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

Integrating ZooMS and Zooarchaeology to Assess the Châtelperronian and Carnivore Occupations at Cassenade (Dordogne, France)

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submitted: 28 November 2023; revised: 24 May 2024; accepted: 29 May 2024

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Handling Editor in Chief: Erella Hovers

ABSTRACT

Archaeological animal bone assemblages are often highly fragmented, meaning that for over 70% of the recovered bone fragments we do not know what animal (or human) species they belonged to. This is especially problematic in Paleolithic contexts, when both humans and carnivores repeatedly occupied the same caves and rock shelters. Identifying bone fragments from these dual occupation contexts through Zooarchaeology by Mass Spectrometry (ZooMS) can provide additional insights into both carnivore and human behavior. In this paper, we apply ZooMS to the majority of morphologically unidentifiable bone fragments larger than 20mm (n=840) recovered from the 2012–2013 excavated Châtelperronian layer of Cassenade (France). Collagen was extracted using an ammonium-bicarbonate (AmBic) buffer and over 99% of the sampled bone fragments could be identified taxonomically. While the proportion of Equidae is similar in both ZooMS and zooarchaeological components, *Bos*/*Bison* is represented by a threefold increase in the ZooMS fraction (50.5% vs. 16.6%). Conversely, Ursidae, the dominant taxa in the morphologically identifiable remains (36.4%), only formed 7.3% of the ZooMS fragments. Carnivores are also present, but in low numbers (0–2%), and include Hyaenidae, *Panthera* and Canidae.

In the ZooMS fraction, only few bones show traces of human activity (ca. 2%), which is most likely related to low bone surface readability. These show that human groups at Cassenade were processing Bovinae and Equidae, as well as Ursidae and rhinoceros. Conversely, traces of carnivore activity are abundant, and we were able to

taxonomically identify 334 bone fragments that were digested by carnivores (as indicated by acid etched surfaces). While large proportions of the Rhinocerotidae (63.79%), Elephantidae (52%), Equidae (48%), and *Bos*/*Bison* (45%) remains were digested by carnivores, this is only the case for 1.7% of the Ursidae ZooMS fragments. Three-dimensional data are available for all the ZooMS-identified fragments and confirm the near-exclusive presence of cave bear in the lower part of the sequence. Further, the ZooMS spatial data identified a restricted presence of mammoth in the middle part of the sequence and a diverging presence of reindeer and Cervid/saiga remains at the bottom and top.

Overall, this study illustrates the added value of integrating zooarchaeological and ZooMS datasets to obtain additional insights into past ecologies, changing site use, carnivore diets, and human subsistence practices.

INTRODUCTION

Across the Paleolithic, humans and large carnivores (such as cave hyaenas and bears) repeatedly occupied the same caves and rock shelters (e.g., Brugal and Jaubert 1991; Daschek and Mester 2020; Discamps et al. 2012; Hussain et al. 2022; Kindler 2012; Rossel and Blasco 2009; Smith 2015; Stiner 1994; Smith et al. 2021; Toniato et al. 2024; Zilio et al. 2021). While at some of these localities remnants of human activities dominate, elsewhere carnivores were the main accumulation agents through hibernation and/or denning, and traces of human occupation are sparse (Airvaux et al. 2012; Discamps et al. 2012, 2019; Smith et al. 2024; Villa and Soressi 2000) or absent (Currant and Jacobi 2011; Jimenez et al. 2022; Schreve 2004). During MIS 3 there is a general increase in the hyaena population across western Europe (Discamps 2011, 2014), and carnivore dens with a low signature of human occupation become more common. Understanding these types of low-density archaeological sites is of importance to fully understand different aspects of human subsistence behavior, including patterns of mobility and human-carnivore interactions.

The Châtelperronian technocomplex groups lithic assemblages across France and northern Spain that are characterized by the production of blades and retouched backed knives (so-called Châtelperronian points) and can be placed around 43,000 to 39,000 cal BP (Rios-Garaizar et al. 2022; Roussel et al. 2015; Ruebens et al. 2015), a time during which both Neanderthals and *Homo sapiens* were present in Europe (Devièse et al. 2021; Fewlass et al. 2020; Higham et al. 2014; Hublin et al. 2020; Mylopotamitaki et al. 2024). The makers of the Châtelperronian remain heavily debated (Bar-Yosef and Bordes 2010; Gravina et al. 2018; Hublin et al. 2012; Ruebens et al. 2015; Gicqueau et al. 2023), with the largest set of fossil evidence coming from Grotte du Renne (58 Neanderthal remains [MNI=7] from three Châtelperronian layers; Bailey and Hublin 2006a, b; Bailey et al. 2009; Hublin et al. 1996; Spoor et al. 2003; Welker et al. 2016; but also see Gicqueau et al. 2023). Consensus is that finding additional human remains from newly excavated faunal assemblages from secure Châtelperronian contexts is key to fully understanding the replacement of Neanderthals by groups of early *Homo sapiens* in western Europe.

Bone fragments 10–30mm in length dominate the vast majority of Paleolithic bone assemblages and, often, cannot be identified taxonomically through traditional approaches based on comparative morphology. Recent advances in soft-ionization mass spectrometry have shown that collagen protein varies by taxa and can be used to identify these bone fragments through peptide mass fingerprinting (Buckley et al. 2009). This method, known as Zooarchaeology by Mass Spectrometry or ZooMS has now been successfully applied to a series of Paleolithic faunal assemblages, both to identify new human remains (Brown et al. 2016; Devièse et al. 2017; Hublin et al. 2020; Welker et al. 2016), as well as to provide additional data on Paleolithic subsistence practices (Brown et al. 2021c; Morin et al. 2023; Pothier Bouchard et al. 2020; Raymond et al. 2024 [this volume]; Ruebens et al. 2022, 2023; Silvestrini et al. 2022; Sinet-Mathiot et al. 2019, 2023; Torres-Iglesias et al. 2024).

In this paper, we apply large-scale ZooMS screening to the unidentifiable bone fraction of the site of Cassenade (Dordogne, France). The focus is on the recently excavated Châtelperronian layer, which has a strong occurrence of carnivores, and a moderate signature of human occupation (Discamps et al. 2019). In a first step, we apply ZooMS to provide additional taxonomic identifications to enhance the faunal spectrum. These ZooMS identifications are then integrated with existing taphonomic (e.g., fragmentation, bone surface preservation, and modification) and zooarchaeological (e.g., species and skeletal element identifications) data. Secondly, we integrate glutamine deamidation values to assess patterns of biomolecular preservation between taxa and in relation to carnivore digestion. In a third step, we use the available spatial data to obtain a more detailed understanding of faunal changes over time. Finally, combined, this extensive ZooMS-zooarchaeology data integration allows further insights into both carnivore and human presence and behavior at this site.

BACKGROUND

The site of Cassenade (Figure 1a; Saint-Martin-des-Combes, Dordogne, France), is an open karstic system and was discovered and first excavated in the 1970s by Michel Besse. He recovered faunal remains and lithics from both a Mousterian and Châtelperronian context. In 2012 and 2013 a new excavation campaign led by E. Discamps focused on the Châtelperronian unit (lithostratigraphic layer 2, Figure 1c) in a 16m^2 area south of the previous excavations (Figure 1b,

