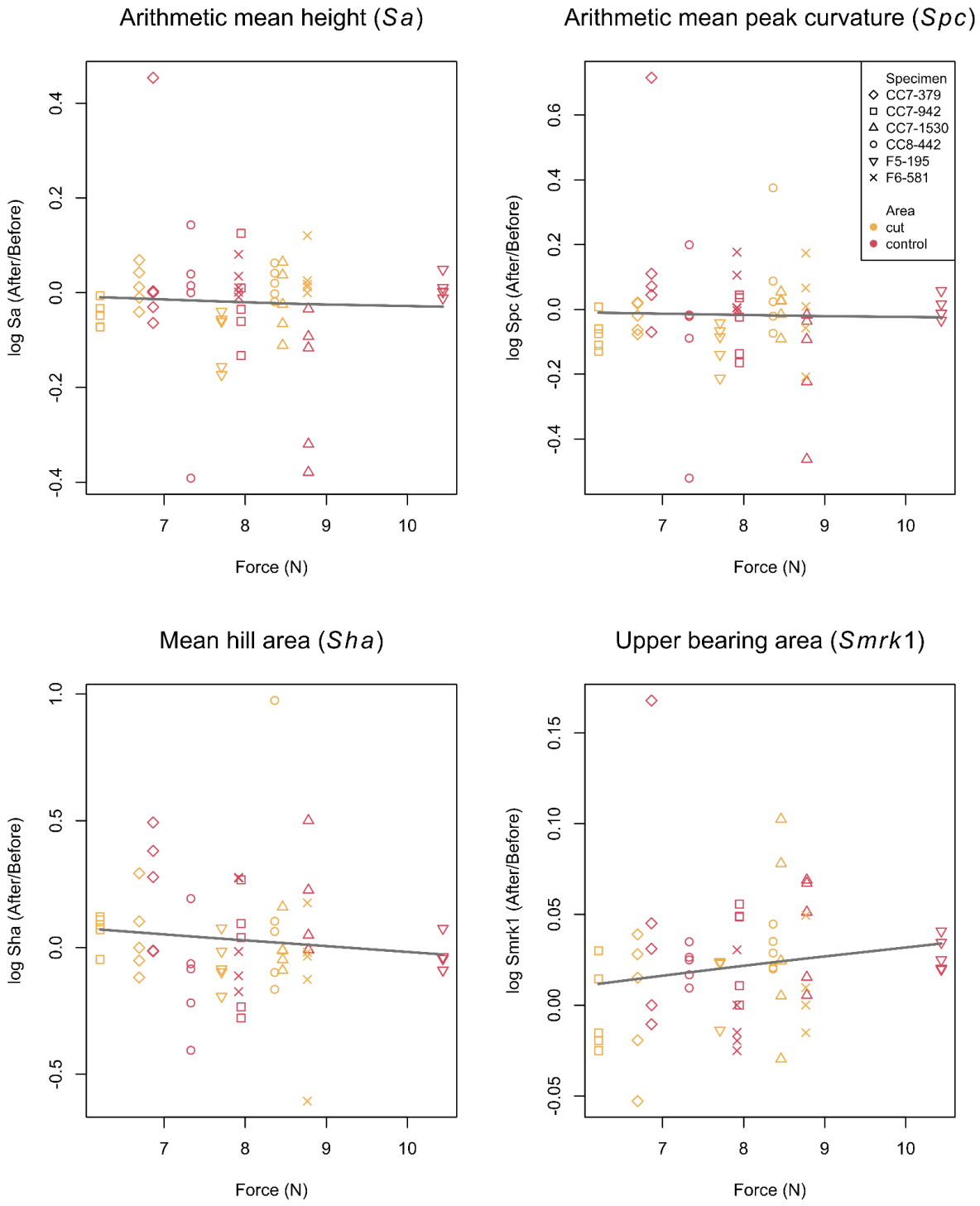

Supplementary Figure S6: signal-to-noise (S/N) ratio for peptide markers COL1α1 508-519, COL1α2 978-990, and COL1α2 484-498 for each specimen. In green are the values obtained for each bone sample, in yellow are the values for each cut area sampled with an eraser and in red are the values measured for each control area sampled with an eraser.



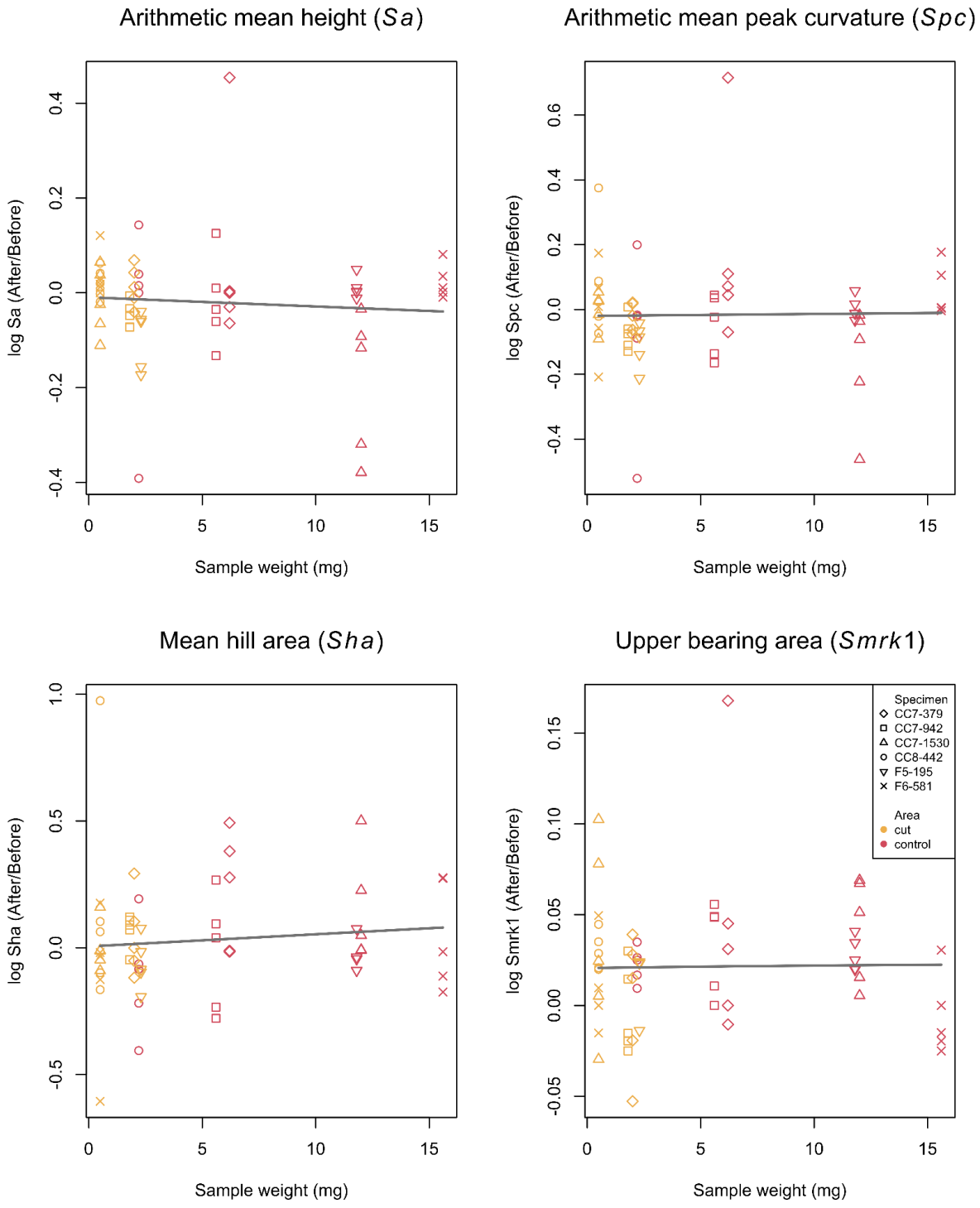

Supplementary Figure S7: Signal-to-noise (S/N) ratio for peptide markers COL1α1 508-519, COL1α2 978-990, and COL1α2 484-498 across sample weight generated during each EEM event. In yellow are the values obtained for each cut area sampled with an eraser and in red are the values measured for each control area sampled with an eraser.



