Article

Current Biology

Tracing early pastoralism in Central Europe using sedimentary ancient DNA

Highlights

- We analyze metagenomic data from a rock shelter using sedimentary ancient DNA
- Ancient DNA reveals domesticated animals and their associated microbiomes
- Phylogenetic analyses detect mitochondrial haplogroups in domestic sheep and cattle
- *seda*DNA can help improve the understanding of our herding practices and forest management

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In brief

Sedimentary ancient DNA buried underneath a rock shelter reveals shifts in the presence of domesticated animals, associated microbiomes, and plants gathered for fodder from the Late Neolithic to the Bronze Age. Zampirolo et al. highlight the potential of using ancient DNA from rock shelter deposits to infer past human-environment interactions.

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Tracing early pastoralism in Central Europe using sedimentary ancient DNA

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SUMMARY

Central European forests have been shaped by complex human interactions throughout the Holocene, with significant changes following the introduction of domesticated animals in the Neolithic (~7.5–6.0 ka before present [BP]). However, understanding early pastoral practices and their impact on forests is limited by methods for detecting animal movement across past landscapes. Here, we examine ancient sedimentary DNA (*sedaDNA*) preserved at the Velký Mamuťák rock shelter in northern Bohemia (Czech Republic), which has been a forested enclave since the early Holocene. We find that domesticated animals, their associated microbiomes, and plants potentially gathered for fodder have clear representation by the Late Neolithic, around 6.0 ka BP, and persist throughout the Bronze Age into recent times. We identify a change in dominant grazing species from sheep to pigs in the Bronze Age (~4.1–3.0 ka BP) and interpret the impact this had in the mid-Holocene retrogressions that still define the structure of Central European forests today. This study highlights the ability of ancient metagenomics to bridge archaeological and paleoecological methods and provide an enhanced perspective on the roots of the "Anthropocene."

INTRODUCTION

The emergence of agriculture marks a radical juncture in the entanglement of humans and Earth systems. In Central Europe, the first agricultural sites are associated with the Linearbandkeramik (LBK) peoples, who rapidly occupied fertile lowlands after 7.5 ka before present (BP), introducing a suite of domesticated plants and animals from the Near East.[1,2] Initially, domesticated animals were kept in proximity to agricultural settlements, typically located on loess soils conducive to cultivation. However, the Late Neolithic (sometimes referred to as the Eneolithic, ~6.4–4.2 ka BP) witnessed the advent of forest grazing, facilitating a full expansion of pastoralism across varied Central European environments.[3–7] By the Bronze Age (~4.1 ka BP), rich and diverse broadleaf mosaics transitioned into the comparatively species-poor structure that continues to define many Central European forests today.[8–10] Human influence likely played a significant role in this profound transformation, as pastoral activities can impact forest succession.[11] However, detecting pastoral movement of domesticated animals across past landscapes is challenging, and the coevolution of forest structure and human agency remains poorly understood.

In this context, the analysis of ancient environmental DNA from sediments offers an opportunity to refine insights into paleoecological changes through time[12,13] by complementing traditional fossil evidence.[14–16] Prior attempts to extract ancient DNA from archaeological deposits have utilized target-capture techniques to detect hominin and mammal DNA in cave sediments[17–19] and rock shelters.[20,21] However, the potential for shotgun sequencing of bulk ancient environmental DNA in a broader range of archaeological settings, such as semi-open to open-air sites, has not yet been fully realized. With this in mind, we identified the Velký Mamuťák (VM) rock shelter as an ideal site for investigation. VM is situated in the Český Ráj region

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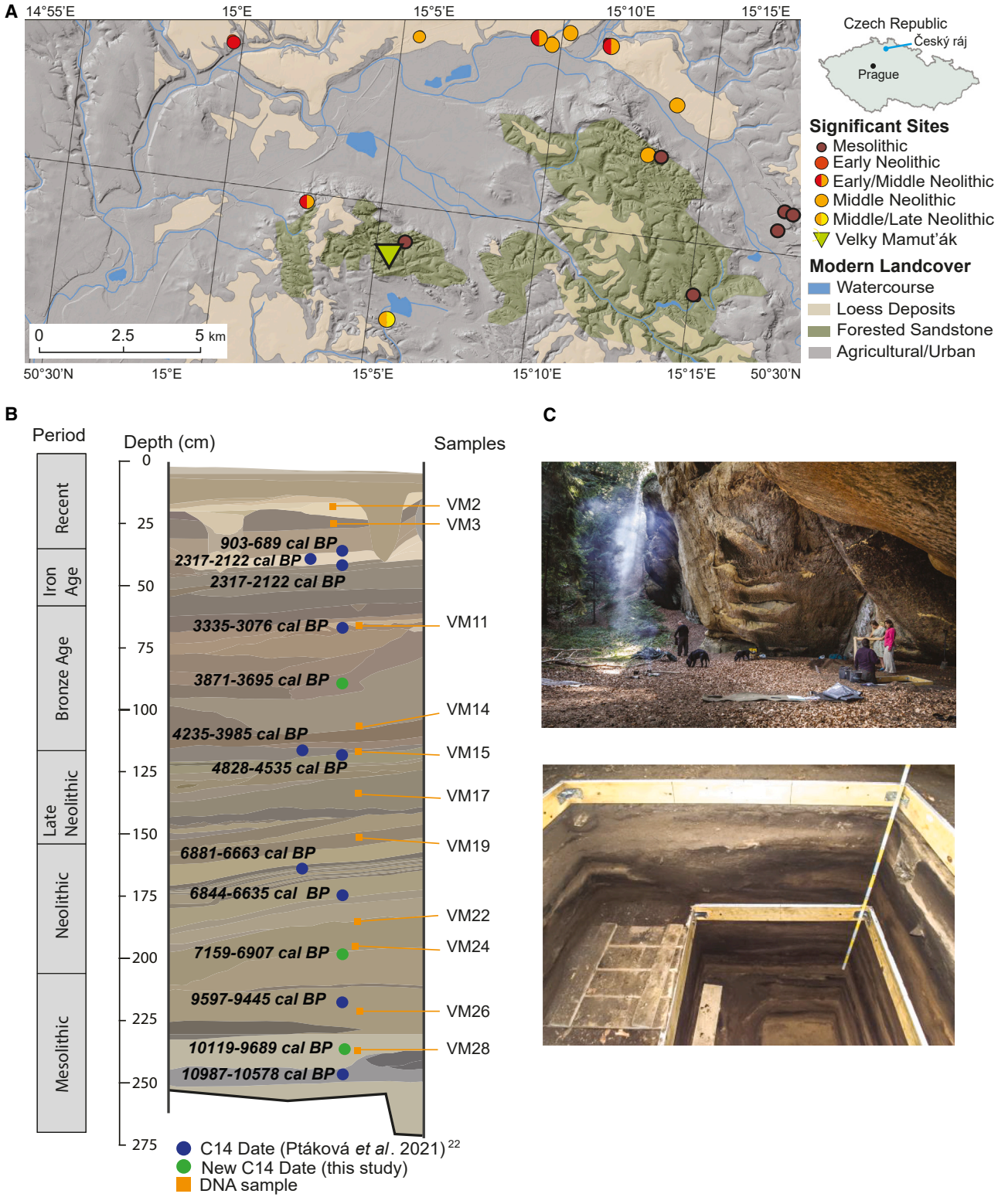

Figure 1. Archaeological setting and age profile

(A) Location of VM and nearby archaeological sites from Mesolithic to Late Neolithic (based on geological map 1:50,000. Adapted by Václav Vondrovský. In: geological map 1:50,000 [online]. Praha: Czech Geological Survey [cit. 2022-10-01]).