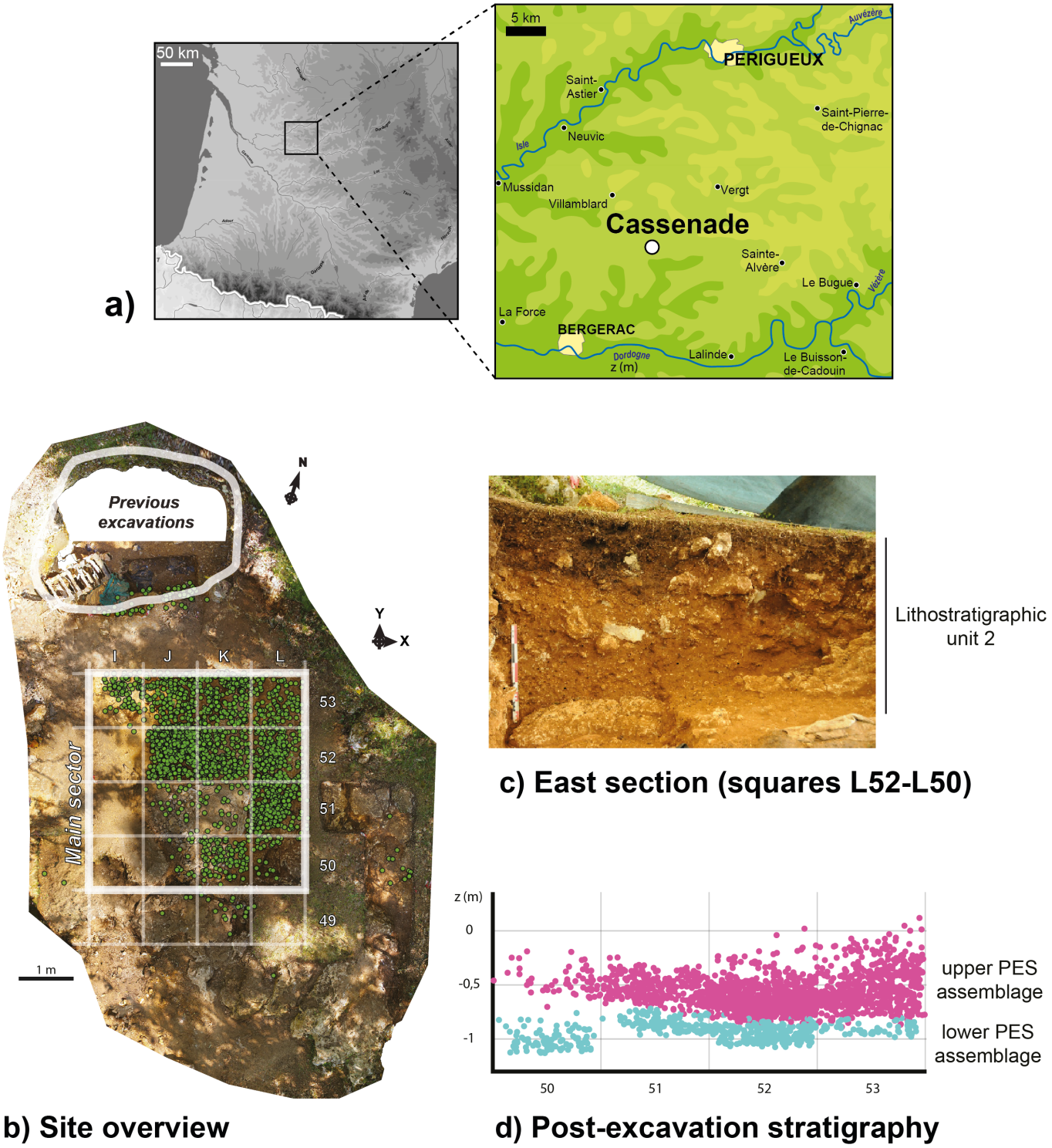

Figure 1. a) General overview of location of the site of Cassenade in the Dordogne (France); b) overview of the location of the new excavations (I53-L50) south of the previous excavations (zenithal orthophotography); c) stratigraphic section of lithostratigraphic unit 2; d) projection of the recovered archaeological material and its division in upper and lower post-excavation stratigraphic (PES) assemblages.

Discamps et al. 2019). These Châtelperronian occupations took place in the upper part of a collapsed karstic corridor. Detailed spatial taphonomy and post-excavation stratigraphic analysis (as defined in Discamps et al. 2023) allowed us to divide layer 2 into an upper part, with hyaena and human presence, and a lower part, during which the site was used as a den by both cave bears and hyaenas (Figure 1d, Discamps et al. 2019). Ten radiocarbon dates place both these human and carnivore occupations of lithostratigraphic layer 2 between 39,000 to 44,000 cal BP.

The 2012–2013 excavations were focused on better understanding the palimpsest nature of the assemblages, and hence all lithic and faunal material larger than 30mm (2012 season) and 10mm (2013 season) was plotted in three dimensions. In total, 2,003 faunal remains, 212 lithics, 125 coprolites, and 9 charcoal fragments were piece-plotted. The lithics are characterized by the use of local raw material to produce blades, six of which were retouched into Châtelperronian points (Discamps et al. 2019). Due to high fragmentation, 70% of the faunal remains could not be identified taxonomically. The morphologically identifiable fauna is dominated by both carnivore (mainly cave bear and cave hyaena) and herbivore (mainly equids and large bovids) remains. Carnivore modifications on the fauna are abundant, while traces of human involvement are difficult to identify and seem limited (e.g., few cut-marked or burnt bone fragments). Because of this ephemeral archaeological signature, Cassenade seems to have been used by Châtelperronian groups for short stopovers, during which they produced and discarded lithic points, as well as processed animal carcasses. In this paper, we will explore how incorporating biomolecular analyses of the morphologically unidentifiable bone fragments, specifically taxonomic identifications through peptide mass fingerprinting, can enhance patterns of faunal diversity and spatial distribution, as well as interpretations of carnivore and human behavior.

METHODOLOGY AND MATERIALS

ZOOARCHAEOLOGY AND SPATIAL ANALYSIS

Zooarchaeological analysis was carried out prior to the ZooMS study (Discamps et al. 2019) on faunal material both piece-plotted (n=1,973) and recovered through screening (n=362). Pieces were morphologically identified as precisely as possible to species, anatomical part, and portion. For plotted bones, unidentifiable fragments were classified according to mammal size classes (see Table 3 below). Taphonomic analysis included the recording of anthropogenic (i.e., cut marks, percussion striae) and carnivore (i.e., gnaw marks, digested bones) modifications, as well as several other bone surface alterations (root etching, concretions, abrasion, dissolution, weathering, manganese deposits). Cortical surfaces were observed under low-angled light using a 20x hand lens and a stereomicroscope when necessary. The preservation, or "readability," of cortical surfaces was recorded also (i.e., percentage of well-preserved cortical surface according to four classes; 0–25%, 25–50%, 50–75%, and 75–100%), as well as the general macroscopic aspect of bones (i.e., hue/patina). Each bone fragment was categorized by 1cm size class (e.g., 2–3cm) using drawn outlines of concentric circles. Three-dimensional coordinates of plotted bone fragments, including the ones analyzed by ZooMS, were used for spatial analysis (see Supplementary Information [SI] 4). Projections in three planes (XY, XZ, YZ, with projection slices of 50cm or less) were reconstructed using QGIS (QGIS.org 2023) and SEAHORS (Royer et al. 2023). Photographs of cut marks (see Figure 5 below) were realized using a Dino-Lite digital microscope.

ZOOMS EXTRACTION AND ANALYSIS

We sampled the majority of the morphologically unidentifiable bone fragments larger than 20mm recovered from the 2012–2013 excavated lithostratigraphic layer 2 (including 626 that could be assigned to the upper part and 209 to the lower part). This approach resulted in 840 individual bone samples (10–30mg), including 32 from the sieves and 808 from the piece-plotted sample, representing 78.6% of the plotted bones above 20mm (n=1,028). Subsequent ZooMS extractions and analyses were conducted at the paleoproteomics lab at the Palaeoanthropology section of the Collège de France (Paris) following existing protocols (Buckley et al. 2009; van Doorn et al. 2011; Welker et al. 2016).

Collagen was extracted using the ammonium-bicarbonate (AmBic) extraction method (van Doorn et al. 2011). All bone samples were first immersed in 100µl of AmBic overnight at room temperature to clean and remove any soluble contamination. After this buffer was removed, the samples were incubated in AmBic for one hour at 65°C to extract soluble protein through gelatinization (van Doorn et al. 2011). Next, 50µl of the supernatant containing denatured protein, was incubated in trypsin at 37°C allowing for the protein to be digested and cleaved into peptides. After 17–18 hours trifluoroacetic acid (TFA) was added to stop the digestion and the peptides were purified, extracted, and spotted in triplicate with an α-cyano-4-hydroxycinnamic acid (CHCA) based matrix solution onto a MALDI plate. Spotted plates were analyzed at the École Supérieure de Physique et de Chimie Industrielles (ESPCI, Paris). Nine empty wells were processed as blanks alongside the bone samples for quality control. All returned empty spectra, illustrating that no contamination was introduced during the lab work.

MALDI spectra were automatically acquired with an AB SCIEX 5800 MALDI-TOF spectrometer (Framingham, MA, 01701, USA) in positive reflector mode for MS acquisition. A droplet of each sample (0.5µl) was deposited on an OptiTOF 384 MALDI plate insert (AB Sciex, Framingham, MA, 01701, USA) in triplicate with α-cyano-4-hydroxycinnamic acid matrix (0.6µl, dissolved at 4mg/mL in a 6:4(v/v) ACN/H2O solution and TFA 0.1% (v/v)). Before sample acquisition, an external plate model calibration was achieved on 13 adjacent MS standard spots with a standard peptide mix (Proteomix Peptide calibration mix4, LaserBioLabs, Sophia Antipolis, France). This calibration is validated according to the laboratory specifications (resolution above 10000 for

573Da, 12000 for 1046Da and 15 to 25000 for other masses, error tolerance <50ppm). For MALDI MS sample measurements, laser intensity was set at 50% after optimization of signal to noise ratio on several spots, then operated at up to 3,000 shots accumulated per spot, and covering a mass-to-charge range of 1000 to 3500 Da.

The obtained mzxml files were exported into txt files in R and merged into single spectra in R using the packages MALDIquant and MALDIquantForeign (Gibb and Strimmer 2012). First, the intensity of the peaks was smoothed applying a moving average function. Second, the baseline was removed using the TopHat method. Third, the spectra were aligned with the SuperSmoother function with a signal to noise ratio set to 3. Finally, the three replicates were summed into a single spectrum and the baseline removed again with the TopHat approach. The obtained .msd files were analyzed in the open source mass spectrometry tool mMass (http://www.mmass.org/, Strohalm et al. 2010) with a signal to noise ratio set to 3. Taxonomic identifications were made through comparisons with existing databases (Brown et al. 2021a; Welker et al. 2016). Glutamine deamidation values, seen as an indicator of biomolecular preservation, were calculated using the Betacalc3 package (Wilson et al. 2012).

