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Uncovering Neanderthal Subsistence through Proteomics: Methodological Insights into ZooMS Applications at Oscurusciuto Rock Shelter

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into stars for you. Perhaps, in time, we will become stars—visible only to those who take the time to look closely.

Abstract

This thesis conducts a methodological examination of the applications and theoretical contributions of Zooarchaeology by Mass Spectrometry (ZooMS) in reconstructing Neanderthal subsistence strategies and ecological adaptations during the Middle Paleolithic, specifically focusing on the Oscurusciuto rock shelter in southern Italy. Oscurusciuto, a prominent Neanderthal site in Europe, offers a distinctive chance to investigate methodological progress in paleoproteomics, particularly with the analysis of deteriorated and fragmented faunal remains. ZooMS targets the collagen protein COL1, allowing for species identification and providing insights into ancient faunal communities and ecological contexts, which is crucial for examining sites such as Oscurusciuto, where collagen preservation is minimal. This study underscores a theoretical framework for ZooMS, accentuating its capacity to provide substantial taxonomic and ecological insights in scenarios where conventional biomolecular methods may be hindered by preservation constraints.

ZooMS, a proteomic method focused on collagen proteins, facilitates species identification from non-diagnostic bone fragments, addressing difficulties commonly faced in locations with poor DNA preservation or extensively fragmented remains. This thesis offers a sequential description of ZooMS implementation, encompassing sample preparation, collagen extraction, and peptide mass fingerprinting, while highlighting the method's ability to rebuild faunal assemblages and ecological contexts from samples with low integrity. The study methodically evaluates the advantages and disadvantages of ZooMS, focusing on its taxonomic resolution for closely related species, dependence on collagen preservation, and the interpretive capacity of proteomic data within wider paleoenvironmental contexts.

The thesis examines the wider implications of ZooMS for enhancing archaeological science, namely in finding faunal elements that elucidate Neanderthal subsistence strategies, resource allocation, and possible rivalry with anatomically modern humans.

This methodological approach establishes ZooMS as a crucial asset in paleoproteomics, adept at tackling significant inquiries on Neanderthal adaptability and persistence in variable habitats. This study endorses the incorporation of ZooMS into multidisciplinary archaeological methodologies, especially for sites facing significant fragmentation and preservation difficulties, suggesting that proteomic techniques can address essential deficiencies in the paleoecological and subsistence accounts of prehistoric hominins. This study lays the groundwork for future research to utilize ZooMS more extensively in archaeological investigations, aiming not only to reconstruct ecological interactions but also to enhance our comprehension of hominin behavioral adaptations across diverse

paleoenvironments.

The present phase of the project examines the implementation of the ZooMS technology on bone samples obtained from stratigraphic units SU 4, SU 5, SU 6, and SU 7 of the Oscurusciuto Rock Shelter. The research remains under progress.

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1 Introduction

One of the most important and challenging questions in archaeology is the study of ancient faunal assemblages and their relationships with early hominins. Deep insights into our evolutionary past can be gained by reconstructing the settings that influenced the social dynamics, survival tactics, and behavioral patterns of early human societies. In particular, Middle Palaeolithic sites hold a unique significance, as they provide a window into the lifeways of Neanderthals—a hominin species that, for millennia, thrived across vast regions of Eurasia before their eventual extinction. However, a precise understanding of the interactions between Neanderthals and the animals with which they coexisted is hindered by incomplete remains and deteriorated materials at numerous sites, complicating access to specific faunal data. This thesis aims to tackle these challenges by employing innovative methodologies to extract concealed data from deteriorated materials, particularly within the archaeological framework of the Oscurusciuto rock shelter, a prominent Middle Palaeolithic site in southern Italy.

This research focuses on the application of Zooarchaeology by Mass Spectrometry (ZooMS), an innovative approach that has transformed the analysis of ancient proteins (Buckley et al., 2009). ZooMS is especially effective for examining morphologically uncertain bone pieces (Desmond et al., 2018; Pothier Bouchard et al., 2019; McGrath et al., 2019; Antonosyan et al., 2024; Wang et al., 2024). Proteins, especially collagen, function as durable macromolecules that can endure for millennia, offering crucial taxonomic and evolutionary insights that would otherwise be unattainable (Warinner et al., 2021; Buckley, 2018). ZooMS facilitates species identification from a minimal quantity of specimens by extracting and analyzing collagen from bone, thereby advancing faunal reconstruction and providing insights into historical ecological and subsistence patterns. Nonetheless, the advantages of employing this rapid, cost-effective, and time-efficient, high-resolution technique far surpass these drawbacks, particularly in scenarios where DNA is frequently too compromised for dependable analysis.

The Oscurusciuto rock shelter in the Apulian region of southern Italy serves as an intriguing case study for the implementation of ZooMS. Oscurusciuto, a significant Middle Palaeolithic site in the region, has produced essential archaeological layers that may enhance our comprehension of Neanderthal lifestyles (Marciani et al., 2016, 2018, 2020). However, prior investigations of some layers have been limited by the scarcity of zooarchaeological material, with incomplete faunal remains posing challenges to fully reconstructing the faunal community and understanding the habitat of these Neanderthals (Boscato et al., 2020; Boscato & Crezzini, 2011). Among the recognized species, aurochs (*Bos primigenius*) is the most prevalent taxon; yet, the general scarcity of identified specimens results in deficiencies in our comprehension of species diversity and broader ecological processes. Although ZooMS provides significant taxonomic insights, it cannot autonomously resolve essential inquiries on species diversity, age-at-death distributions, and overarching ecological dynamics.

This research is significant for its contribution to Neanderthal ecology and its methodological refinement. The utilization of ZooMS in this context offers a novel perspective on the site's faunal assemblage, enabling the analysis of even the most damaged samples and the extraction of essential taxonomic information.

Notwithstanding advancements in protein analysis technology, substantial questions persist concerning the robustness and interpretive constraints of ancient protein data. Although collagen is an extraordinarily durable biomolecule, dependence on its existence for taxonomic identification presents difficulties in settings where collagen preservation is inadequate or non-existent. This thesis aims to address these discussions by employing ZooMS in an environment characterized by limited faunal data but significant potential for new discoveries.

The purposes of this thesis are threefold:

- **Identify Unknown Species and Analyze Faunal Community:** Using

ZooMS on fragmentary faunal remains from Oscurusciuto to identify previously unknown species and get a better knowledge of the site's faunal community.

- **Contextualize Neanderthal Ecology and Subsistence:** These discoveries will be used to complete and integrate the information concerning the ecological context in which Neanderthals lived, focusing on their subsistence tactics and interactions with local animal species.

- **Contribute to Methodological Debates in Zooarchaeology:** This study seeks to contribute to broader methodological debates in the field of zooarchaeology by critically analyzing the benefits and limits of ZooMS in comparison with other zooarchaeological methods.

This study specifically inquires: To what extent can ZooMS elucidate the significance of both small and big faunal species in Neanderthal subsistence strategies as evidenced by the Oscurusciuto assemblage? Additionally, what insights may ZooMS offer regarding the adaptations of Neanderthals' hunting and foraging techniques in response to changing climatic conditions in southern Italy during the Middle Paleolithic? This thesis seeks to validate ZooMS as a reliable methodological instrument for deducing ecological and behavioral adaptations in fragmented archaeological environments, especially where conventional approaches are hindered by preservation limitations. Three hundred bone fragments extracted from the specified layers of the Oscurusciuto Rock

Shelter have been studied for this purpose, and the results are awaiting.

2

Literature Review

2.1 Fossil Bone Collagen: Type I Collagen (COL1)

Composed of both cellular and extracellular components, bone tissue exhibits a dynamic nature (García-Vázquez et al., 2023). Embedded within lacunae, various cell types orchestrate critical functions in bone formation, maintenance, and remodeling (Davies & Hosseini, 2000). The extracellular matrix, the predominant constituent of bone, can be further subdivided into two key elements: the mineral fraction and the organic matrix. The mineral fraction, primarily composed of calcium phosphate in the form of hydroxyapatite, provides structural rigidity to bone (Davies & Hosseini, 2000). In contrast, the organic matrix, accounting for roughly one-third of bone weight, offers flexibility and tensile strength. Notably, collagen type I proteins constitute approximately 90% of the organic matrix, with the remaining 3-5% comprised of non-collagenous proteins that play a crucial role in binding mineral crystals (Davies & Hosseini, 2000).

This intricate interplay between the cellular and extracellular components underscores the remarkable durability and adaptability of bone tissue.

Richter et al. (2022) describe collagen as a fundamental building block for animals, highlighting its abundance and crucial role in their structure. Type I collagen (COL1), which constitutes a significant part of bone and connective tissues, is stated as the most common form. At a microscopic level, COL1 consists of three protein chains (COL1A) twisted together in a triple helix. Notably, the structure of COL1 is highly restricted to ensure its functionality. Each chain features a repeating sequence where glycine, the smallest amino acid, occupies a central position within the helix. The remaining positions are dominated by proline and hydroxyproline, the latter being a unique modification of proline specific to collagens. These hydroxyproline residues contribute to the stability of the triple helix through hydrogen bonding. Interestingly, bulky amino acids are virtually absent in COL1 as they hinder the formation of the helix. During its production, COL1 goes through a maturation process involving signal and propeptide sequences that are later removed to reveal the functional A-chain. In bones, collagen fibers provide the foundation for mineral deposition, a process critical for bone formation. Additionally, the tight organization of collagen fibers is essential for its structural role and contributes to its exceptional preservation in archaeological finds (Richter et al., 2022).