(legend continued on next page)

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of northern Bohemia, which is a forested area enclosed by sandstone outcrops that create dramatic relief (Figure 1C). VM is remarkable for its exceptional organic preservation and deep occupation layers spanning all significant periods from the Mesolithic to the present (Figures 1B and 1C). Furthermore, VM benefits from a comprehensive multi-proxy account of environmental practices, derived from prior archaeological and paleoecological examinations.[22,23] At VM, we collected a sequence of sediment samples from stratigraphic layers to examine metagenomic changes spanning the early Holocene to recent times.

We identified shifts in domesticated animal DNA at the site from the onset of the Late Neolithic (~6.0 ka BP) through the Bronze Age (~3.0 ka BP) and show, using phylogenetic placement, that key taxa are ancestral lineages to modern species. In addition, microbial source analysis[24,25] confirms the presence of bovine species, sheep, and pigs within the same sediment layers. We even find evidence of human-associated microbes in a Bronze Age (~3.0 ka BP) layer. Furthermore, we track changes in plant DNA that likely correspond with both grazing practices and a wider understanding of environmental retrogressions in this period. Our results at VM support the understanding that the full expansion of herding to forested ecoregions did not take place until the Late Neolithic, with initial management practices focused on sheep (*Ovis*). Importantly, we identify a gradual change in dominant species from sheep to pigs (*Sus*) by the Late Bronze Age. This correlates with the mid-Holocene transformation of forest structure and can potentially be the consequence of this shift in forest succession patterns, nutrient depletion, and habitat connectivity. Overall, our results demonstrate the possibilities for sedimentary DNA (*seda*DNA) to explore past human-environment systems and emphasize the value of archaeological deposits as genetic archives for historical ecology.

RESULTS

Site, samples, and age-depth model

VM (50°31.10945′ N, 15°4.26233′ E) is located in the Český Ráj region of northern Bohemia (Figure 1A), with a strong history of research on paleoenvironments and prehistoric human settlements in the immediate vicinity (5 km).[23,26–28] VM is a 450-m^2 area, sheltered by 10 m overhanging cretaceous sandstone, and is enclosed in a small canyon with limited drainage (Figure 1C). Such conditions create a cooled and stable microclimate, where sediment accumulated throughout the Holocene. The stratified deposit consists of cultural layers with organic remains producing an extensive assemblage of artifacts and ecofacts. Analyses of charcoal, vertebrate faunal remains, insect remains, plant macro-remains, malacofauna, pollen, phytoliths, and microcharcoal are reported in prior publications.[22,23] VM yielded abundant well-preserved coprolites in layers as early as the Late Neolithic (Figure 2B), as further described in the full study by Ptáková et al.[22] In subsequent periods, the presence of stabled animals' enriched pollen and phytolith contributions to sediments have been interpreted to outline changes in both the local environment and grazing practices.[23] However, representation of domesticated animals among recovered archeozoological remains was generally sparse, likely due to the temporary nature of sheltering events that took place at the site. Given this limitation, *seda*DNA emerged as a promising technique to enhance the fossil record with further insight into species' presence.

Our sampling strategy was therefore built on this prior knowledge, assisted by additional 14C dating. We collected 28 sediment samples from different occupation layers, spanning from the Mesolithic to recent times. Of the 28 samples, 22 were extracted for DNA analyses and 11 of these were successfully converted to double-stranded dual-indexed Illumina libraries (Data S1A). Challenges in DNA extraction are addressed in the discussion below. Radiocarbon dating of macro botanical remains was used to establish a depositional age-depth model for the entire profile (Figure 1B), with OxCal (v 4.4.4)[29] calibrated years BP using the IntCal20.[30] Overall, the resulting model demonstrated a relatively steady rate of deposition that was beneficial for understanding age ranges represented by the sediment samples (Figure S1; Table S1). Fluctuations have been noted to align with general changes in precipitation during the Holocene.[23]

Metagenomic analyses

Libraries were sequenced on an Illumina HiSeq 4000 (80 bp single-end) and Illumina Novaseq 6000 (100 bp paired-end) platforms, obtaining a total of 409,863,279 reads, which were parsed for quality control and removal of low complexity reads and duplicates (Figure S2; Data S1A). Some libraries show an increased DNA complexity (VM 14–19), while others a greater number of duplicates (VM 2, 11, and 22–28) (Figures S2A–S2D; Data S1A). The high duplication rate seems to correlate with a relatively higher number of cycles needed to amplify the libraries, as found by quantitative PCR (Figure S2A). For the reasons stated in the discussion, this is commonly caused by a low abundance of DNA molecule templates within the extracts. A total of 171,216,656 reads were then parsed through the Holi pipeline for competitive mapping.[13] Taxonomic profiling and post-mortem DNA damage was estimated using metaDMG.[31] We used a data-driven filtering approach to investigate and set a minimum threshold for the DNA damage (for details, see STAR Methods), which was applied to obtain the final taxonomic profiles (Figure 2). In summary, we find post-mortem DNA damage to vary between 5% and 16% for animals and 5% and 14% for plants (Figure S7), increasing by depth and age. The five layers of the taxonomic profile, reported as *NA* (VM 2, 3, 22, 26, and 28, Figure 2), fell below the threshold values for authenticated DNA damage, primarily driven by a low significance in the fit to the beta-binomial model used by metaDMG (*significance* < 2) and/or low degree of damage detected (*damage* < 5 %) (see STAR Methods; Figures S3D–S3G and S7B; Data S2B and S2D). Post-mortem DNA damage was further validated by plotting the damage frequency using the metaDMG dashboard

(B) Sediment profile with radiocarbon ages from a previous study[22] (blue) and new dates from this study (green) together with samples processed for *seda*DNA (orange).
(C) Rock shelter and trench where sediment was sampled.
See also Figure S1 and Table S1 for further details on the chronometric data.