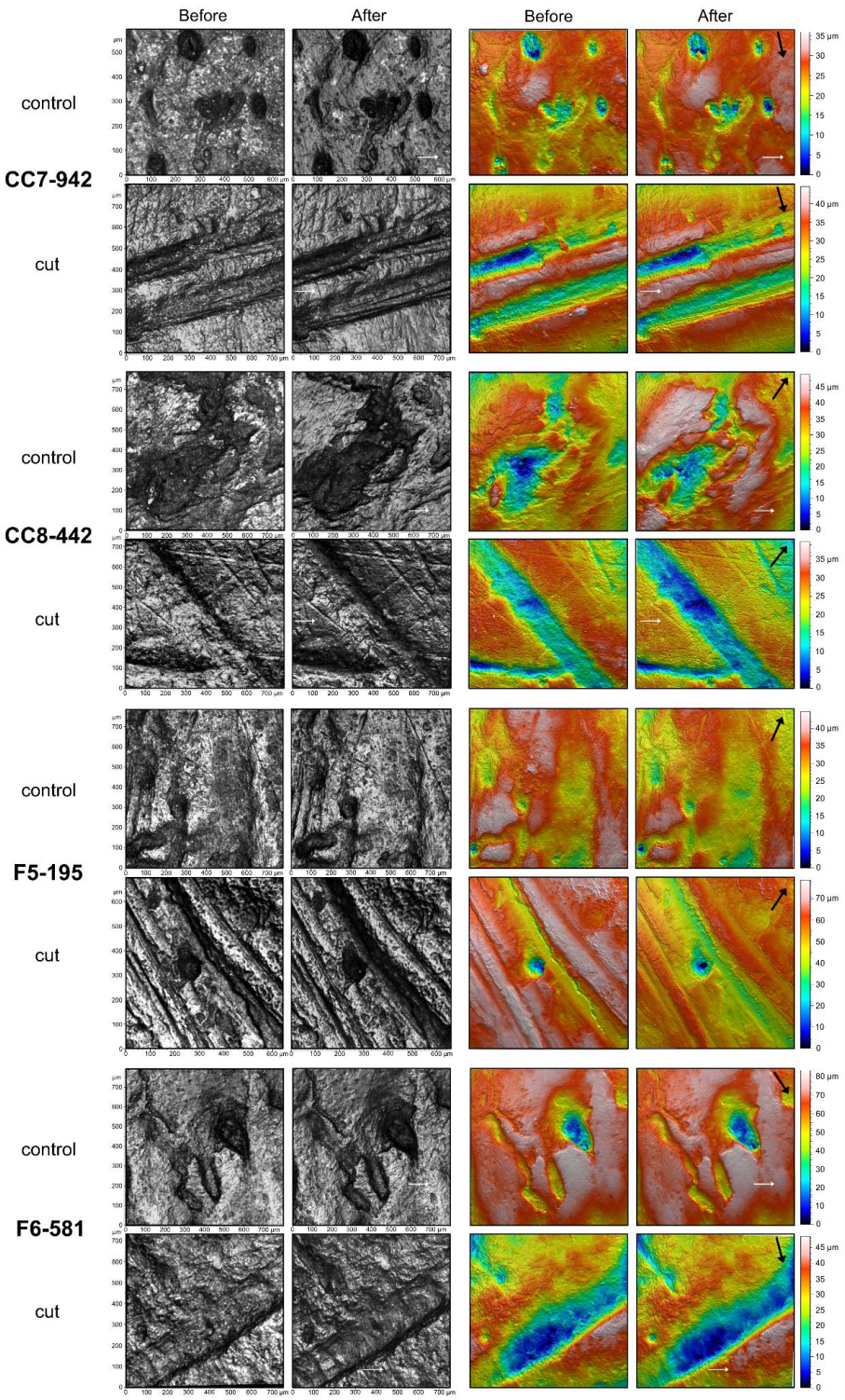

Supplementary Figure S8: After to before differences of the four ISO 25178 parameters (*Sa, Spc, Sha* and *Smrk1*) by the number of eraser movements. Each specimen is represented by different symbols, cut areas are in yellow and control areas are in red.




Supplementary Figure S9: After to before differences of the four ISO 25178 parameters (*Sa, Spc, Sha* and *Smrk1*) by force (in Newton). Each specimen is represented by different symbols, cut areas are in yellow and control areas are in red.




Supplementary Figure S10: After to before differences of the four ISO 25178 parameters (Sa, Spc, Sha and Smrk1) by sample weight (in mg). Each specimen is represented by different symbols, cut areas are in yellow and control areas are in red.




Supplementary Figure S11 : 2D intensity images (left) and 3D models (right) of the bone surface microtopography of four specimens (CC7-942, CC8-442, F5-195, F6-581), before (left) and after (right) EEM in both control (upper) and cut (lower) areas. Orientation of the eraser movements are indicated by the black arrows. Depth of the bone microtopography is color-coded with blue indicating the lowest valleys and white the highest peaks. We note the generation of microstriations after the use of EEM with some examples indicated by the white arrows.

Supplementary Table S1: Raw ISO 25178 data before and after EEM for each surface texture parameter on each ROIs of all specimens. Sa is expressed in µm, Spc in 1/µm, Sha in µm2 and Smrk1 in %

