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Pliocene–Early Pleistocene climatic trends in the Italian Peninsula based on stable oxygen and carbon isotope compositions of rhinoceros and gomphothere tooth enamel

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Abstract

The Pliocene and Early Pleistocene (5.2 to 1 Ma) palaeoclimate for localities in Italy is evaluated using stable carbon and oxygen isotope compositions of tooth enamel of fossil specimens from Rhinocerotidae *(Stephanorhinus* sp.) and Gomphotheriidae (*Anancus* sp.) taxa. Carbon isotope composition was measured in the structural carbonate ($\delta^{13}C$), while oxygen isotope values were determined both in the structural carbonate ($\delta^{18}O_{CO3}$) and the phosphate ($\delta^{18}O_{PO4}$) of bioapatite.

The $\delta^{13}C_{CO3}$ values indicate that the taxa were grazers-browsers of a pure C_3 vegetation. Low $\delta^{13}C_{CO3}$ values for Central and North Italy indicate a humid climate with woodlands and forest cover in the Pliocene. For northern localities the $\delta^{13}C$ values increase between MN16a and MNQ16b biozones most likely linked to the Northern Hemisphere Glaciation at 2.7 Ma after the "Mid-Pliocene Warm Period". For Central Italy the values have a wide range with a long term increasing trend in the Early Pleistocene, indicating more arid climate and / or more open vegetation.

Overall, the $\delta^{18}O_{PO4}$ values in Central Italy change together with the $\delta^{13}C_{CO3}$ values and are taken to reflect the warmer / wetter interglacials and cooler / more arid glacial phases. The $\delta^{18}O_{PO4}$ values in North Italy are lower than those in Central Italy and show no clear temporal trend. One explanation for the low values especially in MN14-15 biozone is that these $\delta^{18}O_{PO4}$ values do not reflect entirely the isotopic composition of local precipitation but river waters from the Alps with ^{18}O-depleted isotopic compositions or a N-S directed rain-shadow effect on the precipitation. In general the new isotope data agree well with palaeoclimate reconstructions based on palynological and other proxies.

47 Keywords: stable isotopes, enamel, climate, Pliocene, Early Pleistocene, Italy

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1. Introduction

There is an increasing interest in understanding Pliocene and Early Pleistocene terrestrial climates and environments because it can help to predict future climate changes more accurately. Reconstructions of terrestrial palaeoenvironments, palaeoecology, and palaeoclimates are commonly based on proxies like paleosols, fossil plants (including pollen), vertebrate occurrences, speleothems, travertines, tufas, as well as the geochemistry and isotope geochemistry of biogenic carbonates and phosphates such as mammal teeth and bones. (Sullivan and Krüger, 1983; Luz et al., 1984; 1990; Luz and Kolodny, 1985; Lee-Thorp and van der Merwe, 1987; Lee-Thorp et al., 1989; Ayliffe et al., 1992; 1994; Bryant and Froelich, 1995; Bocherens et al., 1996; Fricke et al., 1998; Cerling and Harris, 1999; Kohn et al., 1999; Kohn and Cerling, 2002; Arppe and Karhu, 2006; Levin et al., 2006; Tütken et al., 2006; Martin et al., 2008; Kohn, 2010; Pellegrini et al., 2011; Kocsis et al., 2014; Pushkina et al., 2014; García-Alix, 2015; Kovács et al., 2015; Hartman et al., 2016; Metcalfe et al., 2016 and references therein).

In this study 51 Pliocene and Early Pleistocene tooth enamel samples of fossil rhinoceros (*n* = 44) and gomphothere (n = 7) were studied from Italy. Most of the samples are from Central and North Italy and a few samples from South Italy (Figure 1). The age of the fossils covers the Early Pliocene to late Early Pleistocene, from about 5.2 to 1 Ma.

The carbon and oxygen isotope compositions of structural carbonate and the oxygen isotope composition of phosphate in enamel bioapatite were used to explore variations in past climate and the environment of the animals. The aims of this study are: 1) Reconstructing the isotope composition of environmental water ($\delta^{18}O_w$) and estimating the changes in mean annual surface air temperature (MAT) on the basis of $\delta^{18}O_{PO4}$ values of fossil tooth enamel; 2)

interpretation of the diet of the species; 3) detect changes in vegetation based on the enamel carbon isotope analyses.