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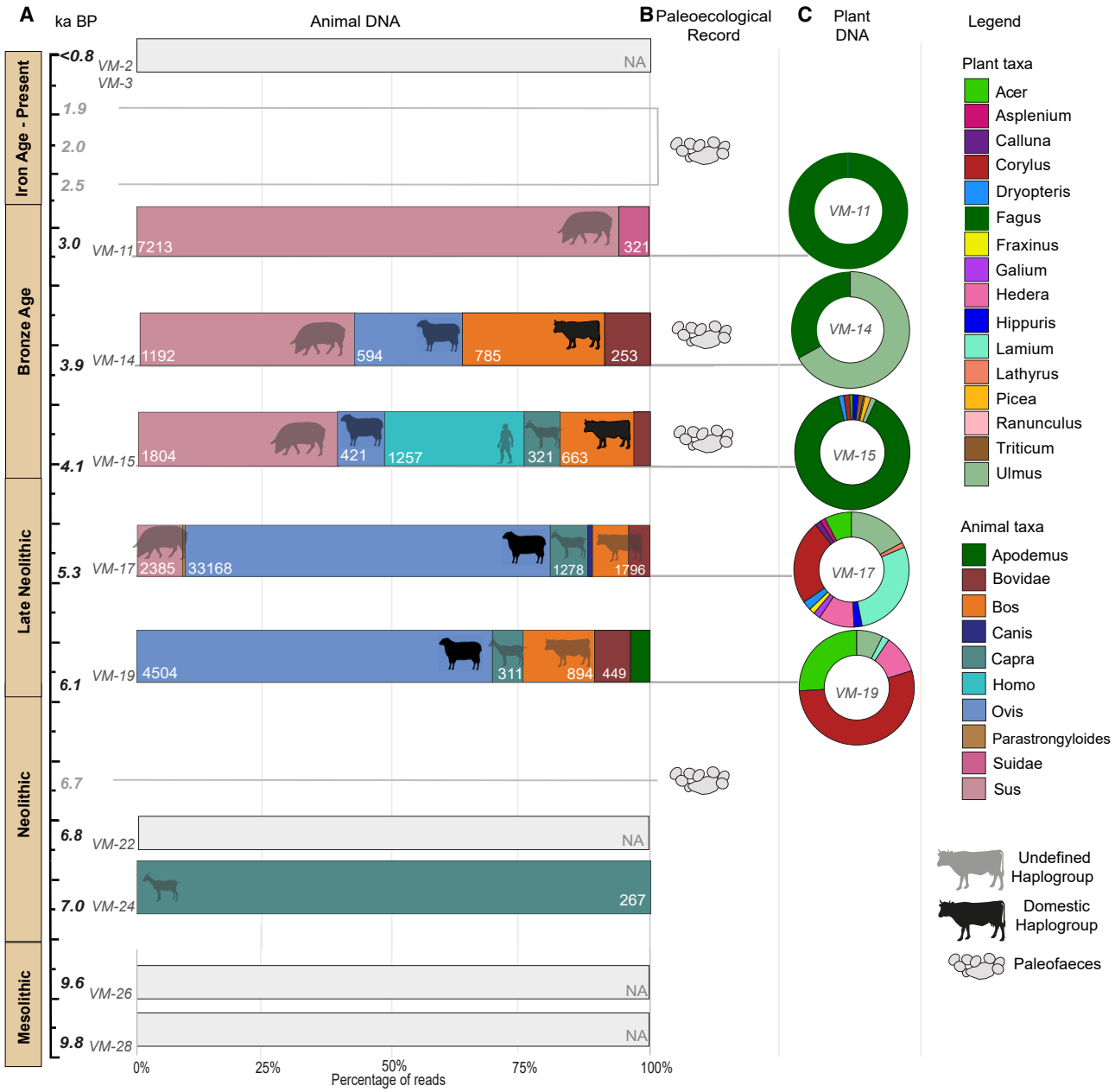

Figure 2. Plant and animal DNA taxonomic profiles

(A) The relative proportion of DNA reads assigned to animals, with total unique reads for each taxon.

(B) The paleoecological data were reproduced from previously published studies.[22,23]

(C) The relative proportion of DNA reads assigned to plants at the genus level. The legend includes domestic (black outline) and undefined haplogroups (gray outline).

See also Figures S8 and S9 for further details on the haplogroup identification.

(Figure S4), which shows the typical damage pattern with increased deamination at the fragment ends. However, some genera within the Bovidae and Suidae families do not exhibit the typical deamination pattern. Instead, they show high uncertainty in the model fit, and the nucleotide misincorporation for the forward (C > T) and reverse (G > A) strands are scattered (Figure S5). These taxa are also unexpected in the study region and likely represent a signal of overmatching due to little genetic diversity within the genera that is not well represented by the reference genome.[32] Therefore, we placed these reads at the family level.

To determine whether the animals found were carrying a domestic or wild haplogroup of origin, we extracted all reads at the family level and mapped these against the mitochondrial genomes of each of the animal species. For cattle and sheep, having a mean breadth of coverage ranging from 0.02 to 0.32, we

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first performed a phylogenetic placement using a Bayesian evolutionary analysis by sampling trees (BEAST)[33] and hereafter identified unique single-nucleotide polymorphisms (SNPs) using pathPhynder[34] (Figures S8 and S9).

Animal DNA

We find a clear shift and appearance of DNA from domestic animals during the progression of the Neolithic (~7.0–6.0 ka BP, see Figure 2). Prior to this, no animal DNA was found in the earliest layers (~9.9–9.6 ka BP), and goat or possibly ibex (*Capra* sp.) appears as the only taxon in the Early Neolithic layer (~7.0 ka BP). In the Late Neolithic period (~6.1–5.3 ka BP), we record the first appearance of a wider range of animals, including cattle or aurochs (*Bos* sp.), pig or wild boar (*Sus* sp.), goat (*Capra hircus*), and sheep (*Ovis aries*). We also detect between 125 and 285 reads assigned to wisent (*Bison*) and buffalo (*Bubalus*); however, both species and their geographical distribution in this area remain debated.[35–41] We proceeded with further investigation of these reads (see STAR Methods and Figure S5), which we report in Figure 2 as belonging to the Bovidae family. In the same layer, we also detected reads assigned to the genera *Oryx* (antelopes) and *Budorcas* (takin), which we also explain to be an over-match due to little genetic diversity within the genera.[32]

During the Bronze Age (~4.1 ka BP), we find a composition of animals similar to the Late Neolithic period, with the difference being that pig/wild boar increased in abundance. Furthermore, we find ancient human DNA (*Homo sapiens*) that shows ancient DNA damage characteristics similar to the other taxa.

In contrast to the animal diversity in the *seda*DNA record from the earlier periods, the Late Bronze Age (~3.0 ka BP) is less diverse, with only domestic pig/wild boar present. We also detect in this layer a total of 321 reads aligned to genus *Phacochoerus* (warthogs), which is unlikely to be found in the region, but possibly explained by limited genetic diversity within the genus *Sus*. We have reported these reads as being part of the family Suidae (see STAR Methods and Figure S5). The uppermost layers are from much more recent periods (Iron Age and later). Here, we find domestic pig/wild boar but with limited evidence for DNA damage (Figure S3D).

