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Diet and environment of *Mylodon darwinii* based on pollen of a Late-Glacial coprolite from the Mylodon Cave in southern Chile

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**Abstract**

We studied the pollen content of a well-preserved coprolite of a Late-Glacial giant ground sloth (*Mylodon darwinii*) from the Mylodon Cave, province Última Esperanza, southern Chile. The specimen was obtained in 1909 and has been stored in a museum in the Netherlands since. It was radiocarbon dated to 13,140 ± 55 BP (15,927–15,522 cal BP), which fits with other radiocarbon dates showing the early Late-Glacial presence of *M. darwinii* in the province Última Esperanza. Contemporaneous oxygen isotope data from Antarctic EPICA Dome C indicates that our Mylodon specimen lived during a warming phase of the Late-Glacial, ca. 1000 years before the start of the Antarctic Cold Reversal. We compared our pollen data with pollen records showing contemporaneous regional vegetation and discuss the uncertainties in the interpretation of pollen spectra from faeces. To expand on the pollen data, we tested ancient DNA preservation in the sample; we sequenced ~9.4 million DNA reads and found that the concentration of ancient plant DNA is below detectable levels. Pollen analysis confirms earlier findings that the *Mylodon* was a grazer, but the discovery of large amounts of *Fragaria* and *Azorella* pollen in the faeces may indicate that *Mylodon* was also able to select and consume specific plants, and therefore could also be regarded as a selective feeder.

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1. Introduction

1.1. History of research

The Naturalis Biodiversity Center in Leiden, the Netherlands, hosts an extraordinary collection of Pleistocene mammal bones and other remains from southern South America. The Dutch biologist Jan Herman Kruimel (1885–1916) obtained this collection in 1909, including bones, skin fragments, and coprolites (fossilised faeces), in Punta Arenas. The material originates from the Mylodon Cave (Cueva del Milodón), also known as Cave Última Esperanza, Eberhardt Cave, and Last Hope Cave. The cave is in the southern part of Chile (province Última Esperanza; coordinates 51°33′S, 72°37′W) 160 m above sea level, and 24 km from Puerto Natales (Fig. 1). It is situated in the Benítez mountain, north of the Strait of Magellan that separates the island Tierra del Fuego from mainland South America. The entrance of the cave is 170 m wide, 30 m high, and the cave is 270 m deep.

Landowner Hermann Eberhardt (1852–1908) made several discoveries in the cave and unearthed large bones and skin. He showed the specimens to the Swedish explorer A.E. Nordenskjöld in 1896. Nordenskjöld subsequently visited the cave and dug up more bones, and also some skin fragments, similar to the remains unearthed by Eberhardt (Nordenskjöld, 1898, 1899a, 1899b, 1899c, 1900). He also found a keratin nail sheath of a ground sloth. The discoveries of Nordenskjöld were described by Lönnberg (1899, 1900).

Shortly after the departure of Nordenskjöld in 1899, the geologist R. Hauthal carried out an excavation and found some more pieces of skin, as well as faeces from the giant ground sloth *Mylodon darwinii* (Hauthal et al., 1899; Hauthal, 1900, 1904). Hauthal also dug in a smaller cave, three kilometres east of the main cave. Both Hauthal and Nordenskjöld mentioned up to one-meter thick layers of faeces, which they attributed to *M. darwinii*, and they concluded that the animals used the cave over a long period.

J.H. Kruimel visited the Mylodon Cave in 1909 but did not excavate any remains himself. Rather, he obtained a collection from Mr. Charles Milward, the British Consul in Punta Arenas, who also sold a collection of bones and other remains from the cave to the Natural History...
Fig. 1. Location of Mylodon Cave in southern Chile, and palaeoenvironmental sites discussed in the text (Markgraf, 1993; Kilian and Lamy, 2012, and references therein). Indicated are the estimated extent of the Patagonian Ice Sheet and palaeolakes around 15 ka BP (after Davies et al., 2020), annual precipitation (1950–2000) calculated with WorldClim version 1.4 (Hijmans et al., 2005), and modern occurrence of Fragaria species (GBIF, 2021). Abbreviations: V = Viamonte, PE = Puerto Eden, H = Haberton, PG = Paso Garidaldi, M = La Misión, FC = Fells Cave, L = Lynch, PV = Punta Yartos, PDH = Puerto del Hambre, EE = Estancia Esmeralda, PA = Punta Arenas, RR = Rio Rubens, G = Guanaco.

