Sedimentary ancient DNA reveals Neolithic pastoralism and plant community interactions at Southern European high altitudes

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Abstract

The Neolithization process introduced significant ecological impacts, especially in Mediterranean mountain areas. We generated a comprehensive sedimentary ancient DNA record from the central Pyrenees, spanning 12.2 to 1.3 ka BP, revealing the earliest continuous presence of sheep (6.5 ka BP) and cattle (5.9 ka BP) in alpine southern Europe. This evidence suggests pastoralism nearly concurred with the Neolithic in the Iberian lowlands, challenging prior assumptions of only sporadic occurrence and confirming Neolithic pastoralist use of mountain ecosystems. A notable plant community shift arosed at 6 ka BP, with deciduous forests transitioning into diverse open grasslands. This change became pronounced at 4.2 ka BP, aligning with continuous presence of domesticates and a regional cooling climate, suggesting a synergistic relationship between past climate change and human-induced plant community alteration. These findings highlight the complex interactions between climate, human activities, and landscape dynamics during the Neolithic in Mediterranean mountains.

1. Introduction

The timings of the transition from foraging to farming economies is a pivotal focus of current transdisciplinary research. This transition, known as the Neolithization process, marked a significant turning point in both human and landscape evolution [1]. It started in the Fertile Crescent ca. 11.5 thousand calibrated years ago (ka BP) and reached Europe ca 8.5 ka BP in Greece, via the introduction of domesticated plants and animals. These early introductions have spurred extensive research into the mechanisms of expansion, their cultural significance, and, more recently, their environmental impacts and long-term ecosystem effects [2,3]. In the western Mediterranean basin, the advent of the Neolithic period instigated notable cultural, symbolic, economic, genetic, and environmental transformations [4,5]. Nevertheless, there is a pronounced knowledge gap regarding the repercussions of early pastoralism on ecosystem dynamics in the Mediterranean region, where some of the most ancient complex societies originated, making the enduring impacts of agriculture more discernible [6]. Numerous hypotheses have been proposed regarding alterations of the landscape due to intensified land use, particularly in central Europe [7–9]. However, a lingering uncertainty persists concerning the potential long-term ecological impact of domesticated animals and even more so in mountain areas [10–12].

Mountains play a crucial role in our comprehension of early animal husbandry, as they likely enabled the continuous presence of domesticated animals for a substantial part of the Holocene period [13]. The presence of productive pastures in summer likely facilitated the early adoption of seasonal resource utilisation strategies such as the pastoral mobility in altitude, known as transterminance [14,15]. It has been generally accepted that montane Mediterranean environments have long been transformed by human activities [16–21]. However, identifying clear unequivocal evidence of long-term human impact on vegetation in the high-altitude regions of the central Pyrenees, has often been difficult and a true challenge, only becoming noticeable from the Mediaeval period onwards [22–24]. This study addresses the knowledge gap on the interaction between past climate changes and human landscape management in the Pyrenees, being one of the very few available studies in southern European mountains, by showcasing the early occurrence of domesticated animals and their impact on plant communities [25–29].

Archaeological data from the Iberian Peninsula lowlands show a rapid integration of domesticated animals at 7.5 ka BP [30] and the subsequent occupation of mountain areas such as the Pyrenees from around 7.3 ka BP [31,32]. However, while archaeological research provides a sound perspective on the importance of human presence in high altitude areas of the Pyrenees, it does not definitively establish clear connections between ecosystem responses and human influence there. The presence of a cattle bone at the Coro Trasito archaeological site (1600 m a.s.l., **Fig. 1**), dating ca. 7 ka BP, represents the earliest known instance of cattle at such high altitudes in the Iberian Peninsula [33]. Nevertheless, a generally scattered archaeological record of cattle bones in NE Iberia, including the Pyrenees, compared to the very abundant presence of sheep bones, has prevented us from knowing the complete scenario of domesticated animal management in the mountains, thereby generalising the idea of a sparse, discontinuous presence of cattle in these mountains [34]. Despite the wealth of archaeological data on the Neolithization process in the Pyrenees [31,33,35,36], there is an absence of spatially-coincident direct evidence of both ecosystem change and the presence of different animal herds. This lack of evidence under a robust quantitative framework hinders testing hypotheses regarding the main types of herds associated with Pyrenean landscape management.

The motivation for this study is thus to establish the early presence of domesticated animals in the southern Pyrenean region and their impacts on the landscape.

Our objectives are twofold: i) to test the archaeological timing of arrival for the first domesticated animals at high altitudes in the central Pyrenees, with a particular focus on distinguishing between various herbivore groups; and ii) to assess the potential impact of these domesticated animals on the plant communities of the current subalpine area of the Pyrenees. To achieve these aims, we present the first record of both animal and plant sedimentary ancient DNA (*seda*DNA) metabarcoding from a lacustrine sequence in the Pyrenees, Tramacastilla Lake (TRAM21), located at 1682 m a.s.l. (**Fig. 1**).

