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Strong bias towards carcass product processing at Neolithic settlements in northern Greece revealed through absorbed lipid residues of archaeological pottery

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Abstract

The emergence of agriculture in Greece denotes the start of the Neolithic in Europe, however, little is known about dietary practices in the region. Archaeobotanical and zooarchaeological remains indicate reliance on cereals and pulses, together with meat-based subsistence practices, including sheep/goat and pig husbandry. Preliminary investigations of dietary practices obtained through lipid residue analysis of pottery of a small number of sites in the region have confirmed primarily carcass products were processed. The weak evidence for dairy products contrasts with finding of dairy-based subsistence strategies in NW Anatolia, which is surprising given its close proximity. This paper aims to build on this earlier work to provide a more detailed model for the dietary changes throughout the region, both chronologically and spatially. To achieve this >900 potsherds from 11 sites spanning the Early (EN) to Late Neolithic (LN) periods from the north of Greece have been investigated using the lipid biomarker approach involving high temperature-gas chromatography (HT-GC), GC-mass spectrometry (GC-MS) and GC-combustion-isotope ratio MS (GC-C-IRMS) to determine the nature and origins of organic residues preserved in the fabric of pottery vessels. Lipid residue analysis of pottery vessels revealed ruminant and non-ruminant carcass fats comprise the majority of animal fat types identified, reflecting the high abundance of sheep/goat and pig in faunal assemblages. The emergence of dairying in northern Greece can now be dated to the site of EN/Middle Neolithic (MN) Ritini (5900/5700 - 5500 cal. B.C.E.), however, the frequency of dairy fat residues was low, overall, indicating that dairying was not intensively practised. The δ^{13}C values of the fatty acids extracted from potsherds reflect a predominately C_3 diet, however, in the EN and MN there is greater variation with some lipids exhibiting enriched δ^{13}C values indicating a significant abundance of C_4 plants in the ecosystem(s) covered by the study. Significantly, plant-derived *n*-alkanes (C_{22} to C_{34}) detected in pottery vessels provide the first evidence

for plant processing identified in lipid residues from ceramic vessels in Neolithic northern Greece, supporting the abundant archaeobotanical evidence for the processing of cereals and pulses.

1. Introduction

The adoption of farming practices (and other elements of the 'Neolithic Package') in Greece denotes the start of the Neolithic in Europe, yet little is known about the relationships between Neolithic people in northern Greece and their environment. The emerging view is that early farming practices developed in varying ways in different regions, depending on local conditions and cultural practices (Thomas, 1999; Perlès, 2001; Kotsakis, 2003; Çilingiroğlu, 2005). Preservation of plant and seed remains at Neolithic sites indicates that several taxa were cultivated (Valamoti *et al.*, 2011). Glume wheat species dominate plant domesticate assemblages in northern Greece, with einkorn (*Triticum monococcum*) and emmer (*T. dicoccum*) being the most abundant at several Neolithic settlements (Valamoti, 2011). Difficulties remain in being able to distinguish cereals used for human consumption versus that used for fodder. By-products of cereal processing are identified by the abundance of glume bases present in assemblages in the north of Greece, which shows that cereals were de-husked before consumption (Valamoti *et al.*, 2011). It should be noted that C_4 plants are rarely observed in the archaeological record before the Late Bronze Age (LBA; Valamoti, 2016), with the earliest occurrences of broomcorn millet (*Panicum miliaceum*) recorded in north-central Greece found in storage pithos at Assiros (Jones *et al.*, 1986; Halstead, 1987) and Kastanas (Kroll, 1983).

Faunal skeletal evidence indicates the predominance of domesticated sheep, goat and pigs, with cattle being minor components; kill-off patterns suggest herds were managed for meat rather than milk (Tzevelekidi, 2012; Halstead and Isaakidou, 2013). The predominance of sheep and goat and a scarcity of wild animals in the faunal assemblage is a typical feature of open-air settlements during the EN in Greece (Halstead and Isaakidou, 2013). No firm faunal evidence has been obtained for the exploitation of ruminant animals for secondary products (Halstead and Isaakidou, 2011; Tzevelekidi, 2012). During the EN and through to the early MN animal bones are highly fragmented implying marrow and bone grease was intensively recovered, suggesting that carcasses were intensively processed to avoid wastage (Halstead, 2012; Tzevelekidi, 2012; Halstead and Isaakidou, 2013). Such high levels of fragmentation in faunal assemblages are often associated with subsistence stress (Outram, 2001, 2003). Carcasses of cattle were seen to be more intensively processed in this way than sheep and goat throughout the Greek Neolithic (Halstead and Isaakidou, 2013).

Archaeological evidence of marine product consumption exists at several sites in the region lying within close proximity to the sea (Vika and Theodoropoulou, 2012) but the extent to which these resources were exploited is still debated. Molluscs are found in relatively high abundance in the archaeological record, particularly at coastal and semi-coastal locations (Veropoulidou, 2014). However, the variety of species is often low, with the common cockle (*Cerastoderma glaucum*), which is native to brackish environments, accounting for up to 83 % of the molluscan assemblages (Veropoulidou, 2014). Despite this, and the presence of fishing hooks and nets (Perlès, 2001), low bulk $\delta^{13}C$ and $\delta^{15}N$ values of human bone collagen, support the idea of a diet of largely terrestrial C_3 origin (Papathanasiou, 2003; Triantaphyllou, 2015), plants and animals with higher $\delta^{13}C$ values being attributed to the inclusion of C_4 plants (Triantaphyllou, 2001; Vika and Theodoropoulou, 2012).

Organic residue analysis of lipids preserved in archaeological pottery vessels from Greece has provided complementary evidence to that derived from zooarchaeological and palaeobotanical remains. The high abundance of animal fats detected in ceramic vessels reflects the importance of animals to the diet. Investigations of pottery in northern Greece (Evershed *et al.*, 2008b) have revealed that ruminant and non-ruminant carcass fats were prevalent in pottery vessels with little evidence for the exploitation of secondary products. These findings are consistent with the high numbers of sheep, goat and pig in the faunal assemblages. This contrasts with subsistence practices observed in the Near East (6500-5500 B.C.E.; Evershed *et al.*, 2008b) and south-eastern Europe (6200-5650 B.C.E.; Ethier *et al.*, 2017). These regional differences imply that milk exploitation was influenced by environmental and/or cultural variations rather than chronology (Evershed *et al.*, 2008b). Despite the large numbers of charred cereals and pulses identified in archaeobotanical remains (Valamoti, 2009) processing of domesticated plants has yet to be detected in lipid extracts from Greek pottery (Evershed *et al.*, 2008b). This agrees with finding from Neolithic pottery vessels from other regions of Europe, and is likely a consequence of the relatively low concentrations of lipids in plants compared to animal products (Charters, 1996; Evershed *et al.*, 1999).

In summary, the available archaeological evidence provides a current picture of dietary practices in Neolithic in northern Greece revolving around a predominately C_3 terrestrial diet, despite the close proximity of some sites to the coast. There is evidence of the consumption of cereals and pulses and meat-based subsistence practices focussing largely on sheep/goat and pig. This paper aims to explore the veracity of this model for the subsistence and diet in the region, extending investigations chronologically and spatially. Lipid residue analysis of ceramic material is extensively used to determine the nature and origins of organic residues preserved in the fabric of pottery vessels and provide insights into the exploitation of animal, plant and aquatic dietary resources. The application of organic residue analysis of pottery sherds in this paper will expand the knowledge of dietary practices during the Neolithic of northern Greece and provide new insights into the relationships between humans, animals and their environment.

2. Materials and Methods

A total of 912 potsherds were analysed from 11 sites spanning the EN – LN of Neolithic of northern Greece (Table 1; Figure 1). Preliminary analyses of pottery from Makriyalos (*n* = 103), Stavroupoli (*n* = 100) and Paliambela (*n* = 101) were analysed as part of an investigation into milk use across the Near East and south Eastern Europe (Evershed *et al.*, 2008b). Analyses of pottery from Apsalos (*n* = 26), Ritini (*n* = 48) and Toumba Kremastis Koiladas (*n* = 42) were originally conducted by Debono Spiteri *et al.* (2016). Re-analysis and interpretation of these sherds were conducted in an effort to increase lipid recovery using a modified extraction technique and to screen for an increased range of biomarkers, particularly APAAs.

Where possible rim and upper body sherds from cooking vessels were selected for analysis as previous research has shown these to contain the highest concentrations of lipids (Charters *et al.*, 1993). Cooking pots were recognised through the presence of sooting clouds indicating vessel heating over a fire (Rice, 1987). Lipid analysis and interpretations were performed using established protocols described in detail in earlier publications (Correa-Ascencio and Evershed, 2014). Approximately 2 g of cleaned and ground potsherd were transferred into furnaced culture tubes. A known amount of internal standard (*n*-tetratriacontane, 40 μL, 0.1 mg mL^{-1} solution) was added to the powder, the lipids were then esterified and/or transesterified using 5 mL of 2 % sulfuric acid/methanol solution (δ^{13}C measured) and heated for 1 h at 70 °C mixing every 10 min. The supernatant was removed to a clean test-tube and 2 mL of (DCM) extracted double-distilled water added. The remaining potsherd was washed with 5 mL of hexane and transferred to test-tubes before centrifuging (2500 rpm, 10 min). The hexane supernatant was then transferred to the sulfuric acid-methanol solution and whirlimixed to extract the lipids before being transferred to a vial. A further 3 × 3 mL of hexane was added to the H_2SO_4-methanol solution. The hexane extracts were combined and the solvent was then removed under a gentle stream of nitrogen in a heating block at 40 °C. An aliquot of the extract was treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1 % *v/v* trimethylchlorosilane (Sigma Aldrich) prior to analysis by GC, GC-MS and GC-C-IRMS.