RESULTS

FAUNAL SPECTRUM

With the AmBic ZooMS protocol we were able to taxonomically identify over 99% of the sampled bone fragments, indicating excellent collagen preservation (SI 1, SI 2). Eleven different mammalian taxa or taxonomic groups were identified (Table 1, Figure 2). The ZooMS faunal spectrum is dominated by *Bos*/*Bison* (50.5%), followed by Equidae (19.8%). While the proportion of Equidae is similar in the ZooMS and zooarchaeological collections (which include both *Equus ferus* and *Equus hydruntinus*), *Bos*/*Bison* is represented at a threefold increase in the ZooMS fraction (50.5% vs. 16.6%, see Figure 2). Ursidae, the dominant taxa in the morphologically identifiable remains (36.4%), only formed 7.3% of the ZooMS fragments. Other herbivore taxa identified include Rhinocerotidae (7.0%), Cervidae (6.0%) and Elephantidae (2.7%). Morphological identifications indicate the presence of four types of cervids—red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), giant deer (*Megaceros giganteus*), and reindeer (*Rangifer tarandus*). Carnivores are also present in the ZooMS fraction, but in low numbers (0–2%), including Hyaenidae, *Panthera*, and Canidae. *Panthera*, most likely cave lion (*Panthera spelaea*) or potentially the much less common Ice Age leopard (*Panthera pardus spelaea*), are absent in the morphologically identified fauna (see Table 1). Overall, the faunal diversity at the site is high with 10 herbivore and 5 carnivore species.

BONE PRESERVATION

The Cassenade bone fragments are generally poorly preserved due to both heavy fragmentation and taphonomic alterations to the cortical surfaces (chemical alterations, manganese deposits, and root marks) (Discamps et al. 2019). This contrasts with the excellent level of collagen preservation, as indicated by the 99% success rate for ZooMS identifications. Further, at Cassenade, no link can be observed between poor surface preservation (as expressed by cracking, exfoliation, and surface readability) and the ability to provide a precise ZooMS identification, which is in line with other Paleolithic ZooMS studies (Ruebens et al. 2023).

For all ZooMS-identified taxa, the majority of the fragments measure between 20–40mm in length (Figure 3). There does not appear to be any link between fragment size and taxonomic identification. While fragments larger than 30mm are more common for hyaenidae and non-reindeer cervids, their sample sizes are relatively small (respectively n=15 and n=39). A chi-square test illustrates no statistically significant differences between fragment size and taxonomic identity (X^2 [df=21, n=762] = 16.7, p=0.73). Correlations with the masses of the bone fragments are discussed in further detail in Discamps et al. (2024 [this volume]).

SKELETAL ELEMENT REPRESENTATION

Skeletal-part representations are particularly difficult to interpret when ZooMS identifications are used, as the fragmented nature of the sampled bones preclude the use of MNE-based numbers. Despite this issue, inter-species differences can still be explored using NISP values, keeping in mind that they are potentially biassed by fragmentation. In general, teeth are very dominant in the Cassenade faunal assemblage, representing over 70% of the morphologically identifiable assemblage. These teeth are strongly dominated by Ursidae (n=214), Equidae (n=132), Hyeanidae (n=76), and Bovidae (n=61). Part of the ZooMS identified specimens could be identified to broad skeletal groups (e.g., "axial," "long bone," SI 3). In the ZooMS dataset cranial fragments are generally absent or rare (e.g., Equidae (n=1) and Hyaenidae (n=1)), but more common for Ursidae (n=11). However, most of the unidentifiable tooth fragments were not sampled for this study. Ursidae also has the broadest range of skeletal elements represented in the ZooMS dataset, followed by *Bos*/*Bison* and Equidae (see SI 3). Combined with the morphologically identifiable fauna this shows that cranial/postcranial proportions vary widely according to taxa (Figure 4), potentially because small teeth fragments of certain taxa (such as equids) are easier to identify. Cave bear and Hyaenidae remains have all parts of the skeleton represented, dominated by head elements. Juvenile individuals are particularly abundant within these carnivores (68% of bear teeth are deciduous, 42% for cave hyaena), and a large proportion of their teeth probably accumulated as deciduous teeth were shed as part of denning activities. Conversely, for cervids and bovids, long bone fragments are dominant, an observation that fits well with the model of preferential transport to the site of nutrient-rich elements of prey. However, the overrepresentation of bovid long bone fragments in %NISP might be linked to differences in bone fragmentation by species, as discussed in Discamps et al. (2024 [this volume]).

TABLE 1. OVERVIEW OF THE ZOOMS AND MORPHOLOGICAL IDENTIFICATIONS FROM THE CHÂTELPERRONIAN LAYER AT CASSENADE*.

ZooMS ID	Species at Cassenade	ZooMS		Morphological		Combined	
Bos/Bison	*Bison sp. or Bos sp.*	424	50.5%	117	16.6%	**541**	**35.3%**
Equidae	*Equus ferus or Equus hydruntinus*	166	19.8%			**314**	**20.5%**
	Equus ferus			150	21.3%		
	Equus hydruntinus			5	0.7%		
Ursidae	*Ursus spelaeus, Ursus arctos*	61	7.3%	256	36.4%	**317**	**20.7%**
Rhinocerotidae	*Coelodonta antiquitatis*	59	7.0%	14	2.0%	**73**	**4.8%**
Cervid/Saiga/Capreolus	*Cervus elaphus & Megaloceros giganteus & Capreolus capreolus*	10	1.2%			**60**	**3.9%**
Cervid/Saiga	*Cervus elaphus or Megaloceros giganteus*	40	4.8%				
	Cervus elaphus			8	1.1%		
	Megaloceros giganteus			3	0.4%		
	Capreolus capreolus			1	0.1%		
Elephantidae	*Mammuthus primigenius*	23	2.7%	2	0.3%	**25**	**1.6%**
Reindeer	*Rangifer tarandus*	20	2.4%	17	2.4%	**37**	**2.4%**
Hyaenidae	*Crocuta crocuta*	15	1.8%	94	13.4%	**109**	**7.1%**
Hyaenidae/*Panthera*	*Crocuta crocuta/Panthera spelaea*	6	0.7%			**6**	**0.4%**
Panthera	*Panthera spelaea, Panthera pardus, Panthera uncia*	5	0.6%			**5**	**0.3%**
Canidae	*Canidae (not vulpes vulpes)*	4	0.5%			**31**	**2.0%**
	Canis lupus			1	0.1%		
	Vulpes sp.			27	3.8%		
Suidae	*Sus scrofa*	2	0.2%	3	0.4%	**5**	**0.3%**
Ursidae/Felinae		2	0.2%			**2**	**0.1%**
Bird		1	0.1%	1	0.1%	**2**	**0.1%**
Bovidae/reindeer		1	0.1%			**1**	**0.1%**
Leporids				5	0.7%	**5**	
Unidentifiable		1	0.1%			**1**	**0.1%**
Grand Total		**840**	**100.0%**	**704**	**100.0%**	**1534**	**100.0%**

*Morphological data from Discamps et al. (2019). ZooMS spectra and identifications are available in Supplementary Information 1 and 2.

BONE SURFACE MODIFICATIONS

Poor preservation of the cortical surfaces hinders a clear identification of bone surface modifications across the Cassenade assemblage. On about 3% of the bones larger than 30mm, anthropogenic cut marks could be identified (Discamps et al. 2019), yet this number is most likely an underestimation due to the low surface readability (67% of the bones have less than a quarter of their cortical surface well preserved). Evidence of anthropic modifications on faunal material also include three piece-plotted bones that were combusted (in addition to some small burnt fragments present in the sieve residues), as well as one piece-plotted bone with percussion striae. In the ZooMS fraction analyzed for bone surface modifications (n=803), nine bones present notches, but impact notches could not be distinguished from carnivore-induced ones. Most of the ZooMS-identified specimens with cut marks (n=16) or percussion striae (n=1) correspond to Bovinae (n=7) or Equidae (n=7). These taxa already had evidence of anthropic activity in the morphologically identifiable fraction. However, we also identified additional cut marks in the ZooMS fraction on rhinoceros (n=1, Figure 5a) and bear (n=2, Figure 5b, 5c) that should be, in our opinion, considered as evidence of exploitation of these taxa by human groups at Cassenade.