| specimen | area | erasing | n_measured | Sa | Spc | Sha | Smrk1 | matching |
|---|---|---|---|---|---|---|---|---|
| **CC7-942** | control | before | 1 | 0.408 | 2.16 | 125 | 20.1 | good |
| **CC7-942** | control | after | 1 | 0.412 | 2.11 | 130 | 21.1 | good |
| **CC7-942** | control | before | 2 | 0.177 | 0.791 | 121 | 18.6 | good |
| **CC7-942** | control | after | 2 | 0.155 | 0.69 | 133 | 18.8 | good |
| **CC7-942** | control | before | 3 | 0.285 | 1.97 | 333 | 20 | good |
| **CC7-942** | control | after | 3 | 0.323 | 2.06 | 252 | 20 | good |
| **CC7-942** | control | before | 4 | 0.376 | 1.66 | 121 | 19.9 | good |
| **CC7-942** | control | after | 4 | 0.363 | 1.72 | 158 | 20.9 | good |
| **CC7-942** | control | before | 5 | 0.306 | 1.78 | 177 | 19.2 | good |
| **CC7-942** | control | after | 5 | 0.288 | 1.51 | 140 | 20.3 | good |
| **CC7-942** | cut | before | 1 | 0.919 | 3.42 | 82.4 | 20.5 | good |
| **CC7-942** | cut | after | 1 | 0.876 | 3.22 | 90.1 | 20.8 | good |
| **CC7-942** | cut | before | 2 | 0.662 | 2.73 | 99.7 | 19.7 | good |
| **CC7-942** | cut | after | 2 | 0.658 | 2.75 | 107 | 20.3 | good |
| **CC7-942** | cut | before | 3 | 0.919 | 4.04 | 75.7 | 19.8 | good |
| **CC7-942** | cut | after | 3 | 0.855 | 3.55 | 84.4 | 19.5 | good |
| **CC7-942** | cut | before | 4 | 0.676 | 2.77 | 98.7 | 20.2 | good |
| **CC7-942** | cut | after | 4 | 0.654 | 2.48 | 94.1 | 19.7 | good |
| **CC7-942** | cut | before | 5 | 0.613 | 2.48 | 86.9 | 20.6 | good |
| **CC7-942** | cut | after | 5 | 0.57 | 2.3 | 98.1 | 20.2 | good |
| **CC8-442** | control | before | 1 | 0.198 | 1.18 | 241 | 18.7 | good |
| **CC8-442** | control | after | 1 | 0.201 | 1.16 | 226 | 19.2 | good |
| **CC8-442** | control | before | 2 | 0.271 | 1.4 | 148 | 19.7 | good |
| **CC8-442** | control | after | 2 | 0.271 | 1.37 | 136 | 20.4 | good |
| **CC8-442** | control | before | 3 | 0.728 | 3.08 | 78 | 21.1 | good |
| **CC8-442** | control | after | 3 | 0.84 | 3.76 | 94.6 | 21.3 | good |
| **CC8-442** | control | before | 4 | 0.274 | 1.41 | 134 | 19.8 | good |
| **CC8-442** | control | after | 4 | 0.285 | 1.29 | 89.3 | 20.3 | good |
| **CC8-442** | control | before | 5 | 0.176 | 0.874 | 173 | 17.8 | good |
| **CC8-442** | control | after | 5 | 0.119 | 0.519 | 139 | 18.1 | good |
| **CC8-442** | cut | before | 1 | 0.473 | 1.97 | 89.9 | 19.9 | good |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **CC8-442** | cut | after | 1 | 0.464 | 1.83 | 76.2 | 20.3 | good |
| **CC8-442** | cut | before | 2 | 0.475 | 1.93 | 117 | 19.1 | good |
| **CC8-442** | cut | after | 2 | 0.474 | 1.89 | 106 | 19.5 | good |
| **CC8-442** | cut | before | 3 | 0.618 | 2.52 | 73.4 | 19.6 | good |
| **CC8-442** | cut | after | 3 | 0.644 | 2.75 | 81.4 | 20.3 | good |
| **CC8-442** | cut | before | 4 | 0.642 | 2.5 | 81.4 | 19.7 | good |
| **CC8-442** | cut | after | 4 | 0.655 | 2.56 | 86.7 | 20.6 | good |
| **CC8-442** | cut | before | 5 | 0.819 | 3.58 | 123 | 20.6 | good |
| **CC8-442** | cut | after | 5 | 0.872 | 5.21 | 326 | 21.2 | good |
| **CC7-1530** | control | before | 1 | 0.238 | 1.12 | 122 | 19.3 | good |
| **CC7-1530** | control | after | 1 | 0.217 | 1.08 | 153 | 19.6 | good |
| **CC7-1530** | control | before | 2 | 0.208 | 0.95 | 119 | 18.7 | good |
| **CC7-1530** | control | after | 2 | 0.201 | 0.934 | 118 | 20 | good |
| **CC7-1530** | control | before | 3 | 0.264 | 1.47 | 160 | 19 | good |
| **CC7-1530** | control | after | 3 | 0.235 | 1.34 | 168 | 20 | good |
| **CC7-1530** | control | before | 4 | 0.371 | 1.45 | 88.5 | 18.4 | good |
| **CC7-1530** | control | after | 4 | 0.254 | 1.16 | 146 | 18.5 | good |
| **CC7-1530** | control | before | 5 | 0.245 | 1.12 | 103 | 18.2 | good |
| **CC7-1530** | control | after | 5 | 0.178 | 0.705 | 102 | 19.5 | good |
| **CC7-1530** | cut | before | 1 | 1.43 | 5.61 | 96.1 | 17.6 | good |
| **CC7-1530** | cut | after | 1 | 1.28 | 5.77 | 87.8 | 19.5 | good |
| **CC7-1530** | cut | before | 2 | 0.785 | 3.79 | 91.2 | 20.2 | good |
| **CC7-1530** | cut | after | 2 | 0.766 | 4 | 107 | 20.7 | good |
| **CC7-1530** | cut | before | 3 | 1.2 | 5.88 | 67.7 | 19.7 | good |
| **CC7-1530** | cut | after | 3 | 1.28 | 6.03 | 67 | 21.3 | good |
| **CC7-1530** | cut | before | 4 | 0.736 | 3.13 | 84.2 | 19.7 | good |
| **CC7-1530** | cut | after | 4 | 0.764 | 3.08 | 83.2 | 19.8 | good |
| **CC7-1530** | cut | before | 5 | 0.726 | 3.21 | 75.1 | 20.5 | good |
| **CC7-1530** | cut | after | 5 | 0.68 | 2.93 | 71.6 | 19.9 | good |
| **F5-195** | control | before | 1 | 0.464 | 2.32 | 154 | 20.1 | good |
| **F5-195** | control | after | 1 | 0.469 | 2.36 | 166 | 20.5 | good |
| **F5-195** | control | before | 2 | 0.349 | 1.65 | 99.8 | 19.2 | good |
| **F5-195** | control | after | 2 | 0.345 | 1.63 | 96.2 | 20 | good |
| **F5-195** | control | before | 3 | 0.388 | 1.82 | 116 | 19.8 | good |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **F5-195** | control | after | 3 | 0.389 | 1.76 | 106 | 20.3 | good |
| **F5-195** | control | before | 4 | 0.526 | 2.44 | 129 | 19.4 | good |
| **F5-195** | control | after | 4 | 0.527 | 2.41 | 118 | 19.8 | good |
| **F5-195** | control | before | 5 | 0.437 | 2.03 | 115 | 19.9 | good |
| **F5-195** | control | after | 5 | 0.459 | 2.15 | 110 | 20.6 | good |
| **F5-195** | cut | before | 1 | 0.971 | 4.47 | 113 | 21.9 | good |
| **F5-195** | cut | after | 1 | 0.914 | 3.89 | 122 | 21.6 | good |
| **F5-195** | cut | before | 2 | 0.754 | 3.32 | 96.2 | 21.2 | good |
| **F5-195** | cut | after | 2 | 0.645 | 3.05 | 94.7 | 21.7 | good |
| **F5-195** | cut | before | 3 | 0.824 | 2.97 | 82.5 | 20.6 | good |
| **F5-195** | cut | after | 3 | 0.779 | 2.85 | 74.9 | 21.1 | good |
| **F5-195** | cut | before | 4 | 0.811 | 3.65 | 110 | 21.4 | good |
| **F5-195** | cut | after | 4 | 0.682 | 2.95 | 101 | 21.9 | good |
| **F5-195** | cut | before | 5 | 0.489 | 2.33 | 120 | 20.6 | good |
| **F5-195** | cut | after | 5 | 0.47 | 2.18 | 98.9 | 21.1 | good |
| **CC7-379** | control | before | 1 | 0.322 | 1.92 | 168 | 19 | good |
| **CC7-379** | control | after | 1 | 0.302 | 1.79 | 166 | 19.6 | good |
| **CC7-379** | control | before | 2 | 0.148 | 0.616 | 113 | 17.5 | none |
| **CC7-379** | control | after | 2 | 0.233 | 1.26 | 185 | 20.7 | none |
| **CC7-379** | control | before | 3 | 0.199 | 1.48 | 390 | 19.2 | good |
| **CC7-379** | control | after | 3 | 0.199 | 1.59 | 384 | 19 | good |
| **CC7-379** | control | before | 4 | 0.291 | 1.29 | 94.7 | 19.5 | good |
| **CC7-379** | control | after | 4 | 0.292 | 1.44 | 125 | 19.5 | good |
| **CC7-379** | control | before | 5 | 0.27 | 1.76 | 276 | 19.5 | good |
| **CC7-379** | control | after | 5 | 0.262 | 1.84 | 404 | 20.4 | good |
| **CC7-379** | cut | before | 1 | 0.577 | 2.7 | 119 | 21.1 | good |
| **CC7-379** | cut | after | 1 | 0.602 | 2.75 | 113 | 21.7 | good |
| **CC7-379** | cut | before | 2 | 0.764 | 3.15 | 81.6 | 20.1 | good |
| **CC7-379** | cut | after | 2 | 0.756 | 3.09 | 81.5 | 20.9 | good |
| **CC7-379** | cut | before | 3 | 0.555 | 2.57 | 102 | 19.7 | good |
| **CC7-379** | cut | after | 3 | 0.533 | 2.38 | 90.6 | 20 | good |
| **CC7-379** | cut | before | 4 | 1.65 | 6.48 | 99.2 | 23.3 | good |
| **CC7-379** | cut | after | 4 | 1.67 | 6.62 | 110 | 22.1 | good |
| **CC7-379** | cut | before | 5 | 0.785 | 3.44 | 82.8 | 20.9 | good |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **CC7-379** | cut | after | 5 | 0.841 | 3.23 | 111 | 20.5 | good |
| **F6-581** | control | before | 1 | 1.1 | 5.24 | 128 | 20.2 | good |
| **F6-581** | control | after | 1 | 1.09 | 5.27 | 126 | 19.9 | good |
| **F6-581** | control | before | 2 | 0.451 | 2.59 | 187 | 19.3 | good |
| **F6-581** | control | after | 2 | 0.467 | 2.6 | 157 | 19.3 | good |
| **F6-581** | control | before | 3 | 0.78 | 3.94 | 105 | 20.2 | good |
| **F6-581** | control | after | 3 | 0.781 | 4.38 | 138 | 19.7 | good |
| **F6-581** | control | before | 4 | 0.552 | 2.46 | 123 | 20.6 | good |
| **F6-581** | control | after | 4 | 0.558 | 2.45 | 110 | 20.2 | good |
| **F6-581** | control | before | 5 | 0.474 | 2.38 | 132 | 19.4 | good |
| **F6-581** | control | after | 5 | 0.514 | 2.84 | 174 | 20 | good |
| **F6-581** | cut | before | 1 | 0.722 | 3.88 | 143 | 19.8 | good |
| **F6-581** | cut | after | 1 | 0.731 | 3.15 | 78 | 19.8 | good |
| **F6-581** | cut | before | 2 | 0.896 | 3.52 | 84.7 | 19.9 | good |
| **F6-581** | cut | after | 2 | 0.919 | 3.76 | 101 | 19.6 | good |
| **F6-581** | cut | before | 3 | 1.08 | 5.37 | 91.3 | 20.4 | good |
| **F6-581** | cut | after | 3 | 1.08 | 5.07 | 89.6 | 20.4 | good |
| **F6-581** | cut | before | 4 | 0.767 | 3.25 | 76.5 | 20.6 | good |
| **F6-581** | cut | after | 4 | 0.781 | 3.28 | 73.9 | 20.8 | good |
| **F6-581** | cut | before | 5 | 0.857 | 3.58 | 81.2 | 19.7 | good |
| **F6-581** | cut | after | 5 | 0.967 | 4.26 | 71.6 | 20.7 | good |

Supplementary Table S2: Model effects including their representation in each model's design space and LOO expected log predictive density (elpd) and standard error (se) differences relative to M1.

| Model | Effects | Design matrix | Δ elpd | Δ se |
|---|---|---|---|---|
| M1 | 2 fixed + random | Area + erasing + specimen + measurement location + error | 0 | 0 |
| M0 | 1 fixed + random | Area + specimen + measurement location + error | -15.5 | 7.2 |

