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Mycological evidence of coprophagy from the feces of an Alaskan Late Glacial mammoth

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ABSTRACT

Dung from a mammoth was preserved under frozen conditions in Alaska. The mammoth lived during the early part of the Late Glacial interstadial (ca 12,300 BP). Microfossils, macroremains and ancient DNA from the dung were studied and the chemical composition was determined to reconstruct both the paleoenvironment and paleobiology of this mammoth. Pollen spectra are dominated by Poaceae, *Artemisia* and other light-demanding taxa, indicating an open, treeless landscape ('mammoth steppe'). Fruits and seeds support this conclusion. The dung consists mainly of cyperaceous stems and leaves, with a minor component of vegetative remains of Poaceae. Analyses of fragments of the plastid *rbc*L gene and *trn*L intron and nrITS1 region, amplified from DNA extracted from the dung, supplemented the microscopic identifications. Many fruit bodies with ascospores of the coprophilous fungus *Podospora conica* were found inside the dung ball, indicating that the mammoth had eaten dung. The absence of bile acids points to mammoth dung. This is the second time that evidence for coprophagy of mammoths has been derived from the presence of fruit bodies of coprophilous fungi in frozen dung. Coprophagy might well have been a common habit of mammoths. Therefore, we strongly recommend that particular attention should be given to fungal remains in future fossil dung studies.

1. Introduction

During glacial periods Beringia, the vast east-Siberian west-Alaskan area linked by the Bering Land Bridge, was vegetated by an extinct biome, the so-called 'mammoth steppe' (Guthrie, 1990, 2001) with the centre of the land bridge probably being moister than the more arid continental regions on either side. Detailed studies using a variety of proxies indicate that the vegetation was not uniform but locally differentiated into communities under the influence of factors such as exposure or shelter influencing snow lie and water availability (Elias et al., 1996; Goetcheus and Birks, 2001; Kienast et al., 2001, 2005; Yurtsev, 2001; Zazula et al., 2006b; Elias and Crocker, 2008).

During the Late Glacial period the site of Cape Blossom still formed part of the ice-free Bering Land Bridge. Originally this land bridge effectively blocked moisture from entering much of interior Alaska and the Yukon. Between 13,000 and 8000 BP, Beringia underwent a dramatic ecological change from a complex of arid adapted vegetation to a more mesic ensemble (Anderson et al., 2001). At the highest latitudes, the Late Pleistocene arid Mammoth Steppe dominated by graminoids and *Artemisia* developed into predominantly shrub birch (*Betula*) tundra, *Carex* wetlands, and uplands rich in lichens and mosses. Further south and inland, arid grasslands were replaced by climax conifer forests and succession trees of the now familiar taiga vegetation (Mann et al., 2002). Details of the proximate reasons for this overall shift are somewhat controversial, but the consensus is that they were the result of seasonal alterations if not annual net changes of temperature and moisture. The vegetation shifts were paralleled by dramatic changes in the mammalian faunas (Graham and Lundelius, 1984; Grayson, 1991; Guthrie, 2006). Not only were there major shifts in species distribution including the colonization

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of new species, but there were also several dramatic extinctions. The most familiar of these was the extinction of woolly mammoth (*Mammuthus primigenius*). Simplified, extinction arguments boil down to (1) direct dietary stresses from changing vegetation character and/or changing intraspecific forage competition with other herbivorous large ungulates in this newly emerging ecological setting, or (2) invasion of the north by human hunters that wiped out vulnerable large mammal species, or (3) abrupt ecosystem disruption caused by a cosmic impact event (Firestone et al., 2007; Kennett et al., 2009; but see van der Hammen and van Geel, 2008).

Finding frozen mammoths with forensically identifiable food, parasites, and microorganisms in their gastrointestinal tracts or feces has the potential of adding data to the extinction debate, in addition to the more obvious contribution it makes to our ecological understanding of the extinct woolly mammoths. The habitats of ice-age mammoths and other herbivores are broadly known from vegetation reconstructions from preserved gut contents and dung with complementary information coming from plant macrofossils and pollen from sediments (Solonevich et al., 1977; Ukraintseva, 1979, 1993; Gorlova, 1982a, b; Liu and Li, 1984; Davis et al., 1985; Mead et al., 1986; Vasil'chuk et al., 1997; Kienast et al., 2001, 2005; Ager, 2003; Zazula et al., 2003, 2006a, b, 2007; Sher et al., 2005; Aptroot and van Geel, 2006; Mol et al., 2006; Drescher-Schneider et al., 2007; Kienast, 2007; van Geel et al., 2007, 2008). Additional information about climate, environment and diets is based on stable isotope studies of mammoth tusks, teeth and hairs and bones of Pleistocene herbivores and carnivores (Iacumin et al., 2006; Rountrey et al., 2007; Tütken et al., 2007; Fox-Dobbs et al., 2008; Chritz et al., 2009).

While gut contents contribute strong evidence of mammoth diet, undoubtedly the food of woolly mammoths was diverse in time and space. Also long 'fingers' (big bites) of the trunk tip probably inadvertently collected a mix of minor components while feeding on target forage. The distribution of identifiable plants in all other mammoth gastrointestinal tract studies supports the picture of a mammoth diet dominated by grasses (van Geel et al., 2008 and references therein), which is also what paleontologists have long concluded, namely, that the highly complex mammoth molars indicate a grass diet (Haynes, 1991).

We studied the chemistry, botanical microfossils, macroremains and ancient DNA present in mammoth dung to reconstruct both the paleoenvironment and paleobiology of this mammoth. In an earlier study (Aptroot and van Geel, 2006; van Geel et al., 2008) remains of fungi, combined with chemistry results, appeared to be useful for the reconstruction of the food sources of the Siberian Yukagir Mammoth. The conclusion could be made that the Yukagir Mammoth had consumed some mammoth dung. Therefore, the study of fungal remains and chemical analysis were also included in the present study. From fungal records in pollen samples (van Geel, 1978, 2001, and references therein) it is evident that only a selection of taxa is extant (mainly Ascomycetes and Dematiaceae with spores that have dark, thick walls). So many taxa that originally may have been present are not preserved and cannot be recorded morphologically. Nevertheless, fossil fungi can be important paleoenvironmental indicators and further study of their fossil remains may lead to more detailed identifications.

2. Material and methods

2.1. Fossil material analyzed

The fecal sample of a woolly mammoth we examine here was obtained from the high bluffs of Cape Blossom in Kotzebue Sound, a large embayment of the Chukchi Sea. Cape Blossom is located at the head of the Baldwin Peninsula (Fig. 1). The peninsula is a push moraine into the sound, with its core, the lower Baldwin Member, a result of the largest Pleistocene glacial extension in western Alaska dating to isotope stage 10. There are additional depositions from less dramatic glacial expansions identified from subsequent glaciations 8, 6, 4, and 2 (Roof, 1995). Unfortunately, we cannot place our fecal specimen in situ on the cliff, as our sample was obtained indirectly. It was among a partial skeleton of a woolly mammoth that was located high in the bluff and the various bones became souvenir and collectors' items. Constantly eroded by the sea the high vertical silt cliffs (>30 m) expose new bones every summer, so mammoth fossils from this area are not an unusual phenomenon. Authorities were not informed about the find, which is the fate of virtually all Pleistocene bones and ivory found annually in Alaska. What is unusual in this case is that amongst the bones of this mammoth there were several short columnar fecal sections. Fecal material has a low collector value so one of these was forwarded from Kotzebue, a village near the site, to the University of Alaska. This is fortunate as the fecal specimen was likely the most scientifically valuable part of the mammoth. The mass accelerator radiocarbon date (AA-77015) from the University of Arizona of $12{,}300 \pm 70$ BP (bulk $\delta^{13}C$ value is -28 ± 0.1 ‰) indicates that the mammoth lived during the early part of the Late Glacial interstadial (Greenland Interstadial I; Björck et al., 1998). The 2-sigma calibrated age interval of the sample is between 14,890 and 13,950 cal BP (Reimer et al., 2009). The late radiocarbon date corroborates the information that the mammoth was located in the very highest bluff sediments.