These climatic and ecological parameters derived from the isotope results are compared with palaeobotanical proxies, palaeontological and palaeoecological information (Fauquette et al., 1999; Pontini and Bertini, 2000; Bertini, 2001; 2010; Bredenkamp et al., 2002; Ji et al., 2002; Fauquette and Bertini, 2003; Klotz et al., 2006; Palombo, 2007; Magri et al., 2010; Rook and Martínez-Navarro, 2010; Petronio et al., 2011; Combourieu-Nebout et al., 2015; Loftus et al., 2015; Rivals et al., 2015).

2. Background

2.1. Bioapatite and preservation of its isotopic compositions

Biogenic hydroxyapatite (i.e., bioapatite) is the main inorganic fraction of the skeletal tissues of mammals with up to 6 wt.% of carbonate as structurally bound carbonate. The simplified formula is $Ca_5(PO_4, CO_3)_3(OH, CO_3)$ (e.g., Kohn et al., 1999; Elliot, 2002). There are two types of carbonate forms in bioapatite, structural and labile carbonate. Structural carbonate substitutes for PO_4^{3-} and OH^-, while the labile CO_3^{2-} component's structural identity is ambiguous as it is often considered adsorbed onto the surface. Oxygen is present in three different ions in bioapatite: phosphate, hydroxyl and carbonate ions. Because of the strong P–O bonds phosphate oxygen is considered more resistant to low temperature inorganic alteration processes, than carbonate oxygen (Kohn et al., 1999; Kohn and Cerling, 2002). However, during microbiological reactions even the phosphate oxygen isotope composition can be changed due to enzymatic catalysis between PO_4^{3-} and water (e.g., Blake et al., 1997; Zazzo, 2004a,b; Liang and Blake, 2007). Nevertheless, it was shown experimentally that

when enamel (i.e., well-crystalized bioapatite) is subjected to bacterially mediated conditions, the oxygen isotopic composition of the phosphate group was not affected (e.g., Zazzo, 2004a,b). Moreover, enamel is the preferred tissue for isotopic investigations because it contains the highest proportion of apatite (96%), and structurally compact with little pore space and large phosphate crystallites (up to 1 μm long), arranged in a decussate texture (Kohn et al., 1999). As a result enamel is more resistant to diagenesis than other tissues in the same taphonomic context (Lee-Thorp and van der Merwe, 1991; Quade et al., 1992; Ayliffe et al., 1994; Wang and Cerling, 1994; Koch et al., 1997).

There is a widely used method to monitor diagenetic effects. Oxygen from the body fluids is in isotopic equilibrium with both the carbonate and phosphate ions of the inorganic tissue. Several studies have found a constant offset between the $\delta^{18}O_{CO3}$ and $\delta^{18}O_{PO4}$ values for non-altered mammal tooth enamel and the $\delta^{18}O_{CO3} - \delta^{18}O_{PO4}$ slopes are similar for different species (Bryant et al., 1996; Iacumin et al., 1996; Arppe and Karhu, 2006, 2010; Tütken et al., 2007; Pellegrini et al., 2011; Domingo et al., 2013). Because it seems quite improbable to find isotopically altered skeletal remains showing a good correlation and a similar offset to modern samples, it is plausible to use the parallel $\delta^{18}O_{CO3} - \delta^{18}O_{PO4}$ measurements as a monitor of diagenetic alteration (Iacumin et al., 1996). Most studies found an offset 8.4 – 9.1 ‰ for modern, unaltered samples (Bryant et al., 1996; Iacumin et al., 1996; Martin et al., 2008; Pellegrini et al., 2011). Nevertheless, Martin et al. (2008) found ~2 ‰ intra tooth differences in two upper molars (M1 and M2) of extant *Rhinoceros unicornis* and further variations can be present due to environmental, physiological and tooth growth process differences. The final recommendation is that if the $\delta^{18}O_{CO3} - \delta^{18}O_{PO4}$ difference is in the range of 7.2 ‰ to 10.6 ‰ the values of the samples can be considered as unaltered (Martin et al., 2008).