Supplementary Table S3: Sample information for all bone specimens included in this study and analysed through destructive sampling and EEM (control and cut area), including sample weights, average peak forces, number of erasing movements, taxonomic identifications obtained through ZooMS, peptide marker masses (rounded to whole m/z values), signal to noise ratio (S/N) for the three dominant peptide markers and qualitative observations through digital microscopy.

| Sample | ZooMS ID | Method | Sample Weight | Average Force | Erasing Count | BarcodeID | α1 508 | α2 978 | α2 484 | α2 502 | α2 292 | α2 793 | α2 454 | α1 586 | α2 757 | S/N α1 508 | S/N α2 978 | S/N α2 484 | Residues removed | Presence of microstriations | Increased shininess |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CC8-442-control | BK-1243.1a | EEM | 2.2 | 7.329 | 215 | Bos/Bison/Ovibos | 1105 | 1208 | 1427 | x | x | x | x | x | x | 62.07 | 6.99 | 9.50 | Yes | No | No |
| CC8-442-cut | BK-1243.2a | EEM | 0.5 | 8.363 | 209 | Bos/Bison/Ovibos | 1105 | 1208 | x | x | x | x | x | x | x | 13.78 | 6.54 | 7.17 | Yes | No | Yes |
| CC8-442 | BK-1243 | Destructive | | | | Bos/Bison | 1105 | 1192+1208 | 1427 | 1580 | 1648 | 2131 | 2792 | 2853+2869 | 3017+3033 | 80.40 | 19.44 | 53.79 | - | - | - |
| F6-581-control | BK-1284.1a | EEM | 15.6 | 9.843 | 196 | Bos/Bison/Ovibos | 1105 | 1208 | 1427 | 1580 | 1648 | 2131 | x | x | x | 27.03 | 5.10 | 13.39 | Yes | No | Yes |
| F6-581-cut | BK-1284.2a | EEM | 0.5 | 8.766 | 172 | Bos/Bison/Ovibos | 1105 | 1208 | 1427 | 1580 | 1648 | x | x | x | x | 17.69 | 5.93 | 18.64 | Yes | No | Yes |
| F6-581 | BK-1284 | Destructive | | | | Bos/Bison | 1105 | 1208 | 1427 | 1580 | 1648 | 2131 | 2792 | 2853+2869 | 3017+3033 | 45.31 | 14.82 | 87.53 | - | - | - |
| F5-195-control | BK-1297.1a | EEM | 11.8 | 10.437 | 314 | Bos/Bison/Ovibos | 1105 | 1192+1208 | 1427 | x | x | x | x | x | x | 7.46 | - | 6.54 | No | No | Yes |
| F5-195-cut | BK-1297.2a | EEM | 2.3 | 7.708 | 287 | Bos/Bison/Ovibos | 1105 | 1192+1208 | 1427 | 1580 | 1648 | x | x | x | x | 29.02 | 10.13 | 16.75 | Yes | No | No |
| F5-195 | BK-1297 | Destructive | | | | Bos/Bison | 1105 | 1192+1208 | 1427 | 1580 | 1648 | 2131 | 2792 | 2853+2869 | 3017+3033 | 66.41 | 18.06 | 73.28 | - | - | - |
| CC7-379-control | BK-75.1a | EEM | 6.2 | 6.864 | 229 | Bos/Bison/Ovibos | 1105 | 1208 | 1427 | x | x | x | x | x | x | 43.27 | 8.71 | 5.75 | Yes | Yes | Yes |
| CC7-379-cut | BK-75.2a | EEM | 2.0 | 6.693 | 242 | Bos/Bison/Ovibos | 1105 | 1208 | 1427 | x | x | x | x | x | x | 27.80 | 9.64 | 9.17 | Yes | No | Yes |
| CC7-379 | BK-75 | Destructive | | | | Bos/Bison | 1105 | 1208 | 1427 | 1580 | 1648 | 2131 | 2792 | 2853 | 3033 | 66.63 | 10.54 | 131.38 | - | - | - |
| CC7-942-control | BK-85.1a | EEM | 5.6 | 7.949 | 281 | Bos/Bison/Ovibos | 1105 | 1208 | 1427 | 1580 | 1648 | x | x | x | x | 35.31 | 8.72 | 17.13 | Yes | No | No |
| CC7-942-cut | BK-85.2a | EEM | 1.8 | 6.211 | 284 | Bos/Bison/Ovibos | 1105 | 1192+1208 | 1427 | x | x | x | x | x | x | 33.03 | 10.26 | 9.08 | Yes | No | Yes |
| CC7-942 | BK-85 | Destructive | | | | Bos/Bison | 1105 | 1208 | 1427 | 1580 | 1648 | 2131 | 2792 | 2853 | 3033 | 193.23 | 11.49 | 62.89 | - | - | - |
| CC7-1530-control | BK-91.1a | EEM | 12.0 | 8.779 | 291 | Bos/Bison/Ovibos | 1105 | 1192+1208 | 1427 | 1580 | 1648 | x | x | x | x | 22.30 | 15.17 | 8.25 | No | No | Yes |
| CC7-1530-cut | BK-91.2a | EEM | 0.5 | 8.461 | 250 | Bos/Bison/Ovibos | 1105 | 1192+1208 | 1427 | 1580 | 1648 | x | x | x | x | 70.92 | 8.39 | 18.14 | No | No | Yes |
| CC7-1530 | BK-91 | Destructive | | | | Bos/Bison | 1105 | 1208 | 1427 | 1580 | 1648 | 2131 | 2792 | 2853 | 3033 | 55.89 | 15.51 | 188.86 | - | - | - |

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4. Roth, M. *Linköping university electronic press report: On the multivariate t distribution*. (2013).

Chapter Five

# Discussion and conclusion

The aim of this dissertation has been to provide an integrative approach that incorporates both bone surface modification and peptide mass fingerprint analysis of Late Pleistocene faunal assemblages. Such an approach attempts to unite both the morphologically identifiable and unidentifiable components using complementary zooarchaeological and taphonomic methodology and assess the effect of non-destructive extraction methods (eZooMS) on bone surfaces and the implications for its use on special finds such as bone tools and ornaments. Below, I will discuss the results of the three thesis projects and outline how these findings contribute to our understanding of human subsistence during the MUPT, along with possible future directions.

## 1. Doctoral projects conclusions

### 1.1. Project 1: Combining ZooMS and zooarchaeology at Fumane Cave (Italy)

The bone assemblages recovered at Fumane Cave are highly fragmented resulting in 3% of the bone specimens morphologically identifiable and with a taxonomic composition dominated by red deer (*Cervus elaphus*) and ibex (*Capra ibex*). Restraining interpretations about subsistence strategies and taxonomic abundance on a small proportion of faunal remains can lead to an incomplete picture of past human behaviours. This study has combined taxonomic identification provided by the ZooMS analysis of the morphologically unidentifiable components of the Late Mousterian and Uluzzian layers, with the bone surface modification analysis of these specimens, in order to address taxonomic composition and taphonomic history of these components of the faunal assemblages (Sinet-Mathiot et al., 2019). For the first time, we highlight a quantitative difference between the bone component analysed through ZooMS and the bone component identified by comparative bone morphology. While taxonomic composition is similar, taxonomic abundances vary between both components, including a six-fold increase in the number of *Bos/Bison* remains in the ZooMS component. Addressing the potential causes for this compositional difference has revealed that such increased fragmentation may result from a specific hominin behaviour during food

procurement, such as marrow extraction, thus providing a potential behavioural explanation for the different taxonomic abundance between the two components. While the morphologically-identified component of a faunal assemblage may be dominated by a single species, this may not necessarily reflect true assemblage abundance. Without ZooMS, *Bos/Bison* were previously considered a minor component of the Fumane fauna.
This study has demonstrated that the analysis of the morphologically unidentifiable component of a faunal assemblage can complement our understanding of species proportions at a site, but more importantly can highlight previously unrecognised specific subsistence behaviours such as variation in the intensity and treatment of different prey sizes at Fumane Cave.

Both the Final Mousterian and the Uluzzian faunal assemblages have provided comparable results in terms of taxonomic representation showing no regional evolution or changes in the subsistence strategies from late Middle Palaeolithic to the MUPT. However, the application of a combined palaeoproteomics and zooarchaeological analysis of the faunal material from multiple sites covering the MUPT has the potential to provide new insights into subsistence and site formation during this period of changes. The combined analysis of three transitional assemblages has helped us address this problematic in Project 2.