Analyses of acid extracted FAMEs TLEs were performed using an Agilent 7820A gas chromatograph, using manual injections. The FID used to monitor column effluent was set to 300 °C. Trimethylsilylated FAMEs were introduced to the system *via* on-column injection (1.0 µl). The analytical column was a 50 m × 0.32 mm (Agilent J&W Scientific) fused silica capillary column coated with a 100 % dimethylpolysiloxane HP-1 non-polar stationary phase (0.17 µm). The GC temperature programme was set to hold at 50 °C for 1 min, followed by a gradient increase to 300 °C 10 °C min^{-1}, the oven was then run isothermally for 10 min. Helium was used as the carrier gas set to constant flow of 2.0 mL min^{-1}. Data was acquired using HP Chemstation software (Rev. C.01.07 [27] Agilent Technologies) and eluted peaks were identified by comparison of retention times with those of an external standard, quantification was calculated using a known amount of internal standard introduced during sample preparation.

GC-MS analyses of trimethylsilylated FAME TLEs aliquots were performed using a ThermoScientific Trace 1300 gas chromatograph couple to an ISQ single quadrupole mass spectrometer. Samples were introduced *via* a PTV injector set to splitless mode onto a 50 m × 0.32 mm fused silica capillary column coated with an Rtx-1 stationary phase (100 % dimethylpolysiloxane, Restek, 0.17 µm) for non-polar analyses. The GC temperature programme for was set to hold at 50 °C for 1 min, followed by a gradient increase to 300 °C at 10 °C min^{-1}, once at 300 °C the oven was run isothermally for 10 min. Helium was used as the carrier gas, set to a constant flow of 2 mL min^{-1}. The MS was operated in electron ionisation (EI) mode operating at 70 eV, with a GC transfer line temperature of 300 °C and a source temperature of 300 °C. The emission current was set to 150 µA and the MS was set to acquire in the range of *m/z* 50-650 at 2 scans s^{-1} in full scan mode.

For the detection of APAAs and isoprenoid fatty acids samples were injected onto a 60 m × 0.32 mm fused silica capillary column coasted with a VF-23ms stationary phase (50 % cyanopropyl-methylpolysiloxane, Varian, Factor Four, 0.15 µm). The GC temperature programme for was set to hold at 50 °C for 2 min, followed by a gradient to 100 °C at 10°C min^{-1} and then to 240 °C at 4 °C min^{-1} before a final isothermal at 240 °C for 15 min. Helium was used as the carrier gas and maintained at a constant flow of 2 mL min^{-1}. The MS was operated in electron ionisation (EI) mode operating at 70 eV, with a GC transfer line temperature of 250 °C and a source temperature of 200 °C, the emission current was set to 150 µA. The MS was set to operate in selected ion monitoring (SIM) mode, acquiring at *m/z* 105, 262, 290, 312 and 346 at 1.2 scans s^{-1}.

Data acquisition and processing were carried out using XCalibur software, version 3.0. Compounds were identified by comparison with the NIST mass spectra library (version 2.0) or with reference to external sources such as The Lipid Library (www.lipidlibrary.aocs.org), for the identification of APAAs samples were compared to an archaeological standard known to contain C_{16}, C_{18}, C_{20} and C_{22} APAAs.

Compound specific carbon stable isotope analyses were performed using an Agilent Industries 7890A gas chromatograph coupled to an IsoPrime 100 mass spectrometer. Samples were introduced *via* a split/splitless injector in splitless mode onto a 50 m × 0.32 mm fused silica capillary column coated with a HP-1 stationary phase (100 % dimethylpolysiloxane, Agilent, 0.17 µm). The GC oven temperature programme was set to hold at 40 °C for 2 min, followed by a gradient increase to 300 °C at 10 °C min^{-1}, the oven was then run isothermally for 10 min. Helium was used as a carrier gas and maintained at a constant flow of 2 mL min^{-1}. The combustion reactor consisted of a quartz tube filled with copper oxide pellets which was maintained at a temperature of 850 °C. Instrument accuracy was determined using an external FAME standard mixture (C_{11}, C_{13}, C_{16}, C_{21} and C_{23}) of known isotopic composition. Samples were run in duplicate and an average taken. The $\delta^{13}C$ values are the ratios $^{13}C/^{12}C$

and expressed relative to the Vienna Pee Dee Belemnite, calibrated against a CO_2 reference gas of known isotopic composition. Instrument error was ± 0.3 ‰. Data processing was carried out using Ion Vantage software (version 1.5.6.0, IsoPrime).

3. Results and Discussion

The archaeological sites were chosen to chronologically span a large period of the Neolithic and, in addition, cover a range of geographical environments and terrains from coastal to freshwater locations and fertile basins to mountainous localities (Fig. 1). This allowed temporal study of settlement dietary patterns and comparison between settlements which are geographically adjacent with those spatially apart. Furthermore, it made possible investigations into subsistence patterns and herd management strategies across varying terrains, highlighting the differences in the human-environment relationship occurring in different localities. A total of 912 potsherds were analysed from 11 sites spanning the EN -LN of Neolithic of northern Greece (Table 1). The findings presented herein combine those from new ceramic materials integrated with previously published work in this area (Evershed *et al.*, 2008b; Debono Spiteri *et al.*, 2016).

A suite of different lipid classes were detected within the pottery vessels, the most abundant of which were degraded animal fats in the form of saturated fatty acids. Other lipid classes detected comprise aliphatic lipids including *n*-alkanes and *n*-alcohols. A summary of the lipids detected is given in Table 2. For the purpose of data analysis the study sites are grouped into the main phases of the Neolithic shown in Table 3. Lipid preservation in the region remains consistent with that previously observed for Neolithic pottery from central and south-eastern Europe (Evershed *et al.*, 2008b; Ethier *et al.*, 2017). The overall recovery rate of lipid residues from the pottery analysed was 23 %, although recoveries varied somewhat between the late EN – early MN = 20 %, MN = 16% and LN = 32 %. It is difficult to determine if the lipid recoveries at a site where no residues were recovered, such as EN Revenia, is a result of poor preservation or, as evidence suggests, that pottery in the Greek early Neolithic was not used for cooking (Urem-Kotsou *et al.*, 2002; Yiouni, 2004; Urem-Kotsou *et al.*, 2014a).

3.1 Reconstructing diet in the Early to Late Neolithic northern Greece through biomolecular and isotopic analyses of absorbed lipid residues from potsherds

Degraded animal fats were the most common class of lipid detected. Characterisation was achieved through determination of stable carbon isotope ($\delta^{13}C$) values of the major fatty acids (n-$C_{16:0}$ and n-$C_{18:0}$). The $\delta^{13}C$ values obtained for modern reference animal fats from animals raised on a pure C_3 diet (Copley *et al.*, 2003) are grouped within confidence ellipses (± 1σ), onto which the values from the archaeological pottery have been plotted (Fig. 2 to 4). The $\delta^{13}C$ values of the lipid residues indicate animals during the Neolithic of northern Greece were raised on a predominately C_3 diet. When compared to reference values of animals raised on a purely C_3 diet the $\delta^{13}C$ values of fatty acids extracted from pottery vessels exhibit an isotopic shift (increase in $\delta^{13}C$ value). This isotopic shift is likely due to environmental factors such as aridity as these shifts have been observed elsewhere in the Europe (Evershed *et al.*, 2008b; Özbal *et al.*, 2012) and are usually observed in warmer environments such as on the African continent (Dunne *et al.*, 2012) and Syria (Nieuwenhuyse *et al.*, 2015). As a result all lipids have been classified using their $\Delta^{13}C$ ($=\delta^{13}C_{18:0} - \delta^{13}C_{16:0}$) values.