Collagen, while abundant and structurally robust, is susceptible to taphonomic processes that convert organic remains into fossils, as well as diagenetic changes that take place post-burial (van Doorn et al., 2012; Cleland et al., 2015). Various factors influence these changes, including environmental conditions like temperature, moisture levels, soil pH, and microbial activity, which either accelerate or alter the chemical processes involved in collagen preservation. In these processes, individual amino acids, which are the essential building blocks of proteins, experience several modifications, such as deamidation, glycation, oxidation, and cross-linking. Deamidation involves the elimination of amide groups from amino acids, specifically glutamine and asparagine, resulting in changes to their chemical structure and functionality. The alterations lead to the creation of diagenetiforms—altered amino acids that interfere with the triple helical configuration of collagen, crucial for its stability (van Doorn et al., 2012). These activities collaboratively erode the collagen matrix, affecting its preservation in the archeological record. The deterioration of this helical structure enhances collagen's vulnerability to hydrolysis, a process in which water chemically degrades the collagen backbone. As collagen integrity decreases in archaeological materials, its degradation products are released, resulting in more fragile and chemically altered remnants. Archaeologists commonly evaluate the preservation state of collagen by measuring the percentage of collagen by dry weight (%wt), the percentage of nitrogen (%N), or the atomic ratio of carbon to nitrogen (C:N) in the sample (Schwarcz and Nahal, 2021). Fresh bone typically comprises 20-35% organic material, with collagen constituting about 90% of this organic fraction, varying by species and bone type. The organic content is associated with a nitrogen level of approximately 3.5-4.5% in bone and a C ratio of 3.2 in human remains (Schwarcz and Nahal, 2021).

The extent of collagen preservation is essential for archaeological examinations, especially for species identification and other biomolecular investigations. These parameters estimate the degree of collagen degradation and assess the adequacy of remaining collagen for subsequent molecular investigation.

In conclusion, although collagen exhibits greater resilience than other macromolecules, its vulnerability to taphonomic and diagenetic processes presents considerable obstacles for archaeological investigation. Comprehending the environmental conditions that affect collagen degradation is crucial for enhancing the recovery and analysis of ancient biological materials.

2.2 Zooarchaeology by Mass Spectrometry

A major subsection of paleoproteomics research is Zooarchaeology by Mass Spectrometry (ZooMS), it is an analytical technique using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Buckley et al., 2009; Wang et al., 2021). It is used in archaeology, forensic sciences, ecology, and cultural heritage to identify collagenous materials like bone, ivory, antler, parchment, leather, and other soft tissues (Parker, 2021; Cappellini et al., 2018; Welker, 2018; Warinner et al., 2022). ZooMS allows for the identification of species from fragmentary remains by analyzing the collagen peptides present, offering insights into various topics such as faunal assemblages, ecological niches, and human-animal interactions (Richter et al., 2022).

Collagen, the most genetically prevalent biomolecule in contemporary bone, increases in abundance in prehistoric bone with time due to the fast breakdown of DNA and non-collagenous proteins. This process transpires in bone samples with less collagen, contingent upon the fulfillment of particular conditions that facilitate collagen preservation (Buckley, 2018). Zooarchaeology by Mass Spectrometry (ZooMS) is generally utilized for genus- and family-level identifications, yet it can also yield species-specific results. This capability is influenced not only by the type of material being analyzed, such as bone, parchment, or other collagenous substrates, but more significantly by the preservation quality of the proteins contained within the material (Buckley et al., 2016, 2014, 2009; Harvey et al., 2019; Welker et al., 2016). The durability and consistency of collagen—especially in bone—enable ZooMS to remain a reliable technique, even amidst diverse preservation scenarios. Collagen, specifically type I collagen (COL1), is the principal focus of ZooMS owing to its robustness. The quantity and resilient nature of collagen, marked by densely arranged fibrils and cross-linkages, allow it to persist in the archeological record for prolonged durations, even in adverse settings (Henriksen and Karsdal, 2019; Shoulders and Raines, 2009). The ZooMS methodology generally entails collagen extraction utilizing hydrochloric acid or ammonium bicarbonate, succeeded by enzymatic digestion. The isolated peptides are purified and concentrated prior to examination by soft ionization mass spectrometry (Buckley et al., 2009; van der Sluis et al., 2014; van Doorn et al., 2011; Welker et al., 2015). The resultant peptide spectra are further juxtaposed with a reference library to attain precise taxonomic identification (Buckley et al., 2016, 2014, 2009; Welker et al., 2016).

Buckley and Collins (2011) assert that the identification of animal fossils contributes to understanding not only how humans interacted with them in the recent past but also how they managed and were influenced by changing environmental conditions in the distant past. Bone fragmentation often leads to the complete loss of morphological diagnostic criteria used for species identification. According to Welker et al. (2015), these taxonomically unidentified bone specimens may contain significant behavioral, ecological, or molecular information. Silvestrini et al. (2022) emphasize that in Paleolithic contexts, the high degree of fragmentation of bone assemblages is a common feature. In archaeological contexts, fragmented bone remains frequently cannot be accurately recognized at the species level only through morphological inspection because of the absence of differentiating characteristics. These remnants generally offer indirect evidence of the impacts of numerous factors, including human or carnivore activities. Nevertheless, they rarely provide comprehensive insights into particular hunting habits (e.g., prey species, transit methods, anatomical preferences) or into paleoclimatic and paleoenvironmental conditions. Moreover, The current merger of classic zooarchaeological methods, such as taphonomy and taxonomy, with ZooMS provides significant and thorough insights. Zooarchaeology by Mass Spectrometry (ZooMS) can overcome this constraint by identifying low-frequency faunal elements from otherwise unrecognized bone fragments (Welker et al., 2015). Initial advancements in ZooMS concentrated on the isolation of the collagen $\alpha 2(I)$ telopeptide, which posed significant difficulties for morphologically analogous species such as sheep and goats (Zeder & Lapham, 2010). Taxonomic differentiation among these taxa is contingent upon variations in their $\alpha 2(I)$ collagen sequences, necessitating a minimum evolutionary divergence of 5 million years for accurate identification (Buckley et al., 2010; Buckley & Collins, 2011). Collagen fingerprinting, utilized by ZooMS, has traditionally been employed in the analysis of mammalian bones (Buckley et al., 2009, 2010, 2014). Evans et al. (2022) highlight that the integration of ZooMS with conventional zooarchaeological techniques has considerably expanded the range of recognizable bone pieces, especially in fragmented faunal assemblages. This methodological advancement has enabled precise differentiation between morphologically analogous taxa—such as sheep and goats or cetaceans—by employing molecular evidence to surmount the constraints of visual morphological analysis (Evans et al., 2022; Buckley et al., 2010, 2014; Sinet-Mathiot et al., 2019; Welker et al., 2015). Furthermore, a primary advantage of ZooMS is its capacity to taxonomically classify processed bone artifacts, a capability augmented by the implementation of minimally invasive sampling methods (Desmond et al., 2018; McGrath et al., 2019; Martisius et al., 2020; Luik et al., 2020; Evans et al., 2022). This breakthrough has created new opportunities for the detection of human remains in Paleolithic contexts, providing essential insights into early human behavior (Silvestrini et al., 2022; Welker et al., 2022).

The global implementation of ZooMS is limited, with fewer than 50,000 samples analyzed (Richter et al., 2022). While its integration into conservation is still in the early stages, further development is required for its full incorporation into archaeological and conservation frameworks as it expands to new regions, species, and contexts (Wang et al., 2023; Peters et al., 2021).

2.3 Last Neanderthals to Anatomically Modern Humans

The extinction of Neanderthals (*Homo neanderthalensis*), close relatives of anatomically modern humans, is estimated to have occurred around 40,000 years ago. A major question in paleoanthropology is pinpointing the exact timing of two crucial events: the disappearance of Neanderthals from Eurasia and the arrival of anatomically modern humans (AMH) (Villa & Roebroeks, 2014) and eventually their relationships.

This discussion hinges on two main questions: 1) When exactly did Neanderthals and modern humans coexist in Eurasia? and 2) In which specific locations did their paths overlap? Although Neanderthals were skilled hunter-gatherers with complex toolkits and inhabited various environments for hundreds of thousands of years, they went extinct around 40,000 years ago (Todd, 2006; Ko, 2016; Hawkes et al., 2018; Nielsen et al., 2020)

Anatomically Modern Humans (AMHs) arrived Europe a much earlier than previously thought, with evidence from Grotte Mandrin in France indicating their presence between 56,800 and 51,700 years ago (Slimak et al., 2022), and in Italy, approximately 44,000 to 41,000 years ago at sites such as Grotta del Cavallo and Riparo Bombrini (Benazzi et al., 2011; Benazzi et al., 2015). The emergence of the Uluzzian technocomplex, dated to approximately 43,000 BP, provides compelling evidence of anatomically modern humans in southern Europe, specifically in sites like Grotta del Cavallo, which is associated with this culture (Higham et al., 2024). In contrast, sites such as Riparo Bombrini are linked to the Protoaurignacian (Benazzi et al., 2015), highlighting distinct cultural and chronological phases. The migration of modern humans across Europe ultimately led to the gradual replacement of Neanderthals; however, this process varied geographically and temporally, as evidenced by prolonged coexistence and potential interaction between the two populations in certain regions (Kolodny & Feldman, 2017).