## 1.2. Project 2: Contribution of ZooMS to the understanding of subsistence strategies during the MUPT

Following upon Project 1, the work detailed in Chapter three presents three large-scale applications of ZooMS screening on MUPT assemblages integrated with the zooarchaeological analysis of the faunal assemblages (Sinet-Mathiot et al. under review). We addressed the implications of integrating morphologically unidentifiable components into the general understanding of the formation of these bone assemblages and subsistence strategies during a period of changes by including transitional assemblages from across Europe. This chapter demonstrates that differences in taxonomic abundances between the ZooMS- and morphologically-identified bone components previously reported within the final Mousterian and the Uluzzian layer at Fumane Cave do not seem to be an exception. Indeed, this study shows an under-representation of the proportion of the large ungulates, e.g. *Bos/Bison* and equids, at Les Cottés and La Ferrassie across the MUPT. The zooarchaeological assessment of these bone remains highlights a potential differential identification rate between taxa notably towards reindeer, possibly creating a reporting bias in the representation of the dominant taxa depending on their ease of identification. Previous interpretations of the fragmented components rely on the categorisation of unidentifiable bone fragments into body size classes

(based on cortical thickness and fragment size), and on the assumption that the morphologically unidentifiable component would reflect the taxonomic frequencies of the material identified morphologically. With the addition of ZooMS identities, we highlight discrepancies in the assignment of bone fragments to the body size classes, confirming a pattern previously noted on the material of Fumane Cave. These results bring caution on the interpretation of such categorisations and stress upon the necessity to complement them with secure ZooMS taxonomic assignments.

The correlation of taxonomic identities, provided through ZooMS, with particular agents contributing to the bone accumulation on site, e.g. humans and carnivores, highlight the exploitation of a more diverse range of taxa. The assessment of the fragmented component of the bone material from Les Cottés refines shifts in taxonomic proportions of large ungulates across the MUPT with the progressive decrease of *Bos/Bison* abundances counterbalanced by a progressive increase of an underestimated exploitation of Equidae. In addition, the incidence of carnivores could be assessed confirming a progressive decrease of carnivore activity alongside the expansion of early modern humans at Bacho Kiro Cave and Les Cottés.

Worked bones represent another fraction of the bone assemblages that are morphologically unidentifiable due to heavy modification. Several non-destructive methods, such as the eraser sampling technique, have been developed in order to analyse these specimens while preserving their integrity. However, the effect on bone surfaces has not been addressed yet.

## 1.3. Project 3: Testing the effect of a non-destructive collagen extraction method on Palaeolithic bone surfaces

Traces preserved on the surfaces of anthropogenically worked bones aid in the interpretation of the fabrication process and/or use of the tool, which directly relates to past human behaviour. To address human decision-making related to raw material selection, taxonomic assignments of worked bones is crucial. However, most modified bones found at Palaeolithic archaeological sites are either highly fragmented or their morphological features that would allow for identification of the skeletal element or taxa have been removed as a result of the manufacturing or use processes. Therefore, biomolecular methods become the only option for addressing questions of raw material selection, but destructive sampling of rare and culturally significant bone artefacts is usually problematic. Recent developments in non-destructive extraction techniques allow for the analysis of these finds while preserving the integrity of the piece. One of these methods consists of rubbing a PVC eraser on the surface of an organic

tissue. Although the advantages of a so-called non-invasive method for the analysis of worked bones are clear, the effect of such method on bone surfaces and bone surface modifications has not been assessed. The controlled sampling experiment demonstrates that the eraser extraction method (EEM) generates some alterations that should bring caution before using this method on bone artefacts (Sinet-Mathiot et al., 2021). The EEM creates friction that cleans the bone surfaces and removes residues that may be of interest for further studies related to human behaviour, flattens the bone microtopography, but more importantly the friction and particle movement create microstriations that are comparable to use-wear traces on bone tools. Combined with the marginal success of the taxonomic assignment, the results of this study alert the community on the invasiveness of the EEM on bone surfaces and advise not to use this technique on ancient worked bones.

Addressing past human activities and behaviours rely heavily on the preservation of bone surface modifications or traces. The results from Project 3 raise awareness on the importance of addressing the effects on bone surfaces of non-invasive biomolecular extraction methods prior to large-scale application.

## 2. Implications for the understanding of subsistence strategies

The assessment of the hominin diet plays a major role for our understanding of human response to technological, climatic and biological changes occurring during the Middle to Upper Palaeolithic transition. However, our ability to measure taxonomic abundances of the faunal assemblages influences our perception of prey selection or environmental/ecological adaptation, as it relies solely on the identifiability of the bone remains.

Zooarchaeological and isotopic analyses have suggested that Neanderthal and modern *Homo sapiens* subsistence relied mainly on the consumption of a range of medium and large herbivores (Discamps et al., 2011; Gaudzinski-Windheuser & Niven, 2009; Gaudzinski-Windheuser & Roebroeks, 2011; Jaouen et al., 2019; Niven et al., 2012; Rendu et al., 2019; Richards et al., 2008; Smith, 2015). Ungulates constitute the dominant taxonomic groups hunted by humans across the MUPT in Europe, almost exclusively represented by large bovines (*Bos/Bison*), horse, reindeer, and red deer, and any variability of the subsistence strategies would result in an abundance difference between these taxa (Morin et al., 2016; Soulier, 2013). Late Mousterian faunal assemblages are frequently reported with a high proportion of large bovines and a low occurrence of reindeer remains. This is notably the case at La Quina (Debénath et al., 1998), Mauran (Farizy et al., 1994) or at La Rouquette (Rendu et al. 2011). Faunal assemblages resulting from human occupation during the MUPT such as

the Châtelperronian, have been poorly addressed due to a low number of morphologically identifiable specimens, but provide a reindeer-dominated faunal spectrum, such as at La Quina, Roc de Combe or Saint-Césaire (Grayson & Delpech, 2006, 2008; Morin, 2004; Soulier, 2013). The abundance of reindeer remains progressively increased throughout the MUPT and until representing the dominant taxa among the Aurignacian faunal assemblages. This is the case for example within the Protoaurignacian and Aurignacian assemblages from Le Piage (Champagne et al., 1981), La Quina (Soulier, 2013), Roc de Combe (Soulier, 2013), Castanet (Castel, 2011), La Ferrassie (Delpech, 2007), Les Cottés (Rendu et al., 2019) and Abri Pataud (Sekhr, 1998).

Our integrative work on the Late Mousterian and Uluzzian faunal material from Fumane Cave have shown that certain specific subsistence behaviours can be retained within the morphologically unidentifiable bone component, highlighting here a differential carcass treatment between taxa previously unrecognised at the site, with *Bos/Bison* carcasses processed more intensively for marrow extraction. These results relate the importance of the acquisition of fat from bone marrow in Neanderthals foraging decisions, also noted by other scholars (Morin & Ready, 2013; Stiner, 1994). The assessment of the fragmented fraction of the bone assemblages from Les Cottés and La Ferrassie emphasised, in the morphologically unidentifiable component, the under-representation of large ungulates in the human diet throughout the MUPT, notably equids, due to differential identification rates. Bone fragmentation of large ungulates tends to generate a larger amount of morphologically undiagnostic bone fragments. Other taxa such as reindeer have been reported by zooarchaeologists as easily identifiable in comparison to large ungulates, possibly leading to a biais in the representation of certain taxa within the faunal spectrum of an assemblage. In addition, the incidence of carnivore modifications during late Neanderthal occupation of the sites suggests a context where both humans and carnivores were important in faunal accumulation and modification. The interaction between human groups and large carnivores seems to change during the MUPT and might indicate an increasing predatory pressure of human groups on their environment or a shorter duration of site occupation by Neanderthals compared to Late Pleistocene *Homo sapiens*. The multiplication of large-scale analysis integrating ZooMS of the fragmented component with the zooarchaeological assessment of faunal assemblages will provide further insight into the variability of the taxonomic proportions between components, enhancing our understanding of the formation of these faunal assemblages.

# 3. Future perspectives

The reliability of ZooMS to correctly assign a taxonomic identification to morphologically identifiable bone fragments has been numerously demonstrated on various collagenous materials. First applications on faunal assemblages aimed to improve the number of identified specimens and potentially enrich the faunal spectrum with taxa previously unrecognised based on morphology (**Figure 5**; Welker et al., 2015). ZooMS has been primarily used as an easy and rapid method to identify hominin remains among fragmented morphologically unidentifiable bone fragments. The isolation of hominin specimens through ZooMS permit subsequent palaeoproteomic and aDNA analyses in order to revisit their taxonomic attribution and stratigraphic context (e.g. Grotte du Renne, Welker et al., 2016), or contribute to our chronological understanding of the arrival of *Homo sapiens* in eastern Europe.