The $\Delta^{13}C$ and $\delta^{13}C$ values vary over a wide range suggesting a variety of vegetation types existed in the environment. In the EN and MN $\delta^{13}C$ values of herbivore fatty acids ranged from -30.4 to -18.6 ‰, suggesting sometimes substantial contributions of C_4-plants to an otherwise C_3 graze or browse. This wide range of $\delta^{13}C_{16:0}$ values is much greater than previously observed in Neolithic Europe. Towards the LN $\delta^{13}C$ values become less varied (-29.7 to -21.0 ‰) and exhibit a more uniform C_3 origin (Fig. 2 to 4). Domesticated C_4 crops are believed to have been absent in the north of Greece during the Neolithic, as millet does not appear in the archaeological record until the LBA (Jones, 1987; Valamoti, 2016). However, as yet unidentified wild C_4 vegetation appears to have existed. One possible explanation for broad range of $\delta^{13}C$ values observed include animals grazing on coastal environments, such as salt marshes. Salt marshes contain species that photosynthesise using both the C_3 or C_4 pathways; this is a species adaptation to environmental stress caused by high salinity (Drake, 1989). Salt marsh plants have been shown to display enriched $\delta^{13}C$ values (Couto *et al.*, 2013). Seasonal changes in diet have been observed in coastal grazing of Neolithic sheep (Balasse *et al.*, 2006; Schulting *et al.*, 2017), although evidence for coastal and estuarine grazing based on bulk stable isotope analysis has been found to be inconclusive (Britton *et al.*, 2008; Müldner *et al.*, 2014; Jones and Mulville, 2015). Another possibility is that the signal arises from plants which utilise the C_3 pathway but are growing under drought-stressed conditions signifying arid conditions were present during the Neolithic in northern Greece, however, the variations observed here are larger than those normally associated with this phenomenon (Mukherjee *et al.*, 2005).

Lipid extracts with less depleted $\delta^{13}C_{16:0}$ and $\delta^{13}C_{18:0}$ values (VG-10, MV-39, LIT-5, PAL-67, PAL-214, STAV-6 and STAV-214) all appear to have originated from an animal fat source. There is no evidence of mixing with plant resources due to the absence of plant biomarkers such as *n*-alkanes and *n*-alcohols. Similarly, the absence of *ω*-(*o*-alkylphenyl)alkanoic acids (APAAs) in these lipid extracts suggests that the aquatic commodities were not heated to high temperatures in the pottery vessels. The mixing of animal fats with aquatic commodities can often exhibit an enrichment in the major fatty acid $\delta^{13}C$ values (Craig *et al.*, 2007; Cramp *et al.*, 2014). As discussed above, the enrichment in $\delta^{13}C$ values causing offset from the confidence ellipses, are likely a result of environmental factors, such as aridity or the inputs of C_4 plants to the herbivore diet. Lipid residues at Paliambela show an exceptional range of $\Delta^{13}C$ values. Interestingly, residues with less depleted $\delta^{13}C$ values are all ruminant dairy fats with $\Delta^{13}C$ values ranging from 0.9 to -9.8 ‰. The exceptionally low $\Delta^{13}C$ values reported in this paper have been observed previously in both reference fats from cattle grazing on a mixed C_3/C_4 diet $\Delta^{13}C$ = -6.6 ‰ (Dunne *et al.*, 2012) and archaeological fats residues in pottery from south-eastern Europe $\Delta^{13}C$ = -6.6 ‰ (Evershed *et al.*, 2008b) and the Nile Delta $\Delta^{13}C$ = -8.2 ‰ (Dunne *et al.*, 2017).

3.2 Tracing primary and secondary product exploitation throughout the Neolithic of northern Greece.

Of the animal fats detected ruminant and non-ruminant carcass fats were found to be the most abundant fat types recovered from pottery vessels from all of the studied sites, comprising 88 % of the lipid residues. The abundance of carcass products processed within pottery vessels is consistent with the meat-based subsistence practices identified from kill-off patterns and the large number of sheep/goat and pig identified in faunal assemblages (Pappa *et al.*, 2004; Tzevelekidi, 2012; Halstead and Isaakidou, 2013). These findings are comparable with previous lipid residue analysis studies performed on pottery from the Neolithic of northern Greece which revealed ruminant and non-ruminant carcass fats were the prevalent commodity detected in pottery vessels (Evershed *et al.*, 2008b; Decavallas, 2011; Debono Spiteri *et al.*, 2016). Ruminant and non-ruminant carcass fats are consistently the predominant fat types present in pottery vessels throughout the Neolithic in northern Greece, with ruminant adipose fats being the most abundant followed by non-ruminant adipose fats and finally ruminant dairy fats (Fig. 2 to 4 and Table 4).

The incidence of dairy products in pottery in Neolithic northern Greece was low, suggesting dairying was not intensively practised. The re-analysis of pottery using an acidified methanol extraction (Correa-Ascencio and Evershed, 2014) and further investigation of pottery from across northern Greece has pushed back the date for the emergence of dairying to the late EN – early MN phases of Ritini, although, at none of the sites in the region does dairying appear to have been as intensive as it was in the east and west of the Mediterranean (Evershed *et al.*, 2008b; Debono Spiteri *et al.*, 2016). Interestingly, the exploitation of dairy products observed in northern Greece EN and MN sites appears to decrease in the LN (Fig. 2 to 4). In fact, dairy fat residues are absent from all LN sites with the exception of Stavroupoli, where a small proportion of dairy fat residues were detected. An increase in the abundance of dairy residues has been detected at the MN sites of Paliambela and Apsalos, where previously none were detected (Evershed *et al.*, 2008b; Debono Spiteri *et al.*, 2016). A dairy lipid signal can be masked by the high abundance of pigs present in faunal assemblages across the Neolithic northern Greece where the processing of greater than 50 % non-ruminant fat yielding products in ceramic vessels would shift the $\Delta^{13}C$ values higher than -3.1 ‰, leading to false negatives. The Neolithic in Greece predates the earliest evidence for the presence of lactase persistence allele (-13,910*T; Itan *et al.*, 2009; Gerbault *et al.*, 2013), thus inhabitants were likely to have been lactase non-persistent (Hofmanova *et al.*, 2016).

The occurrence of dairy fats across the 11 studied sites was low with 12 % of residues with appreciable lipid concentrations containing dairy fats. Previous studies using organic residue analysis in the surrounding regions have shown extensive use of secondary products in the regions of north-west Anatolia and south-east Europe where dairy fats comprised 80 % and 53 % of the lipid residues, respectively (Evershed *et al.*, 2008b). In western Turkey during the Neolithic the exploitation of dairy fats is comparable to those in northern Greece where only 17 % of lipid residues identified in pottery vessels derived from dairy fats (Özbal *et al.*, 2012). Comparison of the results obtained in this paper with the wider region reveals that the subsistence patterns observed in Greece also contrasts with those observed across the rest of the Mediterranean (Debono Spiteri *et al.*, 2016). The high number of carcass fats residues within the pottery vessels and the predominance of meat-based subsistence strategies are unique to northern Greece. Evidence for dairying is observed in both the lipid residues from pottery and slaughter profiles from both the eastern and western regions of the Mediterranean (Debono Spiteri *et al.*, 2016).

3.3 Assessment of changes in subsistence patterns across the temporal span of the Neolithic within northern Greece.

An assessment of temporal changes within settlements cannot be observed due to the small numbers of lipid extracts recovered from pots for each settlement phase and no clear stratigraphy between phases make statistically significant interpretations difficult. Similarly, there are no apparent trends in subsistence patterns between inland, coastal and lake settlement locations but instead the main changes observed are the result of chronological variations. It has been suggested that seasonal movement between different pastures i.e. between the hot lowlands and cooler mountain highlands was practised during the Neolithic (Efstratiou *et al.*, 2006). But the only evidence for this has been inferred from the lack of wild plants in dung in archaeobotantical assemblages. The absence of wild plants, which would have been in seed during the summer months in fields surrounding the pasture, suggests that animals were not present at the settlement during this period (Valamoti, 2007). The fact that glume wheat chaff is solely associated with dung suggests that animals were grazed close to the settlement on managed land during the winter months (Valamoti, 2007). This seasonal movement to different pastures could explain the enrichment of $\delta^{13}C$ values observed in ruminant dairy fats compared to ruminant and non-ruminant adipose fats.

3.4 Did aquatic commodities contribute to the diets of the inhabitants of settlements close to the coast and estuaries?

All residues containing an appreciable lipid concentration were screened using GC-MS in selected ion monitoring (SIM) mode for the presence ω-(*o*-alkylphenyl) alkanoic acids (APAAs) by scanning for the molecular ions ($M^{+\bullet}$) for APAAs of carbon chain lengths C_{16}–C_{22} at *m/z* 262, 290, 318 and 346 and the fragment ion of the base peak *m/z* 105 (Fig. 5). Despite the close proximity of several sites to the coast, no aquatic biomarkers (APAAs, isoprenoid and dihydroxy fatty acids) were detected in extracts at 8 of the studied sites inferring aquatic commodities were not being processed within pottery vessels (Table 5). At the remaining 3 sites a small percentage (~ 7 %) of extracts contained C_{18} APPAs and in some cases C_{20} but these alone (without C_{22} APAA and isoprenoid fatty acids) are not characteristic enough to conclude that aquatic products were processed within ceramic vessels (Evershed *et al.*, 2008a). The absence of aquatic biomarkers within the pottery vessels complements the low abundance of fish bones found in faunal assemblages and isotopic evidence (bulk collagen $\delta^{13}C$ and $\delta^{15}N$ value determinations) conducted on human skeletal remains (Vika and Theodoropoulou, 2012; Berg, 2013). The rejection of aquatic resources with the arrival of the domestication of plants and animals is observed elsewhere in Neolithic Europe (Richards and Hedges, 1999; Richards *et al.*, 2003; Cramp *et al.*, 2014; Eriksson *et al.*, 2016).