We next phylogenetically placed each consensus mitochondrial genome of *Ovis* and *Bos* onto their respective phylogenetic tree (Figures S8 and S9) in order to determine whether the animals were falling closer to domestic or wild living relatives and counted supporting and conflicting SNPs along each of the branches (see STAR Methods; Figures S8 and S9).

We found supporting evidence of domestic alleles for a minimum of one taxon in all layers between 6.1 and 3.0 ka BP. In the earliest layer from the Late Neolithic (~6.1 ka BP), *Ovis* reads cluster basal to currently living *Ovis aries* haplogroup B and European mouflon (*Ovis aries musimon*), both domesticated, but with only one supporting SNP (Figures S8A and S8C; Data S4B). In the younger layer of the Late Neolithic (~5.3 ka BP) we find strong support (40 SNPs) for a placement within the domestic sheep (*Ovis aries*) branch and the lowest placement within haplogroup B (5 supporting SNPs and 2 conflicting) (Figures S8A and S8B; Data S4B). Little support (posterior probability = 0.81) was found for the basal placement of the cattle mitochondrial DNA in this layer (Figure S9A); however, pathPhynder analysis placed *Bos* reads ancestral to both domestic cattle and aurochs (1 supporting SNP) (Figure S9D).

The mitochondrial reads from the Early Bronze Age periods (~4.1–3.9 ka BP) fall basal to the cattle haplogroups Q and T, but with a low posterior probability between 0.58 and 0.65 (Figure S9A). However, despite this, the SNPs support the Bronze Age (~4.1 ka BP) cattle placement as ancestral to the *Bos taurus* haplogroups Q and T (3 supporting SNPs and 1 conflicting one), with a single SNP supporting the placement within *Bos taurus* haplogroup T (Figure S9C). Similarly, in the upper layer of the Early Bronze Age (~3.9 ka BP), reads are placed at the basal node (1 unique SNP) of domesticated species carrying haplogroups Q and T (Figure S9B). This placement was also confirmed by the analysis of transversions only, although fewer SNPs were found (Figure S9E).

The coverage of the mitochondrial reads for *Sus*, *Capra*, and *Homo* was below 0.01, with a breadth of coverage of 136 base pairs, which we deemed to be insufficient to confidently proceed with the phylogenetic investigation of their respective haplogroups (labeled as "undefined haplogroup" in Figure 2; additional details can be found in the STAR Methods).

Plant DNA

We find a relatively low diversity of plants and only 5 samples to yield taxa in the period between the Late Neolithic (~6.1 ka BP) and Early Bronze Age (~3.9 ka BP). The Late Neolithic was initially (~6.1 ka BP) dominated by hazel (*Corylus*) and maple (*Acer*), with a low abundance of elm (*Ulmus*), which likely indicates densely forested conditions. This finding is in agreement with previously published results of pollen and charcoal analyses.[22,23] As the Late Neolithic progressed (~5.3 ka BP), we find evidence for the continuous presence of hazel and elm trees, including deadnettles (*Lamium*) as the most abundant taxon. Deadnettles grow naturally in the forest and are typically most abundant from spring to early summer. In this period, we find the richest plant diversity.

In the Early Bronze Age (~4.1–3.9 ka BP), beech (*Fagus*) became a dominating plant taxon in the assemblage, while hazel (*Corylus*), elm (*Ulmus*), spruce (*Picea*), and maple (*Acer*) retain their positions; leaves of broadleaf trees also have high nutritional value and historically were favored fodder.[42] All this is again in accordance with previously published results of pollen and charcoal analyses[22,23] and constitutes further evidence for deep forest ecosystem changes related to retrogressive succession in the respective period. It is also worth mentioning the occurrence of wheat (*Triticum*), the presence of which could indicate the feeding of domestic animals with agricultural waste.

In the Late Bronze Age (~3 ka BP), the captured diversity decreases and is mainly limited to two genera of trees, beech and elm; the retrogression was clearly already at an advanced stage.

Microbial sources

Lastly, we taxonomically profiled the microbial composition in our samples and compared these to potential source microbiomes to estimate the contributions of each source to each sample. We selected metagenomic sources from different environments, including forest, wetland, river sediment, and grassland metagenomes, as well as selected mammalian gut and fecal metagenomes. The reads from all samples were mapped,

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classified, and their post-mortem DNA damage estimated (see STAR Methods and Table S4). We then calculated the proportion of sources using Sourcetracker2,[24,43] both with and without the DNA damage filtering criteria, which were also applied to animal and plant data, and found identical trends. However, filtered data also meant removing the majority of the diversity, most likely due to a low number of reads, hence the proportion without filtering is shown in Figure 3 (see also Figures S10C and S10D; Data S5; Table S4).

We find the presence of pig, human, sheep, and bovine fecal microbiomes and that their presence systematically follows the layers in which the animal DNA is also found (Figure 3). During the pre-and Early Neolithic period, the sources are classified primarily as "unknown," which likely reflects that the source soil and sediment microbiomes are not similar to those inhabiting the soil in the rock shelter. A small proportion of bovine rumen-fecal microbiome (1%) is found in the Early Neolithic sample at ~7.0 ka BP, which likely derives from the goat/ibex (*Capra* sp.), as found with the animal DNA in this period. A major change occurs during the Late Neolithic (~6.1–5.3 ka BP), where a large proportion (77 %) of the microbiome comes from bovine and sheep rumen-fecal matter, a trend that continues up until the Early Bronze Age periods (~4.1–3.9 ka BP), where it comprises of 33%. By the Late Bronze Age ~3.0 ka BP, a change in the metagenome occurs as the pig fecal matter becomes the dominant microbiome, with more than 30% of the metagenome, though we still find a small proportion (10%) of bovine and sheep rumen-fecal microbiota remaining. In the same layer, we also find a low abundance of human fecal microbiome (~1.3%), which is not the layer where ancient human DNA is found. Representations of the mammalian gut and fecal metagenomes clearly follow the same patterns identified in animal DNA. The taxa assigned as mammalian gut and fecal microbiome include the genera *Methanobrevibacter*, *Methanosphaera*, *Prevotella*, *Acinetobacter*, and *Clostridium* (Table S4).

Overall, we find only a small proportion of the soil and sedimentary microbial communities to be of wetland-acidic soil, temperate forest, and temperate grassland (1%–3%), which is most likely explained by reference source metagenomes being dissimilar to the sediment microbiome.