Conversely, traces of carnivore activity are high in the Cassenade assemblage, with 7% of the bones showing trac-

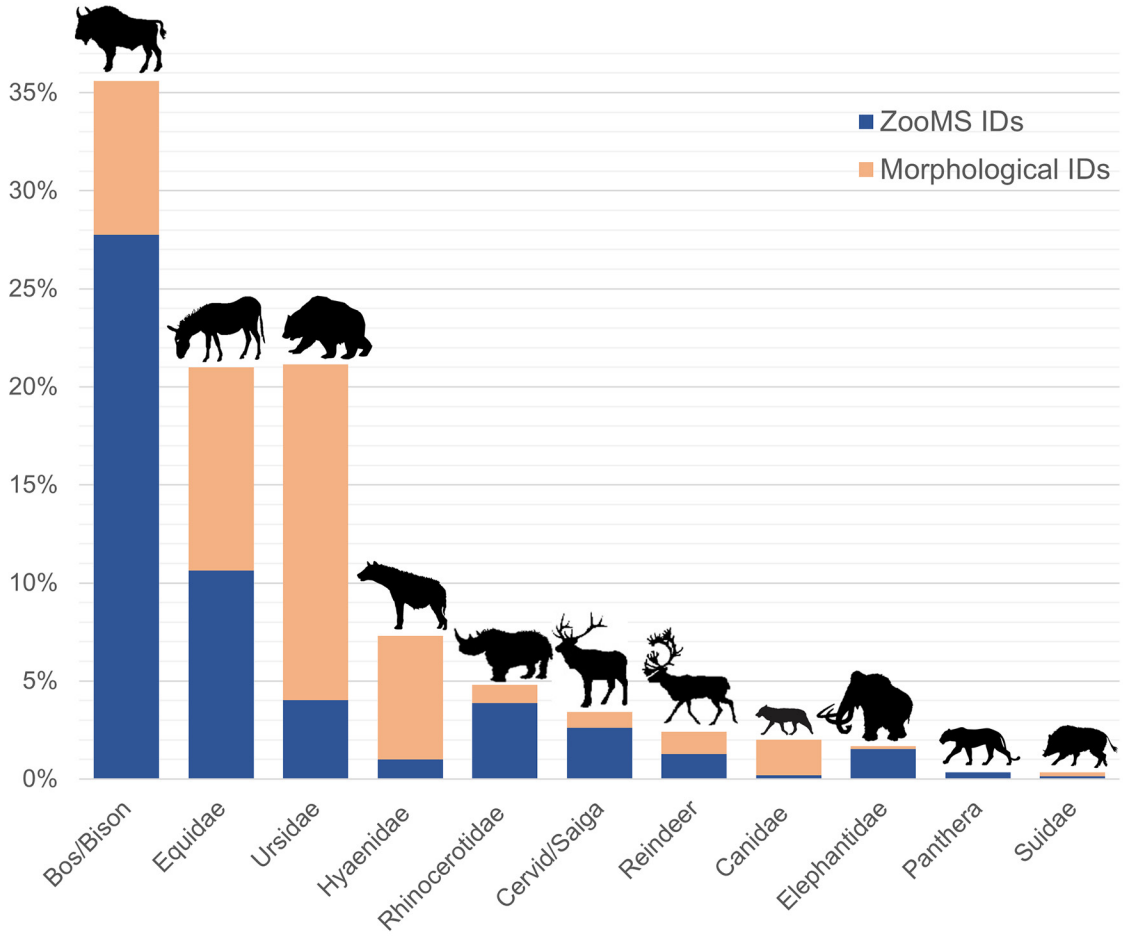

Figure 2. ZooMS (n=800) and morphological (n=697) taxonomic identifications of Châtelperronian faunal material recovered and piece-plotted during the 2012–2013 excavations at Cassenade (raw data available in SI 2).

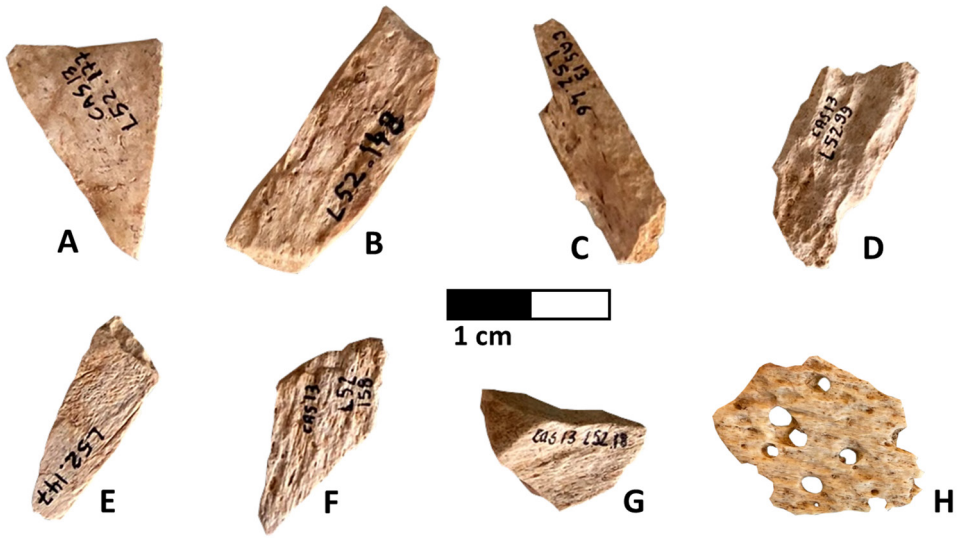

Figure 3. Examples of bone fragments 20–40mm in length from Cassenade identified by ZooMS: A) Bos/Bison *(CAS-283), B)* Bos/Bison *(CAS-285), C) Rhinocerotidae (CAS-286), D) Cervid/Saiga (CAS-289), E)* Bos/Bison *(CAS-291), F) Rhinocerotidae (CAS-292), G) Equidae (CAS-293), H) carnivore digested Rhinocerotidae (CAS-540).*

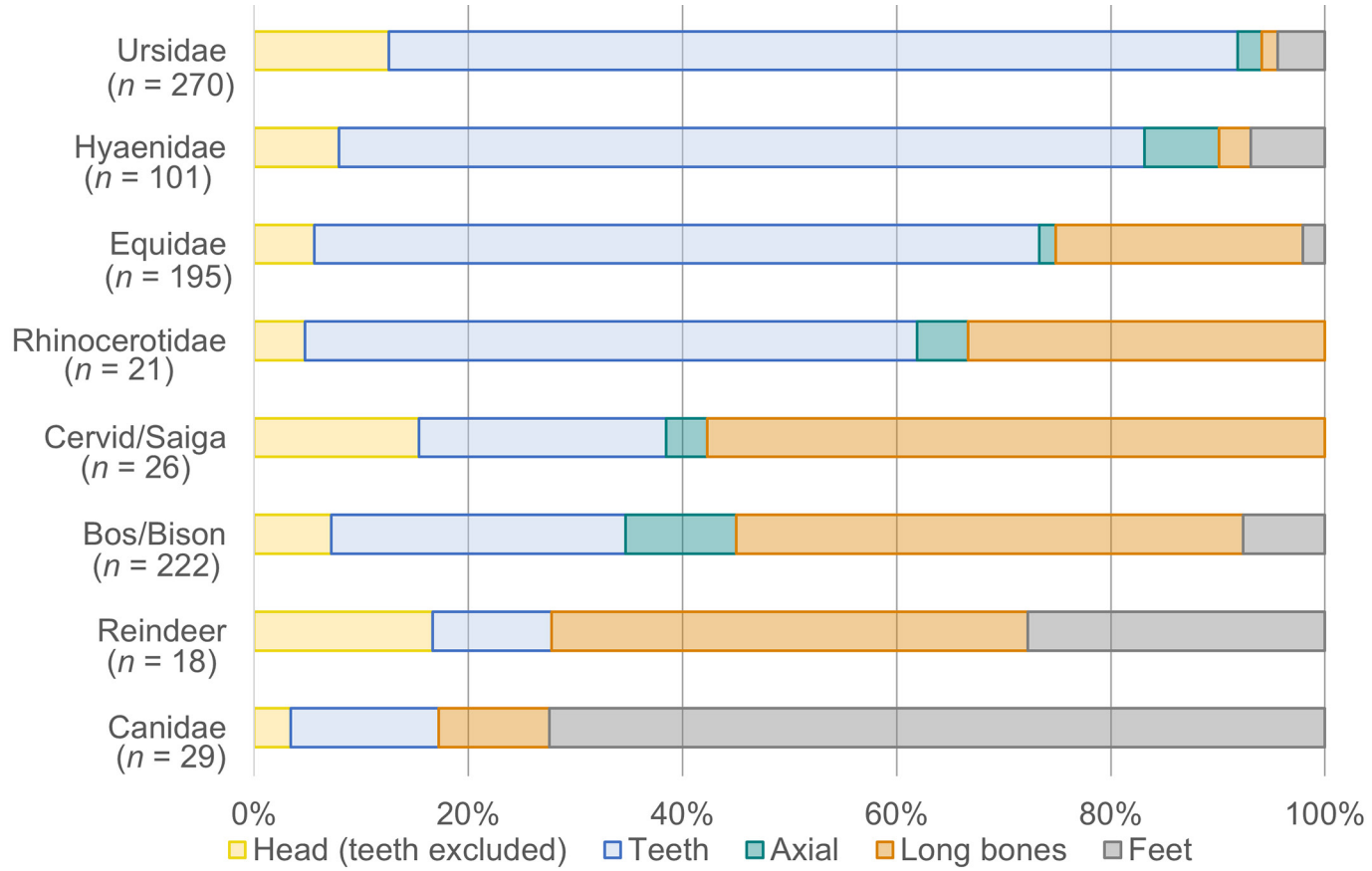

Figure 4. Overview of the skeletal elements (NISP) represented in the combined ZooMS and zooarchaeological datasets for the main taxa present at Cassenade. Morphologically identifiable broken teeth are included. Elephantidae and Suidae are not plotted because of low sample sizes (each have n=3) (for a full breakdown see SI 3).