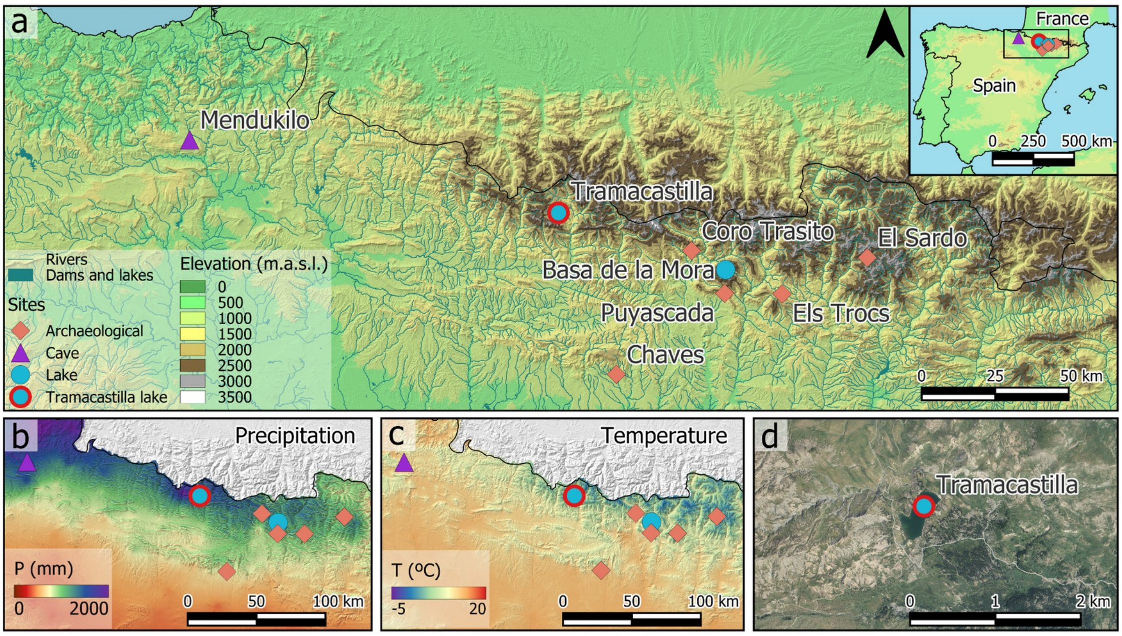

Figure 1. ***Location map of Tramacastilla Lake, highlighted with a red circle, and other records (cave, lake, and archaeological sites) cited in this article****: a) altitude map; b) mean annual precipitation; c) mean annual temperature; d) orthophoto of Tramacastilla lake. Map sources: elevation data from ASTER GDEM v2 (METI/NASA, 2011), hydrology from IGN-CNIG 1:200k base map (BCN200), climate data from Digital Climatic Atlas of the Iberian Peninsula [37], and orthophoto from IGN-CNIG (PNOA). Projection UTM 30 Datum ETRS89 (EPSG: 25830). All coordinates in* ***Supp. Data 12****.*

2. Results

We developed an independent age-depth model for the Tramacastilla sediment cores acquired in 2020 and 2021, from which *seda*DNA was extracted (Methods and **Figs. 1, 2 and Table 1 Supp. Mat**). The sedimentary archive from Tramacastilla spans from 25 to 1.3 ka BP, but we present here results for the period between 12.2 and 1.3 ka BP, as this period offers the optimal time resolution of the record, with a mean sedimentation rate of 30 yrs/cm (Standard Deviation (SD) 19 yrs/cm, see also **Table 1 Supp. Mat.**).

2.1 Molecular evidence of plant and animal community changes

We present the *seda*DNA findings using a Relative Abundance Index (RAI), which is a ratio that represents the relative abundance of each taxa, obtained by multiplying the relative proportion of total reads and the proportion of weighted PCR replicates in which the sequence was identified [25]. A total of 9.8 million reads of 529 plant sequences were initially acquired in all 46 samples analysed (**Supp. Data 2**), with 423 sequences meeting post-filtering criteria (**Supp. Data 3**). Within this set, 192 taxa were identified at the species level, 171 at genus level and 57 at family level. Regarding animal sequences, a total of 2.9 million reads of 62 sequences were obtained in 42 samples, with no animal *seda*DNA detected in four samples (**Supp. Data 8**). Within this group, 32 taxa were excluded from analysis (31 worms and one bryozoan), retaining then 30 taxa that comprises 11 wild mammals, 10 other wild animals, 3 domesticated mammals, and 6 categorised as 'others' that could be either domesticated or wild mammals. Notably, 27 animal taxa were identified at the species or subspecies level, demonstrating a high degree of taxonomic precision, with the remaining three classified at the genus level. We highlight the precise identification of domesticated mammals, all of which belonged to the ruminant category, including cattle, sheep and goats. The proportion of weighted PCR replicates where plant and animal sequences were found are represented in **Figs. 3-12 Supp. Mat.**

The analysis of *seda*DNA from Tramacastilla Lake reveals the prevalence of deciduous forest communities (**Fig. 2a**) since the onset of the Holocene, with RAI values fluctuating between 40 and 75% (**Supp. Data 5**, total mean and SD 28.1±26.4%, **Table 2 Supp. Mat**). Then, at 6 ka BP, these communities started to decrease, until the Northgrippian to Meghalayan transition (4.2 ka BP), at which point the deciduous forest practically disappears. During the first interval of the studied period (12.2-9.7 ka BP), *Pinus* sp. is consistently present and reaches up to 40%, but thereafter declines to almost negligible RAI values, aside from some expansions ca. 3.6 and 1.6 ka BP (**Fig. 2b** and **Supp. Data 5**, total mean 4.3%±9.3%, **Table 2 Supp. Mat**). Throughout the transition period between 6-4.2 ka BP, open landscape RAI increased (**Fig. 2a** and **Supp. Data 5**), with values from 30 to 50% until the end of the record, at approximately 1.3 ka BP. Parallel to the progressive expansion of open communities, silver fir (*Abies alba* Mill.) is first detected at ca. 6 ka BP with relatively low RAI abundances until 3.2 ka BP (0.1-22%). Between 3.2-2.4 ka BP there is an increase in *Abies* RAI values, reaching 75% at 2.4 ka BP, and then maintains its percentages stable at around 30% until the end of the record (**Fig. 2b, Supp. Data 5**).