DNA taphonomy in the archaeological context

The challenges we encountered during the extraction of the DNA and the conversion to sequencing libraries may be related to several factors. Only 11 of our 22 samples were successfully converted into Illumina libraries and sequenced (Data S1A and S1B). In addition, we found six of the libraries that required a higher number of PCR amplification cycles (>18 cycles) to exhibit less complexity in plant and animal taxa, which ultimately resulted in high levels of duplication and is likely explained by too few template DNA molecules (Figure S2). The lack of template DNA molecules can be connected to several factors, such as the amount of deposited material with DNA in the sediments, faster degradation, or localized factors leading to poor preservation in these specific samples. On the other hand, given the organic-rich composition of the sediments,[22,23] inhibiting substances such as humic acids co-extracted during the DNA extractions, despite efforts of removal, may also explain the challenges in both DNA isolation[44–46] and eventual downstream molecule preparation.[47,48]

Several lines of evidence demonstrate that the DNA we recovered stayed in its respective deposited layers through time. If both upward and downward leaching had occurred, the proportion of DNA in the source layer should be larger than in the layers to which it leaches. This does not seem to be the case in this deposit. For example, the youngest layer in the Late Neolithic shows less cattle DNA than the layers both above and below. Early Neolithic caprine DNA was recovered in a single layer without traces above or below. We also find layers beneath the Neolithic period to show the absence of animal DNA, and, furthermore, no domesticated animal DNA was detected beneath the Neolithic boundary. Furthermore, using the microbial source tracking, we could authenticate the DNA record, showing that the layers in which we recover animals also show the presence of their respective ruminant and fecal microbiomes in varying abundances, which are likely related to the intensity of the presence at the rock shelter.

Accounting for post-depositional taphonomy of DNA molecules is important for the interpretation and hence application of *sed*aDNA in archaeological contexts. We expected that bioturbation and/or changes in groundwater level would facilitate some post-depositional movement of metagenomic DNA between some of the stratigraphic layers. Indeed, several studies from other sedimentary deposits have shown that DNA can leach between sediment layers in both caves[49] and open-air settings.[50] We interpret the apparent stratigraphic integrity of our results as an indication that DNA molecules recovered likely were those bound to mineral particles[51] or contained within organic structures that do not leach, such as micro and macrofossils. We also find cytosine to thymine misincorporations due to DNA damage to increase with depth and age (Figures S7 and S10B), which we would not expect with the implied mixing of metagenomic material that would take place with post-depositional movement.

DISCUSSION

Our results complement prior paleoecological analyses from VM and its surroundings by demonstrating a patterned progression in animal, microbial, and plant DNA. While this dataset is representative of a single site, it provides valuable insights into broader connections between human practices and environmental outcomes in Central European forests through time. This includes an apparent shift in forest grazing practices during the Bronze Age, which we interpret as a potential factor in the retrogressions of the mid-Holocene.[8,9,52] As such, VM underlines the potential of *sed*aDNA to open new source deposits for understanding human agency in the postglacial and Holocene succession of Central European forests.

The earliest indication of forest grazing at VM may be the caprine DNA (*Capra* sp.) in association with the bovine rumen-fecal microbiome from the Early Neolithic layer (~7.0 ka BP). However, further DNA data are required to distinguish domestic goat (*Capra hircus*) from wild species such as ibex (*Capra ibex*), whose occurrence in the area (Český Ráj) cannot be fully excluded, despite being undocumented in the fossil record for this period. Clearer genetic representation of domesticated species at VM begins during the Late Neolithic (~6.1 ka BP), long after their initial introduction to Central Europe. This supports the

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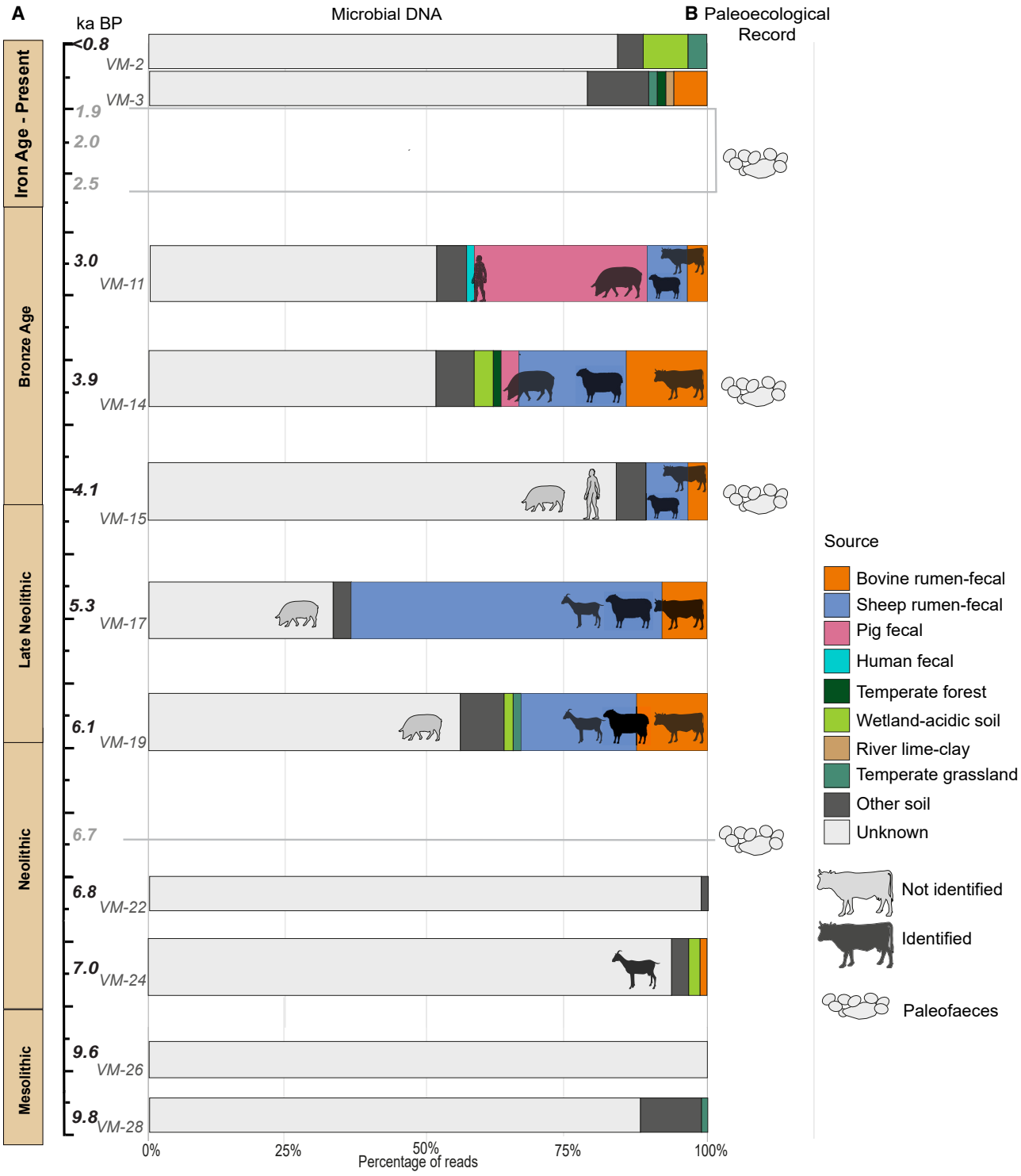

Figure 3. Microbial DNA taxonomic profiles

(A) The relative proportion of DNA assigned to different sources.