The emergence of AMHs instigated substantial cultural transformations, evidenced by the introduction of new cultural indicators, including art, ornamentation, and advanced bone tools, which have been scarce in earlier Neanderthal assemblages (Arrighi et al., 2019). These innovations are evident in several archeological discoveries throughout the region. AMHs may have had a competitive advantage over Neanderthals during their coexistence due to their complex and diverse subsistence strategy (Peresani et al., 2011; Arrighi et al., 2019; Holt et al., 2018; Vallerand et al., 2024).

Central inquiries are to the methods by which Neanderthals procured resources, especially dietary adaptations, subsequent to the emergence of Homo sapiens, and how these elements influenced their final extinction.

ZooMS, in conjunction with conventional zooarchaeology, has enabled researchers to examine morphologically non-diagnostic osseous pieces from multiple Italian locations, such as the Uluzzian strata at Uluzzo C Rock Shelter and Roccia San Sebastiano cave (Silvestrini et al., 2022). Recent research employing the ZooMS approach has elucidated the subsistence strategies and faunal exploitation patterns of Neanderthals in Italy (Silvestrini et al., 2024).

Nevertheless, data for Neanderthal subsistence practices, especially during the era concurrent with the emergence of Homo sapiens in Europe, is limited (Romandini et al., 2023). Recent zooarchaeological research provides evidence that small mammals, such as fox and hare, were consumed in specific contexts, particularly within the Uluzzian strata at Uluzzo C and Roccia San Sebastiano caves (Romandini et al., 2018; Silvestrini et al., 2022). These findings highlight the variability in human subsistence strategies across different ecological contexts.

2.3.1 Last Neanderthals in Italy

The particular geography of the Italian Peninsula serves as a natural conduit between Alpine Europe and the central Mediterranean, providing a singular viewpoint on the interactions between Neanderthals and Homo sapiens across varied ecological and climatic environments. The peninsula served as a potential obstruction for human and animal mobility, playing a strategic role throughout the Late Middle Paleolithic period (50,000–40,000 BP) (Benazzi et al., 2011; Higham et al., 2011; Moroni et al., 2018; Romandini et al., 2019). This region is posited to have functioned as a refugium during glacial periods, especially in southern Italy, where specific Neanderthal populations may have sought sanctuary from severe climatic conditions (Richter, 2022; Silvestrini et al., 2022; Boschin et al. 2022; Silvestrini et al., 2024). The concept of refugium is crucial for comprehending how Neanderthals endured in specific regions of Europe during environmental extremes.

The southern region of Apulia, in particular, exhibited additional difficulties owing to its proximity to Greece and Albania. This site likely facilitated potential migration routes and served as a nexus for initial interactions with emerging technocomplexs associated (Moroni et al., 2013; 2018) with the earliest Homo sapiens populations (Romandini et al., 2023). Italian archaeological research has examined the interaction between Neanderthal behavior and the diverse geological, ecological, and cultural factors that shaped settlement patterns throughout the Italian Peninsula and central Mediterranean (Guidi & Piperno, 1992; Stiner, 1994; Kuhn, 1995; Milliken, 2000; Mussi, 2001; Peresani, 2011; Romandini et al., 2023).

Moreover, Apulia's southeastern position during the Mousterian period (about 50,000–40,000 BP) situated it at a nexus between various regions in southern Italy and the Gulf of Taranto area. ,

Southern Italy was one of the first European regions inhabited by *Homo sapiens*. In fact, one of the oldest archaeological remains attributed to our species was excavated in Apulia (Grotta del Cavallo, ~45 thousand years ago - kya) (Moroni et al. 2018; Higham et al 2024). Southern Italy, particularly Apulia, is highlighted as a key region for investigating the interaction between late Neanderthals and early Modern Humans (MHs) due to the evidence of co-existence. Archaeological sites like Grotta del Cavallo (MH presence) and Riparo L'Oscurusciuto (late Neanderthal presence) in Apulia suggest this co-existence may have lasted several hundred years (Higham et al., 2014; Boschin et al., 2021). This extended period of overlap allows researchers to study the influence of the environment on both groups during this critical period (Boschin et al., 2021).

2.4 Oscurusciuto Rock Shelter

The Oscurusciuto rock shelter (Fig. 1), an important location of ongoing academic inquiry, systematic investigation, and careful excavation since 1998, provides a unique context for evaluating the effectiveness of Zooarchaeology by Mass Spectrometry (ZooMS) in reconstructing Neanderthal subsistence strategies. The research at Oscurusciuto, directed by the U.R. Preistoria e Antropologia of the University of Siena's Department of Physical Sciences, Earth, and Environment, has been carried out with the approval of the Soprintendenza Archeologia Belle Arti e Paesaggio for the provinces of Brindisi, Lecce, and

Taranto. Following the local discovery of lithic artifacts and skeletal remains in 1997, Professors Annamaria Ronchitelli, Paolo Boscato, and Paolo Gambassini from the University of Siena incorporated Oscurusciuto into their comprehensive research on Middle Paleolithic habitation in Southern Italy.

Figure 1: Panoramic view of the Ginosa ravine and the Oscurusciuto rock shelter (red arrow). Photo by: A. Ronchitelli.

The Oscurusciuto rock shelter in Ginosa, Apulia, Southern Italy, is a crucial archaeological site for comprehending the behavioral and environmental adaptations of Neanderthals throughout the Middle Paleolithic (Marciani et al., 2016, 2018, 2020). Located at a pivotal geographical intersection, the site provides understanding of how the region's distinctive environment shaped Neanderthal survival tactics in a transitional landscape between the Mediterranean and the European mainland (Spagnolo et al., 2020). The site's stratigraphy, consisting of various occupation strata, offers essential information on Neanderthal habitation patterns, technical progress, and subsistence methods.

Recent chronological analyses have impacted our comprehension of Oscurusciuto's occupational chronology. A new chronological model presented by Higham et al. (2024) suggests that Neanderthals continued to live in Southern

Italy well into the Upper Paleolithic, challenging previous assumptions. Their research, utilizing advanced radiocarbon and Bayesian calculations, suggests the existence of Neanderthals approximately 43,000 years ago, corresponding with the emergence of early modern human communities. The application of these new dating methods, particularly the reanalysis of SU 1 and other layers, reveals a brief but considerable chronological overlap between Neanderthals and the emerging Uluzzian technocomplex. This evidence demonstrates that the interactions between these communities were more complicated and longer than previously thought, providing a more nuanced understanding of Neanderthal habitation in the region (Higham et al., 2024).

Recent dating has enhanced our comprehension of the chronology, with tephra study verifying the existence of Mount Epomeo green tuff in SU 14, dated to around 55,000 BP, placing this layer inside MIS 3 (Allen et al., 2000; Marciani et al., 2020). Radiocarbon dating of collagen in SU 1 indicates that Neanderthal habitation occurred approximately 42,724 ± 716 BP (Marciani et al., 2018). The stratigraphy of Oscurusciuto is notable for its depth, surpassing six meters, and its preservation of multiple occupational layers (Spagnolo et al., 2020).

The faunal remains reveal deliberate subsistence tactics centered on large ungulates like aurochs and horses (Boscato et al., 2017; Boschin et al., 2021). Neanderthals utilized a methodical strategy for hunting and carcass preparation, as demonstrated by marrow extraction methods and the presence of a substantial hearth for communal activities (Spagnolo et al., 2020). Strata 4 to 7 contain faunal remains suggesting that Neanderthals engaged in subsistence activities, primarily targeting species adapted to steppe and forest-steppe habitats (Boscato & Crezzini, 2012).

Excavations at Oscurusciuto have uncovered a sophisticated spatial arrangement within the rock shelter. The existence of numerous hearths, arranged in particular configurations, indicates intentional spatial organization, with designated zones for slaughtering, tool fabrication, and potentially resting (Spagnolo et al., 2020). The identification of a potential sleeping area, together with the deliberate arrangement of hearths, underscores the shelter's function as a semi-permanent dwelling, particularly during times of climate volatility (Spagnolo et al., 2016).

Magnetic susceptibility (MS) data from Oscurusciuto, along with speleothem records from the adjacent Pozzo Cucù cave, suggest that the region underwent alternating periods of aridity and humidity throughout MIS 3 (Columbu et al., 2020). The environmental variations presumably affected resource availability and Neanderthal habitation patterns. Notwithstanding these hurdles, faunal evidence indicates that Neanderthals managed to endure in the region by utilizing the varied habitats afforded by the ravine system, which presented both open steppe conditions and more sheltered, vegetated zones (Boschin et al., 2022).