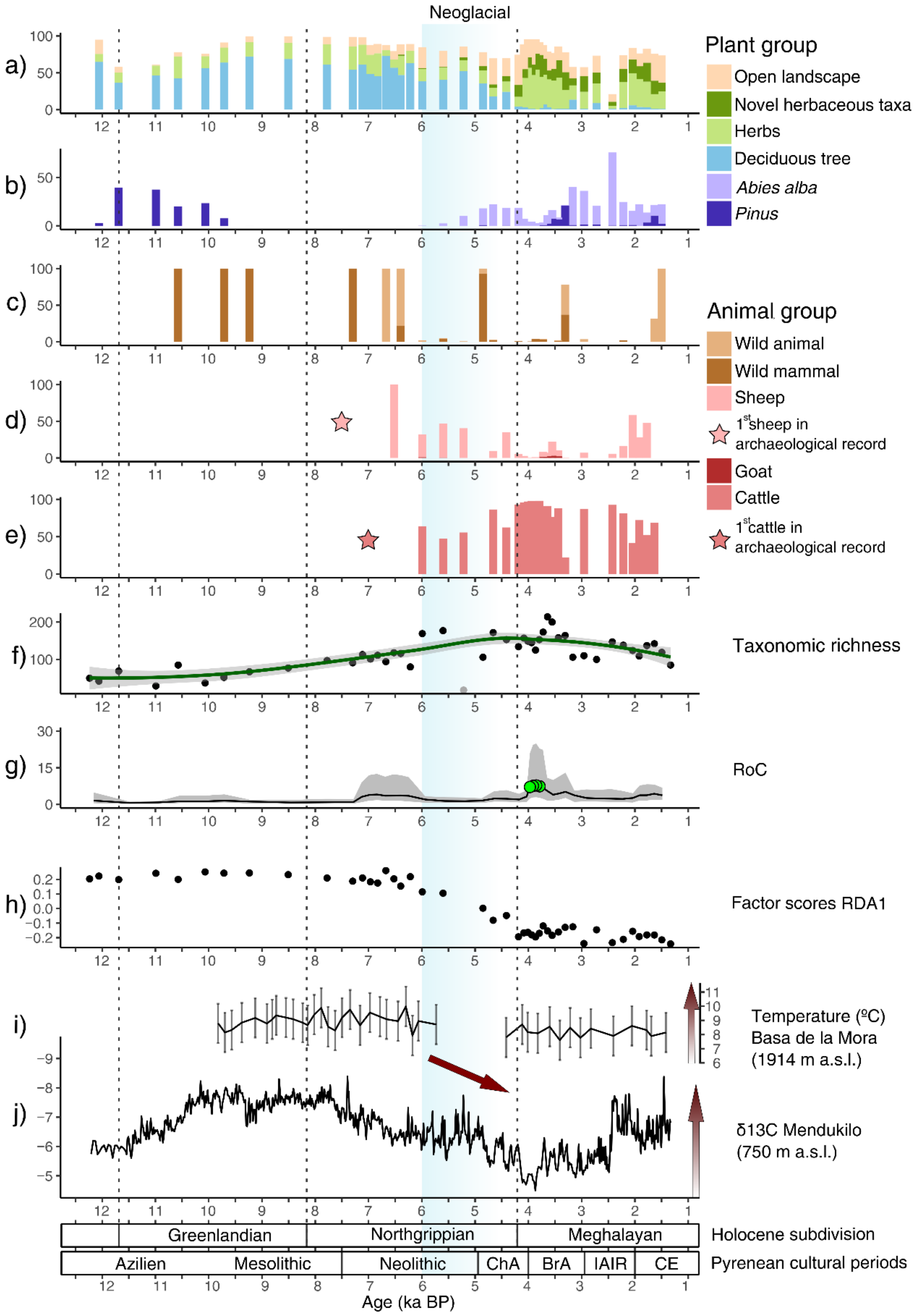

Figure 2. *Palaeological synthesis for Tramacastilla Lake throughout the Holocene*. *Indicated in light blue is the beginning of the period known as Neoglacial* [38]*, and vertical dashed lines represent 11.7, 8.2 and 4.2 ka BP Holocene subdivisions. RAI percentages for: a-b) selected plant groups; c) wild*

mammals and wild animals; d) sheep and goats and e) cattle, with the star symbols indicating the chronology of the first sheep or cattle occurrence in the Pyrenean archaeological record (sheep: Chaves cave, 7.5 ka BP 39; cattle: Coro Trasito cave, 7 ka BP[33]*; f) taxonomic richness of plants and animals with loess smooth area, with grey point representing low-DNA-quality sample TRAM_1b_4U_34.35 (6.5 ka BP); g) rate of change (RoC) analysis results, with uncertainty range in grey, and green circles representing statistically significant points when community composition changes; h) RDA scores for first axis, seen as an open landscape index; i) July temperature reconstruction for Basa de la Mora lake based on chironomid analysis, with sample-specific standard error bars*[40]*; j) Mendukilo cave isotope composites for* $\delta^{13}C$*, that represents a temperature variability reconstruction*[41]*. The accepted subdivisions of the Holocene*[13] *and cultural periods for the Pyrenees are shown at the base of the figure: Chalcolithic Age (ChA), Bronze Age (BrA), Iron Age and Ibero-Roman (IAIR) and Current Era (CE). All colours are suitable for colour blindness.*

The landscape around Tramacastilla Lake would have remained open for most of the Meghalayan period (4.2 ka BP-present), with mean herb RAI abundances of 32.4±13.5% (**Supp. Data 5**) (**Fig. 2a**). Amongst the *sed*aDNA of herbs, there is a group whose onset happens at ca 6.5 ka BP and becomes prevalent at 5 ka BP, that were never found before in our time series; consequently, this plant group has been designated as 'Novel herbaceous taxa' (**Fig. 2a**) indicating its unprecedented presence, but not linked to human introduction, nor an invasive character. Wild mammal and wild animal RAI values (**Fig. 2c**) are found scattered across most of the Holocene (particularly between 10.5-9.2 and 7-6.4 ka BP). These mainly correspond to red deer *(Cervus elaphus* L.) at ca. 10.5, 9.7 and 7.3 ka BP (RAI 100%, **Supp. Data 10**) and the sporadic presence of other large mammals such as brown bear (*Ursus arctos* L.), which was observed at ca. 9.2 ka BP (RAI 100%) and 3.3 ka BP (RAI 2%). Amphibians, such as the common toad (*Bufo bufo* L.), are notably prevalent around 6.6 and 6.3 ka BP, while the common frog (*Rana temporaria* L.) becomes the dominant species in the wild animal assemblage at approximately 1.5 ka BP (RAI 100%). We found the first *sed*aDNA detections of sheep at 6.5 ka BP with varying RAI values since then, interspersed with *sed*aDNA from goats at lower values (**Fig. 2d**). The evidence for domesticated animals is further supported by the consistent detection of cattle *sed*aDNA in this lacustrine record for nearly the entire period spanning 6 to 1.3 ka BP (**Fig. 2e**), exhibiting elevated RAI values approaching 100% (mean RAI of 65.3±33.5%, **Table 3 Supp. Mat** and **Supp. Data 10**).