Externally the fecal segment (CBA) is a stubby cylinder with smoothed edges on both ends. Its surface is a fine homogeneous network of tightly packed, thin monocot (poaceous and cyperaceous) stem and leaf remains (Fig. 2). The direct association with the fossil mammoth and the large pellet's shape indicate that it was derived from the distal colon and had already undergone physiological moisture removal. Fecal pelletization in winter is a universal method in northern ungulates of reducing moisture loss. This is mostly a thermoregulatory adaptation as moisture loss in feces must be made up by increased water intake and it takes calories to melt snow or warm ice water. Pelletization occurs in late autumn, winter and early spring, so we can exclude summer as the time of death. Fortunately, the pellet state helped increase taphonomic preservation and also made the specimen more identifiable to an amateur.

2.2. Preparation of microfossils and macroremains

Two microfossil samples of ca 1 cc were taken from inside the dung ball and treated with KOH, sieved and subsequently acetolyzed (Fægri and Iversen, 1989). Pollen and other microfossils were identified and counted (magnification ×400 and ×1000), including fossil fungal spores (Aptroot and van Geel, 2006; van Geel and Aptroot, 2006; van Geel et al., 2008). The material on the sieve was scanned for botanical macrofossils.

A macrofossil sample of 70 cc was gently boiled with 5% KOH (0.9 M $KOH_{(aq)}$; deflocculation to dissolve humic and fulvic acids) and disaggregated. During sieving on a 150 μm sieve the plant remains were kept just under the water surface to avoid damage of delicate remains. Macrofossils retained on the sieve were then transferred into a petri dish, a little at a time, and distilled water was added to float the remains. The subsample was inspected, then more of the material was added to another petri dish, until the whole sample had been scanned (Mauquoy and van Geel, 2007). The material was studied using a low power microscope (×10–×50) stereozoom. Various categories of remains (among

Fig. 1. Location map.

which epidermis samples and small fungal fruit bodies) were mounted onto slides and examined at high magnification (×100–×400).

2.3. Pyrolysis–gas chromatography/mass spectrometry (GC–MS)

Macromolecules were thermally degraded in an inert atmosphere. The resulting low molecular weight fragments were subsequently analyzed by GC/MS and are representative of the overall composition of the macromolecular fraction of the sample (Wampler, 1999). Pyrolysis was carried out according to the procedure described by Nierop and Jansen (2009).

2.4. Lipid extraction and isolation

Lipids were solvent extracted and isolated from freeze-dried dung following the methods of Bull et al. (1999b) and a slightly modified version of the methodology proposed by Elhmmali et al. (1997) for bile acids.

2.5. Gas chromatography/mass spectrometry (GC/MS)

Analytes were derivatised to their respective trimethylsilyl (TMS) ethers by adding 50 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane

Fig. 2. Fecal segment (object of the present study) from Cape Blossom, Alaska.

(TMCS), and 50 µl pyridine to the sample and heating at 70 °C for 1 h. The samples were dissolved in ethyl acetate prior to analysis by gas chromatography (GC; Carlo Erba HRGC 5300 equipped with a non-polar fused silica capillary column, CPSil-5CB, 50 m × 0.32 mm × 0.12 µm, Varian Chrompack). Gas chromatography mass spectrometry (GC/MS) was conducted using a ThermoQuest TraceMS GC–MS equipped with an identical column. In both cases the following temperature program was used: initial temperature 70 °C, rising to 130 °C at 20 °C min^{-1}, then rising to 300 °C at 4 °C min^{-1}, holding at 300 °C for 25 min. The ion source was maintained at 200 °C and the transfer line at 300 °C. The emission current was set to 150 µA and the electron energy to 70 eV. The analyzer was set to scan *m/z* 50–650 with a scan cycle time of 0.6 s.

2.6. DNA extraction, PCR amplification and sequencing

Hundred milligram aliquots of air-dried dung samples, stored at 4 °C, were ground to fine powder, in liquid nitrogen, in a sterilized mortar and pestle. Two different extraction methods were used. First of all, freshly prepared CTAB buffer (2% CTAB, 2% PVP, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0, 1.42 M NaCl, 2% 2-mercaptoethanol) was added before incubation for 1 h at 65 °C under agitation. DNA was subsequently extracted using an equal volume of chloroform:isoamyl alcohol (24:1), precipitated with ice-cold iso-propanol and resuspended in TE buffer. The suspension was then reprecipitated with NH4 acetate and pure ethanol at −20 °C for 30 min, washed twice in 76% ethanol 10 mM NH_4 acetate and the resulting pellet was air dried and resuspended in TE. Secondly, ground samples were soaked in an extraction buffer consisting of 0.45 M EDTA, pH 8.0 and 0.25 mg/ml protenase K overnight at room temperature under agitation. After centrifugation at 4000 rpm for 2 min the supernatant was mixed with 8 ml of a silicabuffer (5 M GuSCN, 0.05 M Tris, Ph 8.0, 0.025 M NaCl, silica) in which 40 µl of silica was dissolved. After rotation for 3 h in the dark, samples were centrifuged again at 4000 rpm for 1 min. The supernatant was then mixed with a wash buffer (51% EtOH absolute, 125 mM NaCl, 10 mM Tris, 1 mM EDTA). After a brief spin, the supernatant was discarded and samples were air dried at room temperature and redissolved in 1× TE buffer. Subsequently, aliquots of each extraction were further purified using Promega PCR purification columns. All extractions were carried out in the special ancient DNA facility of Leiden University following established protocols to avoid contamination (Cooper and Poinar, 2000).

Amplifications were carried out in an MJ Research thermal (Biozym, Oldendorf, Germany) with a 5 min activation step at 98 °C, followed by 35 cycles at 98 °C for 5 s, 63–69 °C for 20 s and 72 °C for 30 s, with a concluding step at 72 °C for 1 min using *Phire* Hot Start DNA Polymerase (Finnzymes). Primers rbcLA1 and rbcL19b (Hofreiter et al., 2000), A49425 and B49466 (Taberlet et al., 2007) and ITS1 and ITS2 (White et al., 1990) were used in 20 microliter reactions. All amplification products were cloned using the TOPO-TA Cloning Kit (Invitrogen). From each amplification, 5 clones were sequenced after tailing. DNA sequencing was carried out on an ABI 3730 XL (Applied Biosystems) at Macrogen. The polymerase chain reactions were carried out in laboratories in Leiden physically separated from the ancient DNA facility and partly replicated in the molecular laboratories of IBED at Amsterdam University. Extraction blanks were always included to monitor contamination. DNA sequences obtained were assembled with Sequencher 4.8 (Gene Codes). The resulting sequences were compared with data in the NCBI GenBank using BlastSearch. Identifications were only accepted in cases where the GenBank sequences had 100% query coverage of the PCR products obtained. The closest match was determined by calculating bootstrap supports (Felsenstein, 1985) with the 10 closest hits, using 1000 replicates in PAUP* 4.0b.10 (Swofford, 2001).