The emergence of AMHs in southern Italy has been linked to the extinction of Neanderthals at Oscurusciuto, which was previously estimated to have occurred around 42,000 BP, as indicated by the presence of Uluzzian assemblages found at neighboring sites such as Grotta del Cavallo (Benazzi et al., 2011). Nonetheless, no direct evidence of interaction between the two populations has been observed at Oscurusciuto.

Faunal remains from the final Neanderthal layers at Oscurusciuto suggest that climatic change was not a major contributor to their extinction, as ecological conditions remained consistent (Columbu et al., 2020). The reduction in Neanderthal numbers in the area may have resulted from demographic pressures or competition with anatomically modern humans, who had advanced hunting implements, such as projectile weapons (Sano et al., 2019).

In conclusion, the Oscurusciuto rock shelter provides a unique insight into the final phases of Neanderthal occupancy in southern Italy. The integration of stratigraphic, faunal, and paleoenvironmental data from the site offers significant insights on the endurance and persistence of Neanderthal communities amid climatic and demographic adversities. As research progresses in elucidating this pivotal epoch in human prehistory, Oscurusciuto remains an essential site for investigating the complex interactions between Neanderthals and early modern humans in the Mediterranean geographical region.

2.4.1 Geological Features and Paleoenvironmental Conditions

The region of Oscurusciuto predominantly consists of sedimentary rocks, which are created through the compaction of accumulated sediments such as sand, silt, and clay into solid strata over time. The earliest geological strata in this region originate from the Upper Cretaceous period, approximately 100 million years ago, and are referred to as the Calcare di Altamura formation. This formation primarily consists of limestone, a sedimentary rock that originates in marine environments from the remnants of ancient marine animals such as corals and shellfish. The limestone is overlain by younger strata from the Pleistocene epoch, a more recent geological period that spanned around 2.6 million to 11,700 years ago. The Calcareniti di Gravina and Conglomerato d'Irsina formations were created through the accumulation of marine sediments and fluvial deposits throughout periods of sea level fluctuation caused by glaciations. The Pleistocene was characterized by substantial climatic fluctuations, notably the Ice Ages, during which glaciers enveloped a considerable portion of the northern hemisphere. In areas such as southern Italy, the sea level would have fluctuated in accordance with the expansion and retreat of glaciers, resulting in the deposition of various sediment types as evidence of these alterations (Boenzi et al., 1971; Martini et al., 2020).

The Conglomerato d'Irsina formation, located near Oscurusciuto, is noteworthy for its inclusion of pebbles from several rock types, such as radiolarite, granite, and quartzite. The stones were conveyed from the Apennine Mountains, a proximate mountain range, by rivers and streams during the Pleistocene epoch. This formation signifies the dynamic characteristics of the environment, wherein rivers were actively sculpting the terrain and depositing materials at the location (Boenzi et al., 1971; Marciani et al., 2020). The varied geological formations in Oscurusciuto not only serve as a habitat for humans but also yield essential insights into the environmental circumstances encountered by Neanderthals. The limestone of the Calcare di Altamura is readily eroded, resulting in the formation of caves and rock shelters that provide natural dwellings for both humans and animals. Limestone, abundant in calcium carbonate, reacts with acidic rainwater, gradually eroding the rock and forming voids. These natural shelters would have been optimal for Neanderthals, providing protection from the elements and a strategic perspective of the surrounding environment (Martini et al., 2021). The presence of superior stone resources would have impacted the habitation patterns and technological approaches of these ancient human groups (Spagnolo et al., 2020). Consequently, the region's geology not only sculpted the terrain but also affected the conduct of its human residents.

Sediments at the site have been accumulated by several natural processes, including rockfalls, aeolian deposition, and fluvial activity. These procedures have safeguarded artifacts and bones by fast burial, shielding them from weathering and erosion. The deposits from the Mount Epomeo volcanic eruption, located in Stratigraphic Unit 14 (SU 14), signify a notable geological occurrence that resulted in a layer of tephra—comprising volcanic ash and debris. This ash layer functions as a natural landmark, enabling researchers to accurately date the adjacent strata and enhance their comprehension of the Neanderthal habitation timeframe. The tephra offers a "snapshot" of the conditions of deposition, as volcanic eruptions can significantly transform the environment, affecting climate, flora, and animal populations. The swift accumulation of volcanic ash likely contributed to the remarkable preservation of artifacts and bones in this layer by rapidly entombing and shielding them from subsequent disturbance (Marciani et al., 2020; Martini et al., 2020).

Alongside sedimentary studies, ichnology—the examination of trace fossils, including footprints, burrows, and other indicators of animal activity—offers additional understanding of the environment at Oscurusciuto. The examination of ichnofabrics, or the physical structures imprinted in sediment by organisms, indicates intervals of non-deposition or less sedimentation, during which organisms inhabited the surface. These trace fossils are essential for recreating the paleoenvironment, as they signify intervals when the site was not entirely covered by silt and was accessible for habitation by Neanderthals or other species (Martini et al., 2021).The existence of vertical burrows and tunnels inside specific sediment layers indicates that the site was intermittently accessible to surface-dwelling creatures, presumably during periods of reduced or halted sedimentation. These intervals of non-deposition may signify alterations in climate or local environmental circumstances that momentarily diminished sediment influx into the shelter.

The Pleistocene climate variations, especially during Marine Isotope Stage 3 (MIS 3), significantly influenced Neanderthal populations throughout Europe, including those at Oscurusciuto. MIS 3 was marked by a succession of climatic fluctuations, featuring intervals of relative warmth (interstadials) succeeded by colder phases (stadials). These changes would have impacted the availability of resources, including water, game, and vegetation, hence influencing Neanderthal subsistence tactics (Bond et al., 1992).

Notwithstanding these adverse climatic conditions, the evidence indicates that Neanderthals exhibited remarkable resilience, consistently returning to Oscurusciuto following volcanic occurrences such as the Mount Epomeo eruption (Marciani et al., 2020). The shelter's advantageous location and plentiful resources likely contributed to its durability, rendering it an appealing site for human habitation even during environmental stressors. The existence of distinct occupational layers, interspersed with sterile tephra deposits, underscores the Neanderthals' capacity to adapt to and utilize fluctuating environmental conditions, thereby reinforcing the notion of their profound spatial memory—an affinity for particular locales that furnished essential resources for survival (Spagnolo et al., 2016, 2019, 2020).

The geological and paleoenvironmental factors of Oscurusciuto significantly influenced Neanderthal habitation and survival in Southern Italy. The shelter's distinctive geological structures, comprising limestone and marine terrace deposits, established an optimal habitat, while the sedimentary record offers a crucial archive of environmental alterations and human endeavors. Through the integration of sedimentological, ichnological, and paleoenvironmental investigations, researchers (Boscato et al., 2011; Spagnolo et al., 2016, 2019; Martini et al., 2020, 2021) elucidated the intricate connections between Neanderthals and their environment, providing a more profound comprehension of how these ancient humans adapted to the fluctuating Pleistocene terrain.

2.4.2 Stratigraphic Structure and Faunal Exploitation

The scientific significance of the Oscurusciuto rockshelter is attributed to its rich and well-preserved Mousterian sequence. The characteristics of the recovered finds and the stratigraphic sequence of the Oscurusciuto rockshelter were determined to be well-suited for a time-perspectivism approach (Bailey, 2006). This approach incorporates both high-resolution temporal and diachronic perspectives, offering valuable clues for reconstructing the evolution of technical and economic behaviors, hunting strategies, diet, campsite spatial organization, and mobility patterns (Boscato et al., 2011; Marciani et al., 2016, 2018, 2020; Spagnolo et al., 2016, 2019; Boscato & Ronchitelli, 2017). Furthermore, the site is considered to be one of the last refuges for Neanderthals in Southern Italy. Dating of the basal part of layer (layer 1) has been conducted, placing it after 45 ka BP (see Marciani et al., 2019 for a comprehensive review of the last

Mousterian in Italy). Analysis of archaeological finds from the upper part of the succession indicates that some Neanderthal groups frequented the shelter even during a period when anatomically modern humans had already replaced other Neanderthals in nearby regions of the Italian peninsula (i.e., the southernmost Apulian peninsula) (Benazzi et al., 2011; Higham et al., 2024; Moroni et al., 2018; Marciani et al., 2019).

Excavations, initiated in 1998, uncovered fifteen distinct stratigraphic units (US) within the first 3 meters of the deposit (Spagnolo et al., 2016). Units at the base were formed from calcarenite blocks due to the collapse of the rock wall (Boscato et al., 2011). Several distinct Middle Paleolithic occupation phases were revealed by excavations at the Oscurusciuto rock shelter (Ginosa, Southern Italy), a crucial site for understanding the presence of Neanderthals on the Italian peninsula (Spagnolo et al., 2016). The sedimentary infill of this rockshelter, which reached a depth of 5 meters in its central section and covered an area of approximately 60 square meters, preserves evidence of these populations before their disappearance and replacement by Modern Humans (Martini et al., 2021, Boscato et al., 2011).