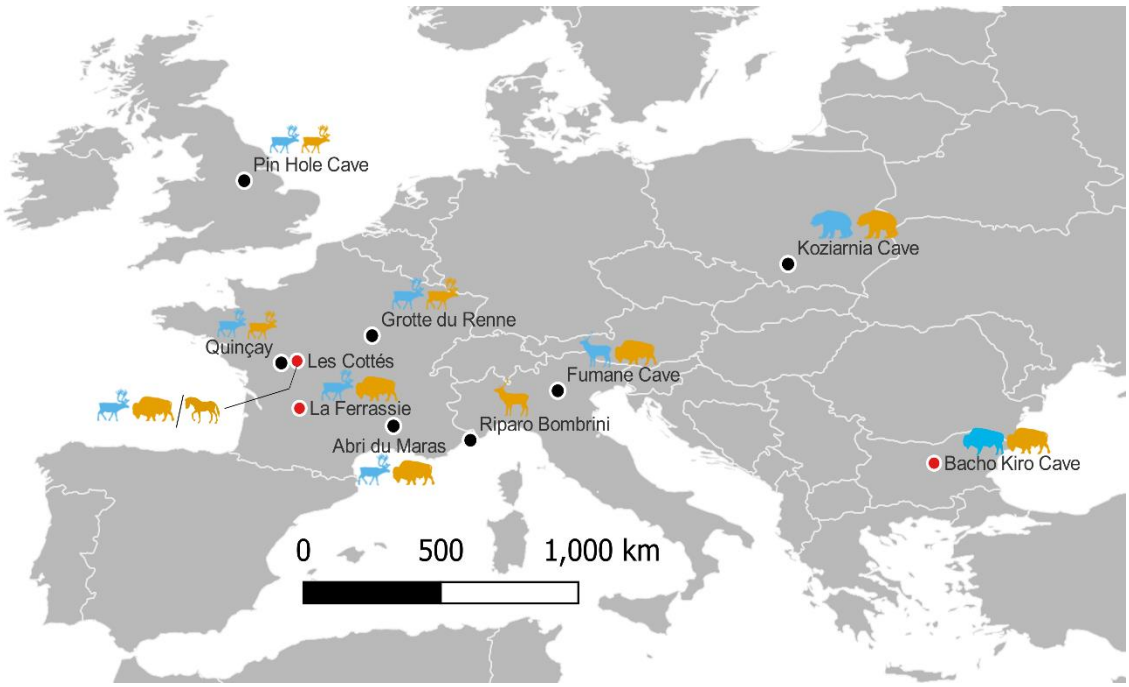


**Figure 5:** Spatial distribution of published European non-targeted ZooMS studies with zooarchaeological data available for the same archaeological layers. Animal silhouettes (phylopic.org) indicate the dominant taxon in each component (ZooMS: orange and morphology: blue) for each site, although the complete faunal spectrum of each site includes various taxa. Sites represented by a red dot were analysed within the framework of this thesis, but also during other research projects not presented in this dissertation. The morphology component from Riparo Bombrini is not illustrated on the map as it is represented by a low NISP (<20 morphologically identified specimens). Data derived from: Pin Hole Cave (UK; Buckley et al., 2017), Quinçay (France; Welker et al., 2017), Grotte du Renne (France; Welker et al., 2016), Les Cottés (France; Sinet-Mathiot et al. under review), La Ferrassie (France; Sinet-Mathiot et al. under review), Abri du Maras (France; Ruebens et al., 2022), Fumane Cave (Italy; Sinet-Mathiot et al., 2019), Riparo Bombrini (Italy; Pothier Bouchard et al., 2020), Koziarnia Cave (Poland; Berto et al., 2021) and Bacho Kiro Cave (Bulgaria; Sinet-Mathiot et al. under review).

ZooMS screening has also been used on the bone material from Quinçay (France) to compare spatial and temporal distribution of glutamine deamidation values in order to assess bone assemblage integrity and inform about differential bone preservation (Welker et al., 2017; Wilson et al., 2012). While the authors observed similar taxonomic composition between components of the faunal assemblage, they identified specimens that have undergone different diagenetic histories, potentially stratigraphically intrusive to the material belonging to the same layer. The decay rate and state of preservation of collagen has been subsequently addressed on other assemblages with varying success (Brown et al., 2021; Ruebens et al., 2022; Sinet-Mathiot et al., 2019). Although the environmental agents possibly influencing glutamine deamidation remain poorly addressed, deamidation calculations can be routinely done, without any additional cost, thus generating data to enlarge our comprehension of the variability within and between archaeological sites.

Due to its potential for a better understanding of faunal ecology and taxonomic composition, ZooMS has been applied to several faunal assemblages across Europe (e.g. Pin Hole Cave, (Buckley et al., 2017); Koziarnia, (Berto et al., 2021); Abri du Maras, (Ruebens et al., 2022)). However, the work presented in this thesis provides evidence, for the first time, that the morphologically unidentifiable bone components do not necessarily reflect taxonomic abundance of the dominant taxa represented in the morphologically identified component. These differences can be explained by specific treatment of carcasses related to bone fragmentation for marrow extraction previously unrecognised (Sinet-Mathiot et al., 2019), or can emphasise methodological limits and biases brought by differential identification rates between taxa, and which ZooMS can help to overcome (Sinet-Mathiot et al. under review). At Abri du Maras (France), the combination of multiple proxies including ZooMS aimed to improve the faunal spectrum (NISP Morphology = 49) and to contextualise one of the largest Neronian lithic assemblage, seeking to untangle old collection curation and lithic technology attribution to the chronology of human occupation during Late Middle Palaeolithic (Ruebens et al., 2022). At Riparo Bombrini (Italy), ZooMS and zooarchaeology have been used to explore mobility strategies and resource management during the Protoaurignacian, seeking to improve our understanding of faunal selection diversity, although with limited results due to intense bone fragmentation (Pothier Bouchard et al., 2020). However, this study enhances a high proportion of large bovines within the fragmented component, interpreted as a result of large-scale marrow extraction and comparable to what has been observed at Fumane cave, but unfortunately based on a small amount of bone specimens morphologically identifiable (NISP ZooMS = 235, NISP Morphology = 16).

Thus, the assessment of the fragmented component has shown that it can provide fresh insights on assemblage composition and prey selection, and when combined with bone surface modification analysis, can inform about subsistence strategies, accumulator agents,

site formation, and butchery practices. The combination of ZooMS taxonomic identification with bone surface modification analyses is crucial to understand the taphonomic history of the fragmented bones.

ZooMS can be used routinely on highly fragmented assemblages as a tool to observe potential biases generated by bone fragmentation, in combination with other analytical methods such as isotopes and radiocarbon dating (**Figure 6**; McCormack et al., 2022; Ruebens et al., 2022). It was demonstrated in this thesis that interpretations based on the categorisation of morphologically unidentifiable bone specimens into body size classes should be used with caution (Sinet-Mathiot et al., 2019; Ruebens et al., 2022; Sinet-Mathiot et al. under review). Faunal assemblages showing monospecific composition should be complemented with ZooMS analysis in order to have a more complete picture of faunal composition and ecology, especially if the dominant taxa is easily morphologically identifiable.

ZooMS is not only useful on fragmented faunal assemblages but also for the taxonomic identification of rare and culturally significant bone specimens such as worked bones. Various non-destructive methods have been developed permitting their analysis without affecting the integrity of the bone specimen (Dekker et al., 2021; Martisius et al., 2020). However, such methods, defined as non-destructive on a specific collagenous material such as parchment, can have an invasive effect when applied on worked bone surfaces (Sinet-Mathiot et al., 2021). Controlled sampling experiments should be undertaken prior to large-scale application of non-destructive sampling methods.

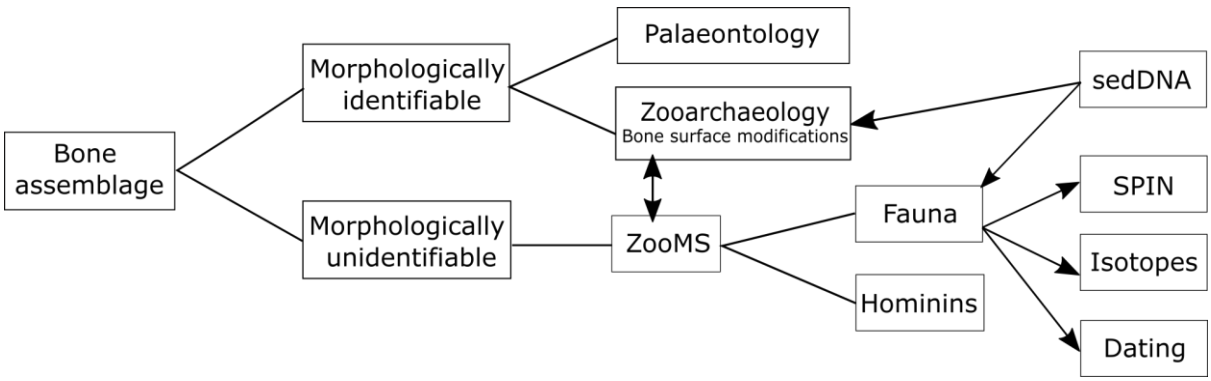


**Figure 6**: Schematic workflow of the integration of the ZooMS analysis of the morphologically unidentifiable component within the general assessment of faunal assemblages. Subsequent analysis can be performed on the faunal fraction identified through ZooMS such as radiocarbon dating, isotope analysis and other palaeoproteomics approaches (for example SPIN). The data generated can also be integrated with other proxies such as sediment DNA.