3. Results

3.1. Reconstruction of the paleoenvironment

3.1.1. Microfossils, macroremains and ancient DNA

The microfossil record (Table 1) shows that the vegetation was dominated by grasses (Poaceae), *Artemisia*, and other light-demanding taxa, representing a mammoth steppe (Guthrie, 1990, 2001). The landscape was open, with only 4–5% *Betula* pollen; probably representing dwarf birch. The pollen spectra, dominated by Poaceae, do not reflect the macroscopic composition of the mammoth dung, because the dung consisted primarily of cyperaceous tissues (Table 2 and Plate 1). Cyperaceous epidermis tissues

Table 1
Microfossil record of two CBA subsamples.

	CBA-1	%	CBA-2	%
Betula	13	4.1	15	4.8
Salix	2	0.6	–	–
Poaceae	220	70.1	216	69.5
Artemisia	22	7.0	23	7.4
Cyperaceae	16	5.1	14	4.5
Asteraceae tubuliflorae	6	1.9	6	1.9
Apiaceae	14	4.5	23	7.4
Caryophyllaceae	1	0.3	4	1.2
Rosaceae undif.	–	–	1	0.3
Potentilla type	5	1.6	1	0.3
Plantago	1	0.3	3	1.0
Rumex acetosella-type	1	0.3	–	–
Rumex aquaticus-type	–	–	1	0.3
Polemonium	4	1.3	1	0.3
Onagraceae	5	1.6	3	1.0
Sanguisorba officinalis	3	1.0	–	–
Pollen sum	313		311	
Sphagnum	1	0.3	–	–
Podospora conica, ascospores	554	176.4	457	146.9
Sporormiella-type (T.113), ascospores	41	13.1	5	1.6
Podospora-type (T.368), ascospores	2	0.6	–	–
Cercophora-type (T.112), ascospores	1	0.3	1	0.3
Chaetomium (T.7A), ascospores	1	0.3	–	–
Gaeumannomyces (T.126)	–	–	2	0.6
Urocystis (T.1403)	147	46.8	220	70.7

Percentages are based on a pollen sum of all pollen taxa. Fungal spores were expressed as percentages calculated on the pollen sum. Type numbers refer to van Geel and Aptroot (2006). Identification of ascospores of *P. conica* is mainly based on morphology of fruit bodies (see descriptions and illustrations). Also for *Urocystis* (Type 1403) see descriptions and illustrations in the present study.

were often infected with *Puccinia*. In addition to the vegetative remains, some fruits and seeds were found (Table 2 and Plates 1 and 2). The most common ones were *Carex* achenes (fruits; Plate 1, 1), probably of the species that produced the vegetative cyperaceous remains that dominated the sample. One achene was still present in its periginium. Some caryopses (grains), glumes/florets and vegetative remains (stems and leaves showing the characteristic epidermis cell pattern) of Poaceae were recorded (Plate 1, 5–10), but poaceous remains were not common (estimated ca 5 volume percent).

The study of ancient plant DNA in the mammoth dung generated plant genera/species belonging to (almost) the same orders/families/genera and species as compared with the microfossils and macroremains (Table 3). Most dominant plant genera were *Artemisia*, *Carex* and *Salix*. The different barcoding markers used retrieved similar plant orders but different genera. For Poales, for instance, *Carex* was identified using the *trnL* intron and *Hordeum* using the nrITS1 region.

3.1.2. PGC–MS

The pyrolysis results of the dung sample are shown in Fig. 3. The pyrogram shows a high abundance of syringols and guayacols derived from monocotyledon lignin-cellulose, with 4-ethenyl-2-methoxyphenol derived from ferulic acid as the most abundant component (van Bergen et al., 1997; Nierop, 2001). The observed pattern of syringols and guayacols confirms the presence of an open, treeless landscape as concluded from the identification of the microfossils and macroremains. Other important compounds in the pyrolysate are a series of even-numbered *n*-fatty acids (C_{16}–C_{24}) with the higher homologues most likely originating from wax esters from roots and leaves of higher plants (van Bergen et al., 1997), while the lower homologues ($<C_{20}$) may also be of bacterial or fungal origin (Amelung et al., 2008). The observed homologous series of *n*-alkanes and *n*-alkenes (C_{10}–C_{31}) could originate from the biopolymer cutan but could also be the result of diagenetic processes (De Leeuw et al., 2006). In a previous study it was tentatively attributed to the latter (van Geel et al., 2008). The presence of the polysaccharide derived compounds 2-furaldehyde and levoglucosan, combined with the abundant presence of lignin derived compounds confirms the excellent preservation of the sample (Nierop and Jansen, 2009).

Table 2
Macrofossils recorded in 70 cc of fecal CBA material.

Cyperaceous vegetative remains (estimated)	90% (Estimated volume)
Carex spec., achenes (mainly *Carex* subgenus Vignea, possibly *C. pachystachya* or *C. macloviana*)	53
Luzula sp., seeds	2
Poaceous vegetative remains	5% (Estimated volume)
Poaceae, parts of inflorescence (empty florets)	8
Poaceae, caryopses	
Alopecurus sp.	4
Poa sp.	5
Elymus sp.	6
Potentilla spec., seeds (among which *P.* cf. *stipularis* and *P.* cf. *hyparctica*)	4
cf. *Chenopodium*, seed	1
Caryophyllaceae, seeds (among which *Cerastium/Silene*)	4
cf. *Draba*, seed	1
Minuartia rubella, seed	1
Unidentified seeds (Plate 2, 18 and 19)	2
Thuidium abietinum (Hedw.) Schimp.	+

Several hundreds of fungal fruit bodies; the majority contained spores; those could be identified as *P. conica* (Plates 2, 21–27; see also description).
Several hundreds of unknown macrofossils (Plate 3, 31a–d; see also description).
Cyperaceous tissues were often infected with cf. *Puccinia* (Plate 3, 30a–c).

3.1.3. *n*-Alkyl lipids

Fig. 4 summarises the *n*-alkane, *n*-alkanol and *n*-alkanoic acid distributions of the dung. *n*-Alkanes range from C_{21} to C_{33} with a strong odd-over-even predominance and maximising at C_{31}. The *n*-alkanols exhibit a similarly narrow distribution, ranging from C_{18} to C_{28} with a strong even-over-odd predominance and maximising about C_{28} although the C_{26} homologue is also a dominant component. Both *n*-alkane and *n*-alkanol distributions are consistent with a major input of higher plant derived organic matter. Whilst it is probable that grass may well be an important component of this vegetation, as indicated by the high concentration of the C_{26} *n*-alkanol, the predominance of the C_{28} homologue indicates that additional inputs from other vegetation are likely to be present (Walton, 1990; Maffei, 1996; van Bergen et al., 1997). *n*-Alkanoic acids range from C_{14} to C_{34} with an even-over-odd predominance and maximising about a predominant C_{16} component. Of particular note is the occurrence of monounsaturated C_{16} and C_{18} homologues with C_{18} monosaturated isomers constituting the third most concentrated component after the saturated C_{16} and C_{18} homologues. Previous investigation of the gut contents of a Yakutian (Yukagir) mammoth (van Geel et al., 2008) yielded only trace amounts of these more labile components attesting to the high level of preservation in this particular specimen and reinforcing the vegetative origin of the organic matter (Killops and Killops, 2005). In addition *iso*- and *anteiso*- C_{15} and C_{17} analogues also occur. These components are known to be derived from bacteria and attest to the large bacterial population that organic matter in the gut of the mammoth will have been exposed to (Akashi and Saito, 1960; Saito, 1960). In summary, the *n*-alkyl lipid composition reflects typical characteristics of higher plants with hardly any contributions from other sources (Walton, 1990; van Bergen et al., 1997).