In general terms, we identified a vegetation composition transition between 6 and 4 ka BP, marked by a gradual shift from a diminishing deciduous tree vegetation type to an expanding grassland community (**Fig. 2a**), that occurs coeval with the arrival of domesticated animals in the lake basin from 6 ka BP. Significantly, an unprecedented shift in the floral composition of pastures takes place since 6.5 ka BP and steadily increased from 5 ka BP, marked by the appearance of novel, not human-introduced, nor invasive herbaceous taxa (**Fig. 2a** and **Supp. Data 5**).

2.2. Taxonomic richness, rate of change and plant community trajectories

Excluding sample TRAM_1b_3U_136.137 (5.1 ka BP), with only 18 taxa identified, the taxonomic richness of both animals and plants, varied between 29 and 214 taxa (mean 118±43) reaching its highest values at 3.6 ka BP for both plants and animals (**Fig. 2f**). The analysis of the rate of change (RoC) of RAI values of Tramacastilla plants and animals indicates significant changes between 4 and 3.5 ka BP (**Fig. 2g**).

A transformation-based canonical Redundancy Analysis (tb-RDA) of plants, and animals as explanatory variables, (**Fig. 3a**) (**Tables 5-10 and Fig. 13 in Supp. Mat.**) yielded a globally significant analysis (p-value < 0.01), where the first axis (RDA1) explains 39% of the observed variance. Cattle RAI is the only explanatory variable that is significant (p-value < 0.01), although it presents a variance inflation factor (VIF) of 1.63, indicating a slight multicollinearity. In a broader context, the tb-RDA illustrates an ecosystem trajectory that aligns with the temporal correlation between the declining deciduous forests and the emergence of open landscapes around 4-4.5 ka BP. This trend is summarised in RDA1, plotted in **Fig. 2h** and can be used as an open landscape index. Notably, the expansion of open landscapes and the appearance of novel herbaceous taxa (**Fig. 2a**) occurred mostly under the presence of cattle (5-4 ka BP, **Fig. 2e**, **Fig. 3a**), while the proliferation of silver fir at 5 ka BP (**Fig. 2b**) aligned with the presence of sheep (4-1 ka BP, **Fig. 2b, d**, **Fig. 3a**).

We targeted a tb-RDA on just open landscape plant groups, namely ‘Open landscape’, ‘Herbs’ and ‘Novel herbaceous taxa’ previously grouped under three functional variables (favoured by fertilisation, grazing or trampling and grazing - see Methods) (**Fig. 3b**) (**Tables 11-16 and Fig. 14 Supp. Mat.**). This is also significant (p-value < 0.01) with RDA1 explaining 30% of the total variance. Cattle is the only significant variable among animals (p-value < 0.01), and similar to the global tb-RDA, exhibits subtle multicollinearity (VIF = 1.6). The ecosystem trajectory of the open landscape community in the tb-RDA exhibits minimal variability along both axes 1 and 2 but suggests an association of herbs favoured by fertilisation and the presence of red deer at 12 to 9 ka

BP (**Fig. 3b**). The community trajectory between 9 to 6 and 3 to 1 ka BP was led by plants promoted by trampling and grazing. Additionally, these plants align with the presence of sheep, goats, and cattle, although not in a distinctly separated manner. The community between 6 to 3 ka BP tend to exhibit plants favoured by grazers and coexisting with other animals.

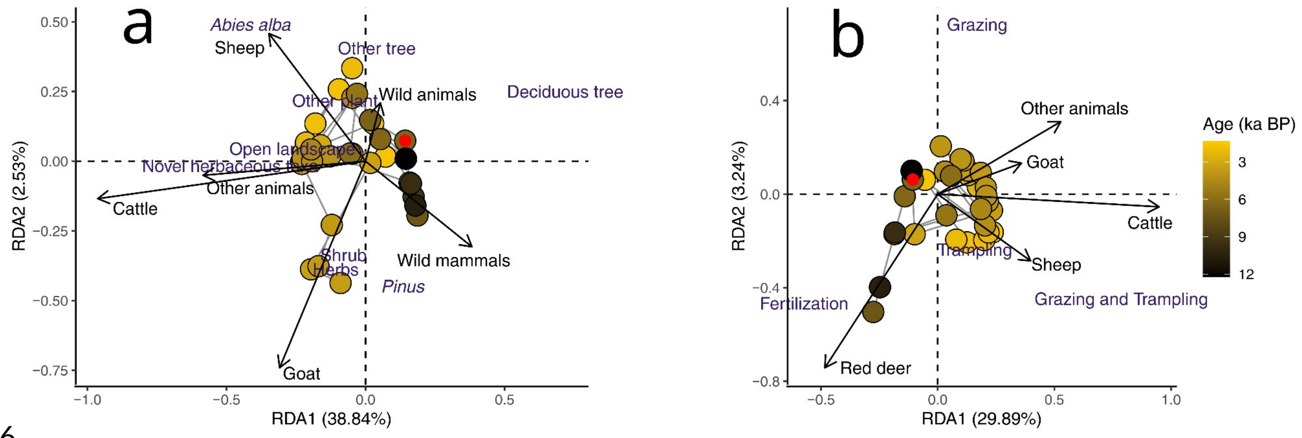

Figure 3. ***Ordination analyses with Hellinger transformation (tb-RDA):*** *a) for all plant taxa with all animals as explanatory variables; and b) for all open landscape plants (i.e. 'Open landscape', 'Herbs' and 'Novel herbaceous taxa') classified according to whether they are favoured by grazing, trampling or fertilisation practices, with selected animals as explanatory variables. Red circles represent the sample where the first appearance of domesticated animals is detected (6.5 ka BP, sample TRAM_1b_4U_34.35).*

3. Discussion

We present, to our knowledge, the earliest molecular evidence of domesticated animal presence and grazing in European mountains based on *seda*DNA (**Fig. 2**). Our results depict plant community dynamics from a mid-altitude natural archive (Tramacastilla Lake, 1682 m a.s.l.) in the Central Pyrenees, offering a continuous record of vegetation and faunal changes from 12.2 to 1.3 ka BP.