3.5 Investigating plant exploitation through organic residues preserved in pottery

The percentage of lipid residues containing plant biomarkers was below 10 % across the whole of the Neolithic (Table 4.3). Four extracts contained *n*-alkanes and wax esters indicating plant use. Long-chain fatty acids (LCFAs) up to n-C_{26} with an even-over-odd carbon chain length predominance were identified in extracts from 10 of the studied sites. Identification of the LCFAs were conducted using GC-MS, the components displayed fragment ions characteristic of fatty acid methyl esters at *m/z* 74, 87 and 143 and molecular ions ($M^{+\bullet}$) at *m/z* 326, 354, 382 and 410. These have previously been observed in pottery vessels containing partially degraded animals fats which yielded LCFAs on extraction with acidified methanol (Correa-Ascencio and Evershed, 2014). The occurrence of LCFAs in the lipid residues from pottery studied in this paper is the first time they have been reported in such high frequency. LCFAs are well-known biomarkers associated with plant cuticular waxes (Kolattukudy, 1976; Post-Beittenmiller, 1996), storage lipids in seeds (Harwood, 1996; Kunst and Samuels, 2003), and have been detected in both mosses (Sphagnum capillifolium; Ficken *et al.*, 1998) and plant roots as a building block of aliphatic biopolymers (Bull *et al.*, 2000). They are formed *via* the fatty acid elongation (FAE) pathway and are either directly incorporated into waxes or processed further into *n*-alkanes, primary and secondary *n*-alcohols, ketones and wax esters (Harwood, 1996; Millar *et al.*, 2000; Kunst and Samuels, 2003). The obvious interpretation for the presence of LCFAs is the processing of plants, however, other plant biomarkers (*n*-alcohols and *n*-alkanes) were absent in all extracts suggesting they do not arise by this means (Fig. 6). Significantly, the majority of residues containing LCFAs co-occurred with $C_{16:0}$ and $C_{18:0}$ fatty acids exhibiting carbon isotope values indicative of an origin in ruminant adipose and ruminant dairy fats. The association with ruminant adipose and dairy fats, coupled with the lack of plant biomarkers, points to the LCFAs in the residues arising through routing from the plant diet into the carcass and milk fats (Halmemies-Beauchet-Filleau *et al.*, 2014). The higher concentration of LCFAs in the residues compared to fresh fats likely relates to their enhanced resistance to leaching and/or degradation compared to their short-chain counterparts.

However, unusually abundant *n*-alkanes were observed in some rare cases, in extracts lacking animal fats, inferring that plants were processed in some vessels. C_{22} to C_{34} *n*-alkanes were detected in extracts from Varemenoi Goulon, Stavroupoli and Thermi. The Carbon Preference Index (CPI) of all samples containing *n*-alkane distributions was calculated to determine if the higher *n*-alkanes present were of plant origin or derived from contamination during vessel burial (Bray and Evans, 1961). The CPI can be used as an indicator of the predominance of odd-carbon-numbered wax *n*-alkanes which are found in terrestrial higher plants is expressed as a high carbon preference index (CPI > 5), whereas petroleum-derived *n*-alkanes have no significant odd-over-even carbon number predominance and, thus, have a CPI of close to 1 (Rommerskirchen *et al.*, 2006; Freeman and Pancost, 2014). Of the 11 samples containing characteristic distributions only 2 were calculated to have a CPI of close to 5 inferring a terrestrial plant origin (Fig. 7). The *n*-alkanes present in the remaining extracts have a CPI of close to 2 and thus are a result of contamination of oil derived *n*-alkanes from burial conditions or post-excavation handling. As a consequence of the differences in the concentrations of lipids in plants and animals, the mixed use of vessels results in the absence of detectable plant biomarkers in archaeological lipid residues from the Neolithic of Europe. The $\delta^{13}C$ values for the series of *n*-alkanes derived from terrestrial plant waxes range from -32.4 ‰ to -30.8 ‰ at Stavroupoli and -33.4 ‰ to -31.6 ‰ at Thermi. These reflect the carbon isotope values from C_3 leaf wax lipids which have been shown to range between -39 ‰ and -29 ‰ (Collister *et al.*, 1994) inferring that these plants originated from a C_3 environment. The presence of plant derived *n*-alkanes detected in pottery from LN Stavroupoli and Thermi is the first evidence for processing of leafy plants identified in lipid residues from ceramic vessels in Neolithic Greece and supports the abundant archaeobotanical evidence for the processing and consumption of plants (Valamoti, 2011; Valamoti *et al.*, 2011).

4. Conclusions

Lipid residue analysis has been applied to investigate dietary changes throughout the Neolithic of northern Greece, both chronologically and spatially to determine the nature and origins of organic residues preserved in the fabric of pottery vessels.

Reconstruction of diet conducted using biomolecular and isotopic analysis of absorbed lipid residues from archaeological pottery has confirmed that ruminant and non-ruminant carcass fats comprise the majority (88 %) of animal fat types identified within pottery vessels reflecting the abundance of sheep/goat and pig in faunal assemblages. Despite the abundance of ruminant animals the occurrence of dairy fats is low indicating that dairying was not intensively practised in the region. This finding is consistent with mortality profiles of cattle, sheep and goat, which indicate that a meat-based subsistence strategy was widely practised. A greater emphasis on a secondary product-based management strategy is observed at Stavroupoli, where mortality profiles indicate goats were maintained for milk (Giannouli, 2002, 2004). Although it must be noted that the faunal evidence at the site is sparse due to the low number of age-able remains. From compound-specific analysis of lipid residues in pottery vessels the emergence of dairying in northern Greece can now be dated to the site of EN/MN Ritini (5900/5700 - 5500 cal. B.C.E.). However, the generally weak evidence for dairying in Northern Greece contrasts with findings from both the east and western regions of the Mediterranean (Debono Spiteri *et al.*, 2016).

The preservation of macro archaeobotanical remains at Neolithic sites in the north of Greece indicate that several taxa were cultivated (Valamoti, 2007; Valamoti *et al.*, 2011). The presence of plant-derived *n*-alkanes detected in pottery is the first evidence for plant processing in ceramic vessels in Neolithic northern Greece identified from lipid residues supporting the abundant archaeobotanical evidence for the processing of plants.

The main changes in subsistence patterns occur chronologically but there are no detectable differences between coastal and inland sites or those from mountainous regions. Environmental differences are apparent through the range of $\delta^{13}C$ values observed for the major fatty acids (n-$C_{16:0}$ and n-$C_{18:0}$) which vary widely suggesting unexpected diversity in the vegetation available of forage to grazing animals. Greater observed variation in plants type was observed in the EN compared to MN, with $\delta^{13}C$ values indicating a both a C_3 and C_4-like origin. During the LN $\delta^{13}C$ values become less varied and exhibit a uniform C_3 origin. The reduced variation in $\delta^{13}C$ values is more apparent in ruminant dairy fats than adipose fats.

There is no evidence for the exploitation of aquatic resources despite the close proximity of sites to the coast and estuarine environments. The absence of aquatic biomarkers within the pottery vessels is consistent with the low abundance of fish bones in faunal assemblages and bulk collagen $\delta^{13}C$ and $\delta^{15}N$ values of human skeletal remains (Triantaphyllou, 2001; Papathanasiou, 2003).

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Figure 1

Map of archaeological sites investigated in this paper, where (1) Apsalos, (2) Liti III, (3) Mikri Volvi, (4) Stavroupoli, (5) Thermi, (6) Paliambela, (7) Makriyalos, (8) Revenia, (9) Ritini, (10) Toumba Kremastis Koiladas, (11) Varemenoi Goulon (base map source Wikimedia commons).

Figure 2

Scatter plot showing $\delta^{13}C$ values for the n-$C_{16:0}$ and n-$C_{18:0}$ fatty acids prepared from lipid extracts from late EN-early MN sites of northern Greece where (a) Mikri Volvi, (b) Varemenoi Goulon, (c) Liti III and (d) Ritini. The values of reference fats are represented by confidence ellipses ($\pm 1\ \sigma$) for animals raised in a strict C_3 diet (Copley *et al.*, 2003). The difference in the $\delta^{13}C$ values of the n-$C_{18:0}$ and n-$C_{16:0}$ fatty acids ($\Delta^{13}C = \delta^{13}C_{18:0}$-$\delta^{13}C_{16:0}$) obtained for the n-$C_{16:0}$ and n-$C_{18:0}$ fatty acids prepared from lipid extracts from the (e) Mikri Volvi, (f) Varemenoi Goulon, (g) Liti III and (h) Ritini. All $\delta^{13}C$ values were adjusted for post-Industrial Revolution effects of fossil fuel burning by the addition of 1.2 ‰ (Friedli *et al.*, 1986). Analytical precision is ± 0.3 ‰.