(B) The paleoecological data were reproduced from a previously published study.[22] Black outlines represent identified gut metagenomes, while gray outlines represent not-identified sources compared with the animal DNA record.

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perspective of slow environmental neolithization, where the full impact of agriculture across varied ecological zones depended on population expansions associated with Late Neolithic innovations in cultivation and land tenure.[5,10] In a peripheral environment like Český Ráj, it is interesting that DNA from the full range of domesticates—cattle, pigs, goats, and sheep—are initially represented; with the exception of a few bone fragments of sheep or goat, these are absent in the archeozoological assemblage from this period at VM due to its scarcity.[22,23] However, despite this range, both animal and microbial DNA demonstrate that sheep were the dominant species in Late Neolithic layers. The phylogenetic analyses allowed us to place cattle (*Bos taurus*) carrying modern haplogroups T and Q, and domestic sheep (*Ovis aries*) carrying haplogroup B, with the species brought to Europe from the Near East by early farmers.[53–59] However, because cross-breeding between European aurochs and introduced Near Eastern cattle was practiced during the Neolithic in Central Europe,[55,60–66] we cannot exclude the presence of wild aurochs at the site.

Český Ráj appears to remain a broadleaf forest mosaic during this stage of Late Neolithic pastoralism.[8] Sheep are selective grazers and prefer feeding on saplings, shrubs, herbs, and grasses in forest environments.[67] However, plant DNA from Late Neolithic layers is initially dominated by hazel (*Corylus*), maple (*Acer*), and elm (*Ulmus*), the leaves of which are high-nutrition fodder, particularly maple.[68,69] This could be a possible indication of seasonality, where animals were brought to the area to graze on select leaf fodder available in the autumn or winter. The later presence of *Lamium* (~5.3 ka BP), typically an early seasonal succession weed, is interesting, as deadnettle (*Lamium purpureum*) can be palatable but closely related henbit (*Lamium amplexicale*) is toxic for sheep.[42,70] Further investigation of DNA from the Late Neolithic may indicate strong control in grazing combined with adjustment and broadening of seasonality through time. The absence of domesticated crops in both the fossil and genetic records throughout the Late Neolithic could indicate that animals were not crossing cultivated areas while grazing in the vicinity of VM. Overall, this snapshot of Late Neolithic pastoralism is suggestive of a pattern of transhumance, where animals were herded to forested hinterlands during brief seasonal episodes. Although further analysis may demonstrate the beginnings of biotic and abiotic impact on forest structure, Late Neolithic pastoralism does not appear to have changed the ecological matrix.

The apparent shift in dominance from sheep to pigs through the Bronze Age (4.1–3.0 ka BP) could be an indication of a change in pastoral practices. We were unable to phylogenetically place the pig DNA found due to insufficient mtDNA coverage, however, it would be challenging to genetically discern these differences due to the complex lineages of Near Eastern domesticates and local domestications from *Sus scrofa* by around 6.0 ka BP.[63,71] In any case, pigs are less selective than sheep in forest grazing patterns, and both feral domestic pigs and wild boars remain a key issue in contemporary forest conservation in Europe.[72] Pigs have the capacity to disrupt normal patterns of succession and pedogenesis through indiscriminate consumption of saplings, herbs, shrubs, grasses, and forest litter. Pigs also practice rooting, which can lead to erosion of soil and nutrient depletion and significantly impact forest resilience.[73]

A corresponding change in woody plant DNA begins in the Early Bronze Age (~4.1 ka BP), consisting of taxa such as beech (*Fagus*) and spruce (*Picea*). These species are less favorable as animal feed due to their low nutritive values.[68] Wheat (*Triticum*) DNA also appears at the site during this period, together with the presence of other taxa such as sedges (*Carex*) and heather (*Calluna*). Here, again, DNA provides extended insight because, while wheat is represented in the fossil record at this point by charred grains, sedges and heather do not appear in the regional pollen or macrofossil record until the start of the Iron Age (~3.0–1.9 ka BP).[9,23] Representation of these species, if deposited with pig feces, suggests that agricultural waste was used as fodder, or that pigs were crossing cultivated fields and pastures in the vicinity of VM. In this case, we interpret this shift as an indication that Český Ráj had by this point become the isolated vestige of forest that it is today, bounded by the sandstone formations that make the area unsuitable for cultivation.

More intensive sampling of *seda*DNA across a range of Neolithic and Bronze Age sites could reveal the timing and sequence of habitat segmentations between Bohemian forests due to expansions in cultivation. These alterations in Bronze Age pastoral practices coincide with the wider mid-Holocene retrogression of Bohemian forest ecosystems, characterized by a shift to comparatively species-poor conditions, as evidenced by pollen records and nutrient depletion in paleosols.[8,9,74] While global-scale Quaternary climatic cycles are known to have influenced local factors such as precipitation, impacts of pastoralism may have affected the resilience and capacity of Central European forests to absorb these changes. In areas adjacent to Český Ráj, rapid agricultural expansion through slash-and-burn land conversion is observed in charcoal records, and our results from VM support the interpretation that this resulted in habitat segmentation of forests by the Bronze Age.[11] No longer used in transhumance, forested islands became a sort of commons predominantly used for grazing pigs; we argue that this could be a prime factor in the observed state shift. Expanded studies of *seda*DNA from archaeological and paleoecological contexts could help confirm a wider range of ecological damage, such as the transformation of mycorrhizal networks.

Shotgun sequencing sedimentary ancient DNA demonstrates here its power to enhance archaeological and paleoecological understanding of coupled human-environment systems. At VM, *seda*DNA successfully complements the fossil record to provide a more nuanced representation of species presence through which complex interactions between environmental practice and impact can be assessed. Wider application could provide many more reference points like VM and help to further unravel the coevolution of forests and pastoral practices in Central Europe while providing a historical frame for understanding forest resilience in the present. Archaeological deposits must be understood as valuable genetic archives for understanding the deep roots of the "Anthropocene."[75]

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mikkel Winther Pedersen (mwpedersen@sund.ku.dk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The raw sequencing data have been deposited at the European Nucleotide Archive (ENA) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
  - All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
  - Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