3.2. Paleobiological aspects

3.2.1. Microfossils and macroremains

The fungal microfossil record of the CBA-mammoth is dominated by the spores of the coprophilous (living on dung) *Podospora conica*. The identification was based on spores and fruit bodies (see Appendix). *Sporormiella*-type spore cells are of regular occurrence. Less frequent coprophilous taxa are *Podospora*-type and *Cercophora*-type (for illustrations of the various spore types see van Geel et al., 2003; van Geel and Aptroot, 2006). Spores of *Urocystis* were also very common. *Urocystis* spores, or spore producing organs were not found in organic connection with host plant tissues.

In the macrofossil sample hundreds of fungal fruit bodies were observed (Plate 2, 21–25); some of them still attached to cyperaceous tissues, but many fruit bodies had become separated from plant remains. About 110 fruit bodies were collected and no changes in the distribution of fruit bodies were observed between surface material and material collected from deep inside the pellet. Also the material that remained on the sieve during the preparation of the microfossil samples contained many fungal fruit bodies. These two samples had been taken from deep inside the dung ball, and therefore it is evident that the observed fruit bodies (only growing on dung surfaces exposed to the air according to Wicklow, 1981; Richardson, 2001; van Geel et al., 2003;) were ingested as part of the food. Fruit bodies were mounted onto slides and subsequently squashed to be able to observe the ascospores. Most of the fruit bodies appeared to be still full of ascospores (Plate 2, 25–27) of the *Sordaria*-type (van Geel and Aptroot, 2006). Based on fruit bodies and spores the material was identified as the coprophilous species *P. conica*. Two fruit bodies of the coprophilous *Sporormiella*-type were observed (Plate 3, 28).

Plate 1. 1: *Carex*, mainly subgenus *Vignea*, achenes; possibly *Carex pachystachya* or *Carex macloviana*; one achene still in perigynium. 2a: Cyperaceous epidermis with stomata. 2b: Cyperaceous epidermis. 2c: Cyperaceous epidermis with marginal prickle hairs. 3a–c: Papillate cyperaceous epidermis; 3b in high and low focus; 3c showing papillae also in lateral view. 4: *Luzula* sp., seed. 5: *Alopecurus* sp., caryopsis. 6a–c: *Poa* sp., caryopses. 7a–d: *Elymus* sp., caryopses; 7d still connected with glumes and rachis. 8a and 8b: Remains of poaceous inflorescens. 9: Poaceous epidermis (from inflorescens) showing long cells and short cells. 10: Poaceous epidermis (from stem) showing elongated long cells and short cells.

Table 3
Plant identifications from the Cape Blossom mammoth dung based on 110-, 133- and 230-bp fragments of the plastid *rbc*L gene and *trn*L intron and nrITS1 region, respectively.

Number of clones	Order	Family	Genus/species	Bootstrap support (%)
3	Apiales	Apiaceae	indet	100
17	Asterales	Asteraceae	*Artemisia* sp.	100
1			*Pulicaria* sp.	100
4			*Tagetes* sp.	63
11			indet	100
1	Brassicales	Brassicaceae	indet	100
2	Caryophyllales	Plumbaginaceae	*Armeria* sp.	100
1	Lamiales	Plantaginaceae	*Plantago media*	63
6	Malpighiales	Salicaceae	*Salix* sp.	100
1	Malvales	Malvaceae	indet	100
1	Myrtales	Onagraceae	*Chamerion* sp.	100
3	Poales	Cyperaceae	*Carex diluta*	63
5			*Carex* sp.	100
1			*Eriophorum* sp.	65
1		Poaceae	*Bromus* sp.	100
1			*Hordeum* sp.	100
2	Rosales	Rosaceae	*Potentilla* sp	66
1			*Sanguisorba* sp.	100
1	Solanales	Solanaceae	indet	100

3.2.2. GC/MS and fecal biomarkers

Fig. 5 depicts a partial gas chromatogram of the sterol fraction isolated from the dung. The presence of a suite of (C_{27}–C_{29}) 5β-stanol components confirms the fecal origin of this material with a predominance of the C_{29} 5β,3β- and 5β,3α-homologues being consistent with the dung having derived from a herbivorous diet (Evershed et al., 1997; Bull et al., 1999a, 2002). In contrast to the Yakutian (Yukagir) mammoth Δ^{22}-5β-stanols are also present at relatively high concentration, further substantiating the well preserved nature of this dung (van Geel et al., 2008). The additional presence of campesterol, sitosterol (as the predominant component present) and the pentacyclic triterpenoid β-amyrin all confirm the higher plant origin of this material (van Bergen et al., 1997; Killops and Killops, 2005). An additional analysis was performed to screen for any bile acid components in the dung. As observed for the Yakutian mammoth no bile acid components were detected. This is consistent with the idea that mammoths, like their modern-day counterparts, elephants (along with hyraxes and manatees), do not produce bile acids, this role being fulfilled by a suite of tetra- and pentahydroxylated bile alcohols (Kuroki et al., 1988; Hagey et al., 1993). This indicates that any coprophagous activity was restricted solely to mammoth dung, as the presence of bile acids would indicate the ingestion of fecal material of a non-mammoth origin.

4. Discussion and conclusions

4.1. Paleoenvironment

The two pollen spectra from the Cape Blossom dung are dominated by Poaceae, with *Artemisia*, Cyperaceae and various other light-demanding herbaceous taxa (among which Asteraceae, Caryophyllaceae, Apiaceae, Onagraceae and *Polemonium*) as minor components. The spectra and the identifications generated by our ancient DNA study reflect the vegetation in an open, treeless landscape ('mammoth steppe'; Guthrie, 1990, 2001). Various studies (e.g., Bigelow and Edwards, 2001; Brubacker et al., 2001) have shown that the vegetation during the early part of the Late Glacial period was herb-dominated in central and western Alaska. At many sites only after ca 12,000 BP a *Betula* dominated vegetation developed, but some sites show an earlier development of *Betula* shrub (Ager, 2003). The CBA-mammoth dung shows *Betula* percentages lower than 5%, and was radiocarbon dated 12,300 ± 70 BP, so indeed from before the *Betula* rise. The fact that no woody remains were found as macrofossils can be explained by several factors, such as the lack of woody taxa in the mammoth's direct surroundings just before the time of death. Material from trees was