Figure 3

Scatter plot showing $\delta^{13}C$ values for the n-$C_{16:0}$ and n-$C_{18:0}$ fatty acids prepared from lipid extracts from MN sites of northern Greece where (a) Apsalos and (b) Paliambela. The values of reference fats are represented by confidence ellipses ($\pm 1\ \sigma$) for animals raised in a strict C_3 diet (Copley *et al.*, 2003). The difference in the $\delta^{13}C$ values of the n-$C_{18:0}$ and n-$C_{16:0}$ fatty acids ($\Delta^{13}C = \delta^{13}C_{18:0}$-$\delta^{13}C_{16:0}$) obtained for the n-$C_{16:0}$ and n-$C_{18:0}$ fatty acids prepared from lipid extracts from the (c) Apsalos and (d) Paliambela. All $\delta^{13}C$ values were adjusted for post-Industrial Revolution effects of fossil fuel burning by the addition of 1.2 ‰ (Friedli *et al.*, 1986). Analytical precision is ± 0.3 ‰.

Figure 4

Scatter plot showing $\delta^{13}C$ values for the n-$C_{16:0}$ and n-$C_{18:0}$ fatty acids prepared from lipid extracts from LN sites of northern Greece where (a) Makriyalos, (b) Stavroupoli, (c) Thermi and (d) Toumba Kremastis Koiladas. The values of reference fats are represented by confidence ellipses ($\pm 1\ \sigma$) for animals raised in a strict C_3 diet (Copley *et al.*, 2003). The difference in the $\delta^{13}C$ values of the n-$C_{18:0}$ and n-$C_{16:0}$ fatty acids ($\Delta^{13}C = \delta^{13}C_{18:0}$-$\delta^{13}C_{16:0}$) obtained for the n-$C_{16:0}$ and n-$C_{18:0}$ fatty acids prepared from lipid extracts from the (e) Makriyalos, (f) Stavroupoli, (g) Thermi and (h) Toumba Kremastis Koiladas. All $\delta^{13}C$ values were adjusted for post-Industrial Revolution effects of fossil fuel burning by the addition of 1.2 ‰ (Friedli *et al.*, 1986). Analytical precision is ± 0.3 ‰.

Figure 5

Mass chromatograms of a) *m/z* 105, b) *m/z* 290, c) *m/z* 318 and d) *m/z* 346 of the acid-extracted FAME from Ritini (RI-64) illustrating the presence of C_{18} and C_{20} APAAs.

Figure 6

Partial GC profile of the acid-extracted FAME from Apsalos (APS-29); illustrating the distribution of LCFA characteristic of partially degraded animal fats. Key: $FA_{X:Y}$ are fatty acids of carbon length X and degree of unsaturation Y. IS is the added internal standard (C_{34} *n*-alkane).

Figure 7

Partial GC profile of the acid extracted FAME from Stavroupoli (STAV-53); illustrating the distribution of compounds characteristic of plant lipids with a CPI of 5. Key: FA_X are fatty acids, AL are *n*-alkanes and OH are *n*-alcohols of carbon length X. IS is the added internal standard (C_{34} *n*-alkane).

Figure 1

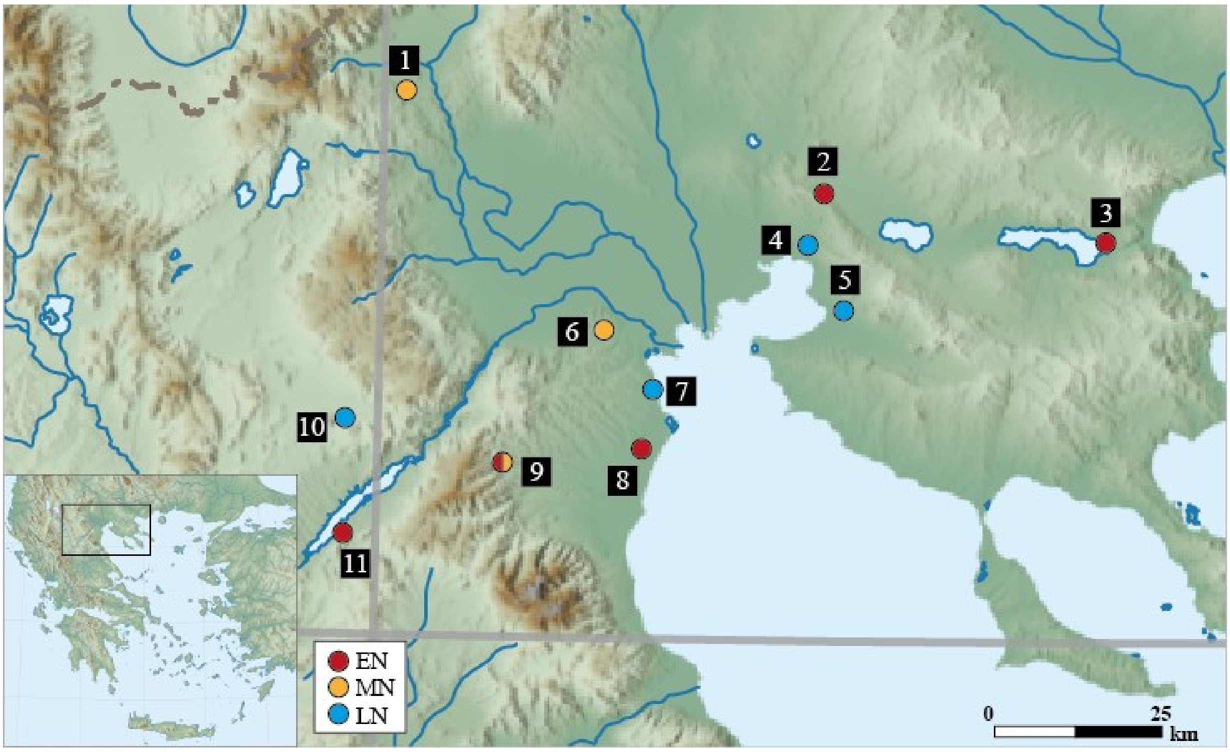
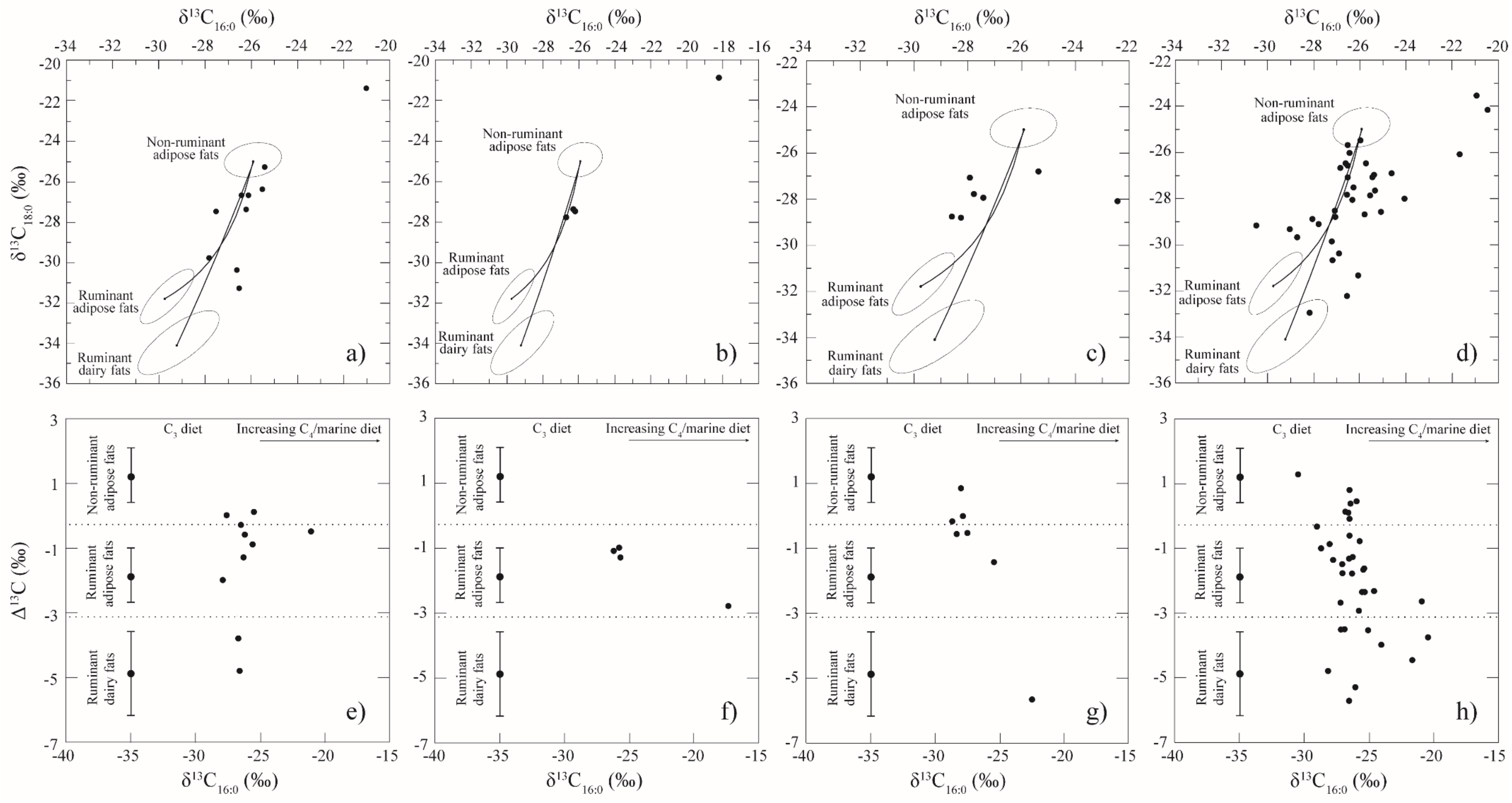