M.W.P., P.P., and M.W. conceived and planned the idea and experiments. G.Z. and L.E.H. carried out the laboratory work. G.Z., M.W.P., R.S., L.E.H., P.P., and M.W. planned and carried out the data analysis. P.S., P.P., M.P., and L.K. contributed to sample preparation. All authors contributed to the interpretation of the results. G.Z., M.W.P., and M.W. took the lead in writing the manuscript. All authors provided feedback and helped shape the research, analysis, and manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Sediment sample	This paper	VM-2
Sediment sample	This paper	VM-3
Sediment sample	This paper	VM-11
Sediment sample	This paper	VM-14
Sediment sample	This paper	VM-15
Sediment sample	This paper	VM-17
Sediment sample	This paper	VM-19
Sediment sample	This paper	VM-22
Sediment sample	This paper	VM-24
Sediment sample	This paper	VM-26
Sediment sample	This paper	VM-28
Sediment sample	This paper	VM-7
Sediment sample	This paper	VM-16
Sediment sample	This paper	VM-20
Sediment sample	This paper	VM-25
Sediment sample	This paper	VM-1
Sediment sample	This paper	VM-5
Sediment sample	This paper	VM-9
Sediment sample	This paper	VM-18
Sediment sample	This paper	VM-21
Sediment sample	This paper	VM-23
Sediment sample	This paper	VM-27
Charred nutshell (*Corylus avellana*)	This paper	UGAMS- 59900
Charred twig	This paper	UGAMS- 59903
Charred nutshell (*Corylus avellana*)	This paper	UGAMS- 59904
Chemicals, peptides, and recombinant protein		
Sodium phosphate buffer (pH 8.0)	MP Biomedical, Irvine, CA, USA	Cat# 6560205
Proteinase K	Roche Diagnostics, Mannheim, Germany	Cat#03115887001
Zymo research inhibitor spin columns	Zymo Research, USA	D6030
Magnetic beads (MagBio HighPrep PCR)	MagBio Genomics Inc., USA	AC-60050
Zymo-spin-based protocol	Wang et al.[76]	https://doi.org/10.1038/s41586-021-04016-x
Deposited data		
Sedimentary DNA sequence data	This paper	ENA: PRJEB59830
Codes and Rscipts	This paper	Zenodo: https://doi.org/10.5281/zenodo.7628960
Microbial sources	MGnify: https://www.ebi.ac.uk/metagenomics	All ENA accessions in Table S3 (this paper)

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
AdapterRemoval	Schubert et al.[77]	RRID: SCR_011834; https://adapterremoval.readthedocs.io/en/stable/
SGA	Simpson and Durbin[78]	RRID:SCR_001982; https://github.com/jts/sga/tree/master
Holi pipeline	Pedersen et al.[14]	https://doi.org/10.1016/j.cub.2021.04.027
Bowtie2	Langmead and Salzberg[79]	RRID: SCR_016368; https://bowtie-bio.sourceforge.net/bowtie2/index.shtml
metaDMG	Michelsen et al.[31]	https://github.com/metaDMG-dev/metaDMG-core; https://github.com/miwipe/metaDMG_installation
R project	http://www.r-project.org/	RRID: SCR_001905
Adobe Illustrator	https://adobe.com/products/illustrator	RRID: SCR_010279
Mafft	Katoh and Standley[80]	RRID: SCR_011811; https://mafft.cbrc.jp/alignment/software/
Raxml-ng	Kozlov et al.[81]	RRID:SCR_022066; https://github.com/amkozlov/raxml-ng
Angsd	Korneliussen et al.[82]	RRID:SCR_021865; https://www.popgen.dk/angsd/index.php/ANGSD
Beast	Drummond and Rambaut[33]	RRID:SCR_010228; http://beast.bio.ed.ac.uk/
FigTree	http://tree.bio.ed.ac.uk/software/figtree	RRID:SCR_008515
pathPhynder	Martiniano et al.[34]	https://github.com/ruidlpm/pathPhynder
Seaview	Gouy et al.[83]	RRID:SCR_015059; https://doua.prabi.fr/software/seaview
Sourcetracker2	Knights et al.[24]; McGhee et al.[43]	https://github.com/caporaso-lab/sourcetracker2
IntCal20	Reimer et al.[30]	https://intcal.org/data.html
Oxcal	Ramsey[29]	https://c14.arch.ox.ac.uk/oxcal.html
Geneious (2021.2.2)	http://www.geneious.com/	RRID:SCR_010519
MGnify	Richardson et al.[84]	RRID:SCR_016429; https://www.ebi.ac.uk/metagenomics
Other		
FastPrep-24™ 5G Homogenizer	MP Biomedicals, Santa Ana, CA, USA	Cat#116005500

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EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

We have analyzed genomic sequences from sediment samples collected from Velký Mamut'ák rock shelter, in the Czech Republic. See the method details section for more information.

METHOD DETAILS

Sampling

In 2019, we collected a total of 28 sediment samples (~10 grams), taken directly in the stratigraphic profile, of the North wall in the excavation trench (Figure 1C). We minimized contamination by removing any exposed layer, prior to transferring material to sterile 15-mL centrifuge tubes using sterile disposable scalpels while wearing face masks and nitrile gloves. All samples were hereafter transported to the ancient DNA dedicated laboratories at Globe Institute, University of Copenhagen, Denmark, and stored at -20°C, until subsequent DNA extraction and library preparation.

Radiocarbon dating

Three additional macrofossils were radiocarbon dated, at depths of 89, 198, and 239 cm (Table S1), to strengthen the previously published chronology,[22] now totaling 14 AMS dates. AMS dates were processed at Poznan Radiocarbon Laboratory, Center for Applied Isotope Studies at the University of Georgia, and Beta Analytic (for laboratory codes, and ages see Table S1).

Extraction, library preparation and sequencing

We extracted DNA from a total of 22 sediment samples (Data S1A and S1B) by subsampling ~0.25 cm^3 of sediment into 15 ml sterile spin tubes and adding 5ml of pH 8.0 sodium phosphate buffer (MP Biomedical, Irvine, CA, USA) together with 80ul (18mg/ml) of Proteinase K (Roche Diagnostics, Mannheim, Germany). The reactions were then homogenized using a FastPrep® (MP Biomedicals, Santa Ana, CA, USA) for 2 times 40 seconds at 4.5m/sec and subsequently incubated overnight at room temperature. All samples were hereafter processed by following the Zymo-spin-based protocol[76] and 20ul of the extracted DNA (including two negative controls) were then converted to double-stranded Illumina libraries following the standard protocol[85]. The final reaction was purified with magnetic beads (MagBio HighPrep PCR, MagBio Genomics Inc., USA) at a 1:1.8 ratio. All libraries were equimolarly pooled and sequenced on a HiSeq 4000 80bp single-end or Illumina NovaSeq 6000 100bp paired-end platform at the GeoGenetics Sequencing Core, University of Copenhagen.