3.1 Pastoral timing in the Pyrenees: cattle and sheep

In many mid-latitude European regions, pastoral timing is frequently proposed to have been instrumental in clearing dense forest cover and shaping landscapes [17,42–47] since the Northgrippian period (8.2-4.2 ka BP following [13]). However, most of the studies are based on indirect palaeoecological indicators, such as pollen assemblages [48–50], coprophilous fungal spores [51], or archaeological sites, including both anthracological data and the presence of bones of domestic fauna [52]. They record episodic presence of domestics during the Neolithic (at 6 ka BP) and the Bronze Age (since 4 ka BP) before agro-pastoralism in the Middle Ages (more information about archaeological context in **Supp. Mat. 9**). The first direct evidence of domesticated animals in European mountains has been recorded in Lake Anterne (northern French Alps), with cattle *sed*aDNA at ca. 5 ka BP, although sporadic until 2.4 ka BP [26]. Complementarily, Lake Sulsseewli (central Alps) presents a more continuous record of direct evidence of domestic animals (cattle and sheep) since 4 ka BP [25].

Among early agro-pastoralist communities inhabiting the Mediterranean basin mountains, sheep (*Ovis aries* L.) and goat (*Capra hircus* L.) held prominence as one of the earliest domesticated animals, primarily due to their remarkable versatility and adaptability to various environments [35]. It is hypothesised that they became the foremost species for economic exploitation, also on both slopes of the Pyrenees since 7.5-7.3 ka BP [33,35,53–56], with sheep forming a significant component of domestic herds throughout prehistoric times [30]. In fact, most of the zooarchaeological domesticate remnants found in the Pyrenees correspond to sheep and goats (in Chaves, Coro Trasito, Els Trocs, Puyascada and El Sardo caves: **Fig. 1**), while cattle (*Bos taurus* L.) herds have been found at low abundance albeit sparsely present during the early Neolithic [34,35,56,57]. Traditionally, archaeological findings of domestic animals at Pyrenean high-altitude caves have suggested that these were seasonally occupied during spring and summer, likely indicating a transterminant landscape management [15], thereby implying periodic movements from lowland areas [56]. This does not generally happen in lowland shelters as it is the case of Chaves Cave, at 640 m a.s.l and in the outer Pyrenean range (**Fig.1**). This is a pivotal site for understanding the arrival of Neolithic and management of early domesticates in northeastern Iberia since 7.6 ka BP [35,36,39], and has been proved to be a permanently occupied habitat. Regarding fauna remnants, cattle is infrequent as well in lowland locations as found in Chaves, where there are mainly sheep (more than 50% including both *Ovis aries*, *Capra hircus* and *Ovis*/*Capra* categories) and only ca. 2% of cattle [55].

Our study reveals the consistent presence of *sed*aDNA from sheep (since 6.5±0.3 ka BP) and cattle (since 5.9±0.3 ka BP) throughout the Northgrippian and Meghalayan Holocene sub-periods in the Tramacastilla Lake record (**Fig. 2d, e**). This finding constitutes the earliest direct molecular and uninterrupted evidence of cattle in European mountainous regions compared to other records, like Lake Anterne or Lake Sulsseewli in the Alps, that constitute other available records of domesticates in high altitudes but nearly two thousand years later (ca. 4 ka BP) [25,26]. Our findings reinforce the historical management of cattle, sheep and goats at high altitudes over the past seven millennia. The limited regional archaeological records of early cattle husbandry in the high altitudes of the Central Pyrenees (Coro Trasito, Els Trocs, Puyascada, **Fig. 1**) have provided a synchronous spatio-temporal scenario of cattle presence to that of Tramacastilla (**Fig. 2e and Supp. Data 10**), confirming its presence at ca. 7 ka BP in subalpine environments. Indeed, cattle could have co-existed with native wild aurochs that were present in the Iberian Peninsula from the Middle Pleistocene to the Roman Period [58]. In any case, Coro Trasito in particular has yielded the oldest dated fragment of cattle in the Pyrenees, with an age of 7.1-6.9 ka BP [33], about a millennium before our molecular evidence in Tramacastilla.

Both the archaeological context and our molecular evidence from Tramacastilla Lake, supports that: i) cattle husbandry onset in the high altitudes of the central Pyrenees probably appeared at ca. 7 ka BP, although this age could be reduced by a thousand years if ages based solely on molecular evidence are considered; and ii) there was a continuous presence of cattle since then, defying the idea of a punctuated presence given the sparse archaeological record (**Fig. 2e**). In this way, both the new molecular data from Tramacastilla Lake and the entire regional archaeological scenario confirm that human use of the high altitude areas of the Pyrenees only occurred three centuries after the first Neolithic human settlements in the region (**Fig. 1 and Supp. Data 7**).

3.2 Landscape transformation: from dense to open vegetation and pasture novelty

In the Tramacastilla record, the floristic and functional change from woodland to open landscape initiated before 6 ka BP, seems to become critical at ca. 4 ka BP (**Fig. 2a and Supp. Data 5**), when significant RoC values appear in our sequence (**Fig. 2g**). Until then, the plant communities around Tramacastilla may have resiliently responded to environmental change, undergoing a critical transition when deforestation and grassland compositional change occurred. Considering that, in our record the primary transformation in the plant community (first appearance of "Novel herbaceous taxa" category: **Fig. 2a**), co-occurs with the emergence of domesticated animals at 6.5 ka BP (first sheep appearance: **Fig. 2b**), being then tempting to propose the logical causation that Neolithic people intentionally cleared woodlands to facilitate the expansion of pastures and, perhaps, key tree taxa.