123 *2.2. Stable carbon isotope composition and diet*

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125 Carbon isotope differences among vertebrates largely reflect the differences in the diet of the
126 animals. Variations in $\delta^{13}C$ values of the plants reflect mostly the three different
127 photosynthetic pathways used by plants. The C_3 pathway is the most common, occurring in all
128 trees, most shrubs and herbs, and grasses in regions with a cool-temperate growing season. C_3
129 plants have a mean $\delta^{13}C$ value of about –27 ‰ with a range from –35 to –22 ‰. C_4
130 photosynthesis occurs in grasses from regions with a warm growing season, and in some
131 sedges (Bruhl and Wilson, 2007) and dicots. C_4 plants have higher $\delta^{13}C$ values (mean ca. –13
132 ‰, range between –19 and –9 ‰). Crassulacean acid metabolism (CAM) is the least common
133 pathway, occurring mostly in succulent plants, with a range between the C_3 and C_4 plants
134 (O'Leary, 1988; Farquhar et al., 1989; Martinelli et al., 1991). Enamel $\delta^{13}C$ values of large
135 non-ruminant herbivores have a bioapatite-plant fractionation offset of about 13.3 ‰ (Passey
136 et al., 2005). In C_3 ecosystems, the variation in $\delta^{13}C$ values can provide further information
137 about mean annual precipitation (MAP), humidity, the type of vegetation, habitat differences
138 or niche partitioning between animals (Van der Merwe and Medina, 1991; Feranec and
139 MacFadden, 2006; Kohn, 2010). Generally C_3 plants show a monotonic increase in $\delta^{13}C$ with
140 decreasing MAP (Kohn, 2010). Effects of vegetation like the canopy effect complicate this
141 relationship though. In forests the limited sunlight availability, high relative humidity and the
142 low $\delta^{13}C_{CO2}$ values from decaying litter of the soil organic matter lowers the $\delta^{13}C$ values (Van
143 der Merwe and Medina, 1991; Bocherens et al., 1996; Cerling and Harris, 1999; Drucker et
144 al., 2008). As the $\delta^{13}C$ values of herbivores reflect the values of the consumed plants,
145 preferential consumption of some specific plants could imply that the $\delta^{13}C$ values of apatite
146 can not be taken to determine the average vegetation. Food preference of the animals is also

not necessarily constant within a taxonomic group, and for a given taxon through space and time.

Because of the complex information encoded in the $\delta^{13}C$ values, several studies applied it to address a variety of palaeodietary, palaeoecological, and palaeoenvironmental problems (e.g. Lee-Thorp et al., 1989; Quade et al., 1992; Bocherens et al., 1996; Cerling et al., 1999; Iacumin et al., 2000; Feranec, 2004; Metcalfe et al., 2009; Arppe et al., 2011; Montanari et al., 2013; Kocsis et al., 2014; Scherler et al., 2014; Zanazzi et al., 2015).

2.3. Stable oxygen isotope composition and environmental water

Oxygen isotope compositions of mammal teeth are linearly related to the body water of the animals (Longinelli, 1984). The isotope composition of body water depends on the total oxygen flux through the body, but water from drinking and food sources have the largest effect compared to other factors (Luz, 1984). In the case of elephants the ratio is approximately 2:1 of water ingested via drinking and that ingested by consumption of plant matter (Ayliffe et al., 1992), while for rhinoceroses this ratio is 4:1 (Clauss et al., 2005; Martin et al., 2008). Thus teeth of these two groups are potentially good indicators of the $\delta^{18}O$ values of local environmental waters (Martin et al., 2008). The drinking water of the animals may include different sources including streams, ponds, rivers, lakes, and leaves. Each of these reservoirs typically can have different $\delta^{18}O$ values relative to precipitation, due to variable mixing of temporally different precipitation and potential evaporative effects on the different reservoirs (e.g., Montanari et al., 2013). Teeth of large (>100 kg) obligate drinking mammals record an average oxygen isotope composition of ingested waters ($\delta^{18}Ow$) from different sources from a longer period. Thus the correlation between the average $\delta^{18}O$ values of teeth enamel and the local precipitation is usually strong (Ayliffe et al., 1992; Bryant and

Froelich, 1995; Koch, 1998). However, there are exceptions for example glacial meltwaters, rivers from mountainous area, or lakes with high evaporation in arid areas can have a systematic $\delta^{18}O$ offset from the local precipitation. Migration of the animals can also complicate the relationship. If animals migrated in order to escape harsh winters or to exploit seasonally available resources, isotope composition of their enamel may not be representative for the area where the fossils are found (Hoppe and Koch, 2007). The $\delta^{18}O$ values can also offer information about the ecology of the different taxa if they lived under the same climatic regime. Browsing taxa that ingest a higher proportion of ^{18}O-enriched water with their food tend to have higher $\delta^{18}O$ values compared to sympatric grazing taxa (Kohn, 1996; Tütken et al., 2013).