Figure 2



$\delta^{13}C_{16:0}$ (‰)
$\delta^{13}C_{18:0}$ (‰)
Non-ruminant adipose fats
Ruminant adipose fats
Ruminant dairy fats
a)
b)
c)
d)
$\Delta^{13}C$ (‰)
C_3 diet
Increasing C_4/marine diet
e)
f)
g)
h)

Figure 3

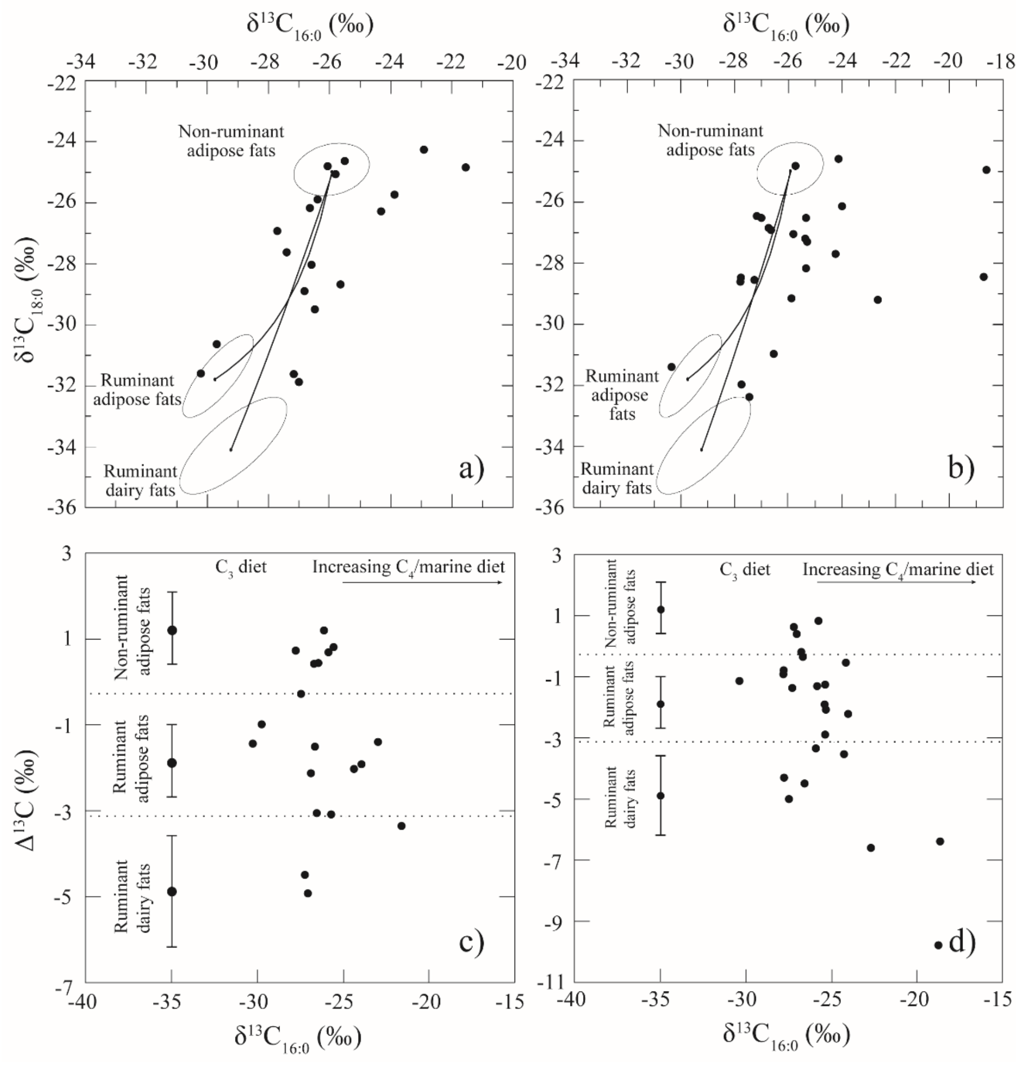

$\delta^{13}C_{16:0}$ (‰)
$\delta^{13}C_{18:0}$ (‰)
Non-ruminant adipose fats
Ruminant adipose fats
Ruminant dairy fats
a)
b)
-34
-32
-30
-28
-26
-24
-22
-20
-18
-36
C_3 diet
Increasing C_4/marine diet
$\Delta^{13}C$ (‰)
3
1
-1
-3
-5
-7
-9
-11
-40
-35
-25
-15
c)
d)

Figure 4

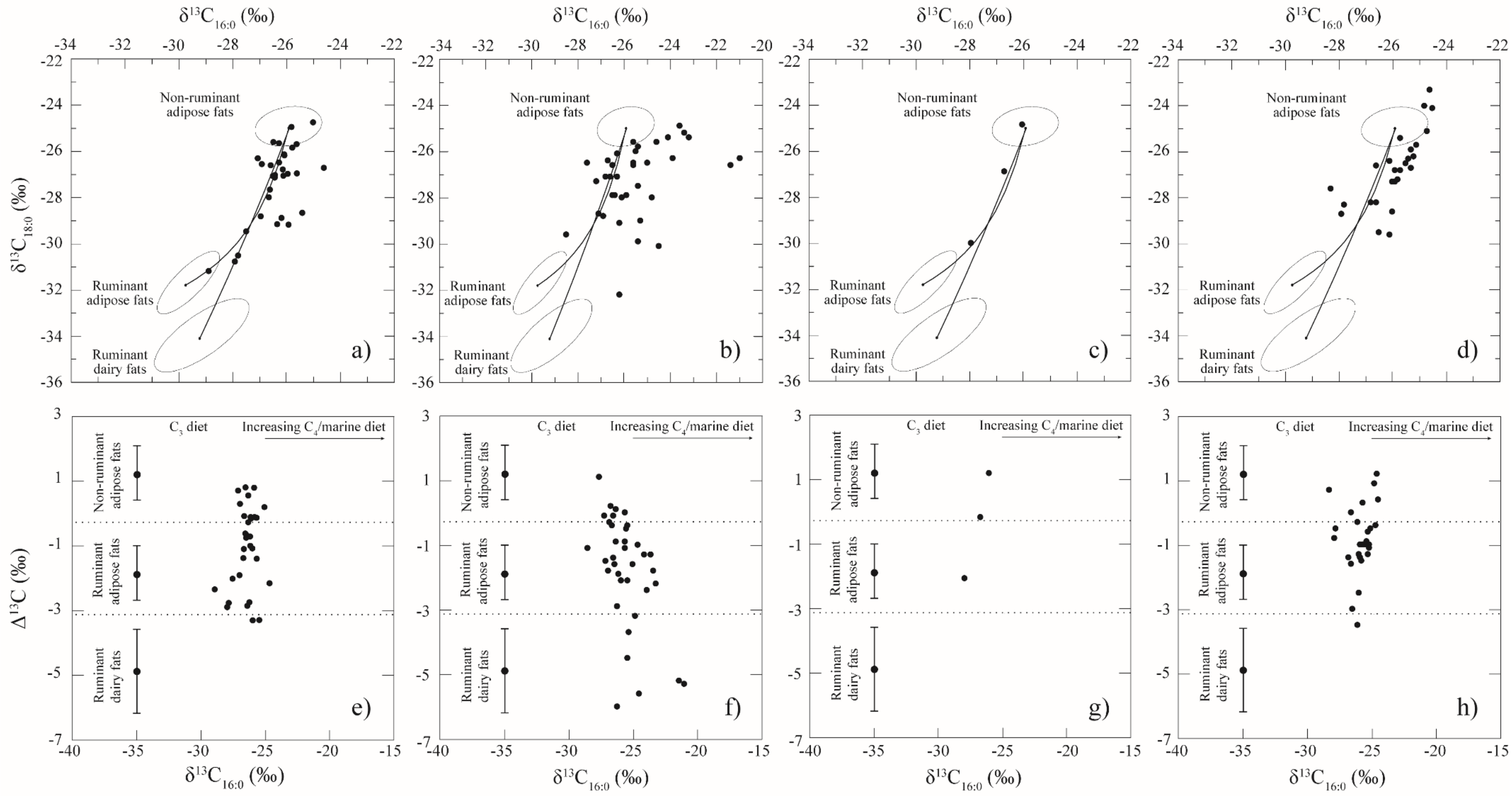

δ13C16:0 (‰)
δ13C18:0 (‰)
Δ13C (‰)
Non-ruminant adipose fats
Ruminant adipose fats
Ruminant dairy fats
C3 diet
Increasing C4/marine diet
a)
b)
c)
d)
e)
f)
g)
h)
-34
-32
-30
-28
-26
-24
-22
-20
-36
3
1
-1
-3
-5
-7
-40
-35
-25
-15