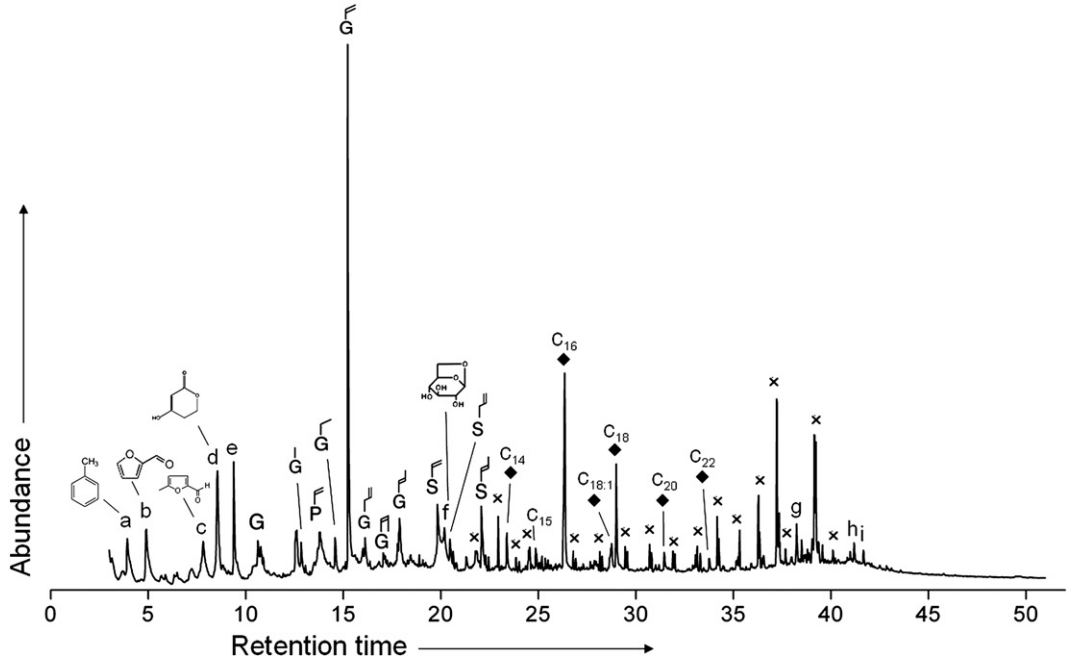

Fig. 3. Partial gas chromatogram of the pyrolysate of the dung. Legend: P = phenol; G = guaiacol; S = syringol; side chains of P, G and S are indicated; × = doublet of *n*-alkene and *n*-alkane; ◆ = alkanoic acid; C_n indicates chain length; a = toluene; b = 2-furaldehyde; c = 5-methyl-2-furaldehyde; d = 4-hydroxy-5,6-dihydro-(2H)-pyran-2-one; e = unsaturated fatty acid; f = levoglucosan; g = unidentified sterol; h = stigmasterol; i = unidentified pentacyclic triterpene.

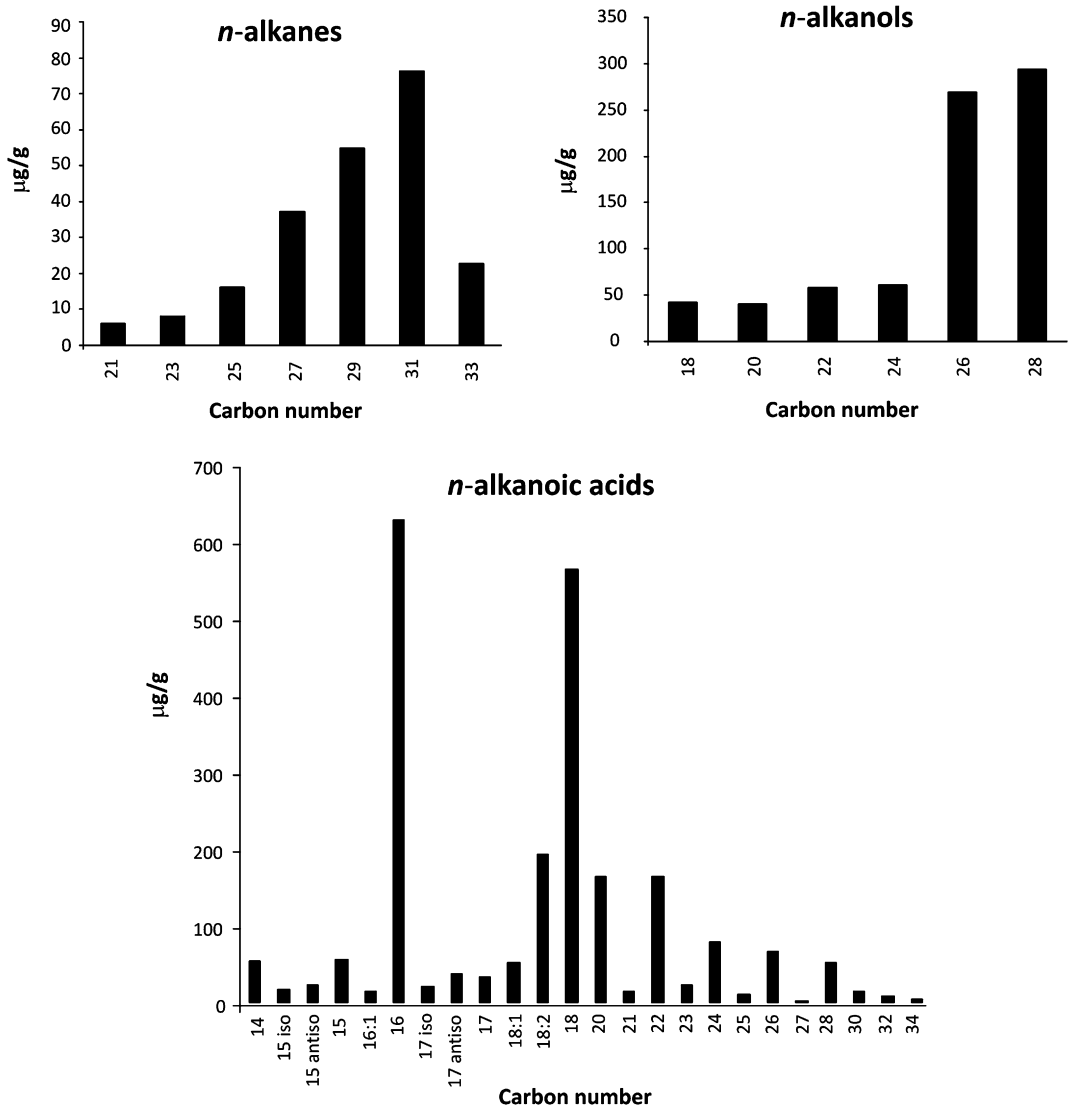

Fig. 4. *n*-Alkane, *n*-alkanol and *n*-alkanoic acid distributions obtained from the dung.

found in many but not all mammoth dung samples analyzed so far (van Geel et al., 2008), indicating that twigs from trees were a regular part of the diet when available. The fact that no woody remains were found in the CBA dung supports the reconstruction of a treeless landscape.

The pollen spectra from the dung might be biased because of locally produced pollen at the site where the mammoth was taking its food. Cyperaceous stems and leaves were the major components of the dung, with vegetative remains of Poaceae as a minor component. Nevertheless, cyperaceous pollen only accounts for percentages lower than 5%, and therefore, we consider the pollen spectra as mainly reflecting the regional vegetation. The season may have played a role in the composition of the pollen spectra, but no inflorescences with pollen were observed and the presence of numerous cyperaceous seeds may indicate that the dung was produced after the flowering season of most plants. Also, the pelletization of the dung points to production in late autumn, winter, or early spring (see introduction).

Leaves and stems of sedges formed the dominant part of the Cape Blossom feces. The presence of so much sedge may be a small piece in the jig-saw puzzle of the Latest Pleistocene ecology. We may be able to account for an abundance of available sedge, because by 12,300 BP sea encroachment onto the Bering–Chukchi Platform was accelerating causing the surrounding regions to become even more mesic (Guthrie 2001), and promoting end-Pleistocene vegetational changes already underway. Furthermore, this region adjacent to the Bering Sea remained moister during the Pleistocene as it was not so continental (Elias and Berman, 2000; Guthrie, 2001).