The oxygen isotope composition of local precipitation ($\delta^{18}O_{ppt}$) is controlled by several factors. The mean annual temperature (MAT), the different moisture source, and air mass trajectories, the latitude, altitude, continental and amount effects, all determine the isotopic compositions of precipitation. The MAT generally has the greatest effect on the $\delta^{18}O$ values of the precipitation ($\delta^{18}O_{ppt}$) at mid and high latitudes and in case of no extreme low or high precipitation or humidity (Dansgaard, 1964; Rozanski et al., 1993).

2.4. Pliocene-Pleistocene palaeoclimates and palaeoenvironments in Italy

According to different proxies and models, the climate in the Early Pliocene was in general warmer and wetter than today (2 – 3 °C higher global MAT with a decreased equator to pole gradient) (Dowsett et al., 1996, 2013; Hill et al., 2011; Salzmann et al., 2011; Haywood et al., 2013). In continental Europe thick forests were present with a temperate climate. Palynological data suggest a relatively stable, warm-temperate climate in Northern Italy with 15 – 17 °C MAT and a MAP above 1000 mm (Bertini, 2001; 2010). Nevertheless, the slight

differences between mega-mesothermic elements and meso-microthermic ones suggest modest fluctuations of temperature (Bertini, 2001). The Climatic Amplitude Method (CAM) applied to other pollen records from Northern Italian sites gave MAT between 16 and 20 °C, with the Most Likely Value (MLV) around 17 – 19 °C; and MAP between 1100 and 1500-1600 mm, with the MLV around 1200 – 1300 mm (Fauquette et al., 1999, Fauquette and Bertini, 2003, 2010). In Sicily the climate was warmer and drier (or with equal humidity) than today (Fauquette et al., 1999, 2006; Combourieu-Nebout et al., 2015).

During the MN16a Neogene Mammalian biozone the climate in general became less stable compared to the Early Pliocene (Hilgen et al., 2012). In SW Europe the modern Mediterranean climatic regime was established and aridity was enhanced (Agustí et al., 2001; Meyers and Hinnov, 2010; Rook and Martínez-Navarro, 2010; De Schepper et al., 2013, 2014; Woodard et al., 2014). The Villanyian/Villafranchian mammal turnover occurred at this time in Europe and a renewal of archaic faunas to mammal assemblages with more modern characteristics indicative to less humid environments under cooler climates documented also in Italy (Pott, 1995; Bredenkamp et al., 2002; Petronio et al., 2011). From 3.3 to 3.0 Ma the "Mid-Pliocene Warm Period" (MPWP) was characterised by 3 °C warmer global temperatures, 10–40 m higher sea-level and less continental ice sheets compared to present day, but even this stable, warm period was preceded by a short-lived, intense global glaciation (3.305–3.285 Ma) (De Schepper et al., 2014). Around 3.3 Ma (MN16a, Triversa FU) in Central Italy the faunas are characterized by the occurrence of new species and by the diffusion of animals also adapted to open habitats. The palaeobotanical data indicates a considerable homogeneity of the vegetation, at least in Northern Italy. Hence, besides the new species, animals strictly relying on the forests still survived. For example *Anancus arvernensis* as a forest living taxon survived from the Ruscinian.

At approximately 2.7 Ma the Northern Hemisphere Glaciation (NHG) occurred, followed by glacial-interglacial cycles of moderate amplitude (orbital periodicity of 41 ka) (Dowsett et al., 2013; Woodard et al., 2014; Salzmann et al., 2011, 2013; Haywood et al., 2013). The resulting increase in aridity and more intense seasonality caused a replacement of forests by tundra-like vegetation in Northern Europe (Pott, 1995; Bredenkamp et al., 2002) and a faunal renewal in Italy, where several forest-dwelling taxa, especially small carnivores and arboreal-scansorial taxa disappeared, whereas new large grazers, mixed feeders or even browsers appeared. This renewal, called the "*Equus*-elephant event" can be regarded as a true turnover phase (Palombo, 2007; Petronio et al., 2011). The irreversible transformation of the climate regime determines rapid variations of the vegetation community, according to a repetitive succession of vegetation types. This event also corresponds to a massive local disappearance of thermophile and humidity-requiring woody plant taxa (Martinetto et al., 2015), even if a few of them survived in Central Italy longer than in the rest of Europe (until the end of the Gelasian, ca. 1.8 Ma), due to a combination of locally high precipitation and high temperatures.