Figure 5

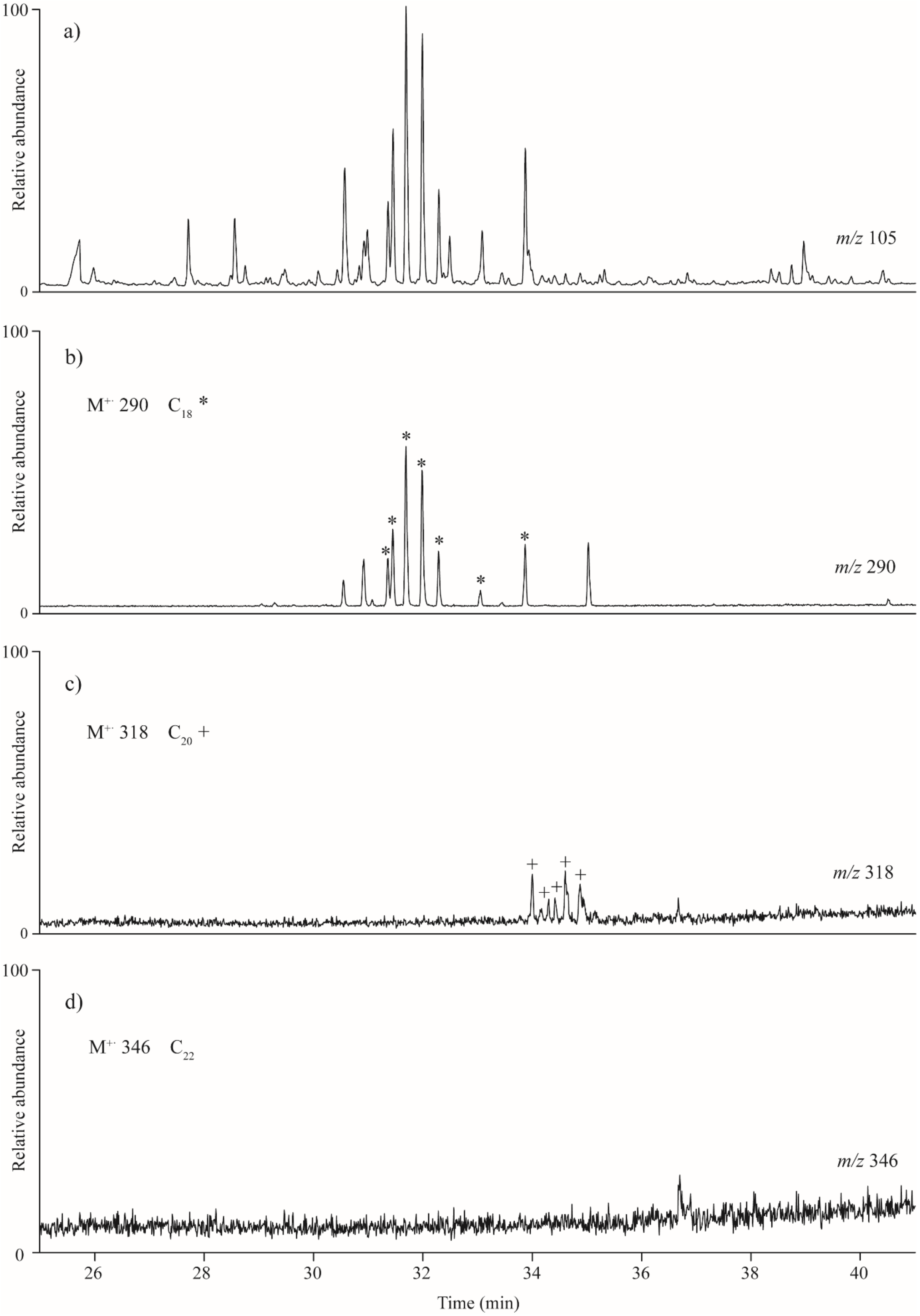

a)
m/z 105
b)
M+· 290 C18 *
m/z 290
c)
M+· 318 C20 +
m/z 318
d)
M+· 346 C22
m/z 346
Relative abundance
100
0
26
28
30
32
34
36
38
40
Time (min)