However there is sound, quantitative, independent regional climate evidence supporting a notable temperature reduction between 6 and 4 ka BP in numerous sites in the Pyrenees (glacier advances during the beginning of the Neoglacial [38]), and particularly in records relatively near to Tramacastilla (Basa de la Mora, 1903 m a.s.l., **Fig. 1**, and **Fig. 2i** [40]) and in lower, more western areas of the Pyrenees (Mendukilo, 750 m a.s.l., **Fig. 1** and **Fig. 2j** [41]). Such a cooling phase could have acted either as a primary cause of the landscape opening at ca. 6 to 5 ka BP, or as a reinforcing factor of the herding activity in Tramacastilla, which would have started ca. 6.5 ka BP, before the onset of the declining temperature (**Fig. 2d, e, i** and **j**). The decrease in local to regional temperatures contrasts with other paleoclimate reconstructions at the Iberian scale, where winter temperatures may have shown a consistent increase throughout the Holocene period [59]. However, these climate reconstructions suggest that both summer temperatures and plant moisture availability from 6 to 4 ka BP lack a discernible positive trend [59], aligning with broader continental models and simulations [60].

We hypothesise that a likely climate-driven landscape opening may have been sustained, initially by small herds of sheep and goats, and later by cattle. The progressive woodland clearing would have facilitated the expansion of herbs that are often frequent in forest margins, all those under 'Novel herbaceous taxa' (**Supp. Data 4**). These plants were native and not from an exotic origin, and occupied a newly created niche, expanding into the open areas near the lake. The major floristic change at 4 ka BP, as indicated by increased richness (**Fig. 2f**) and the sole significant RoC values (**Fig. 2g**), reflects such forest opening. Our hypothesis is additionally supported by the clear presence of human activity plant indicators, like *Urtica dioica*, that only appeared in our *sed*aDNA record after domestic animals arrived. The tb-RDA results further underscore the influence of domesticated animals on herbaceous plant communities. Notably, the sustained presence of cattle is associated with plants indicative of open landscapes ('Open landscape' and 'Novel herbaceous taxa' categories), contributing to the remarkable emergence of new herbs on the vegetation landscape (**Fig. 3a**).

Moreover, the trampling of livestock could have facilitated the proliferation of plant species, even trees, that thrive under such disturbance, a phenomenon commonly observed in mountainous grassland ecosystems (**Fig. 3b**). This is probably the case of Tramacastilla deciduous forest's regression at 6 ka BP, a trampling livestock scenario sharply contrasting with the spread of silver fir *(Abies alba*) at 5 ka BP. These shifts in the Tramacastilla forest community initially reflect broader dynamics observed in the Pyrenees and across Europe. In the Pyrenees, the initial colonisation of silver fir, documented between 9.5 - 8 ka BP, was primarily concentrated in the eastern part of the northern slope [61,62]. Several millennia later, ca 6.5 ka BP, the earliest evidence of *Abies* pollen appeared in the central Pyrenees region, reaching a peak at 4 ka BP [63]. Research carried out in Central Europe has linked the decline of silver fir at 3 ka BP to climatic changes and human influences, particularly the increasing human impact during the Bronze and Iron Ages [64]. While silver fir was extensively used in Central Europe for construction during these periods, leading to its gradual decline due to exploitation and agricultural expansion [64], there is evidence that low-impact pastoralism may have facilitated fir growth during certain periods, such as 5.5 to 4 ka BP in what is now the Czech Republic [65]. In fact, the presence of *Abies* at Tramacastilla coincides with sustained cattle presence in the area (**Fig. 2b, e**), suggesting that pastoral activities did not significantly affect silver fir expansion. Although our findings diverge from broader trends observed in the Alps region [28,66], they align with those of the Czech Republic, indicating that grazing may have facilitated *Abies* regeneration. However, this contradicts modern ecological studies suggesting negative effects of grazing on silver fir dynamics [67]. Our study emphasises the complex interplay between climate change, grazing practices, and forest dynamics since 6 ka BP, highlighting the need for further research to understand this variability.

In summary, our findings present the earliest direct evidence of sustained cattle presence in European mountainous regions, aligning with the concept of continuous cattle occupancy since approximately 6 ka BP. Furthermore, our analysis illuminates the Meghalayan transformation from dense forest to open grasslands, coinciding with the introduction of domesticated animals around 6 ka BP and a pivotal transition at 4 ka BP. Our study underscores the significant influence of climate change, notably a discernible temperature decrease during the beginning of the Neoglacial period, on landscape dynamics. The confluence of cooler climates concurred with the expansion of grazing practices by Neolithic communities, potentially following a transterminant herding model. The synergic effect of climate and grazing likely contributed to the gradual clearing of woodlands and the emergence of open landscapes and novel pasture land communities since 6 ka BP, and a critical plant community change since 4 ka BP in the surroundings of Tramacastilla. This newly established ecosystem probably enabled the presence of mosaic-like landscapes that are currently at risk due to the abandonment of traditional land uses [68].

4. Methods

Geographical setting

Tramacastilla Lake (42°43'31.57"N, 0°22'03.73"W) is located at 1682 m a.s.l. in the subalpine belt of the Upper Gállego Valley of the southern Central Pyrenees (Huesca, NE Spain) (**Fig. 1**), in a glacial valley [69]. Before the construction of a dam in 1956, the lake had a maximum depth of 4.5 m. Following the damming, the depth underwent a substantial increase, reaching 13.5 m. The lake's surroundings are deforested (**Fig. 1d**) due to human exploitation. Consequently, the vegetation predominantly comprises grasslands with scattered shrubs and trees such as *Pinus uncinata* Ramond ex DC., *Pinus sylvestris* L., *Rosa* sp., *and Juniperus communis* L..