Plate I. 1: Two Mylodon darwini coprolite fragments from the Mylodon Cave, province Última Esperanza, southern Chile. The material is stored in a closed container (15 × 10.5 × 15 cm) in the Kruimel Collection at the Naturalis Biodiversity Center in Leiden, Netherlands (collection number ZMA.MAM.27092); 2 and 3: coprolite specimen 1, showing the borehole where the radiocarbon sample and pollen sample 1 were taken.
The present vegetation in the surroundings of the Mylodon Cave is open, with scattered stands of *Nothofagus* woodland. Moore (1978) and Heusser et al. (1994) described general features of the modern vegetation zones of southern Patagonia and the principal plant associations of Última Esperanza. The location of Mylodon Cave is in an area where the natural vegetation would be deciduous forest dominated by *Nothofagus pumilio* and *N. antarctica*. However, during the past 150 years human influence has considerably altered the natural vegetation (Moore, 1978).

Estimates based on EPICA DOME C, east Antarctica (Jouzel et al., 2003; Saxon, 2009; Pedro et al., 2015), indicate ca. 10.3 °C lower 100-year mean surface temperatures during the Last Glacial Maximum (20–18 ka BP) than during the last millennium (Fig. 2). An increase of ca. 6.5 °C between 18 and 14.5 ka BP was followed by the Antarctic Cold Reversal (ACR) between 14.7 and 13 ka BP, when the 100-yr mean dropped by about 2 °C. After 13 ka BP, warming commenced again with an increase of 4 °C by 11.9 ka BP.

![Fig. 2. Oxygen isotope stack of EPICA DOME C, east Antarctica and the calibrated age range (horizontal bar: 15.027–15.522 cal BP) of our radiocarbon dated coprolite. According to Pedro et al. (2015) the oxygen isotope fluctuations indicate an increase of about 6.5 °C between 18 and 14.5 ka BP, followed by the Antarctic Cold Reversal (ACR) between 14.7 and 13 ka cal BP, when the 100-yr mean dropped by about 2 °C. After 13 ka BP, warming commenced again with an increase of 4 °C by 11.9 ka BP.](image-url)
dusty, non-compacted faecal material from the bottom of the glass container (Plate I). The preparation of the material for radiocarbon dating was according to Dee et al. (2019).

2.1.1. Pollen analysis
The five sub-samples used for pollen analysis were prepared according to Faegri and Iversen (1989). The microfossils were embedded in glycerol gelatine and sealed in with paraffin wax on microscope slides. Pollen grains were identified using the pollen reference collection at the Institute for Biodiversity and Ecosystem Dynamics (University of Amsterdam) and the pollen floras by Markgraf and d’Antoni (1978) and Heusser (1971).

2.1.2. Ancient DNA
For the ancient DNA (aDNA) analysis, we sub-sampled the inside of coprolite specimen 1. The outer layer of the specimen was removed before sampling to minimise ‘secondary’ contaminants, as explained above. Sub-subsampling was carried out inside a flowhood at the Centre for Geogenetics - Ancient DNA lab (University of Copenhagen) with proper decontamination and using sterile material for the sampling. All the pre-PCR steps (including DNA extraction, library build, index PCR set up) were also carried out in appropriate facilities for ancient DNA work. Extraction blanks with no coprolite added were used to monitor possible contamination.

To incorporate within-sample variability, three sub-samples of ~200 mg were taken from different areas within coprolite specimen 1. For aDNA extraction we used ~200 mg of starting material. This was achieved by combining up to 70 mg from each sub-sample in an Eppendorf tube.

DNA extraction was carried out following method B (Modified MinElute Protocol) from Hagan et al. (2019) with some modifications. Post-incubation, the supernatant was added to a 30 KDa Amicon® Ultra-4. The sample was spun down at 4000 rpm, until the supernatant was concentrated down to 70 μl. The concentrate was combined with 13 × Qiagen PB buffer and purified using Monarch columns (NEB). We performed two washes with Qiagen PE buffer as recommended by Hagan et al. (2019). DNA elution was performed in two steps to reach 60 μl of eluate. We added 30 μl of Qiagen EB buffer to the Monarch column, incubated for 5 min at room temperature, and centrifuged at 13,000 rpm (max speed) for 1 min.

The aDNA sequencing library was prepared as in Kapp et al. (2021) and double-indexed using KAPA HiFi uracil+ premix (KAPA Biosystems). The number of cycles for the index PCR was determined by qPCR analysis. The resulting indexed library was quantified on an Agilent 2100 Bioanalyzer, combined with other indexed libraries, and shotgun sequenced on an Illumina HiSeq 4000 SR 80 basepairs (bp) at Geogenetics Sequencing Core, University of Copenhagen.