Increased interest in ZooMS by various research groups with different scientific backgrounds will generate an expansion of the applications of this method at a larger geographic and temporal scale (Richter et al., 2022). This method can be used within an educational framework exemplified by the project developed in Denmark where ZooMS analyses of Danish medieval urban specimens are being used to teach high school students laboratory techniques and scientific processes (Brandt et al., 2022). In order to make this technique widely accessible, there is a need from the field to expand the reference library by increasing the number of biomarkers identified through sequencing (LC-MS/MS), in order to provide accessibility to a larger subset of taxonomic groups including small mammals, fish (Harvey et al., 2018) and reptiles. In addition, bioinformatic development on automation in spectral processing will benefit the multiplication of application and standardisation of spectral identification.

While ZooMS has been demonstrated as a powerful tool notably for the assessment of faunal assemblages and worked bones, this method also presents some limitations, which would need to be addressed to further exploit its potential.

## 3.1. Further palaeoproteomics approaches of use in zooarchaeological research

Further integration of ZooMS data into standard zooarchaeological investigation necessitates a better comparability of the metrics employed for both components. Species abundance among the ZooMS component is typically assessed using the number of identified specimens (NISP) (Grayson, 1984), as minimum number of skeletal element (MNE) and minimum number of individuals (MNI) cannot be compared quantitatively with ZooMS data, which is inherently a NISP count. Although this measure is commonly used by zooarchaeologists, the relationship between NISP and bone fragmentation has been widely debated, and for some recognised as a problematic tool (e.g., Brothwell & Chaplin, 1972; Klein & Cruz-Uribe, 1984; Lyman, 2008; Marshall & Pilgram, 1993). The addition of taxonomic identification of bone fragments, independently of their morphology and surface preservation, might permit building experimental models allowing for a better understanding of the measure of bone fragmentation in Palaeolithic faunal assemblages in relation to specimen size and the ability to identify the skeletal elements. ZooMS can help refine alternative methods to calculate NME such as diagnostic landmarks on bone elements or refitting bone shafts (Marean et al., 2001; Morin et al., 2016; Stiner, 1994)

Although the reliability of ZooMS as a method to provide taxonomic identification has been previously demonstrated, in most cases ZooMS cannot provide species level resolution. This is particularly true for Palaeolithic faunal assemblages showing a large variability of potential taxa compared to Holocene periods. The ZooMS method can provide taxonomic identifications up to species (e.g. *Rangifer tarandus*) in some cases, but generally the taxonomic resolution remains limited to family or genus. Taxonomic discrimination is based on a list of nine peptide markers, and peptide marker series can be similar for some closely related species, which is the case for the species belonging to the main taxonomic groups constituting the Late Pleistocene faunal assemblages. However, some species do not share the same ecology and being able to differentiate them would provide valuable information on hunting strategies and prey selection.

The quality of the taxonomic identification depends heavily on the richness of the library of references. Further development of the library, built upon modern and extinct species from around the world, will help expand the resolution of the identification. The recent development of a rapid and cheap LC-MS/MS-based species identification approach (Species by Proteome INvestigation or SPIN, (Rüther et al., 2022)) allows for the differentiation of some of these closely related species. The taxonomic group of *Bos/Bison* comprises wild cattle (*Bos primigenius)* and steppe bison (*Bison priscus).* These species are difficult to distinguish based on their morphology, and cannot be discriminated against based on their collagen type I sequences through ZooMS. However, they show a different ecology. While the steppe bison, living in groups, is characteristic of open habitat and would occupy seasonally steppic ecosystems to more forest-steppe lands (Brugal, 1999; Heptner, 1989), the wild cattle is a more isolated individual and indicate a more temperate and forest conditions. Bovines were an important source of subsistence for Late Pleistocene hominins (Terlato et al., 2019) and large accumulation of carcasses have been recovered notably at the sites of La Quina (Chase, 1999; Rendu & Armand, 2009), Coudoulous (Jaubert et al., 2005), La Borde (Jaubert et al., 1990) and Mauran (David & Fosse 1999, Rendu et al. 2012), among others. As hunting these species might require different skills and organisation, the addition of such information could greatly enhance our understanding of subsistence behaviour, hunting strategies, and palaeoenvironmental conditions during human occupation.

## 3.2. Neanderthal subsistence strategies before the MUPT

When addressing subsistence behaviour and comparing any changes between Late Pleistocene human groups in Europe, e.g. Neanderthals and *Homo sapiens*, it is necessary to consider any cultural sub-divisions prior to and after the MUPT at a regional scale. Variation in species representation occurs throughout the Late Middle Palaeolithic and is illustrated by

a shift from reindeer-dominated faunal assemblages (notably during Quina Mousterian) to large game hunting activities based on a more varied fauna (red deer, horse, wild cattle or bison). The reindeer-dominated faunal assemblages seem to appear again during Proto- and Early Aurignacian. The assessment of reindeer-dominated faunal assemblages across the MUPT in this thesis has highlighted the potential of the inclusion of biomolecular methods such as ZooMS within the zooarchaeological analysis of the bone remains, in order to gather information previously unrecognised by traditional methods. The late Mousterian, such as the Mousterian of Acheulean Tradition (MTA) and the Discoid-Denticulate Mousterian, is characterised by distinct mobility patterns and various hunting strategies possibly reflecting a specific response to the dietary needs of Neanderthal groups (Delagnes & Rendu, 2011; Gaudzinski, 2006; Soressi, 2004). Investigating shifts and prey selection variability during Late Mousterian at a regional scale, before the arrival and spread of modern *Homo sapiens* across Europe, will provide fresh insights on late Neanderthal subsistence behaviour during periods of technological changes and, thus, on Mousterian diversity (Delagnes & Rendu, 2011; Discamps et al., 2011; Rendu et al., 2012; Steele, 2004).

## 3.3. General conclusion

The research presented in this thesis has demonstrated the successful integration of ZooMS analysis with bone surface modification analysis in order to contribute to the general zooarchaeological assessment of Palaeolithic faunal assemblages and verify the effect of a non-destructive collagen extraction technique on ancient bone surfaces.

The results emphasise that the assessment of the fragmented and morphologically unidentifiable portion of Pleistocene bone assemblages through ZooMS can provide different patterns of taxonomic abundances for the dominant taxa, which can be explained by various factors. The study at Fumane Cave points out behavioural factors related to different butchery practices between taxa such as the intensive marrow extraction of *Bos/Bison* remains. The study on the material from Les Cottés and La Ferrassie emphasises a differential identification rate between taxa leading to an under-representation of large ungulates. The integration of ZooMS into the zooarchaeological analysis of Palaeolithic bone assemblages allows to overcome methodological limits in taxonomic identification brought by bone fragmentation and brings new insights on the emergence and the development of subsistence strategies across the MUPT. The recent development of non-destructive proteomic extraction techniques, notably on fragmented Palaeolithic worked bones, urged the necessity to test the effect on bone surfaces, at a microscopic level, of sampling techniques such as the eraser extraction method. A multidisciplinary controlled sampling experiment described in this thesis highlights the invasiveness of this technique with the creation of microstriations and plateauing of the

bone surfaces, and which would lead to misinterpretations about ancient human behaviours if not taken into account in future bone surface analyses.

With a wider range of application of these methods, the taxonomic identification of morphologically unidentified bone and dental specimens through ZooMS analysis combined with zooarchaeology and bone surface modification analysis, will provide an opportunity to assess a larger proportion of faunal assemblages.

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# Summary

Through the analysis of Palaeolithic faunal assemblages, zooarchaeology allows us to reconstruct diet and subsistence strategies. However, taphonomic processes, including both carnivore and anthropogenic activities, can lead to high bone fragmentation. This often prevents taxonomic identification based on morphology and restricts our interpretation to a relatively small proportion of identifiable remains.

The development of biomolecular methods gives, for the first time, the opportunity to identify the biological markers preserved in previously undiagnostic bone remains. Zooarchaeology by Mass Spectrometry (ZooMS) provides a taxonomic identification based on amino acid sequence variation of the protein collagen type I. Up to now, ZooMS applications have largely focused on ecological questions such as improving the faunal spectrum, documenting the spread of domesticated species, or for the identification of additional hominin specimens. However, investigating the relationship between faunal composition and bone fragmentation, especially to assess hominin behaviour during transitional periods like the Middle to Upper Palaeolithic transition, has not been explored.