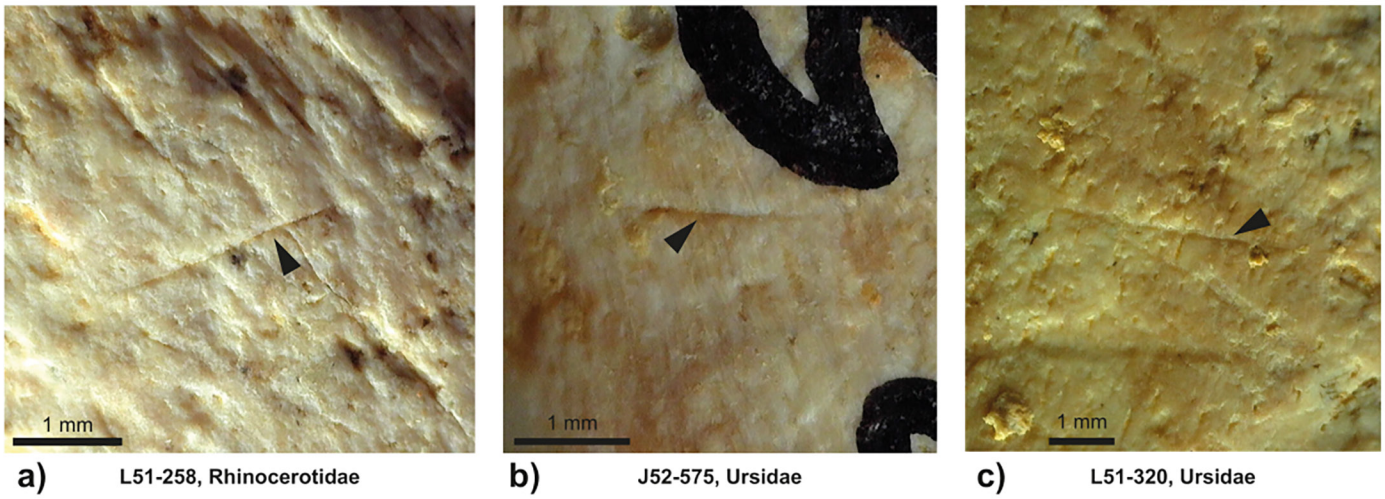

Figure 5. Close-up photographs of the cut marks identified on bone fragments from Cassenade, which were identified with ZooMS as Rhinocerotidae (a), and Ursidae (b and c).

TABLE 2. OVERVIEW OF THE ZOOMS TAXOMOMIC IDENTIFICATIONS OF THE CARNIVORE DIGESTED BONE FRAGMENTS FROM CASSENADE IN RELATION TO THE TOTAL NUMBER OF ZOOMS IDENTIFIED REMAINS FOR EACH TAXA (for a further breakdown see SI 3).

ZooMS ID	Digested	NISP	%
Bos/Bison	187	415	45.06%
Equidae	77	159	48.43%
Ursidae	1	60	1.67%
Rhinocerotidae	37	58	63.79%
Cervid/Saiga	8	39	20.51%
Elephantidae	12	23	52.17%
Reindeer	5	19	26.32%
Hyaenidae	4	15	26.67%
Cervid/Saiga/*Capreolus*	1	9	11.11%
Hyaenidae/*Panthera*	0	5	
Panthera	2	5	40.00%
Canidae (not *Vulpes vulpes*)	0	3	
Suidae	0	2	
Ursidae/Felinae	0	2	
Bird	0	1	
Bovidae/Cervidae	0	1	
Unidentifiable	0	1	
Grand Total	**334**	**817**	**40.88%**

es of gnawing and 38% of digestion. In the ZooMS fraction, eight gnawed pieces are present and we were able to taxonomically identify 334 bone fragments that were digested by carnivores (Table 2, Figure 6). While large proportions of the Rhinocerotidae (63.79%), Elephantidae (52%), Equidae (48%), and *Bos/Bison* (45%) remains have been digested by carnivores, this is only the case for 21% of the cervids (including reindeer) and a mere 1.7% of the Ursidae ZooMS fragments (see Table 2). Two bones have both carnivore and anthropic marks on their surfaces; however, these traces do not overlap, making it impossible to discern the chronology of the access to bones by the two agents.

BIOMOLECULAR PRESERVATION

The biomolecular preservation of the bone fragments was assessed further through calculation of the glutamine deamidation values (following the methodology of Wilson et al. 2012). The rate of conversion of glutamine into glutamic acid depends on multiple factors (such as burial conditions and protein extraction methods) and needs to be interpreted with caution (Brown et al. 2021b). However, glutamine deamidation values can be used as general indicators of protein preservation (van Doorn et al. 2012) and, therefore, allow researchers to broadly assess the homogeneity of an archaeological bone assemblage (Ruebens et al. 2022, 2023; Sinet-Mathiot et al. 2019, 2023; Wang et al. 2023; Welker et al. 2017).

It was possible to obtain deamidation values for COL1α1 508–519 for 88.1% of the identifiable bone remains (AmBic extraction, see SI 2). While deamidation values could be obtained for 85–90% of all main taxa, this was only the case for 39% of the Elephantidae remains. These COL1α1 508–519 deamidation values range from 0.30 to 0.60. Values closer to 0 indicate a full deamidation of the glutamine, and, hence, worse protein preservation. No significant differences in COL1α1 508–519 deamidation could be observed between the faunal remains of the upper and lower assemblages, and hence they were plotted combined (see Figure 7). While the deamidation values of the main taxonomic groupings overlap (Figure 7), a two-sided t-test highlighted significant differences in COL1α1 508–519 deamidation between Ursidae, Bos/Bison, reindeer, and Elephantidae (SI 5). This difference is driven by the lower deamidation values (and hence poorer preservation) of the Ursidae remains, and the higher values (and hence better preservation) of the Elephantidae and reindeer remains. However, it is possible that smaller sample sizes for some groups are driving some of these observed differences, es-

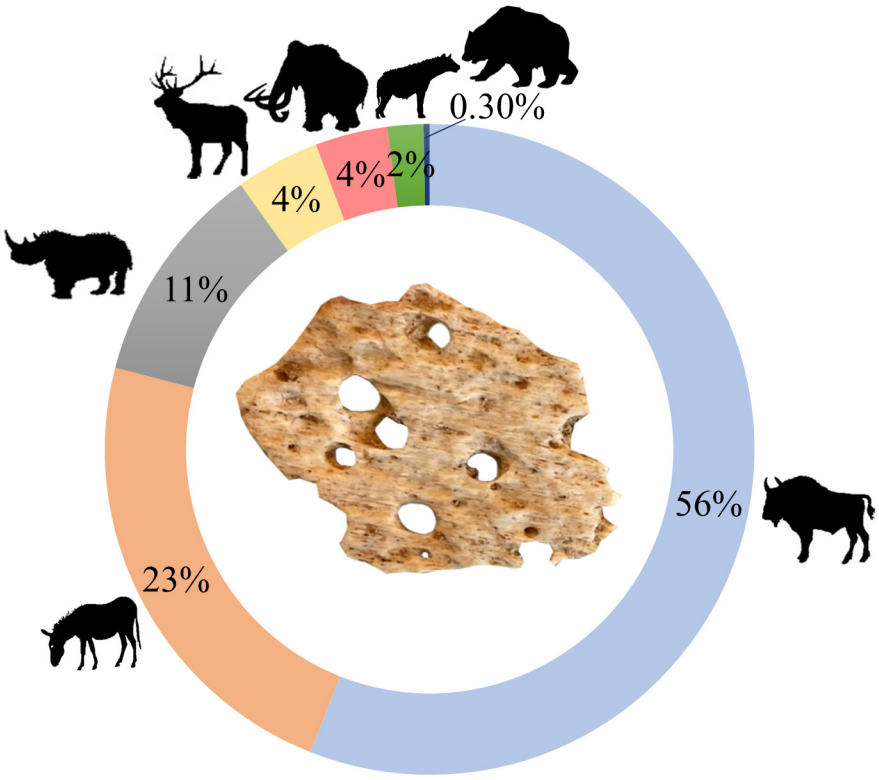

Figure 6. ZooMS taxonomic identification of 334 carnivore digested bone fragments from Cassenade. Cervidae (yellow) includes Cervid/Saiga, Cervid/Saiga/Capreolus, and reindeer. Carnivora (green) include Hyaenidae and Panthera. The rhinoceros bone fragment in the middle (CAS-540) shows a pattern of perforations indicative of digestion and regurgitation by a hyaena.