After sequencing, the raw fastq file was filtered computationally by removing remnant adapter sequences with AdapterRemoval (v 2.3.0), which, in addition, was set to discard reads shorter than 30 bp and to trim stretches of N’s from the 5’ and 3’ ends to reduce false positives from low-complexity regions, low-complexity reads were filtered out using sga preprocessors (v.0.10.15), with a dust threshold of 1.0. The resulting pre-processed fastq files were mapped against databases of all Reference Sequences (ReSeqs) for mitochondria and chloroplasts, respectively, using bowtie2 (v2.3.2) set to report up to 500 alignments per read. Lastly, the lowest common ancestor of the best hit(s) to the database was evaluated using the getLCA script from https://github.com/frederikseersholm/getLCA (Seersholm et al., 2016).

3. Results

3.1. Radiocarbon dating
Our M. darwini material was dated to 13,140 ± 55 BP (GrM-21,338) at the Groningen Centre for Isotope Research. The Late-Glacial climate history as based on Greenland ice cores differs from Antarctic data, and we therefore compare the age interval of our South American coprolite with the chronological and climatological records of the Antarctic EPICA Dome C (Jouzel et al., 2007; Pedro et al., 2015). After calibration using the SHCal20 calibration curve published by Hogg et al. (2020) for samples originating from the Southern Hemisphere, the calendar age was estimated between 15,927 and 15,522 cal BP (95.4% probability).

3.2. Pollen analysis
Fig. 3 shows the results of the pollen analysis. Percentages are expressed on a sum of pollen and the spores of vascular plants. Sample 1 was taken from inside the coprolite, so its pollen content is likely not contaminated with ‘secondary’ pollen grains landing on the surface of the coprolite after its dropping by the ground sloth. Samples 2 and 3 were taken from the surface of the coprolite, and samples 4 and 5 comprise coprolite crumbs from the bottom of the glass container (Plate I) and may contain other pollen from the surroundings of the cave. The sample ‘Σ’ at the top of the diagram shows the combined microfossil record for the five investigated samples. Plates II, III and IV show a selection of the fossil material that we encountered.

3.3. Ancient DNA analysis
We sequenced a total of 9,383,710 DNA reads, which after filtering and pre-processing resulted in 5,374,323 DNA sequences. Consistent with the ancient nature of the sample, the DNA appears highly fragmented, with an average read length of 55.2 bp and a high fraction of DNA reads discarded because of the 30 bp threshold used. We investigated the content of plant and animal remains in the sample by mapping reads against reference mitochondrial and chloroplast genomes. Unfortunately, we did not find evidence of endogenous aDNA for vertebrates or plants. For vertebrates, very few reads had a match in the database, and no taxon was detected more than three times. No DNA sequences mapped to Mylodon, or to the order Pilosa. For plants, 1028 reads could be assigned to a taxon. However, these identifications all represent exotic species that are commonly detected in blank and negative controls. In addition, upon further investigation of the mapping characteristics of the most commonly detected plant taxa, we found that there all had an uneven distribution of mapped reads across the reference genome. This finding suggests that the plant taxa identified are false positives from low-complexity DNA.

4. Discussion
Villavicencio et al. (2016), Hunt and Lucas (2018), and Perez et al. (2021) published a series of radiocarbon dates of M. darwini material from the Mylodon Cave, which encompassed the period from ca 13,560 to 10,200 BP. The coprolite studied by us is one of the oldest found in the cave, and corresponds with many dates on Mylodon bone, hair, and skin recovered from the Mylodon Cave (Pérez et al., 2021). Based on the oxygen isotope curves and our radiocarbon date, we conclude (Fig 2) that the M. darwini faeces specimen was deposited during an early Late-Glacial warming phase, about 1000 years before the start of the Antarctic Cold Reversal.

Faeces are an important source of information for the reconstruction of diets, vegetation, and other environmental aspects (Birks et al., 2018; Carrión et al., 2007; Gil-Romera et al., 2014; Gravendeel et al., 2014; Hunt and Lucas, 2018, 2020; Scott, 1987; Scott et al., 2004; van Geel et al., 2008, 2011a, 2011b), but the pollen content in faecal samples will be biased. Faeces mainly represent a few days at most, and the pollen taxa composition depends on the food choice and (flowering) season. Inflorescences may have been consumed preferentially (van Geel et al., 2019). Consumed plant species may have contained pollen of other species sticking on leaf surfaces, representing the pollen rain for
Fig. 3. Pollen and spore percentages, expressed on a sum of pollen and spores of vascular plants. Sample 1 was taken from inside the coprolite, samples 2 and 3 were taken from the surface of the coprolite, and samples 4 and 5 comprised dusty, non-compacted faecal material from the bottom of the glass container shown in Plate I. The combined record of the five investigated samples (Σ) is included in the top row.
a shorter or longer period. Therefore, some pollen taxa may be present that were not a direct part of the animal’s diet, and some rare taxa may even have arrived via long-distance transport. On the other hand, vegetative parts of some plant species may have been consumed when the flowering period was over, and in such cases no pollen of the consumed plant species was ingested. Regrettably, our ancient DNA analysis of the sample did not provide any useable DNA that could shed further light on this.

