Integrating palaeoproteomics into the zooarchaeological analysis of Palaeolithic bone assemblages

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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.

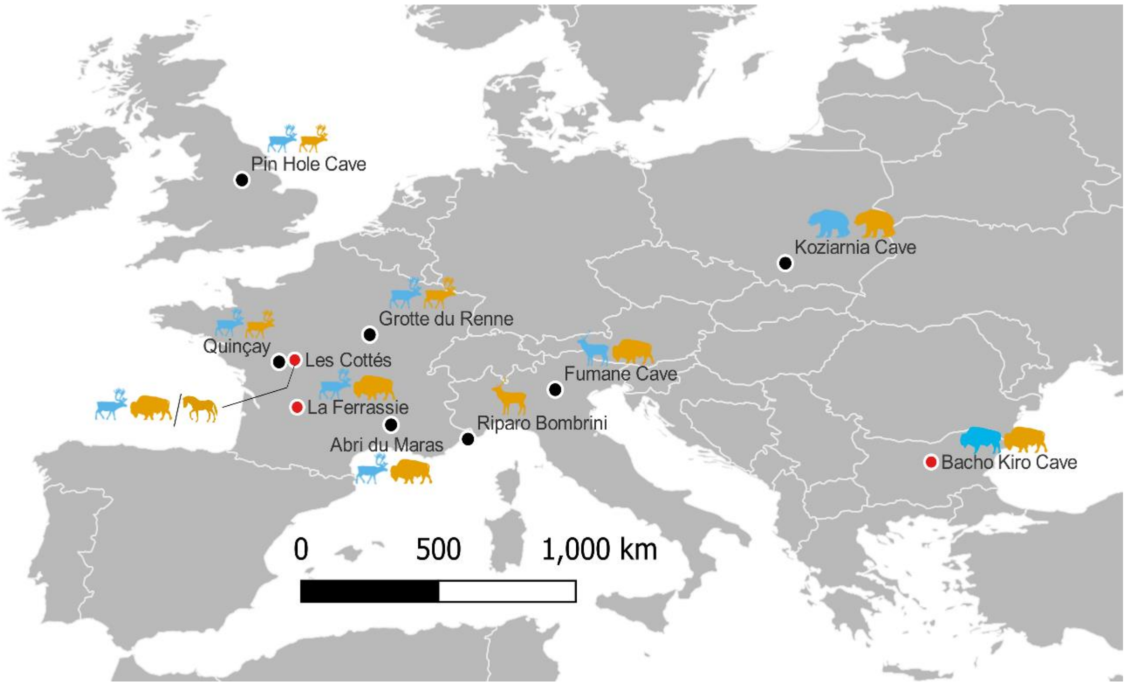

Figure 5: Spatial distribution of published European non-targeted ZooMS studies with zooarchaeological data available for the same archaeological layers. Animal silhouettes (phylopic.org) indicate the dominant taxon in each component (ZooMS: orange and morphology: blue) for each site, although the complete 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.

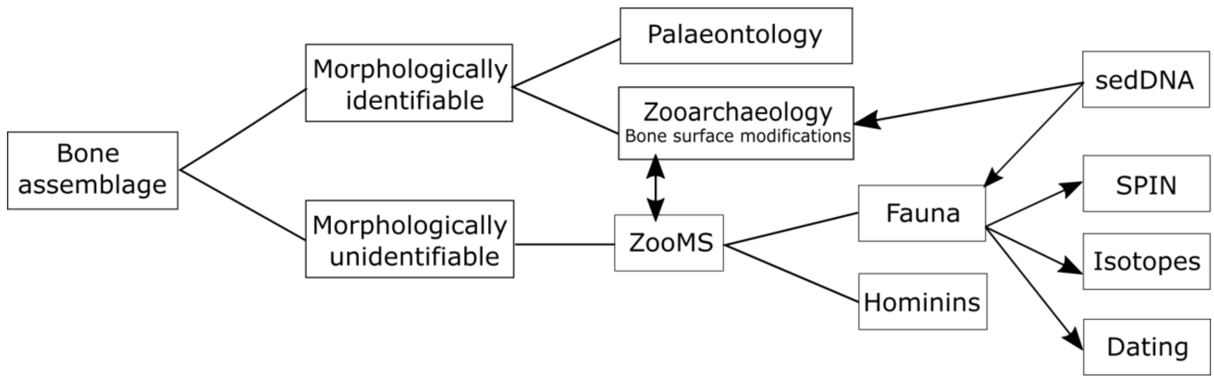

Figure 6: Schematic workflow of the integration of the ZooMS analysis of the morphologically unidentifiable component within the general assessment of faunal assemblages. Subsequent analysis can be performed on the faunal fraction identified through ZooMS such as radiocarbon dating, isotope analysis 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.