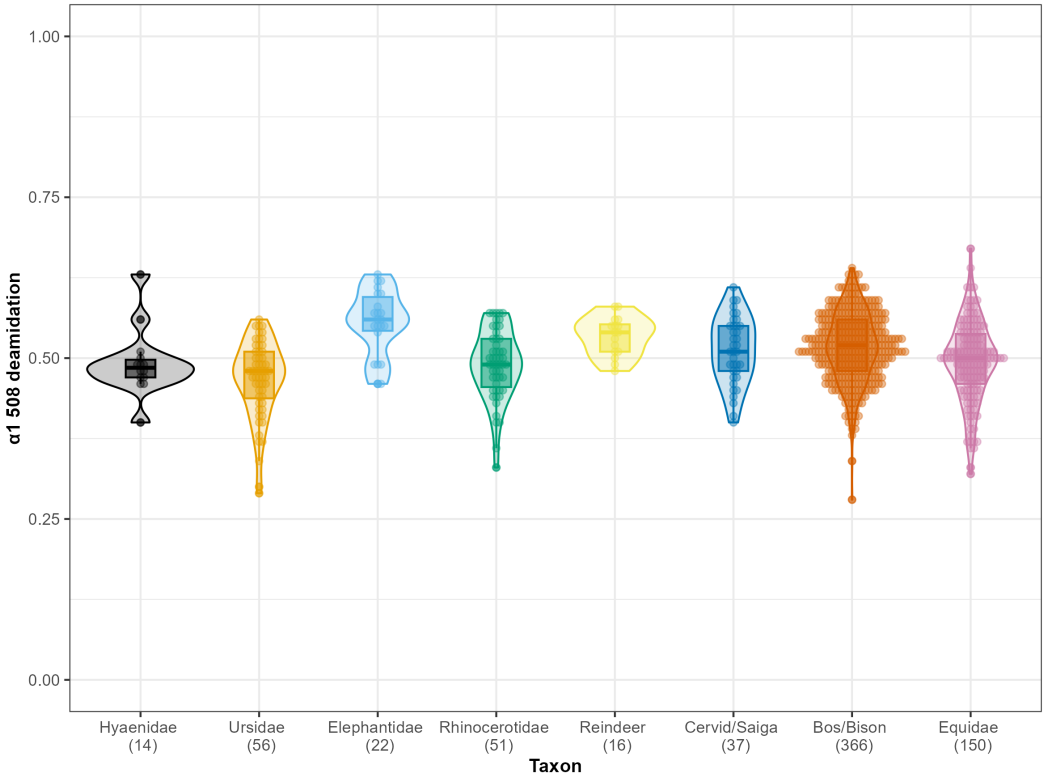

Figure 7. Overview of the distributions of the COL1a1 508–519 deamidation values obtained for the main ZooMS-identified taxa at Cassenade (raw data in SI 2). Figures in parentheses are the number of specimens with deamidation information.

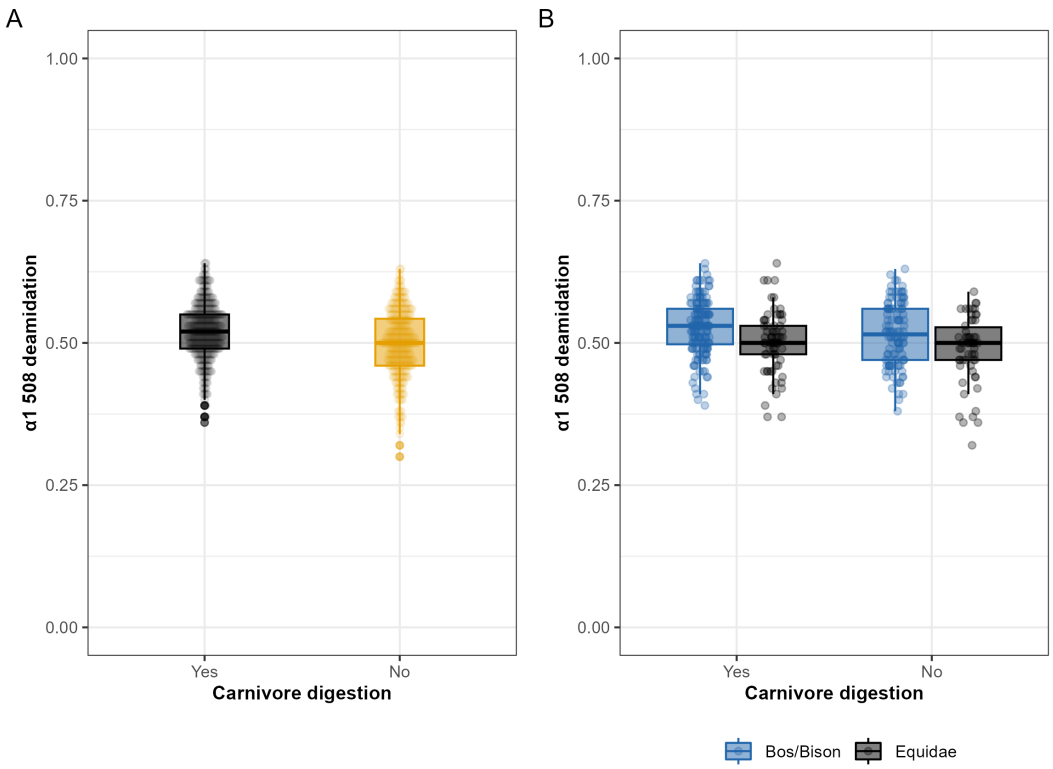

Figure 8. Correlations between COL1a1 508–519 deamidation and carnivore digestion. A) Comparison between all bones with (n=316) and without (n=314) traces of carnivore digestion. Fragments which were not securely assigned to either category were excluded (n=129). B) Comparison between digested and non-digested fragments for two main taxa: Bos/Bison *(n=273) and Equidae (n=120).*

pecially considering the lower number of deamidation values available for Elephantidae.

We tested if there was a link between bone surface preservation or modification and glutamine deamidation. Processes that affect the surface readability of the bone fragments (such as exfoliation, root etching, or staining) do not seem to affect deamidation. Conversely, cracked fragments have a slightly lower deamidation value indicating poorer preservation (see SI 3).

The large quantity of ZooMS-identified fragments that display evidence for carnivore-digested bones (n=334) makes Cassenade a great case study to explore the effect of digestive processes (being immersed in stomach acid) on biomolecular preservation. Our data show that the digested fragments have a slightly higher COL1α1 508–519 deamidation value, and hence better protein preservation (Figure 8a). A t-test shows this is statistically significant (t = (df=573.0277) 4.41, p=0.00011). The effect size, as measured by Cohen's d, was d=0.36, indicating a small effect between the variables. This difference is most pronounced in the main taxa, *Bos/Bison* and Equidae (Figure 8b), but further studies are needed to be able to fully interpret the extent and drivers of such differences in deamidation.

SPATIAL ANALYSIS

Three-dimensional coordinates are available for all fragments analyzed by ZooMS (SI 4), making it possible to test whether ZooMS identifications can alter the definition of post-excavation stratigraphies (Discamps et al. 2023) and/or modify our perception of faunal changes through time. For the main species present among the morphological identifications, there is, as one might expect, no change in the spatial projections when adding the ZooMS identifications. While the ZooMS data do add more data points, the observed trends remain the same—large bovids, equids, and hyaenas are distributed across the stratigraphy, while bear bones are more abundant at the base (as described previously in Discamps et al. 2019). Further, no changes were observed in the spatial projections when including ZooMS identifications for rhinoceros (spread out across the stratigraphy), as well as for very rare species (roe deer, giant deer, cave lion, wild boar, and foxes).

However, when looking at the distribution of three rarer species (reindeer, red deer, and proboscideans [mammoth], Figure 9) some alterations can be observed. Based on morphology alone, reindeer seem mostly present at the top, and red deer at the base in some parts of the cave, but interpretation was difficult owing to the small sample size. Adding the ZooMS data shows that red deer and "Cervid/Saiga" bones are present throughout the sequence and are slightly more frequent at the base compared to reindeer. This spatial analysis of our ZooMS data both confirms (in sagittal 53, see Figure 9f, 9h) and refines (more complex picture in sagittal 52, see Figure 9b, 9d) original observations. Additionally, there seems to be a concentration of mammoth remains in the middle part of the deposits that was