The entire interval between 2.5 and 1.0 Ma is characterized by relatively long interglacial periods, with rather thick forest cover, and short glacial periods, with open vegetation in Central and Southern Italy (Petronio et al., 2011 and references therein; Combourieu-Nebout et al., 2015). In the Mediterranean area the typical glacial-interglacial cycles are characterized by the succession of four main vegetation assemblages. Deciduous forest, subtropical/warm-temperate forest, altitudinal conifer forest and open vegetation. This succession supports four dominant climatic conditions: (1) a first increase of the temperature followed by (2) an increase of the humidity, then (3) a decrease of temperature without variations of humidity and (4) finally a strong decrease of the humidity, corresponding to a gradual transition from warm and humid conditions during interglacials to cold and dry conditions during glacials

(Bertini, 2001, 2010). Pollen data indicate that the steppe-type of conditions, which are longer lasting and more frequent in Southern Italy, developed only during some extreme glacial peaks in Central Italy (Russo Ermolli et al., 2000; Pontini and Bertini, 2000; Petronio et al., 2011). In North Italy the glacial periods led to a progressive expansion of a cooler forest-type of vegetation cover (i.e., progressive increase of *Picea* and *Fagus*), but herbs including steppe taxa remained a minor component (Bertini, 2001, 2010).

Despite the many variations it can be concluded that interglacial phases can be characterised with high MAT and MAP, similar to that in the Zanclean, while during the glacial phases the MAT and MAP were close to modern values. This cyclicity was controlled by the 41 ka rhythm of the orbital periodicity continued in the entire period until 0.9 Ma (Bertini, 2000).

The Pliocene and Early Pleistocene sediments of Northern and Central Italy (often from the same successions that yielded the studied fossil vertebrates) are rich in macrofossil plant assemblages (Forno et al., 2015; Irace et al., 2015; Martinetto, 2015; Martinetto et al., 2015) that have not yet been exploited for a thorough palaeoclimatic analysis, despite their potentially very accurate indications (Teodoridis et al., 2015). The aims of this study are to detect the temporal changes in the vegetation and in MAT and to compare the new isotope results with the palaeobotanical proxies and palaeontological information. The comparisons of the results obtained from the different approaches are summarized in Table 1.

3. Materials and methods

3.1. Fossil materials

Figure 1 and Table A1 give detailed information about the localities, their relative age relationships and the types of investigated fossils. Altogether 51 fossils from 21 localities

were collected from the time period of about 5.2 to 1 Ma. For some localities only one sample whereas for others up to 7 samples were collected. The fossils belong to four different species, three of them are rhinocerotid: *Stephanorhinus etruscus* (n=21), *"Dihoplus" megarhinus* (n=9), *Stephanorhinus jeanvireti* (n=6), and one proboscidean: *Anancus arvernensis* (n=7) (Fig. 1, Table A1). These include rhinoceros samples that cannot be determined at specific level (n=8). Wherever possible, teeth of adult individuals were sampled. Each fossil probably represents a different individual. Samples were acquired from the following institutions: DST, Department of Earth Science, Sapienza University of Roma (Roma); MSNAF, Natural History Museum, Fisiocritici Academy, Siena (Siena); MGC, Museum of Geology G. Capellini, (Bologna), MGPT, Museum of Geology and Paleontology, University of Turin (Turin), MHMB, Natural History Museum Basel (Basel).

Many of the investigated sites and fossils are chronologically well calibrated using magnetostratigraphy, cyclostratigraphy and biochronology. For the interpretation of the results the samples were grouped in the Neogene Mammal biozones. The numbers of samples from different time periods are different in Central and in North Italy. The oldest samples are from MN14 in Central and from MN14-15 in North Italy. The precise stratigraphic and geographic position of the *"Dihoplus" megarhinus* specimens from Dusino San Paolo is uncertain; the MN14-15 interval is based on the well-known record of the taxa.