Core sampling and age depth model

We retrieved four sediment cores at 13.5 m water depth, using a UWITEC platform in two fieldwork campaigns in October 2020 and October 2021, from which the two most continuous ones were selected (TRAM20-1B and TRAM21-1B; each core approximately 10 m long divided in five sections, 1U to 5U, **Fig. 1 Supp. Mat.**). All cores were lengthwise split, photographed, and stored at 4°C at the Pyrenean Institute of Ecology (IPE-CSIC, Zaragoza). All analyses in the present study were performed on sections 3 and 4 (total length of 360cm) from core TRAM21-1B. Sections 1U and 2U exhibit sedimentary features that preclude their use in building a reasonable chronology, while section 5U consisted of sediments of an age beyond the scope of the present study.

The age-depth model for the composite sequence was obtained by combining sections from cores TRAM20-1B and TRAM21-1B, that were sedimentologically correlated (see details in **Supp. Mat. 1** and **Table 1 in Supp. Mat.**). We generated an age-depth model based on Bayesian inference using the R package rbacon v3.2.0 [70] in R (R Core Team, 2022) (**Fig. 2 Supp. Mat.**), which includes the IntCal curve (IntCal20 [71]) that automatically calibrates the original dates and calculates modelled ages.

<u>*sed*aDNA analysis</u>

We sampled TRAM21-1B for *sed*aDNA analysis under clean conditions at the Paleogenomics Laboratory of the IUCA-University of Zaragoza. We took between 2-3g of 95 samples at around 5-cm resolution from the whole core, excluding the first upper section, as it was not dated.

We carried out *sed*aDNA analyses at the Ancient DNA Laboratory of the Arctic University Museum of Norway in Tromsø, following a metabarcoding approach. Data were generated in two batches (**Supp. Data 1**). Altogether, we analysed 46 of the sediment samples from sections 3U and 4U of TRAM21-1B core, which corresponds to the Holocene (**Supp. Data 11**). We included 15 controls (six in batch 1, nine in batch 2) to detect potential contamination, as detailed in **Supp. Mat. 8**. In the case of plant *sed*aDNA, one control in each batch was positive and included a synthetic extract to confirm successful *sed*aDNA amplification [72]. We followed the protocols of [72] for DNA extractions, using a modified DNeasy PowerSoil kit protocol (Qiagen, Germany). We used the 16S P007 primer set to amplify a region of the animal mitochondrial genome [26] and a set that amplified a chloroplast locus (*trn*L p6-loop) for plants [73]. Each primer set was uniquely-dual tagged to allow for downstream demultiplexing (**Supp. Data 1**). We carried out eight PCR replicates each for both primer sets, following the conditions defined by [74], except for 16S PCRs, in which forward and reverse blocking primers were added, following [25]. PCR products were pooled and cleaned, and library preparation was performed using a modified TruSeq PCR-free library kit (Illumina) and unique dual indexing [72]. Libraries were quantified by qPCR using the Library Quantification Kit for Illumina sequencing platforms (KAPA Biosystems, Boston, USA), and sequenced on the Illumina NextSeq platform using paired-end chemistry for 2x 150 cycles at the Genomics

Support Center Tromsø (GSCT) at The Arctic University of Norway in Tromsø. For the bioinformatics pipeline, we used the OBITools software package [75] following [25].

We performed a plant taxonomic assignments of *sed*aDNA metabarcodes using the following stepwise filtering process:

1. Identifying the sequences in relevant databases. We used four reference databases for plant sequences: PhyloAlps [25], PhyloNorway [76], ArctBorBryo [77–79], and EMBL rl143 [80]. Note that the first two only contain vascular plants whereas the latter two also include bryophytes. Algae are generally poorly represented in reference libraries, but some are present in EMBL. Those with a 100% match to the reference sequence, and that appeared in more than one PCR replicate, were kept while the rest were discarded. We prioritised the PhyloAlps database as a confident source in case of 100% match to several databases, as it is a specific database for the flora of the Alps and Pyrenees.
2. Identifying and discarding PCR/sequencing errors. For this task we ran the LULU software [81], which identified erroneous molecular operational taxonomic units (OTUs), so we grouped the information of these assignment errors into the original sequence they derive from.
3. Comparison to expert knowledge. Final taxonomic assignments for plant sequences were contrasted to the Pyrenees Flora Atlas (FLORAPYR, http://www.atlasflorapyrenaea.eu/src/home/index.php?idma=1), plant records from the Herbarium of Jaca (http://floragon.ipe.csic.es/), and personal communication by botanical experts Daniel Gómez and Federico Fillat [68].
4. We finally retained 423 taxa (**Supp. Data 3**) from the 529 originally identified sequences (**Supp. Data 2**). From the former pool, 2 were recognized as non-native, and 10 were 'Positive control' identified, as expected, in positive controls from the post-PCR lab (**Supp. Data 3**).
5. To facilitate data visualisation and interpretation, we organised plant taxa into 13 distinct groups, encompassing both individual taxa and functional categories. Thus, we obtained the following categories: *Abies alba*, Algae, Bryophytes, Hydrophytes, Hygrophytes, Deciduous trees, Open landscape, Novel herbaceous taxa, Herbs, *Pinus*, Shrubs, Other trees and Other plants (i.e. nemoral plants and pteridophyta) (**Supp. Data 3**). Among these, we have classified all plants associated with open

landscapes in three different categories: 1) ‘Novel herbaceous taxa’, that correspond to herbs that appeared for the first time only after domestic animal *sed*aDNA sequences were present in the record (6.5 ka BP onwards), despite these taxa are not introduced or invasive; 2) ‘Herbs’, that are herbaceous taxa associated with current Pyrenean pastures [68]; and 3) ‘Open landscape’, that are not necessarily herbaceous taxa typical from Pyrenean pastures, but generally associated with open landscapes (e.g. *Veronica serpyllifolia*, *Pedicularis sylvatica*). We discarded Algae, Bryophytes, Hydrophytes, and Hygrophytes to retain only terrestrial vascular plants for analysis.