The five dominant pollen and spore types show comparable percentages in all five sub-samples, without major differences between them (Fig. 3). Five pollen types and the fern spores that were not recorded in the centre of the coprolite (sample 1; Alnus, Podocarpus, Malvaceae, Solanum-type, Trilete fern spore) have mostly a single occurrence in the other samples 2–5.

The high abundance of Azorella and Fragaria chiloensis in our data, and probably also of Poaceae and Empertrum rubrum, likely reflect the pollen-bearing plant consumption of the M. darwini individual. Recorded clumps of urinifer pollen of Azorella-type, Empertrum, Poaceae, and Cyperaceae in our samples also point to consumption of these plants. The recorded Azorella may well be A. monantha, a cushion plant of alpine Patagonian plant communities (Arroyo et al., 2003).

Our results primarily reflect the flora, be it selectively, and not so much the vegetation in terms of dominant plant taxa and structure. The pollen types in the coprolite may largely be derived from the vegetation near the cave, but their relative abundance in the coprolite will heavily depend on the season and the animal’s ingestion of pollen-bearing plants. Heusser et al. (1994) summarised this as follows: “pollen (...) assemblages of (...) dunes are not considered entirely reliable to characterise (...) vegetation (...), due to the mixture of ingested and wind-blown pollen.” Nevertheless, faeces are an important source of information for the reconstruction of diets, vegetation, and other environmental aspects.

Markgraf (1985) and Heusser et al. (1994) also studied fossil pollen assemblages of M. darwini coprolites sampled in the Mylodon Cave. The deposits sampled by Heusser et al. were more than 3 m thick. Faeces were dated between 13,470 ± 180 and 10,575 ± 400 BP. The pollen assemblages were dominated by Poaceae (grass steppe) and Empertrum (tundra or dwarf scrub heath). Grazing habits and habitats of M. darwini may have been selective, forming a source of distortion of the vegetation composition as derived from pollen in lake sediments. In our pollen record, secondary (post-depositional) contamination did not take place, as samples 2–5 show similar pollen taxa in comparable percentages relative to sample 1, which was taken from inside the compact M. darwini coprolite (Fig. 3).

A direct comparison of the pollen assemblage in the M. darwini coprolite with pollen records from nearby Lake Eberhard and Pantano Dumestre (Fig. 1; Moreno et al., 2012) is not possible. Around 15 ka BP, a large palaeolake was present at these sites (after Davies et al., 2020), and thick glacial lacustrine clay and silts were deposited that lack sufficient pollen for palaeoenvironmental reconstructions (Moreno et al., 2012).

McCulloch et al. (2021) published a Late-Glacial–Holocene pollen sequence from the closed basin mire Cerro Benítez (51°33′40.45″S, 72°35′10.24″W). The mire is at an altitude of 211 m asl, at only 4 km distance from the Mylodon Cave (Fig. 1). The pollen record at Cerro Benítez provides insights into the changing nature of landscape and resources encountered by the fauna and early humans. The sediment sequence begins ca. 16.3 ka cal BP, after glacier retreat from the area (deglaciation between 18 and 15 ka BP, McCulloch et al., 2021) and the pollen records indicate a treeless tundra steppe, favoured by megaherbivores. At ca. 14.9 ka cal BP, Nothofagus began to migrate into the area, but the landscape remained open with sufficient open ground for grazers. At ca. 12.0 ka cal BP there was a dramatic expansion of woodland, but the decline of large mammals appears to have started about 700 years earlier and is coincident with the arrival of hunter-gatherers in the area ca. 12.7 ka cal BP. For the discussion about man-maga fauna interactions we refer to McCulloch et al. (2021).

Comparison of the Cerro Benítez pollen diagram with our Mylodon pollen spectra shows high percentages of Poaceae, Empertrum and Nothofagus in both records, but there are also major differences in the percentages: Apiaceae (Azorella in the Mylodon samples) and Fragaria respectively show low percentages of are absent in the Cerro Benítez spectra, while high in the Mylodon record. This we consider as an indication for consumption of Azorella and Fragaria.