Two primary dates form the basis of the current chronological framework. The first is the aforementioned radiocarbon date from SU 1. The second date stems from the identification of a tephra layer (SU 14) as Mount Epomeo green tuff, dated to approximately 55 ka BP (Marciani et al. 2020). This positions the upper portion of the stratigraphic sequence within the terminal phase of the

Middle Paleolithic, a period closely linked to the disappearance of Neanderthals in southern Italy – a topic of ongoing scientific debate (Higham et al., 2024). The investigated stratigraphic sequence revealed the presence of numerous combustion structures throughout the middle and lower portions (Spagnolo et al., 2016). Two main hearth typologies were identified in SU 13, SU 11, and SU 9: smaller hearths with diameters around 20 cm and larger ones with diameters approximating 50 cm. In these specific SUs, most hearths were situated along a belt separate from the rock shelter wall. Conversely, a large hearth (approximately 2 meters wide) was found in the northwestern corner of the shelter within SU 7 (Boscato and Ronchitelli, 2008).

Yellowish sandy sediment was identified in subsequent units (Units 7-4), which yielded an abundance of anthropogenic material. A large hearth, approximately 2 meters in diameter, was uncovered within Unit 7 (Boscato et al., 2011). Unit 4 revealed a layer of loose, medium-sized stones lacking any structured organization. A significant quantity of anthropogenic materials were found in a sub-horizontal position within this layer (Boscato et al., 2011).

Analysis of faunal remains from Units 1 and 4 indicated that the assemblage consisted exclusively of ungulate remains, dominated by *Bos primigenius* (aurochs) (Boscato & Crezzini, 2012; Boscato et al., 2011). This suggests a selective targeting of this species by the Neanderthal inhabitants. It was observed that numerous fragmented long bones, especially from unidentified animals, were dominated by diaphysis portions at Oscurusciuto (Boscato et al., 2011). Aurochs

(*Bos primigenius*), the most abundant ungulate identified, were primarily represented by long bones such as humerus, radius, femur, tibia, and metapodials (Boscato et al., 2011). The scarcity of epiphyses, rich in fat and marrow, could be explained by their potential use as fuel (Boscato et al., 2011). Tooth remains from Unit 4 were analyzed, suggesting that hunting targeted adult aurochs. This finding is consistent with other Final Mousterian sites in central and northern Italy (Boscato et al., 2011). However, limitations in sample size from Oscurusciuto restrict definitive conclusions about the hunting strategies employed by Neanderthals at this location (Boscato et al., 2011). The ungulate assemblage in Unit 4 was dominated by *Bos primigenius* (73.5%), indicating open environments and forest steppes (Boscato et al., 2011). Forest ungulates (red deer, fallow deer, and roe deer) comprised only 20% of the total remains. The presence of mountain species like ibex and chamois suggests a diversified hunting territory encompassing various ecological zones (Boscato et al., 2011).

Across all four stratigraphic units, the ungulate assemblage was indicative of open landscapes as the predominant environment. Observed faunal changes hinted at fluctuations towards colder climates in the intermediate units (Unit 3 and Unit group 2), situated between phases with likely more temperate and humid conditions as reflected in Units 4 and 1 (Boscato et al., 2011). Unit 4, being the least eroded deposit, offered a larger excavation area. This provided valuable data for understanding past subsistence practices and environmental context at the Oscurusciuto site (Boscato et al., 2011). Analysis of faunal remains from the lower levels of the deposit (SU 15 to SU 4) revealed a focus on *Bos primigenius* (aurochs) by Neanderthals. Other identified species include *Cervus elaphus* (red deer), *Dama dama* (fallow deer), *Capreolus capreolus* (roe deer), *Equus ferus* (horses), with rare occurrences of *Capra ibex* (ibex) and *Rupicapra* sp. (chamois). Additionally, sporadic remains of carnivores, including *Panthera leo* (lion) and *Canis lupus* (wolf), have been identified. The later upper units exhibit a higher frequency of deer and horse remains (Boscato & Crezzini, 2012; Spagnolo et al., 2016). This faunal assemblage dominated by these taxa suggests a forest-steppe environment with limited areas of wooded coverage.

The dominance of ungulate remains, particularly *Bos primigenius*, suggests a focus on this species for food and potentially other resources. The presence and absence of specific lithic reduction techniques across units hint at a dynamic approach to tool production. Finally, the identified hearths and potential living surfaces provide valuable clues regarding Neanderthal behaviors and spatial organization at the site. The frequency of small limb bones (*carpals, tarsals, phalanges, and sesamoids*) in Unit 4 was significantly higher compared to other Middle Paleolithic cave sites in Apulia (Grotta del Cavallo and Grotta di Santa Croce) (Boscato et al., 2011). This contrasts with the typical Middle Paleolithic pattern observed elsewhere, where marrow extraction focused primarily on long bones (Boscato & Crezzini, 2006).

3

Methodology

3.1 Definition and Rationale for ZooMS

ZooMS, a mass spectrometry technique, has transformed the identification of collagen-rich tissues, especially bone and dentin, in archaeological and paleontological contexts (Buckley et al., 2009). ZooMS provides a distinctive "barcode" for each sample by evaluating peptide masses produced via trypsin digestion, facilitating taxonomic identification even in non-diagnostic bone fragments. This strategy is particularly appropriate for Oscurusciuto, where fragmented and eroded faunal remnants hinder conventional classification methods. ZooMS addresses the challenges of poor DNA preservation by focusing on collagen proteins, particularly COL1, enabling the differentiation of taxonomic groups within faunal assemblages and the identification of probable Neanderthal and anatomically modern human (AMH) remains.

ZooMS has proven effective in pivotal Neanderthal research locations, including Grotte du Renne (Welker et al., 2016), Bacho Kiro (Hublin et al., 2020), Vindija (Devièse et al., 2017), and Denisova Cave (Brown et al., 2021; Douka et al., 2019), greatly enhancing our comprehension of Neanderthal distribution, population dynamics, and interactions with anatomically modern humans. The application at Oscurusciuto may elucidate the evolution of Neanderthal subsistence strategies and ecological adaptations in response to fluctuating environmental conditions.

This research (ongoing) examines 300 bone specimens from stratigraphic units SU 4, SU 5, SU 6, and SU 7 at Oscurusciuto (Figs. 2 and 3).

The bone samples were meticulously collected and cataloged using the OSC (Oscurusciuto Rock Shelter) coding system and processed according to techniques derived from Silvestrini et al. (2022). Collagen extraction and peptide analysis were accomplished with precision to preserve sample integrity, employing a stringent demineralization process in hydrochloric acid, succeeded by thermomixer-assisted collagen gelatinization and enzymatic digestion. This methodological rigor is crucial for ZooMS's taxonomic resolution and for guaranteeing dependable insights into the faunal elements of the Oscurusciuto ensemble.

The expected results of ZooMS analysis on the Oscurusciuto bone samples are diverse:

- **Taxonomic Resolution:** ZooMS facilitates extensive taxonomic classification, differentiating between human and animal remains through collagen markers.

- **Understanding Neanderthal Conduct:** ZooMS's capacity to distinguish bone implements and surface alterations provides significant insights about Neanderthal resource exploitation, hunting methodologies, and ecological adaptations. These findings may clarify Neanderthal reactions to the fluctuating climatic circumstances in southern Italy during the Middle Paleolithic. Nevertheless, further investigation may be required to definitively assign these markers to Neanderthal occupancy strata.

- **Unveiling Faunal Diversity and Hominin Behavior:** ZooMS can contribute to understanding the regional and temporal distribution of Neanderthal and AMH remains, providing insights into hominin interactions when human fragments are identified. However, the success of these analyses depends on adequate collagen preservation, and the use of ZooMS in combination with other analytical techniques (aDNA) can enhance these investigations.

The implementation of ZooMS at Oscurusciuto is poised to enhance paleoproteomic research and enrich our comprehension of late Neanderthal behavioral tactics and ecological adaptations. This methodological paradigm is especially beneficial for locations where conventional methods may be constrained by preservation issues, highlighting the importance of ZooMS in recreating intricate paleoecological and subsistence histories for prehistoric hominins.

3.2 Step-by-Step Implementation of ZooMS

The ZooMS investigation focused on bone samples from stratigraphic units SU 4, SU 5, SU 6, and SU 7 (Figures 2 and 3). SU 4, SU 5, SU 6, and SU 7 were subjected to a comprehensive scanning methodology. The sample excavation dates span from 16 August 1999 to 15 September 2017. The site is culturally associated with the Mousterian period, with age estimations ranging from 55,000 to 43,000 years. Three hundred bone fragments underwent ZooMS analysis.