Animal sequences were taxonomically assigned using the EMBL rl143 database, retaining those with a 95% match or higher, detected in at least one PCR replicate, and with at least 10 reads in the entire dataset. We further removed detections in the PCR replicates if they were represented by two or fewer reads. We discarded sequences that were identified as human (*Homo sapiens*) using a blacklist (v2.1, see https://github.com/pheintzman/metabarcoding), as it is a common contaminant. This resulted in a dataset of 543 taxonomically assigned sequences that were collapsed into 110 taxa based on identical taxonomic information. We then manually curated these assignments and removed five Nuclear-Mitochondrial DNA Segment (NUMTs) sequences and four contaminant taxa that were found in the negative controls and were non-native to Europe (**Supp. Data 7**). We then recollapsed our curated taxonomic assignments, resulting in a final dataset of 62 animal taxa (**Supp. Data 8**).

We classified animal taxa according to two categories: wild or domesticated animals, obtaining the following classifications: ‘Wild mammal', 'Wild animal' (including birds, amphibians and insects), cattle ('*Bos taurus*'), sheep ('*Ovis aries*'), goat ('*Capra hircus*'), 'Other animals', 'Worms' and 'Bryozoan' (**Supp. Data 8**). Assignments of *Bos* sp., *Sus scrofa*, *Canis lupus* and *Equus caballus*/sp., could not be differentiated between wild and domestic animals (aurochs/cattle, wild boar/pig, wolf/dog and wild/domestic horse, respectively). Consequently, these taxa were not considered as domesticates in our analyses, but 'Others', as well as donkey (*Equus* cf. *asinus*), due to its scarce presence. We removed other invertebrates (worms and bryozoans) from our analysis, as they are regarded as by-catch when using a primer targeting mammal DNA. After filtering, we retained 30 taxa.

We used the Relative Abundance Index (RAI) defined by [25] to represent the relative proportion of plant and animal taxa (**Supp. Data 4, 5, 9, 10**), calculated as the product of the proportion of weighted PCR replicates, as defined by [72], and the relative proportion of reads for each taxon.

Ordination

We explored changes in plant communities related to the presence of animals by performing a transformation-based canonical Redundancy Analysis (tb-RDA) using the vegan R package [82], since our data do not have a normal distribution. We included plant RAI data classified in categories for every sample, and animal RAI data as explanatory variables. Plant data were transformed using Hellinger standardisation, in order to remove differences in absolute abundances between samples [83]. We excluded sample TRAM_1b_3U_136.137, which corresponds to 5.1 ka BP, from the ordination analysis because of its limited DNA content (only 18 taxa were identified in this sample, while the mean taxonomic richness excluding this sample was 118.6, see **Table 4** and **Fig. 13 in Supp. Mat.**).

Characterising different grassland communities in the Tramacastilla record, we expanded the whole group of plants associated with open landscapes (i.e. 'Open landscape', 'Herbs' and 'Novel herbaceous taxa') by accounting for every possible regional species within each taxon above the species level, by considering that there is an equal chance of each species being present (i.e. a probability factor of one divided by the total possible species within a taxon) (**Supp. Data 6**). We added one variable to each of these species, following botanical expertise criteria and [68], based on whether they are favoured by domesticates as grazing, trampling, fertilisation, or both grazing and trampling (**Supp. Data 6**).

Using the RAI data of all plant taxa associated with open landscapes (i.e. 'Open landscape', 'Herbs' and 'Novel herbaceous taxa'), and classified according to the way they are favoured by domesticates, we performed another ordination analysis (tb-RDA) to see how related they are with the presence of animals. We included animal RAI data as explanatory variables, although we only kept red deer (*Cervus elaphus*) from the 'Wild mammal' group for this dataset (**Supp. Data 8**) so as to be able to see the specific signal of the most common herbivore in the record on grasslands. As with the previous tb-RDA, plant RAI data were transformed using Hellinger standardisation and sample TRAM_1b_3U_136.137 was excluded.

Rate of change

In order to see how the composition of ecological communities has changed over time we performed a Rate-of-Change (RoC) analysis with R-Ratepol R package [84] using the RAI data of plant and animal taxa. So as to have parsimonious yet consistent results and ensure the uniqueness of all taxa included in RoC analysis, we grouped together those plants that could be nested in other groups that had lower taxonomic resolution. For instance, all sequences identified as species from the Asteraceae family were grouped together under 'Asteraceae', as some sequences could not be identified below the family category, so those plants identified with coarser resolution could be included. This software allows for the detection of points where community composition significantly changes. We performed 10000 randomisations, using 'chisq' dissimilarity coefficient, and 'levels' as working units. Because the median value of our data was 0, we used the mean value of all RAI data (5) as the number of individuals from each working unit to standardise the variables in the assemblage dataset. In addition, and following the RoC workflow, we included age uncertainties from an age-depth model from Bchron R package (v4.7.6 [85]).

Data availability

Raw *seda*DNA sequence data have been deposited at the European Nucleotide Archive (ENA) under BioProject [accession]. Blacklists and data filtering R scripts are on Github (https://github.com/irenejulianposada/tramacastilla_lake_sedaDNA.git). All processed data and the numerical workflow of this article will be stored on Github and Zenodo (https://doi.org/10.5281/zenodo.11509259), for later integration in Neotoma (https://www.neotomadb.org/). All other data and information needed to replicate the findings is in Supplementary Material and Supplementary Data.

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Author contributions

PGS and GGR conceived the study; PGS and AM acquired the funding; GGR coordinated the field expedition; GGR, IJP, AM, BVG, JLR and PGS performed the fieldwork campaign; IJP, SGP and PB sampled the sedimentary archive; IJP, SGP and PDH did the *sed*aDNA sequencing and built the molecular libraries; SGP, PDH and IGA did the *sed*aDNA bioinformatics; GGR and AM performed the depth-age model and the sedimentological analyses; IJP and GGR did all numerical analyses and integrated them in an open workflow; IJP, GGR and PGS led the writing; LM, AS and JLR wrote the archaeological synthesis; DG and FF contributed data and knowledge on current day grass community ecology. Each author contributed to the writing process and provided significant intellectual input.

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