Metagenome analyses

Base called and demultiplexed reads were trimmed using AdapterRemoval (v2.3.0)[77] and parsed through the 'Holi' pipeline[14] for quality control, low complexity filtering ($\leq$ 25 bp) and dereplication (Figure S2) using SGA (v. 0.10.15)[78]. The filtered reads were then mapped competitively using Bowtie2 (v. 2.3.2)[79] (with options *–k 5000 –no-unal*), against a set of publicly available databases including the non-redundant nt-database (NCBI) as well as the RefSeq database (last download January 2024, release 220). The alignments were then parsed through metaDMG (v. 0.38.0)[31] for taxonomic profiling (allowing a similarity between 95%–100% similarity). Using the range of the negative controls as a guidance, a threshold considering the level of damage (*MAP_damage* $\geq$ 0.05) and uncertainty (*MAP_significance* $\geq$ 10) was set with the purpose of targeting only authentic ancient reads. Given the high abundance of taxa with a low number of reads (ranging from 20 to 150), a high standard deviation ($\geq$10), and a phi value ($\geq$100), metaDMG was run again using a fully Bayesian model (–bayesian: true), aiming at a better resolution for the fitting model of those taxa (Figures S3A–S3C). This model adds a parameter called *significance*, which quantifies the certainty of the damage being non-zero based on the number of standard deviations ("sigmas"). A significance > 2 would indicate a 97.7% probability of the damage being larger than zero[31]. We explored the output damage statistics and used a data-driven approach to filter for ancient taxa (Data S2A–S2D) by requiring each genus to have a minimum level of damage $\geq$ 5% (*damage*) and a significance $\geq$ 2 (*significance*). We also filtered the concentration for the beta-binomial distribution (*phi*) $\geq$ 100 and the standard deviation of the damage (damage_std) $\leq$ 0.10. We investigated the degree of damage and significance for the full dataset without any threshold (Figure S3B). The library control (Figure S3A) shows a maximum level of damage of 12% and a maximum significance of 1.07, thus reflecting the non-ancient reads distribution shown in figures 6 and 7 in Michelsen et al.[31]

Post-mortem DNA damage was validated by plotting the deamination patterns on the DNA strand of the most abundant genera and of the unexpected ones (*Bison*, *Bubalus*, *Oryx*, *Budorcas* and *Phacochoerus*) (see Figures S4 and S5). We also explored the fragment length distribution (Figure S6), damage and mean length by depth (Figure S7) of the most abundant taxa found, which independently confirm the authenticity of our parsed reads. Key statistics and taxonomic profiles were plotted using R and edited with Adobe Illustrator (v. 24.0.3, https://adobe.com/products/illustrator). Scripts and workflow are available for download (10.5281/zenodo.7628960).

Phylogenetic placement

Phylogenetic placement of mitochondrial genomes for relevant animal genera was performed to distinguish domestic taxa from their wild relatives. First, we performed a preliminary alignment to the NCBI reference mitochondrial genome of sheep (*Ovis* sp.), cattle (*Bos taurus*), aurochs (*Bos primigenius*), domestic pig (*Sus domesticus*), wild boar (*Sus scrofa*), goat/ibex (*Capra* sp.) to verify the

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abundance of total aligned reads (Data S3A). After this step, we excluded from further phylogenetic analyses all the reads assigned to *Sus* and *Capra* because of low coverage (Data S3B). We then downloaded a set of mitochondrial complete genome fasta sequences of sheep and cattle from NCBI's GenBank and performed a multiple genome alignment with MAFFT (v7.427)[80] reproducing Maximum Likelihood phylogenetic trees (RAXML-NG, v.1.1)[81] from previously published studies[86,87]. We selected a total of 56 mitogenomes of modern sheep from various breeds of *O. aries*, *O. ammon*, *O. vignei*, *O. orientalis ophion*, and *O. aries musimon*, using a modern *Capra hircus* as outgroup[86] (Table S2). As for reads assigned to *Bos*, we used a total of 15 mitogenomes from *Bos primigenius*, *Bos taurus* haplogroups T, Q, and R, using a modern *Bos indicus* as an outgroup[87] (Table S2). We generated a consensus read for each haplogroup using Geneious (v. 2021.2.2) with the criterion of the majority rules. We extracted the readIDs classified within the family Bovidae from metaDMG-lca files following the method described in Kjær et al.[13] and aligned the damaged filtered reads against each consensus sequence separately using Bowtie2 (v. 2.3.2), with a minimum mapping quality of 30. We produced a consensus of the sequences from the bam files with ANGSD (v. 0.928)[82] and an alignment file using MAFFT (v7.427). We performed phylogenetic placement using BEAST (v.1.10.4) (32) with 20,000,000 replicates and a Bayesian inference analysis with the MCMC sampling method, applying the HKY model (Figures S8 and S9). Trees were visualized with FIGTREE (v1.4.4). In addition to that, to avoid mapping bias, we re-mapped all the extracted reads to the consensus sequence following the method described in Kjær et al.[13] and we retrieved a total of 135 and 393 reads for *Ovis* and *Bos* respectively. We then ran metaDMG with the "global" damage-mode setting to assess the degree of damage for all the mapped reads and each sample. For both *Ovis* and *Bos* reads, we estimated a level of damage between 7 and 17% (the full statistics is reported in Data S4A). Finally, we ran pathPhynder (v. 1.a)[34] to identify the unique markers carried by the different haplogroups of sheep and cattle. This algorithm allowed us to place the above-mentioned sequences to branches of a tree based on ancestral and derived SNPs (Single Nucleotide Polymorphisms). We performed both transitions and transversions analysis (*pathPhynder -s all -t 100*) as well as only transversion analysis (*pathPhynder -s all -t 100 -m transversions*), but the latter was not successful in one of our *Bos* alignments (VM-17) and in both *Ovis* alignments (VM-17, VM-19). To further investigate the quality of the placement produced by pathPhynder, we visually inspected the transitions and their position in the mapped sequences by using Seaview software (v. 5.0.5)[83].

Microbial source tracking

All reads were additionally aligned against the bacterial database GTDB[88] release 202 using bowtie2 default end-to-end alignment. DNA damage estimation for each taxonomic level was evaluated using metaDMG with similar settings as above. Source data (7 gut metagenomes and 15 environments) was downloaded from published microbiome datasets using MGnify[84] (accessions are reported in Table S3) and analyzed identically with the exception that taxonomic profiles were parsed at the species level to Sourcetracker2[24,43]. With the option –sink_rarefaction_depth 100 and –per_sink_feature_assignments to estimate the proportion of each source in sample datasets. The output was grouped by source categories (Data S5A). Multiple sources were utilized to cover diverse environments, and then categorized under "Other soils''. These included metagenomes from the desert, freshwater, permafrost, melting permafrost, agricultural soil, tropical forests, and biocrust. Similarly, we parsed the taxonomic profiles with DNA damage (*damage* $\geq$ 0.05, *significance* $\geq$ 2, see Figures S10C and S10D and Data S5B and S5C). We plotted the proportion of source categories per sample and excluded categories with less than 1% proportion. We extracted the top 10 taxa assigned as animal sources (Table S4) from feature tables of Sourcetracker2 results and plotted the degree of damage for each taxon (Figure S10E).

QUANTIFICATION AND STATISTICAL ANALYSIS

Chronometric data and age modeling of DNA samples

All C14 dates were calibrated in Oxcal v 4.4.4[29] using the IntCal20[30]. Samples were taken during the initial excavation of the test trench at Velký Mamuťák in sectors B and D; exceptions include Beta-473738, Poz-97105, Poz-97106, and taken in the microstratigraphy column in sector B profile (Table S1). The age-depth model (Figure S1) was constructed assuming a non-linear deposition (mediated by a Poisson process). The model was created using Oxcal v 4.4.4[29] with r:5 atmospheric data (IntCal20) from Reimer et al.[30]