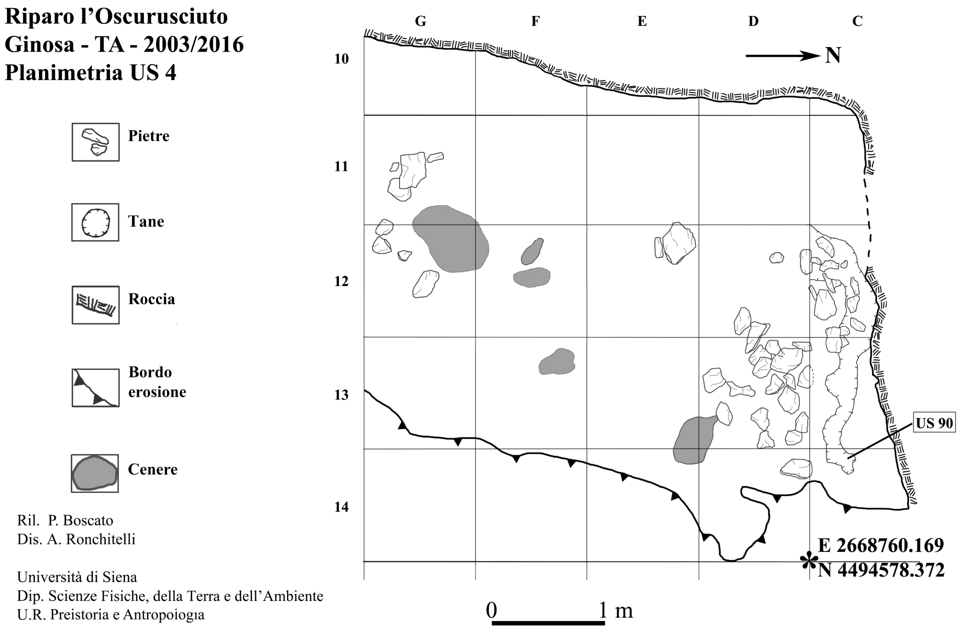

Figure 2: SU 4

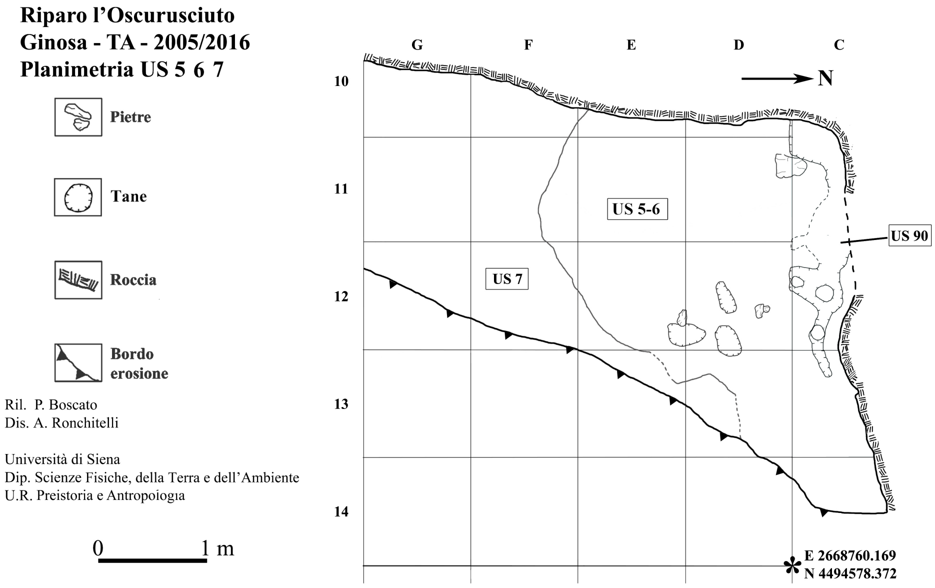

Figure 3: SU 5, 6, 7

Bone samples were collected using a dentist drill at the U.R. of Prehistory and Anthropology within the Department of Physical Sciences, Earth and Environment at the University of Siena. Collagen extraction was performed at the Bones Lab within the Department of Cultural Heritage at the University of Bologna. Each bone was sampled with care, extracting approximately 80-100 mg of material using a disc. For some samples, the mass ranged from 80 mg to 200 mg, depending on the bone's condition, with most falling within the standard 80-100 mg range. The sampling discs were meticulously cleaned with ethanol and bleach after each use to prevent contamination.

Following Silvestrini et al. (2022), the bone samples underwent demineralization in 0.6 M HCl solution. Typically, 250-300 µL of HCl was used per sample, though higher volumes were applied for larger samples. The demineralization period lasted from 1 to 7 days, depending on the state of the samples. After demineralization, samples were centrifuged at 13,000 rpm for 2 minutes to remove the acid, then transferred to the ammonium bicarbonate (Ambic) phase. Each sample was treated with 250 µL of Ambic in two 10-minute intervals, followed by a 3-hour incubation at 65°C in a thermomixer to induce collagen gelatinization. After gelatinization, samples were transferred to new 1.5 mL Eppendorf tubes and prepared for enzymatic digestion.

Trypsin, a protease that cleaves peptide bonds in proteins, was added in volumes of 0.8 µL per sample, and the samples were incubated in the thermomixer for 17.5 hours at 37°C to facilitate digestion. Enzymatic digestion was halted with the addition of 0.8 µL of 5% formic acid (FA). Samples were centrifuged at 13,000 rpm for 2 minutes, followed by purification and concentration of the collagen peptides using stage tips. Stage tips were formed by filling the tip of a pipette with 3 disks of C18 resin.

Then, stage tips must subsequently be conditioned, adding 75 µL of 100% ACN followed by centrifugation at 7,000 rpm for 2 minutes. In this step, performed twice. After removal of the liquid, 75 µL of 0.1% FA was added, followed by another centrifugation. This FA step was also repeated twice. At this point, stages are conditioned and the samples are loaded so that the collagen peptides bind with the conditioned resin. Finally, collagen peptides were eluted with a solution made with 50% ACN/0.1% FA in two steps of 25 µL, to yield a final volume of 50 µL.

Samples were then left uncovered overnight before being transferred to a freezer for the procedure for the MALDI-TOF mass spectrometry. The procedure involved combining the matrix with the sample and putting it to a designated steel plate following sonication for homogenization. The desiccated specimen was analyzed utilizing a RapifleX Tissuetyper MALDI-TOF mass spectrometer (Bruker Daltonics) within the mass-to-charge range of 700-3500 m/z.

3.3 Limitations and Challenges in Applying ZooMS at Oscurusciuto Rock Shelter

The application of Zooarchaeology by Mass Spectrometry (ZooMS) at the Oscurusciuto Rock Shelter can provide significant insights into Neanderthal subsistence practices via the reconstruction of faunal assemblages. Nonetheless, certain methodological problems constrain the efficacy of ZooMS in this situation. These hurdles encompass collagen preservation difficulties, sample fragmentation, contamination hazards, and inherent limitations in taxonomic resolution. Comprehending these constraints is essential for precise data interpretation and for enhancing approaches in paleoproteomic research.

3.3.1 Collagen Preservation Challenges

A principal restriction of utilizing ZooMS at Oscurusciuto pertains to the preservation condition of collagen, the essential protein employed for taxonomic identification by peptide mass fingerprinting. The preservation of collagen is significantly influenced by diagenetic variables, including chemical changes, microbial activity, and physical variations in the burial environment (Buckley et al., 2011). Oscurusciuto is marked by shifting soil pH levels, elevated humidity, and varied hydrological conditions, all of which expedite collagen degradation (Collins et al., 2002). Research at the location has recorded soil pH levels varying from acidic to neutral (pH 5.5–7.0) and relative humidity above 80% during specific intervals (Rinaldi et al., 2019). These circumstances facilitate the degradation of collagen molecules, diminishing the probability of obtaining high-quality peptide sequences crucial for accurate species identification. The degradation of collagen not only reduces peptide yield but also compromises the integrity of the peptide markers utilized for taxonomy matching. Insufficient peptide yield or modified peptide profiles hinder the reliable correlation of samples with reference spectra, thereby diminishing the interpretative significance of the results in reconstructing Neanderthal subsistence tactics (Cappellini et al., 2014).

3.3.2 Sample Fragmentation and Morphological Degradation

At Oscurusciuto, faunal remains frequently exist in a highly fragmented condition due to anthropogenic activity, including butchering and marrow extraction, as well as natural taphonomic processes such as soil erosion and bioturbation (Spagnolo et al., 2020). The significant fragmentation results in inadequate or misleading peptide profiles during ZooMS analysis. The diminutive size and inadequate preservation of bone fragments may yield incomplete or deteriorated peptide "barcodes," complicating the attainment of complete matches with reference libraries (Buckley et al., 2017). This constraint diminishes taxonomic resolution, obstructing our capacity to discern species-level differences within the faunal assemblage.

Recent investigations have shown the influence of bone fragmentation on the effectiveness of ZooMS analysis. Richter et al. (2022) demonstrated that fragmentation impacts peptide recovery and heightens the risk of contamination due to the increased surface area exposed. Innovations in mass spectrometry methodologies, including the creation of microfluidic devices and improved peptide extraction processes, have been suggested to address these challenges (Ha et al., 2021). Nevertheless, even technical advancements may not entirely mitigate the difficulties presented by highly fragmented assemblages such as those at Oscurusciuto.