This dissertation combines the analyses of bone surface modifications with biomolecular analyses in order to provide fresh insights into past human behaviour in relation to faunal species selection and carcass processing. These research questions are addressed by investigating bone assemblages from European sites spanning the arrival of *Homo sapiens* within territories occupied by the last Neanderthals. Site selections focused on recent, well documented excavations with large quantities of unidentifiable faunal fragments and for which there is a modern zooarchaeological analysis, such as Fumane Cave (Italy), Bacho Kiro Cave (Bulgaria), La Ferrassie and Les Cottés (France). This study explores the synthesis and analysis of comparable data for both the morphologically identifiable and morphologically unidentifiable portion of the same faunal assemblages, with the latter assessed using ZooMS. The results at Fumane Cave highlights quantitative differences between taxa, illustrated by a 6-fold increase in the proportion of Bos/Bison remains within the ZooMS component. This is possibly due to specific hominin behavior during food procurement, resulting in increased fragmentation of Bos/Bison remains compared to other fauna. The results for assemblages spanning the Middle to Upper Palaeolithic transiation (MUPT) at Bacho Kiro Cave, Les Cottés and La Ferrassie indicate an underepresentation of Bos/Bison and Equidae due to differential identification rates between taxa, a progressive shift in prey selection from Bos/Bison to Equidae across the MUPT, a reduction in the frequency of carnivore presence, and an increase in carnivore exploitation by Upper Palaeolithic *Homo sapiens* over the course of their dispersal across Europe.

The biomolecular analysis of rare and culturally significant organic archaeological material is often restrained by the invasiveness of the sampling strategy. Recent studies have therefore focused on developing non-destructive collagen extraction techniques. The eraser extraction method (EEM), initially applied to parchments, was developed for palaeoproteomics such as Zooarchaeology by Mass Spectrometry (ZooMS). The friction of a Polyvinyl Chloride (PVC) eraser on an organic tissue, such as a bone surface,releases a small amount of protein from the surface. This doctoral project contributes to the understanding of the impact of the eraser extraction method on ancient bone surfaces by performing a controlled sampling experiment and measuring the microtopography of the bone surface before and after erasing. The results shows that the EEM generates some alterations to the bone surface, including a flattening of the bone microtopography and the creation of microstriations comparable to ancient use-wear traces. Future applications of the EEM should therefore take these observations into account during experiment design.

By combining methods and integrating complementary datasets, this dissertation provides a more comprehensive picture of Late Pleistocene faunal assemblages and hominin subsistence behaviour and show the potential of the inclusion of palaeoproteomic analysis within the current framework of zooarchaeological analysis at Palaeolithic sites.

# Samenvatting

Door middel van de analyse van Paleolithische dierenbotcollecties, stelt zoöarcheologie ons in staat om dieet en overlevingsstrategieën te reconstrueren. De taxonomische identificatie van botresten op basis van morfologie wordt echter vaak verhinderd door tafonomische processen, zoals carnivoor en antropogene activiteiten, die beide kunnen leiden tot hevige bot fragmentatie. Hierdoor is onze interpretatie vaak gelimiteerd tot een relatief klein deel van identificeerbare resten.

De ontwikkeling van bio-moleculaire methoden maakt het voor het eerst mogelijk om biologische markers te identificeren die bewaard zijn gebleven in voorheen on-identificeerbare botresten. De taxonomische identificaties verkregen door middel van Zooarchaeology by Mass Spectrometry (ZooMS) zijn gebaseerd op variatie in de aminozuurvolgorde van het eiwit collagen type I. Tot dusverre heeft de toepassing van ZooMS zich voornamelijk gericht op ecologische vraagstukken om een beter beeld te krijgen van de diersoorten aanwezig op een vindplaats, de verspreiding van gedomesticeerde dieren, of het vinden van nieuwe hominine resten. De relatie tussen de geïdentificeerde fauna op een vindplaats en botfragmentatie, zeker in betrekking tot de studie van menselijk gedrag tijdens transitieperiodes, zoals de overgang van het Middel tot Laat Paleolithicum, is nog niet onderzocht.

Dit proefschrift combineert de analyse van botoppervlak modificaties met bio-moleculaire analyse en hoopt daarmee nieuwe inzichten te geven in het gedrag van mensen in het verleden in relatie tot soortselectie en karkasverwerking. Deze onderzoeksvragen worden beantwoord door middel van botcollecties van Europese vindplaatsen uit de periode van de aankomst van de eerste *Homo sapiens* in de gebieden bewoond door de laatste Neanderthalers. Voor de keuze van de vindplaatsen ging de voorkeur uit naar recente, goed gedocumenteerde opgravingen met grote hoeveelheden on-identificeerbare botresten en waarvoor modern zoöarcheologisch onderzoek beschikbaar is, zoals Fumane Grot (Italië), Bacho Kiro (Bulgarije), La Ferrassie en Les Cottés (Frankrijk). Dit onderzoek richt zich op de synthese en analyse van vergelijkbare data voor zowel de morfologisch identificeerbare botresten als het on-identificeerbare gedeelte van dezelfde faunacollectie, waar de laatste geidentificeerd werden door middel van ZooMS. De resultaten voor Fumane Grot laten een quantitatief verschil zien in soortencompositie, voornamelijk een zes-voudige toename in de proportie aan Bos/Bison in de ZooMS-geidentificeerde component. Dit is mogelijk het gevolg van specifiek menselijk gedrag gedurende voedselverkrijging dat resulteerd in een verhoogde fragmentatie van Bos/Bison botmateriaal. De onderzoeksresultaten voor de vindplaatsen die de overgang van het Middenpaleolithicum naar het Laatpaleolithicum omvatten in Bacho Kiro, Les Cottés, en La Ferrassie laten een onderrepresentatie zien van Bos/Bison en

paardachtigen als het gevolg van verschillen in morfologische "identificeerbaarheid" tussen soortgroepen, een geleidelijke verschuiving in prooiselectie van Bos/Bison naar paardachtigen gedurende de transitie, een vermindering in de aanwezigheid van carnivoren, en een toename van de exploitatie van carnivoren door Laatpaleolitische *Homo sapiens* gedurende hun verspreiding over Europa.

De invasiviteit van de bemonsteringsmethode staat vaak de bio-moleculaire analyse van zeldzame en cultureel waardevolle organisch archeologisch objecten in de weg. Daarom heeft recent onderzoek zich gericht op de ontwikkeling van non-destructieve collageen extractietechnieken. De gum-eiwit-extractiemethode, oorspronkelijk toegepast op perkament, was ontwikkeld voor palaeoproteomics, zoals Zooarchaeology by Mass Spectrometry (ZooMS). De wrijving van een Polyvinyl Chloride (PVC) gum op organisch materiaal, zoals het oppoervlak van een botfragment, maakt een kleine hoeveelheid eiwit vrij van het oppervlak. De mogelijke bewerking van macro- en microscopische kenmerken van het bot en de potentiële verandering van botoppervlakken en specifieke menselijke bewerkingen zijn nog niet eerder beschreven. Dit doctorale onderzoek streeft om bij te dragen aan ons begrip van de invloed van de gum-eiwit-extractiemethode op archeologische botoppervlakken door middel van een gecontroleerd bemonsteringsexperiment en door het vastleggen van de microtopografie van het bot voor en na het extraheren van eiwit met de gum. De onderzoeksresultaten tonen aan dat de gum-eiwit-extractiemethode veranderingen aanbrengt aan het botoppervlak, zoals het afvlakken van de microtopografie van het botoppervlak en de creatie van zeer kleine groeven die overeenkomen met oude gebruikssporen. Toekomstige toepassingen van de gum-eiwit-extractiemethode zullen daarom deze observaties in ogenschouw moeten nemen.

Door methoden te combineren en complementaire datasets te integreren streeft deze dissertatie ernaar om een meer alomvattend beeld van Laat Pleistocene faunacollecties en hominine overlevingsstrategieën te creëren en de potentie van het integreren van palaeoproteomic analyse binnen het huidige raamwerk van de zoöarcheologische studie van Paleolithische vindplaatsen.

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# Curriculum vitae

Virginie Sinet-Mathiot was born on the 9th of January 1991 in Châteauroux (France). She obtained her A-levels in science at Jean Moulin School (Draguignan) in 2008. In 2011, she successfully completed the BSc Biology of Organisms at the University of Bordeaux (France) while volunteering for various archaeological excavations in France and Morocco. She obtained her MSc Physical Anthropology at the University of Bordeaux in 2013. After this, Virginie developed an extensive experience in fieldwork, in field laboratory and collections management but also in restoration of archaeological collections, in France, Germany, Bulgaria, Serbia and Morocco. In 2018, she started her PhD under the supervision of Prof. Jean-Jacques Hublin and the joint co-supervision of Dr. Frido Welker and Dr. Geoff Smith at the Max-Planck-Institute for Evolutionary Anthropology (Leipzig, Germany). After publishing her research in international peer-reviewed journals and presenting her work at various conferences, she submitted her doctoral dissertation to the University of Leiden in the Netherlands in 2022. Virginie has been awarded a Fyssen Foundation post-doctoral Study Grant APD 2022 and will join the laboratory PACEA (University of Bordeaux) and the Proteome platform (Bordeaux, France) in 2023.