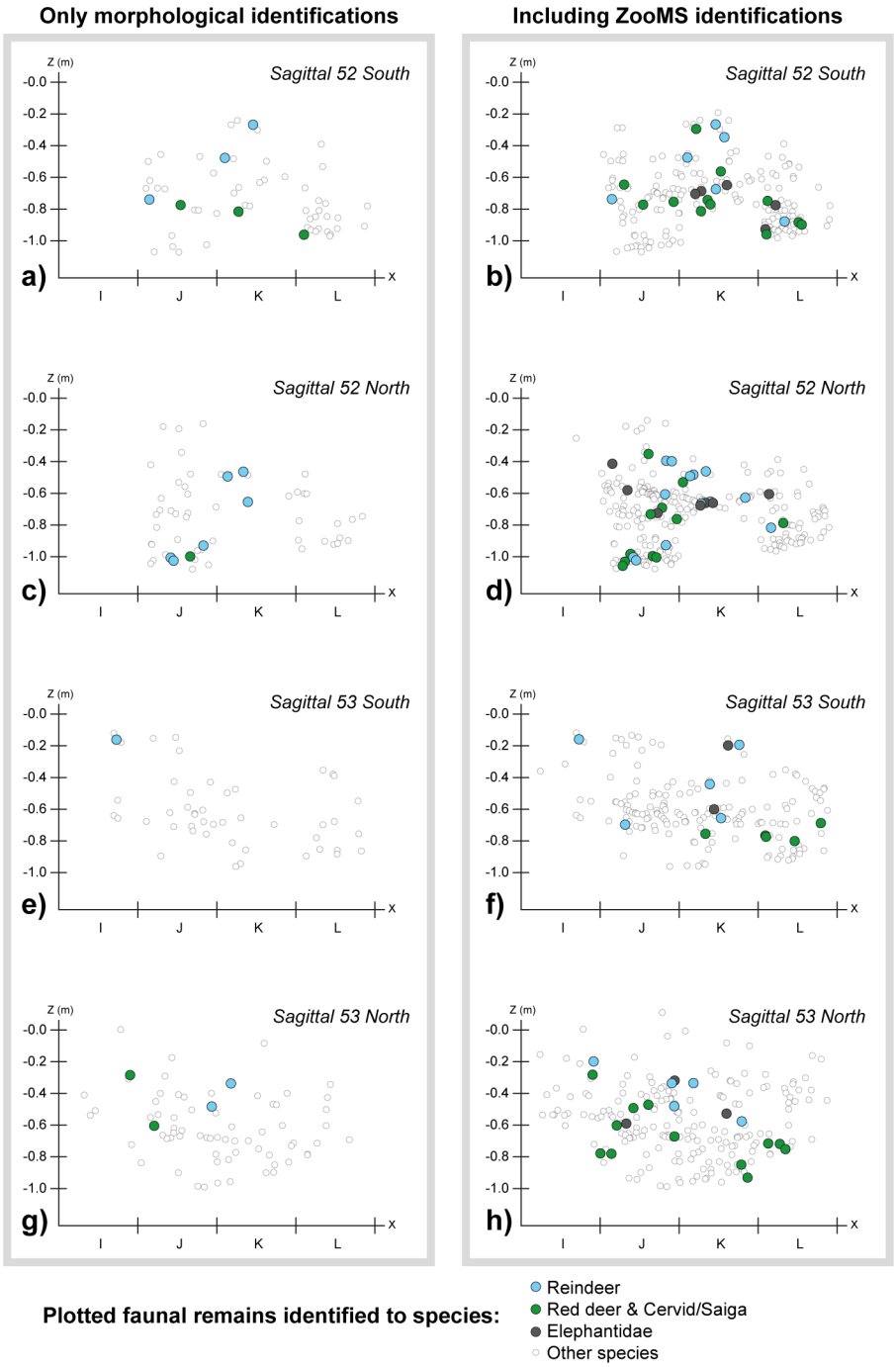

Figure 9: Spatial distributions of identifiable plotted faunal remains (sagittal XY projections in lines 52 and 53, see Figure 1b). Left column only includes morphologically identifiable remains, while the right column includes both morphologically identifiable and ZooMS-identified fragments.

not at all visible with morphological identifications alone.

The depositional context and complex history of the Cassenade deposits (largely reworked by water runoff) render detailed paleoecological interpretations tentative, yet this dataset shows the high potential of ZooMS studies to track the stratigraphic distribution of the less abundant taxa, to refine species-based post-excavation stratigraphies, and to provide a more complex and accurate picture of faunal changes through time.

BODY SIZE CLASSES

When confronted with unidentifiable bone fragments, many zooarchaeologists assign them to mammal size classes (e.g., for Eurasian taxa: Castel 1999; Costamagno 1999; Discamps 2011; Fosse 1994; Morin 2004). These body size classes are sometimes used to better discuss subsistence strategies by integrating unidentified counts to establish the proportion between medium (e.g., reindeer) and larger (e.g., Bovinae and horse) ungulates (Rendu et al. 2019,

TABLE 3. CORRELATION BETWEEN THE ANIMAL TAXA PRESENT AT CASSENADE AND THEIR BODY SIZE CLASSES.

Body size class	Scientific name	Common name
Medium	*Capreolus* sp.	Roe deer
	Canis lupus	Wolf
	Crocuta crocuta spelaea	Cave hyena
	Rangifer tarandus	Reindeer
	Sus scrofa	Wild boar
Medium - Large	*Cervus elaphus*	Red deer
	Equus hydruntinus	Wild ass
	Panthera sp.	Cave lion / Ice Age leopard
	Ursus sp.	Bear
Large	Bovinae	Aurochs and bison
	Equus ferus	Horse
	Megaloceros giganteus	Irish elk
Large - very large	Rhinocerotidae	Rhinoceros
Very Large	Proboscidea	Proboscideans

2023). At Cassenade, unidentifiable bone fragments were, when possible, assigned to size classes (Table 3). This was predominantly achieved by assessing the cortical thickness of the abundant long bone shaft fragments (Discamps et al. 2019). As past studies have shown inconsistencies between body size classes (as identified by zooarchaeologists) and proteomic identifications (Ruebens et al. 2023; Sinet Mathiot et al. 2019, 2023), we also explore this for Cassenade.

Out of the 159 bone fragments that were attributed to a body size class by ED, 11% (17 out of 159) were misidentified (Figures 10, 11). This, for example, includes five reindeer fragments and one hyaena classified as "large mammals," as well as six Bovinae and three equids sorted as "medium mammals." When reviewing these misidentified specimens, some are clear observer mistakes (e.g., small pieces of heavily-altered reindeer antler fragments that were, by mistake, identified as large mammal bone shaft fragments), while others have cortical thicknesses that are at odds with the initial criteria used by zooarchaeologists (see Figure 11).

DISCUSSION AND CONCLUSION

Despite the poor preservation of many of the bone surfaces, the collagen preservation at Cassenade is excellent, with a ZooMS success rate of over 99%. While *Bos*/*Bison*, mammoth, red deer, and rhinoceros are present in higher numbers in the ZooMS fraction, carnivores, including cave bear and hyaena, are more frequent in the morphologically identifiable remains. By integrating these contrasting ZooMS taxonomic identifications with further zooarchaeological, taphonomic, and spatial data, we can now obtain further insights into past ecologies, changing site use, carnivore diet, and human subsistence practices at Cassenade.

SPECIES DIVERSITY AND ABUNDANCE

While ZooMS was able to identify an additional taxon at the site (*Panthera*, n=5, SI Table 1), the zooarchaeological identifications allowed us to identify species such as *Megaceros giganteus* and *Equus hydruntinus*, which are beyond the current taxonomic resolution of ZooMS. Taking all identifications together, the species diversity at Cassenade is high (with 15 mammalian taxa) and this is in line with other sites of MIS 3 age in southwestern France (Discamps and Royer 2017; Discamps et al. 2011). At Cassenade, our zooarchaeological and ZooMS analysis highlights the presence of a range of taxa, including more-cold adapted (reindeer) and warmer-adapted species (roe deer, wild boar). The co-occurrence of such species could indicate a more mosaic/heterogeneous environment around the site, illustrate time averaging phenomenon (i.e., rapid fluctuations in the local climate/environment that cannot be distinguished in the stratigraphic record) or, simply, point to the important ecological plasticity of many Pleistocene large mammals.

Using the morphologically unidentifiable bone fragments to estimate the abundance of the various represented species is methodologically challenging (Discamps et al. 2024 [this volume]). It is common in large-scale ZooMS studies of Paleolithic faunal assemblages to obtain differences between the taxonomic identifications of the morphologically identifiable and unidentifiable fractions. This is also the case at Cassenade where we see a threefold increase in the number of *Bos*/*Bison* remains in the ZooMS