An increasing number of studies show that DNA extraction and amplification from paleofeces is a promising new tool for dietary analysis of extinct mammals (Hofreiter et al., 2000, 2003; Kuch et al., 2002; van Geel et al., 2008). DNA sequences can supplement information from pollen and macrofossils and have the potential to provide more detailed information in cases where plant remains cannot be identified using morphological characteristics due to drastic modification by masticatory and digestive processes, or due to an unspecific seed or pollen morphology. Amplification of relatively short DNA fragments can result in detection of plants missed by conventional detection methods (Rollo et al., 2002; Willerslev et al., 2003). DNA analysis of fossil dung can thus provide a more diverse picture of dietary habits of

Plate 2. 11: cf. *Draba*, seed. 12: *Minuartia rubella*, seed. 13 and 14: *Cerastium* sp. or *Silene* sp., seeds. 15: cf. *Chenopodium*, seed. 16 and 17: *Potentilla*, seeds (16: *P.* cf. *stipularis*; 17: *P.* cf. *hyparctica*). 18 and 19: unidentified seeds. 20a and 20b: *T. abietinum*; 20b showing branched paraphyllia. 21: Fungal fruit body (*P. conica*) attached to cyperaceous tissue. 22: selected fungal fruit bodies. 23–25: *P. conica* fruit bodies, filled with ascospores. 26: detail of squashed *P. conica* fruit body showing ascospores. 27a–c: *Sordaria*-type ascospores; from fruit bodies of *P. conica*.

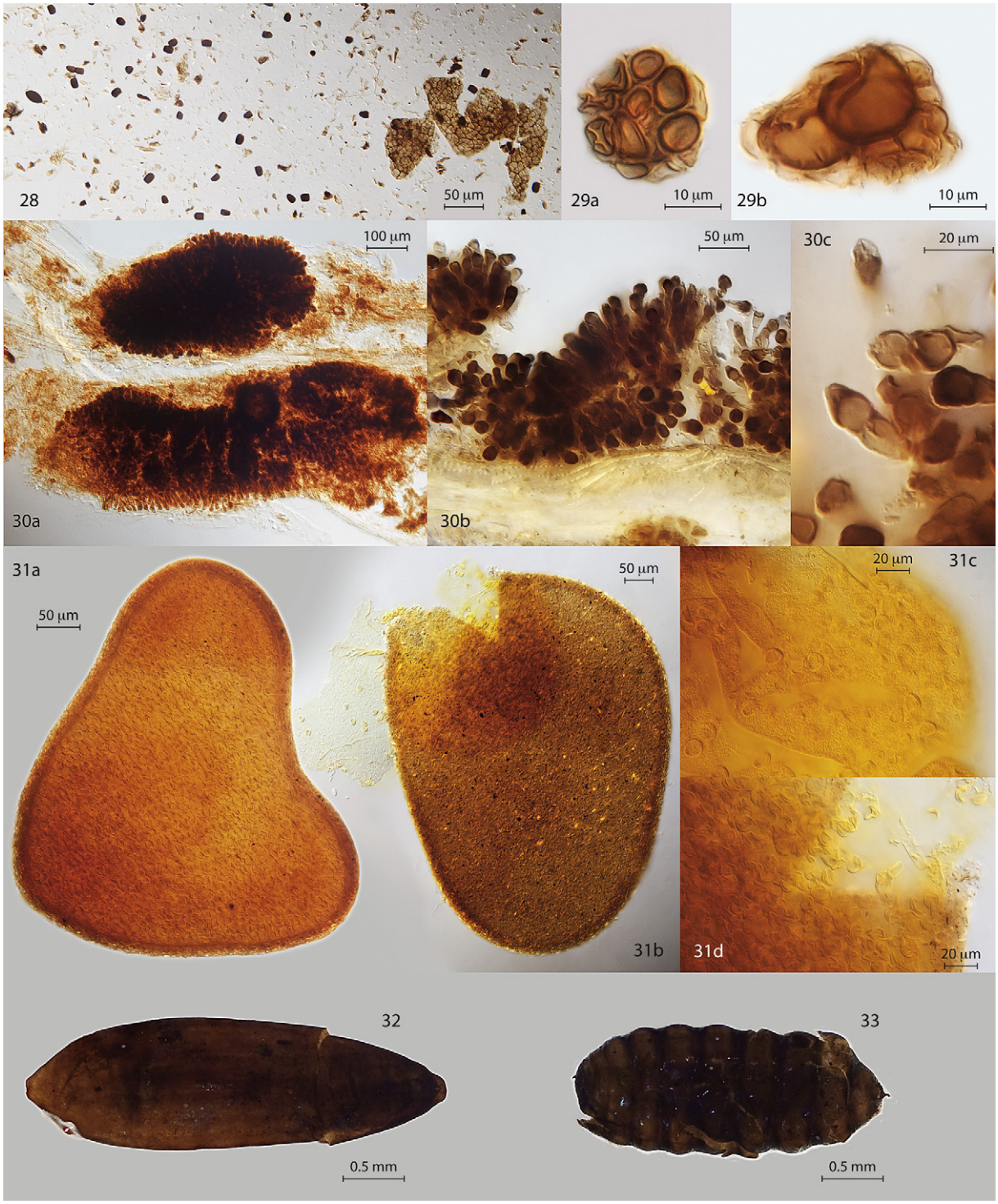

Plate 3. 28: fragment of fruit body with separate *Sporormiella*-type ascospore cells. 29a and 29b: *Urocystis* sp. (Type 1403), spore balls. 30a–c: telium of cf. *Puccinia* (rust fungi) on cyperaceous tissue. 31a–d: not identified macrofossil (fungus?, invertebrate cyst?) with spores. 32 and 33: unidentified zoological remains (pupae?).

extinct animals and also improve our understanding of their ecology (Hofreiter et al., 2003). The mammoth dung analyzed here yielded 16 different plant DNA sequences (Table 3). Identification was often hampered by the fact that no complete reference database was available for comparison. For instance, DNA sequences have not been collected for all species of *Artemisia*, *Bromus* and *Salix* yet, and identifications could therefore often not be narrowed down to the species level. Nonetheless, a total of 9 orders, containing 10 families and 12 different genera could be identified, which were retrieved from 1 to 17 individual clones and could almost always be confirmed by analyses of the micro and macrofossils, making these results very unlikely to be based on contaminations with modern plant DNA. In a total of seven clones, evidence of contamination was found. These particular samples matched most closely with species of Asteraceae, Meliaceae, Musaceae and Phyllanthaceae not native to Alaska

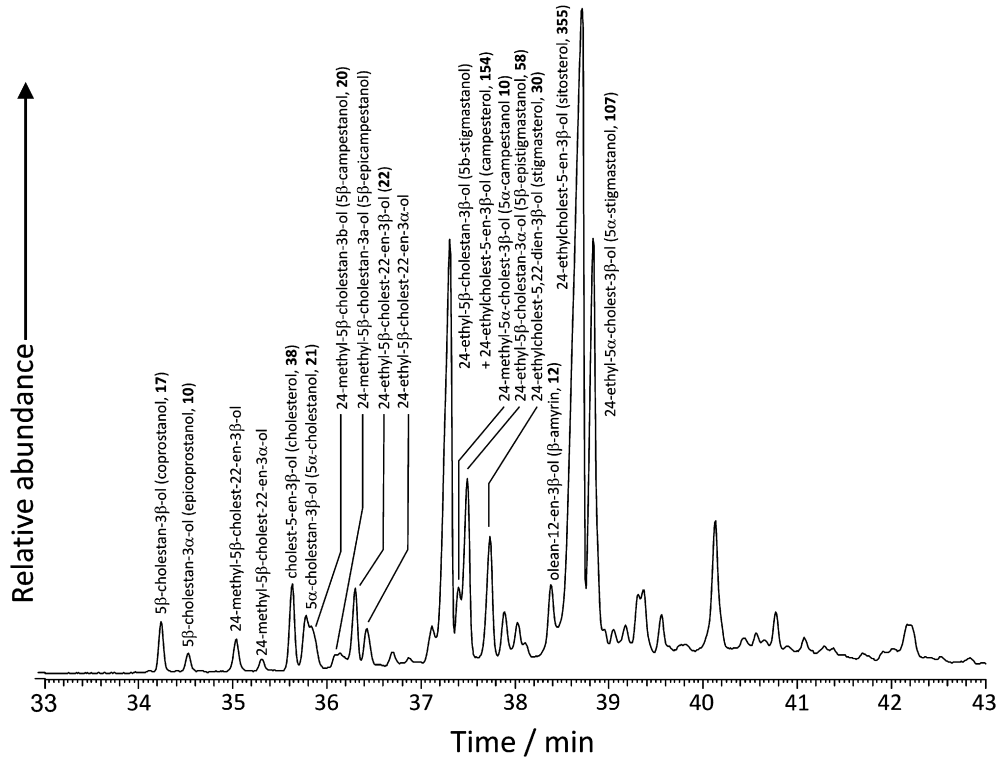

Fig. 5. Partial gas chromatogram of the sterol/triterpenol fraction isolated from the mammoth dung. Where appropriate trivial names are included in parentheses; bold values relate to concentration in units of $\mu g\, g^{-1}$ dry weight.