The failure to differentiate between closely related ungulate species occupying distinct ecological niches may hide changes in Neanderthal hunting methodologies or seasonal resource utilization (Gaudzinski-Windheuser & Niven, 2022). Distinguishing between species such as red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) is essential, as they occupy distinct habitats and their presence may signify particular hunting techniques or seasonal behaviors (Karavanić

& Patou-Mathis, 2009). Our comprehension of Neanderthal subsistence practices is constrained without accurate taxonomic identification.

3.3.3 Contamination Risks and Mitigation

Contamination is a considerable problem in ZooMS investigations, as the introduction of exogenous protein residues may occur during excavation, storage, or laboratory processing, potentially resulting in inaccurate taxonomic identifications (Hendy et al., 2018). Notwithstanding the enforcement of rigorous decontamination protocols—including sodium hypochlorite washes, ultraviolet irradiation, and clean-room facilities—contaminants from laboratory environments, reagents, or handling equipment may jeopardize sample integrity (Saitta et al., 2020; Wilson & Collins, 2019). Exogenous proteins may provide deceptive peptide mass signatures, hindering the distinction between genuine ancient peptides and contemporary contamination (Harvey et al., 2019).

Improvements in disinfection methods and authentication procedures have reduced certain dangers. The inclusion of blank controls and procedural blanks in the analytical workflow aids in detecting and accounting for any contaminants (van der Sluis et al., 2021). Furthermore, the establishment of peptide-based authentication standards, including the evaluation of deamidation levels and other post-translational modifications typical of ancient proteins, improves the differentiation between endogenous peptides and contaminants (Cappellini et al., 2018; Schroeter & Cleland, 2016). Nonetheless, these precautions are not

100% accurate, and the ongoing risk of contamination requires careful analysis of ZooMS data (Richter et al.,2022).

Furthermore, diminished collagen yields in ancient specimens intensify contamination issues, as the endogenous peptide signals may be feeble compared to possible contaminants (Presslee et al., 2018). In these instances, supplemental analyses—such as parallel ancient DNA (aDNA) studies or the utilization of high-resolution mass spectrometry techniques—can bolster confidence in taxonomic identifications (Brown et al., 2021; Demarchi et al., 2016). The amalgamation of several lines of evidence is crucial to mitigate the constraints imposed by contamination and to enhance our comprehension of Neanderthal subsistence tactics within the intricate archaeological framework of Oscurusciuto Rock Shelter.

3.3.4 Taxonomic Resolution Limitations

ZooMS is proficient for extensive taxonomic classification but possesses inherent limits in differentiating closely related taxa, especially at the species level (Richter et al., 2022). This constraint impacts the accuracy of ecological reconstructions, as many species may exhibit unique habitat preferences and behaviors that influence Neanderthal subsistence tactics.

At Oscurusciuto, the failure to reliably differentiate between these taxa may mask subtle elements of Neanderthal hunting and foraging behaviors. In the absence of species-level identification, analyses of resource selection, prey availability, and ecological adaptability remain broad.

3.3.5 Implications for Understanding Neanderthal Subsistence

Limitations in species identification stemming from factors such as collagen preservation, sample fragmentation, and taxonomic resolution impede the reconstruction of comprehensive nutrition profiles, evaluation of hunting preferences, and comprehension of seasonal exploitation patterns (Buckley et al., 2017). Precise species identification is essential for understanding the ecological niches of prey species, which subsequently indicates Neanderthal resilience to environmental fluctuations and their selective hunting strategies (Jones et al., 2018).

Comprehensive investigations into the best possible integration of ZooMS datasets with zooarchaeological and taphonomic data are still in the early stages (Smith et al., 2024). This type of integrations are crucial for determining whether Neanderthals at Oscurusciuto exploited resources opportunistically or selectively targeted specific species based on seasonal availability and nutritional value (Smith, 2020).

Furthermore, constraints in recognizing tiny game species hinder our comprehension of the diversity of Neanderthal diets. Recent research indicates that the exploitation of small prey, such as birds and rabbits, was more crucial to Neanderthal subsistence than previously believed (Hardy & Moncel, 2019).

These issues also affect paleoenvironmental reconstructions. The precise identification of faunal remains enhances comprehension of historical habitats and climatic circumstances, which affect subsistence tactics (Luzi et al., 2022). Misidentification or failure to identify species can result in inadequate or biased reconstructions, hence influencing models of Neanderthal ecological adaptations (Discamps & Royer, 2017).

3.3.6 Alternative Analytical Methods and Advancements in ZooMS Methodology

Integrating ZooMS with other biomolecular methods can compensate for individual limitations. Ancient DNA (aDNA) analysis, for instance, offers species-level identification and can distinguish between closely related taxa with high precision (Hofreiter et al., 2021; Slon et al., 2018). Although aDNA is often more susceptible to degradation and contamination due to environmental factors and the ubiquity of modern DNA, ZooMS can serve as a preliminary screening tool to identify well-preserved samples suitable for aDNA analysis (Harvey et al., 2022). This synergy optimizes resources and enhances taxonomic resolution, enabling more accurate reconstructions of past faunal communities.

Stable isotope analysis is another complementary technique that provides insights into the trophic levels, diets, and migration patterns of ancient animals (Bocherens, 2018; Britton, 2022). While it lacks taxonomic specificity, when combined with ZooMS data, it offers a nuanced understanding of ecological relationships and environmental conditions during the Middle Paleolithic. For example, integrating isotopic data with species identifications from ZooMS can reveal patterns of Neanderthal prey selection and seasonal resource exploitation (Naito et al., 2020).

Recent methodological advancements in ZooMS have further enhanced its applicability and effectiveness. The development of ZooMS allows for the analysis of microsamples from bone fragments and other materials with minimal destruction, preserving valuable archaeological specimens (Fiddyment et al., 2019; Hendy et al., 2018; Naihui et al., 2021;Torres-Iglesias et al., 2024). This technique is particularly useful for highly fragmented assemblages, where sample preservation is a significant challenge.

Enhancements in reference libraries and computational tools have also improved matching accuracy and taxonomic resolution. The expansion of spectral databases, such as the Collagen Peptide Mass Fingerprint Database, facilitates more precise identifications by providing a wider range of reference spectra (Buckley et al., 2019). Advanced bioinformatics approaches and machine learning algorithms have been applied to ZooMS data, increasing the speed and accuracy of species identification (Coutu et al., 2021).

Moreover, the advent of ZooMS-Plus, which integrates ZooMS with advanced proteomic techniques capable of identifying post-translational modifications, offers potential for distinguishing closely related species that traditional ZooMS struggles to separate (Cappellini et al., 2019). This advancement addresses core limitations by enhancing taxonomic specificity and reliability.

4 Discussion

4.1 Evaluation of ZooMS Methodology at Oscurusciuto

The utilization of Zooarchaeology by Mass Spectrometry (ZooMS) possesses considerable theoretical importance in examining subsistence issues at Oscurusciuto Rock Shelter. ZooMS's capability to identify species using collagen peptide mass fingerprinting provides a reliable method for taxonomic classification, even in the absence of distinguishing physical characteristics in bone fragments (Buckley et al., 2017). In scenarios such as Oscurusciuto, where faunal remains are extensively fragmented due to anthropogenic activity (Spagnolo et al., 2020), conventional zooarchaeological techniques encounter constraints. ZooMS can address these challenges by analyzing the preserved collagen in small bone fragments, thus providing insights into species composition and ecological adaptations of Neanderthal populations.

Recent investigations have established ZooMS's efficacy in detecting several taxa from fragmented assemblages (Brown et al., 2021). Welker et al. (2017) effectively utilized ZooMS on Middle Pleistocene bones from the UK, discovering species that were previously indiscernible due to fragmentation. These findings indicate that ZooMS may provide analogous advantages at Oscurusciuto by improving our comprehension of the site's faunal variety and Neanderthal subsistence tactics.

Furthermore, ZooMS has demonstrated efficacy in identifying unusual or unforeseen species within assemblages, providing novel insights about Neanderthal ecology (Harvey et al., 2019)). Identifying such species at Oscurusciuto could uncover previously unrecognized dimensions of resource utilization and movement patterns. Consequently, ZooMS's theoretical significance is in its ability to enhance conventional zooarchaeological assessments, offering a more thorough understanding of Neanderthal lifestyles.

4.2 The Role of Paleoproteomic Methods in Archaeology

Paleoproteomic techniques, such as ZooMS, are gaining acknowledgment for their significance in archaeology, especially in scenarios with inadequate preservation of ancient DNA (aDNA) (Cappellini et al., 2018). Proteins, due to their greater stability than DNA over extended durations and diverse environmental circumstances, can persist in samples when genetic material has deteriorated beyond analytical capability (Cappellini et al., 2019). This resilience renders paleoproteomics an essential instrument for species identification and phylogenetic research in difficult habitats.

ZooMS specifically allows for the swift examination of many fragments, aiding in the reconstruction of faunal assemblages and offering insights on human-animal interactions (Wadsworth & Procopio, 2022). Moreover, proteome investigations can enhance comprehension of historical biodiversity, extinction events, and evolutionary mechanisms, therefore augmenting views of human prehistory (Cappellini et al., 2021).