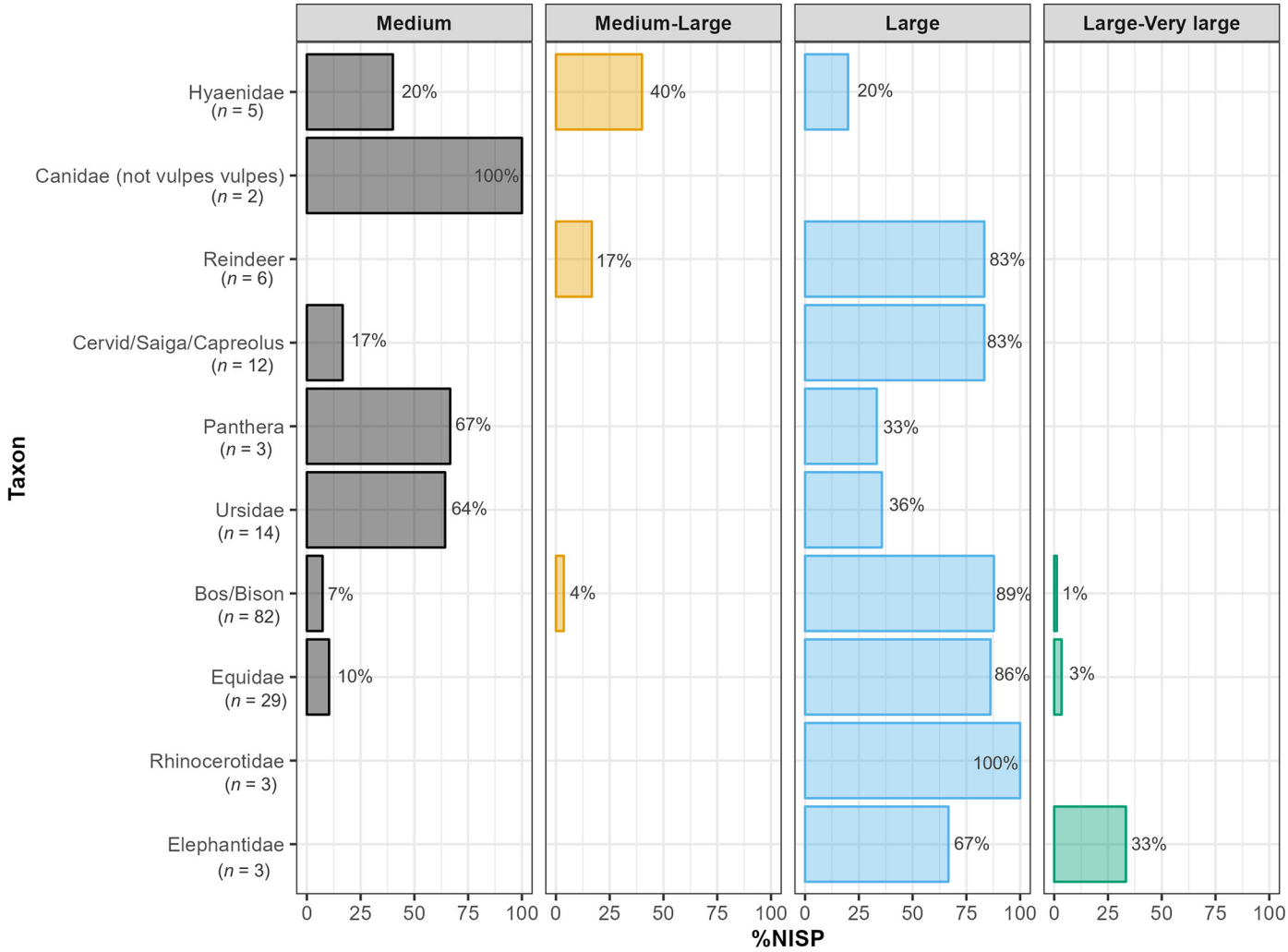

Figure 10. Overview of the ZooMS identified bones and their previous assignment to body size classes (for an overview of which taxa are in which body size class see Table 2).

portion. These differences can relate to a variety of factors, including differential patterns or fragmentation resulting in identification biases (Morin et al. 2023), different processes of faunal accumulation, or differential carcass processing strategies in human groups (Sinet-Mathiot et al. 2019, 2023). At Cassenade, this increase in *Bos/Bison* remains is most likely related to an inflated representation of large ungulate bone fragments, which can be adjusted when considering the mass of the faunal remains (Discamps et al. 2024 [this volume]).

The cross-comparison of ZooMS identifications with body size class assignments indicates that caution is required when estimating species abundance. Most notable, at Cassenade, several *Bos/Bison* long bone fragments were misidentified as smaller body sized animals due to reduced cortical thickness (see Figures 10, 11). Overall, we can question the need for zooarchaeologists to sort thousands of small bone fragments (mostly long-bone shaft fragments) in body size classes, a time-consuming process that provides little extra information and for which data acquisition is often rushed, resulting in higher risks of error. Instead, the use of untargeted ZooMS studies (Ruebens et al. 2023; Sinet-Mathiot et al. 2023), and targeted ZooMS analyses of specific skeletal elements (Arenas-Sorriqueta et al. 2024 [this volume]), could be a more useful tool to clarify patterns of species diversity and abundance.

CARNIVORE SITE USE AND DIET

This ZooMS study confirms changes in the use of the site over time by different groups of carnivores. The spatial distribution of the ZooMS taxonomic identifications indicates a higher presence of ursid remains in the lower part of Layer 2, confirming an initial use of the site by bears (Discamps et al. 2019). The low amount of ZooMS-identified digested ursid remains is a further indication that cave bears denned in the cave prior to the site being used intensively by hyaenas. The poorer biomolecular preservation of the Ursidae remains (as indicated by lower deamidation values) also indicates a slightly different (longer?) process of diagenesis for this taxon.

Figure 11. Examples of comparisons between body size classes (morphology) and proteomic identifications (ZooMS) at Cassenade (from top to bottom: cortical, medullary, and side views). Pieces are sorted from left to right with increasing cortical thickness. Bos/Bison *bones (CAS-470, CAS-153, CAS-612) are 2 to 4 times thinner than reindeer fragments (CAS-611, CAS-318). Note that CAS-318 is probably a fragment of altered reindeer antler that was not recognized as such during zooarchaeological analysis.*

Overall, ZooMS confirms the dominant role of carnivores in the faunal accumulation across the Cassenade sequence. The ZooMS fraction is dominated by faunal taxa that are common prey for cave hyaenas (e.g., bovids, equids, rhinoceros; Jimenez et al. 2021). This can be coupled with a high presence of carnivore damage (ca. 45–60%) on the ZooMS-identified bovids, equids, and rhinoceros, as well as a dominance of these prey taxa in the ZooMS-identified carnivore-digested remains. High levels of fragmentation of these prey species are to be expected in hyaena dens (Kuhn 2005). The identification of mammoth remains among the digested bone fragments shows that, at Cassenade, hyaenas preyed or scavenged upon this species, a behavior that is not always recorded on mammoth remains, for example, in MIS 3 Belgium (Germonpré et al. 2014; Jimenez et al. 2021).

HUMAN PRESENCE AND FAUNAL CHANGES

During the upper part of the Cassenade sequence, both cave hyaenas and Châtelperronian groups intermittently used the site, but indicators of human presence are generally sparse (e.g., stone tools, traces of fire use, humanly processed prey remains). This ephemeral human presence is in line with the low number of human modifications on the ZooMS-identified fauna (n=17), and the lack of human skeletal remains. Because of the low readability of the bone surfaces, it is difficult to securely assess which animal species humans preyed upon. Cut marks could be securely identified on only ca. 2% of the fauna but do indicate the exploitation by humans of a range of large and medium sized herbivores, including bovids and equids. We also identified evidence for the exploitation of bears at Cassenade, alongside an isolated example of a cut-marked rhinoceros bone. Bear exploitation by Châtelperronian groups has been proposed at Fond-de-Gaume (Armand et al. 2003), yet such evidence remains extremely scarce in southwestern France (Armand et al. 2004). Similarly, if confirmed, Cassenade would then constitute one of the very few examples of the exploitation of woolly rhinoceros by Châtelperronian groups. The lower proportion of carnivore modifications on the Cervidae (including reindeer, red deer, roe deer, and giant deer) could suggest a higher human involvement with the accumulation of these taxa. It is difficult to assess if the concentration of mammoth remains in the middle part of the sequence and the increased presence of reindeer in the top part, is driven by human behavior or reflect changes in the locally available fauna.

Taken together, this study has highlighted the added value and future potential of extensively integrating zooarchaeological, taphonomic, ZooMS, and spatial datasets. Cross-comparing taxonomic identifications of both the morphologically identifiable and unidentifiable faunal

remains with taphonomic indicators (e.g., fragmentation, surface preservation, glutamine deamidation) and zooarchaeological observations (e.g., skeletal element and age) can provide additional insights into patterns of site formation, carnivore behavior, and human subsistence practices. Further, in terms of spatial analysis and defining post-excavation stratigraphies (Discamps et al. 2023), the inclusion of ZooMS data can considerably increase the number of data points that can be explored spatially, and, therefore, produce a more complete and more precise picture of faunal changes through time.

ACKNOWLEDGMENTS

The ZooMS part of this study was funded by the Collège de France and the French National Research Agency (ANR) in the framework of the CRADLE project (ANR-23-CE27-0025). We like to thank Joelle Vinh and Emmanuelle Demey for assistance with the use of the MALDI-TOF at the ESPCI (Paris). G.M.S. is funded by the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie scheme (grant agreement No. 101027850). Cassenade excavations were funded by the French Ministry of Culture (Service Régional d'Archéologie Nouvelle-Aquitaine), the Conseil Départemental de la Dordogne, the UMR-5199 PACEA, the UMR5608 TRACES, the Région Nouvelle-Aquitaine project NATCH, and the French National Research Agency project DeerPal (ANR-18-CE03-0007). We wish to thank Marie-Cécile Soulier for her help second-looking at the badly preserved Cassenade cut marks. We like to thank all three anonymous reviewers for their feedback which helped improve this paper.

DATA AVAILABILITY STATMENT

Supplementary data for this manuscript is available through Zenodo (https://doi.org/10.5281/zenodo.11102784). This includes the raw MALDI-TOF data in .mzxml format (SI 1) with an accompanying excel database with their ZooMS identification (SI 2), an excel database with ZooMS identifications, zooarchaeological data (including body size classes), and taphonomic observations (SI 3), an excel database with the spatial coordinates for piece-plotted bone fragments (SI 4), tables for the statistical tests (SI 5) and the R script used for making the figures and statistical tests (SI 6).

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