nowadays. These samples could be traced back to contaminated Promega PCR purification buffers and were excluded from further analyses.

The pyrolysate and lipid content of the dung sample are very similar to those previously obtained from a dung sample of the Siberian Yukagir mammoth (van Geel et al., 2008), both in type of compounds found and in their relative abundance. This indicates a similarity in diet from an organic chemical point of view although the presence of more labile compounds at relatively higher concentrations than observed in the Yukagir mammoth dung indicates better preservation of this specimen.

4.2. Paleobiology

4.2.1. Coprophagy

The indication that the adult mammoth had eaten feces (its own or that of another's) is interesting, but not remarkably strange. Young elephants eat the feces of their mother to obtain the necessary bacteria for the proper digestion of the vegetation found on the savannah. This behavior may have a marked effect upon the type and function of the intestinal flora. Coprophagy is an important means of making a variety of nutrients synthesized by intestinal microflora available to animals. Fermentation products that are synthesized in the intestinal tract may not be adsorbed at their site of formation and the animal might, therefore, be dependent upon the recycling of feces in order to utilize these products. Symptoms of deficiency may develop when coprophagy is prevented (Barnes, 1962). Coprophagy occurs among many mammals and primarily among species, like rodents and lagomorphs, that use hind-gut (cecum) digestion. Coprophagy among foregut digesters, like ruminants, is quite rare. The enlarged incubatory chambers that bulge out from the gut of ruminant and cecalid mammals are elaborate adaptive features that allow them to exploit a highly fibrous diet. The rumen and cecal enlargements are basically microorganism fermentation organs.

We can speculate as to the evolutionary logic of why some herbivores make use of the foregut and others the hindgut (Chivers and Langer, 1994). The advantage of a rumen in deer and bovids is that cell-wall breakdown and vitamin production occur prior to passing through the thin and highly vascularized small intestine walls that allow digestible products to be readily assimilated into the blood stream. The inherent disadvantage of the rumen is that when available fodder is very coarse and of poor nutritional quality, so coarse that the rumen microorganisms cannot easily process it in a timely fashion, the rumen malfunctions and the animal dies. Rhinos, horses, and proboscidians circumvent this difficulty with a simple stomach that rapidly gleans the most easily digestible fraction of the consumed food and relies on a blind hindgut sac into which small sized fibrous material can be diverted for further incubation. The ceclid organization allows coarse fiber to bypass quickly on into the colon where it comes out as very fibrous feces. Cecalids thereby achieve a rapid gut transit time, so they can subsist on lower quality forage than ruminants, but the problem cecalids face is how to absorb enough nutritious material efficiently through the hindgut cecum/colon walls that are less well vascularized and more impervious than the small intestine. One solution has been to just bear the cost of this inefficiency, relying on greater mass of food running through the digestive system. Another potential solution of many cecalids is to defecate cecal material and re-ingest it, a solution roughly analogous to how the ruminant re-ingests its food, regurgitating it from the rumen. Most lagomorph and many rodent species are highly adapted for coprophagy and without it they would die from malnutrition.

Coprophagy is known among living African elephants but it does not seem to be a regular part of their life, at least not during daylight hours when elephants have been studied (Guy, 1977; Lowis, 1991; Leggett, 2004). So, it was not immediately notable to

find the first evidence of coprophagy among mammoths. However, finding evidence now in two cases among the small sample of individual woolly mammoths raises the possibility that coprophagy may have been regular practice. This idea will be tested as more feces are examined in the future, but even in advance of such studies, independent observations may support the same idea. The $\delta^{15}N$ of mammoth collagen is often enriched relative to that of contemporaneous sympatric herbivores (e.g., Bocherens, 2003). The cause of this pattern is not well known, but one factor is especially intriguing in this context - herbivore feces are enriched in ^{15}N over diet by 0.5–3‰ (Sponheimer et al., 2003). Thus, the elevated collegen $\delta^{15}N$ values so often seen in mammoths may reflect a general pattern of coprophagy (Clementz et al., 2009; Rountrey, 2009). If this does turn out to be a regular phenomenon it poses a series of interesting questions. The fact that, by definition, our sample of these fossil feces are all derived from individuals that were to experience death in a few hours, it implies that mammoths under some nutritional stress may have resorted to coprophagy.

Among many cecalids the cecal microorganisms produce a comparatively high amount of several vitamins including K, B12, and B7 that are critical to good health. Research among zoo elephants (Sadler, 2001) has shown that these vitamins are normally in sufficient supply among individuals with a healthy diet but can be critically low on marginal diets. This raises the question as to whether mammoths regularly experienced episodes of marginal diets.

The annual dietary cycle of northern mammoths clearly had seasonal boom-bust aspects. The Berezovka mummy, an individual that died by accident in the autumn of the year, had a large amount of fat, a subcutaneous layer 9 cm thick (Pfitzenmayer, 1939). Such quantity of fat shows winter conditions were regularly severe enough to demand allocation of considerable summer resources in preparation. Additionally, molar cementum seen on many fossil mammoth molars shows marked annual constrictions strongly suggestive of (winter) food shortages (Guthrie, 1990). This is not uncommon among living northern ungulates, and seasonal mortality can be high from starvation in difficult years. Desperate behaviors are often witnessed during this time when animals are resorting to reduced amounts of non-optimal food. Observations on northern ungulates show that many of these late winter animals may become more vulnerable to predation or accident (Guthrie, 1990). Animals may be so ill and parasite infected over the course of winter stress and deprivations that although they live to see spring green up, they are beyond recovery. For mammoths, similarly debilitated from a long winter, feces persisting from the previous summer were likely one of the better forage options for raw energy needs, amino acids and crucial B-complex vitamins.

The straight-line evolution of evermore enamel complexity in northern mammoths implies an intense selection for increasing masticating efficiency, prompted by frequent dietary stress and the competitive advantages of efficient processing of high volumes of silicaceous forage into ever smaller cell-wall particles for microbial access. This mastication of course was to benefit the efficiency of cecal microbes.