4.3 Methodological Contributions and Innovations

ZooMS indicates a methodological enhancement compared to standard morphological and genetic investigations, especially in locations devoid of conventional archaeological material. In comparison to aDNA analysis, ZooMS is less susceptible to contamination and diagenetic degradation (Harvey et al., 2019)). The method necessitates minimal sample preparation and is applicable to small, unidentifiable bone pieces frequently found in archeological sites (Buckley et al., 2017).

Advancements in mass spectrometry, including high-resolution methods and tandem mass spectrometry, have improved the sensitivity and specificity of ZooMS, facilitating more precise taxonomic identifications (Kovač et al., 2022). Technological progress, particularly in machine learning methods, has enhanced data processing and analysis, enabling the management of extensive datasets commonly found in archaeological research (Warinner et al., 2022).

4.4 Hypothetical Applications and Scenarios at Oscurusciuto

Availability of extensive ZooMS data for Oscurusciuto could yield various discoveries, demonstrating the efficacy of this approach in analogous archeological environments. ZooMS can detect small and medium-sized mammals frequently overlooked in morphological investigations due to fragmentation (Melchionna et al., 2020). The discovery of such species may suggest a wider food range for Neanderthals, encompassing the utilization of small game and a more diverse subsistence strategy than previously acknowledged.

Additionally, ZooMS was capable of identifying seasonal fluctuations in species presence, elucidating patterns of site occupancy and resource allocation (Jones et al., 2018). Identifying migratory species or ones with particular habitat preferences could provide insights into Neanderthal movement and resilience to environmental changes. The identification of unusual or non-local species may indicate long-distance acquisition (Spagnolo et al., 2022).

Moreover, employing ZooMS methodologies may elucidate prey selection preferences by distinguishing closely related species with unique ecological functions (Harvey et al., 2019)). This degree of specificity would improve models of Neanderthal hunting tactics, prey accessibility, and environmental utilization at Oscurusciuto. These hypothetical uses highlight the capacity of ZooMS to enhance our comprehension of Neanderthal lifestyles when combined with additional archaeological and paleoenvironmental information.

5 Conclusion

5.1 Summary of Methodological Insights

This thesis has analyzed the technical advantages and theoretical contributions of Zooarchaeology by Mass Spectrometry (ZooMS) and paleoproteomics in enhancing our comprehension of Neanderthal subsistence practices at Oscurusciuto Rock Shelter. ZooMS has shown considerable promise in species identification from severely fragmented faunal assemblages where conventional morphological analyses are constrained (Buckley et al., 2017). ZooMS offers essential taxonomic insights through the analysis of collagen peptide mass fingerprinting, hence improving reconstructions of historical ecosystems and Neanderthal feeding habits.

5.2 Assessment of Methodological Contributions

The utilization of Zooarchaeology by Mass Spectrometry (ZooMS) signifies a notable progression in archaeological techniques, especially in locations with inadequate organic preservation and little conventional morphological data. At Oscurusciuto Rock Shelter, faunal remains are frequently fragmented due to taphonomic processes and human activities, complicating standard morphological identification (Spagnolo et al., 2016). ZooMS employs a molecular methodology to extract taxonomic data from limited and degraded specimens by the analysis of collagen peptide mass fingerprinting, consequently improving species-level identifications (Buckley et al., 2017; Brown et al., 2021).

This methodological advancement enables more comprehensive reconstructions of historical settings, resource utilization patterns, and subsistence strategies, which are essential for understanding human behavioral evolution (Cappellini et al., 2018). ZooMS enhances our comprehension of Neanderthal nutritional diversity, hunting methodologies, and ecological adaptations at Oscurusciuto and other Middle Paleolithic locations by accurate species identifications (Harvey et al., 2019)).

The non-destructive or minimally invasive characteristics of ZooMS sampling safeguard important archaeological artifacts for future research, conforming to optimal methods in cultural heritage preservation (Fiddyment et al., 2019). Additionally, ZooMS can identify unusual or unforeseen species within assemblages, providing novel insights into Neanderthal ecology and movement patterns (Welker, 2018). The integration of ZooMS with additional analytical techniques amplifies its methodological contributions.

Moreover, paleoproteomics enhances ZooMS by providing profound insights into evolutionary linkages and phylogenetic histories via protein sequencing (Cappellini et al., 2019). The examination of ancient proteins can surpass the temporal constraints of ancient DNA (aDNA), offering insights into deep-time evolutionary occurrences and addressing phylogenetic inquiries where DNA preservation is inadequate (Welker, 2018). Utilizing these methodologies in archaeological sites such as Oscurusciuto enhances our comprehension of Neanderthal populations and their environmental relationships.

The incorporation of sophisticated analytical techniques, including high-resolution mass spectrometry and enhanced bioinformatics tools, augments the sensitivity and specificity of ZooMS, facilitating species identification among closely related taxa (Harvey et al., 2019); Buckley & Collins, 2019). The creation of extensive reference databases with a broader spectrum of species and regional variants enhances identification precision and expands the applicability of ZooMS across various archaeological contexts (Brown et al., 2021; Wadsworth & Buckley, 2014).

In conclusion, ZooMS and paleoproteomics are important methodological advancements in archaeology, allowing for species identification from fragmented remains, improving taxonomic precision, and aiding in comprehensive reconstructions of historical human activities and environmental interactions. These developments, especially when combined with other analytical methods, provide significant avenues for addressing the constraints imposed by deteriorated archeological artifacts. They ultimately enhance our understanding of Neanderthal lifestyles and lead to a more thorough comprehension of human evolution.

5.3 Recommendations for Future Research

Future research should prioritize methodological advancements and interdisciplinary collaboration to fully exploit the promise of Zooarchaeology by Mass Spectrometry (ZooMS) and paleoproteomics in enhancing our comprehension of Neanderthal subsistence practices. Advancements in technology, including high-resolution mass spectrometry, refined peptide sequencing algorithms, and sophisticated bioinformatics tools, will markedly enhance the sensitivity and specificity of ZooMS, thereby improving species identification, especially among closely related taxa (Harvey et al., 2019); Buckley & Collins, 2019). Creating extensive reference datasets that include a broader spectrum of species and regional variations would enhance identification precision and expand the use of ZooMS across various archaeological contexts (Brown et al., 2021; Wadsworth & Buckley, 2014).

Combining ZooMS with additional analytical methods, including comprehensive taphonomic analysis, ancient DNA (aDNA) sequencing, and stable isotope research, can yield a comprehensive picture of historical human behaviors and environmental interactions (Cappellini et al., 2018; Welker, 2018). The integration of DNA data with conventional zooarchaeological techniques improves the understanding of Neanderthal hunting behaviors, tool utilization, and ecological adaptations. Identifying specific prey species via ZooMS can elucidate prey selection and hunting strategies, which, when examined in conjunction with cut-mark studies and use-wear analyses, can disclose butchery techniques and resource processing behaviors (Smith et al., 2020; Gaudzinski-Windheuser & Niven, 2017).

Subsequent research ought to investigate the analysis of non-collagenous proteins and peptides to acquire further insights into Neanderthal physiology, health, and environmental stresses (Cleland et al., 2020; Cappellini et al., 2018). Advancements in proteomic technologies may enable the identification of food proteins, infections, or biomarkers that reflect physiological responses to environmental factors. Furthermore, the utilization of non-destructive or minimally invasive sampling methods, including micro-computed tomography (micro-CT) scanning and microZooMS, will enhance the conservation of significant archaeological artifacts while permitting comprehensive molecular analyses (Fiddyment et al., 2019; Hendy et al., 2018).

Collaboration across disciplines is crucial for enhancing methodological strategies. Forming collaborative networks comprising archaeologists, paleoanthropologists, biochemists, and data scientists will improve analytical capacities and data interpretation (Warinner et al., 2017). Investing in training and capacity-building programs will foster methodological standardization and the formulation of best-practice standards, hence improving the reliability and reproducibility of ZooMS and paleoproteomics research (Hendy et al., 2018). Focusing research on underrepresented areas and epochs, such as the Middle Paleolithic in Southern Europe, will enhance the understanding of human evolution. Utilizing ZooMS and paleoproteomics over a wider array of sites, particularly those with difficult preservation conditions, will facilitate the discovery of trends in Neanderthal adaptation and resilience. These investigations provide significant insights into human-environment interactions and the evolutionary processes that have influenced our species (Richter et al., 2022; Sinet-Mathiot et al., 2019).

Moreover, including experimental archaeology helps substantiate interpretations of tool utilization and faunal processing methods. Replicating Neanderthal actions in controlled settings enables scholars to enhance their comprehension of the functional characteristics of bone tools and their implications for cognitive and cultural evolution (Smith, 2020). This method enhances molecular analysis by offering context to the behavioral patterns derived from biochemical data.

In conclusion, the methodological advancements introduced by ZooMS, along with integrative analytical techniques, provide significant avenues for enhancing our comprehension of Neanderthal subsistence tactics and ecological adaptations. Adopting these approaches and promoting interdisciplinary collaboration will allow future research to surmount current limits, thereby making substantial contributions to paleoanthropology and enhancing our understanding of Neanderthal lifestyles.

6

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