The pollen record of Mylodon coprolites published by Markgraf (1985) and Heusser et al. (1994) also show a high abundance of Poaceae (= Gramineae) and Empertrum rubrum. The sum of Asteraceae (named Compositea by Markgraf, 1985 and Tubuliflorae by Heusser et al., 1994) reaches comparable values in both studies. Major differences are shown by Azorella-type (= Umbelliferae in Heusser et al., 1994). This pollen type is most abundant in our samples, but has low values in the samples studied by Markgraf and Heusser.

Our fourth-most abundant type, Fragaria chiloensis, is absent from Heusser’s and Markgraf’s pollen record, and also Velázquez et al. (2015) did not find Fragaria pollen in Mylodonid coprolites from Cerro Casa de Pedra 7 (Fig. 1). Fragaria macrofossils were not present in other Mylodonid coprolites from Mylodon Cave (Moore, 1978). However, Fragaria seeds were encountered in early Holocene cave deposits at Cueva de la Vieja (Fig. 1; Méndez et al., 2018), associated with early human activity. Fragaria species grow in the Andean mountains and foothills, but are nowadays not often described or reported south of 50°S (Fig. 1; GBIF.org). Markgraf (1993) discussed pollen results of numerous ‘pollen profile sites’ and mentioned a dominance of Poaceae and Empertrum rubrum (dry, cold tundra) for Late-Glacial vegetation up to 12.5 ka BP, which is in agreement with our results. Furthermore, Markgraf (1993) mentioned the presence of characteristic herbs that also show up in our results, including Acena, Azorella, and Cunnera. However, a major difference is the high abundance in our material of Azorella and Fragaria chiloensis.

Based on microfossils and macrofossils from coprolites from the Mylodon Cave, Moore (1978) showed that M. darwini fed on a diet of Cyperaceae, Poaceae, and species including Marsippospermum grandiflorum, Plagiobotrys albiflorus, and Oreobolus obrusangulus. These species nowadays occur in the open, cool, wet sedge-grasslands of western Patagonia. Empertrum rubrum occupies a wide range of open habitats, from dry steppe to Magellanic woodlands.

In the pollen record of Musotto et al. (2012), Poaceae pollen dominates the three modern steppe samples, and Empertrum/Ericaceae (equivalent to Empertrum rubrum in our data) dominates the two modern ecotype samples. The closest similarity with our data is one modern ecotype sample that has abundant Poaceae pollen next to dominant Cypereaceae, Poaceae, and species including Marsippospermum grandiflorum, Plagiobotrys albiflorus, and Oreobolus obrusangulus. These species nowadays occur in the open, cool, wet sedge-grasslands of western Patagonia. Empertrum rubrum occupies a wide range of open habitats, from dry steppe to Magellanic woodlands.
suggest that *Mylodon* may have been a selective feeder during part of the year, explaining the high abundance of *Fragaria* and *Azorella* pollen in the faeces studied.

A way to improve the DNA results could be to perform deeper shotgun sequencing of the DNA library. This would increase the amount of sequencing data available for analysis; however, it would not guarantee an increase in the sequencing of target ancient DNA. In samples with poor DNA preservation, this may rather lead to the further sequencing of contaminants and artefacts, but not real ancient DNA. Hybridization by capture prior to sequencing is an effective way to enrich for DNA of
interest, and this approach has successfully been applied to complex samples (e.g., Slon et al., 2017; Schulte et al., 2021). However, it requires designing capture baits, either using a long-range PCR approach (Maricic et al., 2010) or having a company synthesising them. The first approach is challenging for complex samples (such as faeces or sediments), where the target DNA is not a single species, as in our case. This approach would require using long-range PCR for every taxon of potential interest, which is both time and resource consuming.
Commercial baits would be a better solution for a multi-species approach; however, this is also prohibitively expensive.

5. Conclusions

A well-preserved Late-Glacial coprolite of the giant ground sloth Mylodon darwini, which was obtained more than 110 years ago from the Mylodon Cave in southern Chile, was successfully studied for pollen. The specimen was radiocarbon dated to 13,140 ± 55 BP (15,927–15,522 cal BP) and contemporaneous oxygen isotope data from Antarctic EPICA Dome C indicates that our Mylodon specimen lived during a warming phase of the early Late-Glacial, ca. 1000 years before the start of the Antarctic Cold Reversal. We provide a discussion of the contemporaneous vegetation and food choice of the giant ground sloth that produced
the dropping. The absence of tree pollen and the predominance of pol- len of low-growing plants in the Mylopondae (Azorella, Cynancheae, Empetrum, Fragiaria, Poaceae) show that our animal was a grazer, and based on the high representation of Fragiaria and Azorella pollen it was a selective feeder as well.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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