4.2.2. Coprophilous fungi

Efficient dispersal mechanisms are essential for coprophilous fungi, enabling them to colonize fresh substrates. Many species rely on a cyclic process (Wicklow, 1981) involving: (1) Herbivore ingestion of spores with foliage; (2) Germination of spores following passage through the gut. (3) Mycelial growth within dung and eventual sporulation thereon. (4) Dispersal of spores to herbage utilizing phototropic spore discharge mechanisms, whereby some spores are violently discharged (shot). (5) It is essential that discharged spores become affixed to herbage until ingested: many spores show gelatinous outer membranes or appendages and some spores are surrounded by mucilaginous substances (those membranes and appendages do not fossilize). Spore walls often contain dark pigments protecting them from ionizing radiation and also preventing enzymatic degradation of spore walls during, or immediately following gut passage. (6) The spores of many species require passage through the gut of a warm-blooded herbivore to trigger their germination. (7) Many coprophilous fungi are homothallic, an adaptation that has increased survival value when an individual spore becomes isolated during dispersal or gut passage in a single fecal pellet or pat. The mycelium from an isolated spore of a heterothallic species would remain sterile in the absence of an appropriate mating type. (8) Cleistothecial ascomycetous fungi which do not forcibly discharge spores into the air, often produce hooked appendages on their spore-bearing fruit bodies which enable the spore mass to be carried on animal hair from one fecal substrate to another.

Coprophilous fungi are important components of ecosystems, responsible for recycling the nutrients in animal feces (Wicklow, 1981; Ebersohn and Eicker, 1992). Many coprophilous fungi utilize cellulose and hemicelluloses. Lundqvist (1972), as an introduction to his detailed taxonomic study of the coprophilous Sordariaceae, provided information on the preferred hosts and distribution of many species, based on a very large number of samples, mainly from Scandinavia but also from elsewhere in Europe and from other parts of the world. He observed that all species, even those that seemed to be cosmopolitan and catholic in their requirements, showed preferences for particular dung types. Lundqvist identified three groups: (1) those with a wide ecological range and low preference for particular substrates; (2) those with a wide ecological range, but with a high preference for a particular substrate(s); and (3) fastidious species, restricted to particular substrates. He noted that the last group was relatively small, and for the Nordic Sordariaceae, after infrequent species were omitted, included only three species from a total of ca 100. Lundqvist also provided lists of species which seemed to be restricted to two or three kinds of dung, often of related species, e.g. cervids, lagomorphs, rodents.

Richardson (2001) reported on the fungi observed to develop on dung samples collected over five years from a variety of animals and countries, to investigate whether their occurrence could be related to or explained by dung type and chemical composition, or region and season of collection. Lundqvist's observations and conclusions were largely supported by Richardson. Parker (1979) also identified species that were associated with dung of particular animals, some associated with either domestic or wild animals, and others that were widespread.

In the case of fossil material, we should realize that some parts of the fungi (like hyaline appendages of spores, and asci) are not preserved, while being of crucial importance for identification to species level. In other words: identification of the dung-producing mammals, based on specific morphological characteristics of spores of coprophilous fungi, will probably not be possible. Ancient fungal DNA may provide more detailed taxonomic information. Paleomycological studies of Quaternary sites have shown to provide valuable additional information about the presence and population density of herbivores (van Geel et al., 1981, 2003, 2007; Davis, 1987). Fungal remains were important for the interpretation of the diet of the Siberian Yukagir Mammoth (van Geel et al., 2008): The presence of fruit bodies of *Sporormiella*-type in combination with chemical data led to the conclusion that mammoth dung was one of the components of the last meal of the Yukagir Mammoth.

The observation of hundreds of ascospore containing fruit bodies of the coprophilous fungus *P. conica* in the Cape Blossom

fecal material is remarkable and - again - allows far-going conclusions about the diet of the mammoth, as ingestion of dung is the only way to explain the presence of fruit bodies of coprophilous fungi deep inside the dung ball. Finding evidence of coprophagy from one individual is not indicative of a general phenomenon (risking the false trail of a statistical $n-1$ problem), but finding evidence of coprophagy from two mammoths in a very small sample is sufficient cause for us to consider the issue and to alert future researchers to further evaluation of this matter.

The identification of fruit bodies with spores of coprophilous fungi is of crucial importance for the recognition of coprophagy and therefore we illustrate and describe these (Plates 1–3 and Appendix), together with other fungal remains, fruits, seeds, epidermis material and some not identified objects. Preliminary ancient DNA results suggest that nrITS regions can be amplified from these remains and that further identification using DNA barcoding is feasible (Gravendeel et al., unpublished).

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Appendix: Descriptions and illustrations

P. conica, fruit bodies and ascospores (Plate 2, 21–27)

Fruit bodies osteolate, obpyriform, 290–530 × 150–330 μm (excl. mycelium at basal side), neck with agglutinated inflated hairs.

Ascospores (*Sordaria*-type sensu van Geel and Aptroot, 2006) one-celled, ca 20–25 × 12.5–17.5 μm, brown to blackish, ellipsoidal, but slightly inequilateral: one end (with the germ pore) somewhat apiculate. Wall surrounding the germ pore slightly thicker at the inner side of the spore. The other end rounded, but with a ca 2 μm wide flat part, in some specimens still connected with the remains of a short, hyaline pedicel (not illustrated).

Most Sordariales are coprophilous and some species are strongly specialized, such as *Sordaria arctica* on canid dung, and *Sordaria alcina* on elk and moose dung (Lundqvist, 1972; Bell, 2005). According to Dr. Ann Bell (pers. comm., 2009) the fruit bodies and spores fit nicely into the coprophilous *P. conica* (syn. *Schizothecium conica*; compare Bell and Mahoney, 1995).

We should be aware of the fact that the percentage values in pollen slides of spores coming from fruit bodies strongly depend on the roughness of the treatment during the first stage of the sample preparation. Intensely stirring a sample in the KOH solution with a glass rod will squash fruit bodies so that more spores are released and pass through the sieve and finally arrive in the pollen slide. When samples are more carefully treated many spores will remain in fruit bodies that do not pass the sieve. In other words, the microfossil preparation method can strongly influence the frequency of fungal spores coming from fruit bodies.

Urocystis, spore balls (Type 1403; Plate 3, 29a,b)

Spore balls composed of one dark, fertile spore, 12.5–20.5 μm in diameter, surrounded by pale sterile cells that are 4–10 (–15) μm in diameter where attached to the dark central cell. Apart from separate spore balls also clusters of two to five specimens were observed.

Urocystis is a genus with ca 149 species parasitizing many host plant families. The delimination of the extant species is often difficult because of the scanty morphological characters of the spore balls, spores and sterile cells (Vánky, 1994).

In the present case the cyperaceous species that forms the main component of the dung may have been the host of *Urocystis*, but a direct relationship was not recorded as no *Urocystis* spores, or their spore producing organs were found in connection with plant remains.

Telia of cf. Puccinia (Plate 3, 30a–c)

Many cypraceous epidermis fragments were infected with elongated crusts of fungal tissue, probably telia of *Puccinia* (Uredinales).

Cyperaceous epidermis fragments (Plate 1, 2–3)

Recognizable cyperaceous remains showed linear rows of long cells with undulating cell walls. Often stomata were observed. Observed leaf margins showed prickle hairs (Plate 1, 2c). Two types of epidermis cell patterns were observed on different pieces of epidermis: without papillae (Plate 1, 2a–c) and highly papillate epidermis cells (Plate 1, 3a–c).

Poaceous epidermis fragments (Plate 1, 9 and 10)

Recognizable poaceous epidermis fragments showed the characteristic alternation of long cells and short, relatively broad cells. Stomata were also observed.

Unidentified macrofossils (fungal fruit bodies or cysts of invertebrates?; Plate 3, 31a–c)

Globose to ellipsoid closed bodies (230–)330–470(–580) μm, sometimes elongated with several lobes, measuring up to 1600 μm (total length). Outer wall irregularly structured, yellow–brown, ca 12 μm thick. Broken bodies showing ca 1 μm thick inner wall (not attached to outer wall); hyaline with granulate structure, filled up with densely packed round spores (7–)10–11(–14) μm in diameter.

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