Figure 6

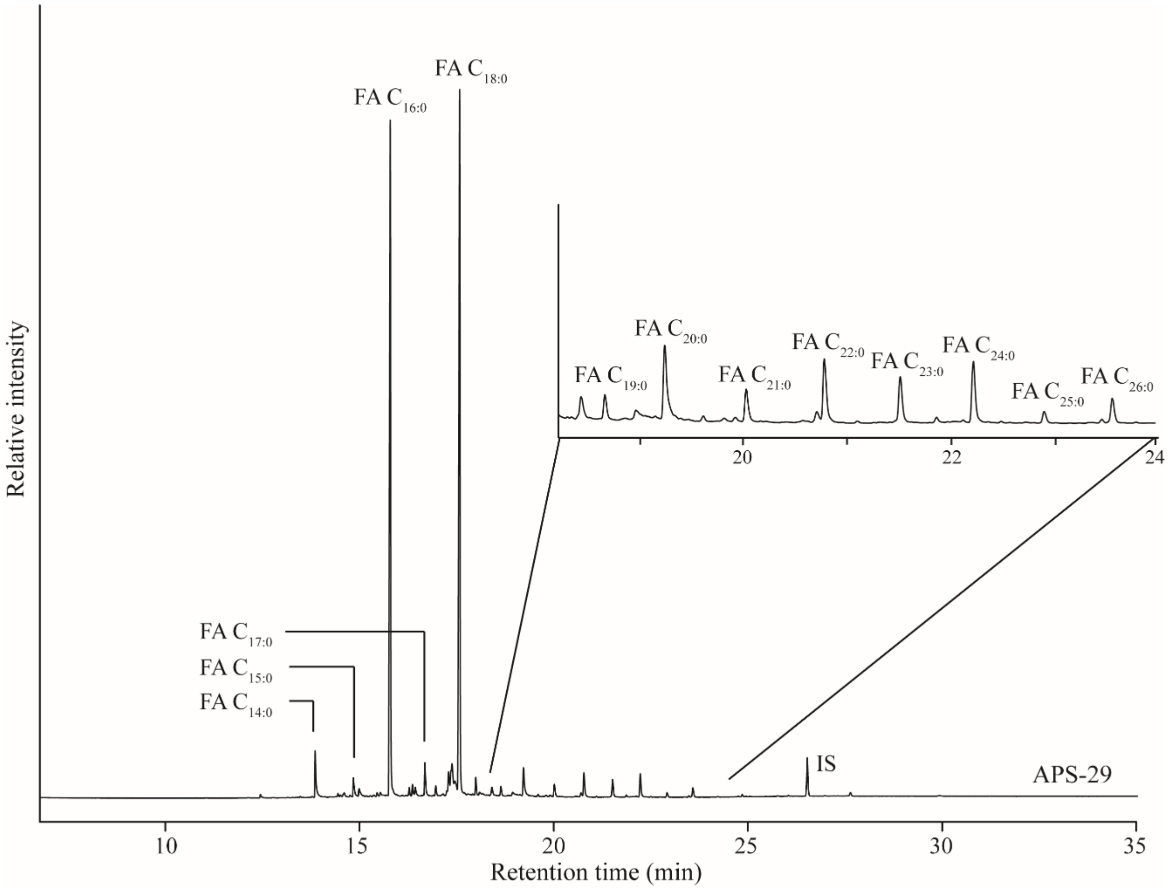


Figure 7

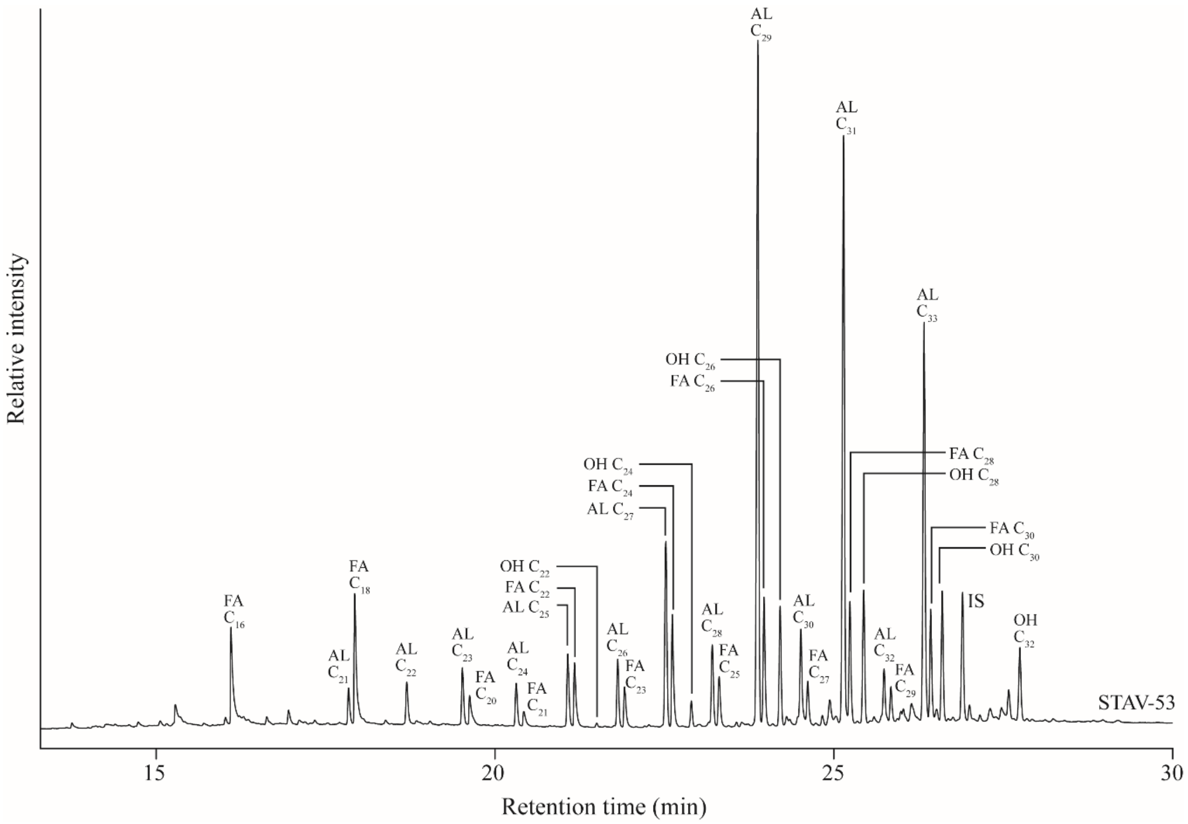


Table 1

Summary of archaeological site characteristics

Table 2

Summary of occurrence of lipid classes detected in pottery vessels at each site. Average lipid concentration of sherds containing a significant lipid concentration (>5 µg g^{-1} of potsherd). NRA = non-ruminant adipose, RA = ruminant adipose, RD = ruminant dairy. Aquatic resources include the co-occurrence of C_{18}, C_{20} and C_{22} APAAs and isoprenoid fatty acids. EN = Early Neolithic, MN = Middle Neolithic and LN = Late Neolithic.

Table 3

Study sites grouped into chronological phases of the Greek Neolithic.

Table 4

Relative proportions of animal fats extracted from pottery vessels throughout the Neolithic in northern Greece determined using $\Delta^{13}C$ values due to the environmental shift observed in the mixing plot of $\delta^{13}C_{16:0}$ and $\delta^{13}C_{18:0}$ values.

Table 5

Occurrence of aquatic biomarkers detected in pottery vessels from the study sites.

Table 1

Site	**Potsherds analysed**	**Longitude**	**Latitude**	**Radiocarbon date (cal. B.C.E.)**	**Pottery**	**Settlement type**	**Houses**	**Size**	**Faunal assemblage (minAU)**	**Faunal management strategy**	**Reference**
Apsalos	97	22.0573	40.8915	5701-5622	red slipped with distinctive black decorations (bitumen)	flat-extended	subterranean	4.5 ha	cattle, sheep/goat, pig		Chrisostomou *et al.* (2003); Urem-Kotsou *et al.* (2014b)
Liti III	8	22.9766	40.7508		red polished wares	flat-extended	pit dwellings	150 m^2			Kotsos and Urem-Kotsou (2006); Tzanavari and Filis (2009)
Makriyalos	103	22.6038	40.4160	5400-4500	Black burnished	flat-extended	semi-subterranean	50 ha	pig: 34 %, sheep/goat: 34 %, cattle: 32 %	meat-based	Pappa and Besios (1999); Pappa *et al.* (2004); Tzevelekidi *et al.* (2012)
Mikri Volvi	91	23.5622	40.6780			flat-extended	wattle-and-daub	10 ha			Kotsos and Urem-Kotsou (2006)
Paliambela	221	22.5035	40.5111	EN: 6609-6461; LN: 5511-5380	red slipped, burnished wares	EN: flat-extended LN: tell mound		500 m^2	sheep/goat: 61 %, pig: 25.1 %, cattle: 13.8%		Maniatis *et al.* (2015; Urem-Kotsou *et al.* (2014b); Halstead and Isaakidou (2013); (Kotsakis and Halstead (2004)
Revenia	37	22.5847	40.3164	6438-6264	red-slipped, monochrome, barbotine and decorated wares, well burnished.	flat-extended	pit dwellings	4 ha	sheep/goat: 70.3 %, pig: 17.4 %, cattle: 12.3 %	meat-based	Hofmanova *et al.* (2016); Urem-Kotsou *et al.* (2014b); Halstead and Isaakidou (2013)
Ritini	125	22.2848	40.2903	5900/5700-5500	red slipped wares	flat-extended	wattle-and-daub				Bessios *et al.* (2005); Kotsos and Urem-Kotsou (2006);

											Urem-Kotsou *et al.* (2014a)
Stavroupoli	125	22.9376	40.6662	5839-5531	ST1: black burnished. ST2: red and often decorated	flat-extended	pit dwellings	10 ha	sheep/goat: 54 %, cattle: 29 %, pig: 17 %	Sheep and cattle meat-based. Goats milk-based	Maniatis (2002); Kotsos and Urem-Kotsou (2006); Giannouli (2002, 2004)
Thermi	22	23.0196	40.5485	5300-5000	MN: red and brown burnished ware. LN: black burnished wares	flat-extended	pit dwellings	6 ha	sheep/goat: 51 %, pig: 28 %, cattle: 22 %	meat-based	Pappa *et al.* (2011); Grammenos *et al.* (1990); Halstead (1996)
Toumba Kremastis Koiladas	72	21.9312	40.3567	5340-4930		low mound			sheep/goat: 62.7 %, pig: 25.6 %, cattle: 8.1%	meat-based	Chondrogianni-Metoki (2009a); Tzevelekidi *et al.* (2014)
Varemenoi Goulon	11	21.9144	40.1603	6430-5670		flat-extended with some tell mound components		12 ha			Chondrogianni-Metoki (2009b)

Table 2

				Animal resources							**Plant resources**
Site	**Period**	**% lipid recovery**	**Av. lipid conc ($\mu g\ g^{-1}$)**	**NRA**	**NRA/RA**	**RA**	**RA/RD**	**RD**	**LCFA**	**Aquatic resources**	**Aliphatic lipids**
Mikri Volvi	EN	12	6.9	0	5	3	0	2	8	0	0
Revenia	EN	0	0	0	0	0	0	0	0	0	0
Varemenoi Goulon	EN	n/a	32.8	0	0	3	1	0	4	0	6
Liti III	late EN - early MN	n/a	20.1	1	4	1	0	1	4	0	0
Ritini	late EN - early MN	33	24.6	4	4	16	4	6	20	0	0
Apsalos	MN	26	35.1	6	1	7	2	2	6	0	0
Paliambela	MN	14	9.1	3	3	9	3	6	11	0	0
Makriyalos	LN	31	46.3	4	12	9	6	0	3	0	0
Stavroupoli	LN	33	25.4	1	9	18	2	6	21	0	1
Thermi	LN	18	10.2	1	1	1	0	0	3	0	3
Toumba Kremastis Koiladas	LN	40	22.8	5	6	12	3	0	11	0	0

Table 3

Phase	**Sites**
late EN– early MN	Liti III, Revenia, Ritini, Mikri Volvi, Varemenoi Goulon
MN	Apsalos, Paliambela
LN	Makriyalos, Stavroupoli, Thermi, Toumba Kremastis Koiladas

Table 4

	Lipid residues (%)		
	late EN – early MN	**MN**	**LN**
Non-ruminant adipose (NRA)	9	21	12
Mixture NRA/RA	25	14	30
Ruminant adipose (RA)	37.5	32.5	42
Mixture RA/RD	12.5	14	10
Ruminant dairy (RD)	16	18.5	6

Table 5

Site	**Period**	**Location**	**APAAs**	**Isoprenoid FA**	**Dihydroxy FA**
Mikri Volvi	EN	Lake	-	-	-
Revenia	EN	Coastal	-	-	-
Varemenoi Goulon	EN	Inland	-	-	-
Liti III	late EN – early MN	Lake	-	-	-
Ritini	late EN – early MN	Inland	C_{18}, C_{20}	-	-
Apsalos	MN	Inland	C_{18}	-	-
Paliambela	MN	Inland	-	-	-
Makriyalos	LN	Coastal	-	-	-
Stavroupoli	LN	Coastal	-	-	-
Thermi	LN	Coastal	-	-	-
Toumba Kremastis Koiladas	LN	Inland	C_{18}	-	-