In Central Italy MNQ18, MNQ19 and MNQ19-20 biozones are treated separately, while in the case of North Italy due to the fewer and less well-dated samples from these time periods, the MN18-19-20 biozones are drawn together (n = 3). In North Italy the MN16 biozone can be separated to MN16a (Triversa FU, 5 samples) and MNQ16b (Montopoli FU, 4 samples) biozones. However recent analyses on plant macrofossils assemblages from the same area where MNQ16b mammal assemblages have been reported, in NW Italy (Irace et al., 2015), noted the absence of several thermophilous plants (HUTEA of Martinetto et al., 2015) already

within the latest Pliocene. This suggests a change of vegetation already in the late Pliocene, and not only at the beginning of the Pleistocene. Since vegetation changes often coincide with mammal turnover events, the actual assignment to the MN16a/MNQ16b mammal zones should be further investigated. In fact the MNQ16b mammals are documented by 19th century findings of uncertain stratigraphic position that could fall within the latest Pliocene as well as within the earliest Pleistocene.

3.2. Sampling

After cleaning the surface mechanically, about 10 mg of bulk enamel was sampled from each fossil using a Dremel diamond-studded drill. Where it was possible, enamel was sampled along a vertical line over the whole enamel length from the crown to the root to get a representative mean sample of the period of enamel formation. Due to sample limitations, in some cases enamel from tooth fragments was collected. Because the complex process of enamel formation and mineralization, which can be 2 – 3 years in the case of rhinos and proboscideans, sampling enamel from a tooth fragment can still represent an average isotope record of a longer period (Tafforeau et al., 2007; Metcalfe and Longstaffe, 2012). Where possible, the teeth from adult individuals were sampled, because physiological effects such as weaning/nursing can modify the $\delta^{13}C$ and $\delta^{18}O$ values of the bioapatite (Martin et al., 2008; Metcalfe et al., 2010). Where possible, several samples were collected from each site to avoid the effects of environmental factors and individual differences. According to Pryor et al. (2014) typical uncertainty in temperature inferred from a single sample can be at least ±4 °C, while analyses of multiple samples can reduce the uncertainty to about 1 to 2 °C.

3.3. Pre-treatment and measurements

Sample powder was pre-cleaned according to the method given in Koch et al. (1997). NaOCl was used to remove soluble organic material and acetic acid-Ca-acetate buffer to remove exogenous carbonates (Koch et al., 1997; Kocsis, 2011). The pre-treatment methods have been recently reviewed by Pellegrini and Snoeck (2016). The pre-treatment procedure suggested is supported by the authors, but it is also clear that some unpredictable contamination cannot be excluded. This may influence the finer crystalline material (e.g., bone, dentine) and could also be the cause for the slightly lower NBS-120c oxygen isotope compositions measured parallel with the teeth.

After pre-cleaning, about 2 mg of sample was used for the carbonate isotopic measurements, while for the $\delta^{18}O$ analyses, another 2 mg was dissolved and the phosphate ions were precipitated as silver phosphate, according to methods adapted after Dettman et al. (2001) and Kocsis (2011).

The carbon and oxygen isotopic compositions of structural carbonate were measured on a Finnigan MAT Delta Plus XL mass spectrometer equipped with a GASBENCH-II preparation unit. The samples were reacted with 99 % orthophosphoric acid and the produced CO_2 was introduced to the mass spectrometer with He-carrier gas following procedures similar to those described in Spötl and Vennemann (2003).

Carrara Marble in-house standards ($\delta^{18}O$ = –1.70 ‰, VPDB; $\delta^{13}C$ = 2.05 ‰, VPDB) were run in the same sequence with the samples and used for correcting the data. The reproducibility of the in-house standard is better than 0.1 ‰ (1σ) for both oxygen and carbon isotopic compositions. NBS120c reference material (Florida phosphate rock) was treated and analysed in parallel with the samples and gave values of $\delta^{13}C$ of –6.3 ± 0.1 ‰ (n=6) and $\delta^{18}O$ of –2.3 ± 0.2 ‰ (n=6) on VPDB scale. These values are identical to those of the long-term average values of NBS-120c in the laboratory.

For the phosphate $\delta^{18}O$ values the silver-phosphate was analysed via reduction with graphite in a TC/EA (high-temperature conversion elemental analyser) coupled to a Finnigan MAT Delta Plus XL mass spectrometer according to the values and method given in Vennemann et al. (2002). The results were corrected to in-house Ag_3PO_4 phosphate standards (LK-2L: 12.1 ‰ and LK-3L: 17.9 ‰) that showed standard deviations (1σ) better than ± 0.3 ‰ during the measurements. As internal standard the NBS-120c was prepared and run together with the samples in order to test sample preparation. The analyses gave an average $\delta^{18}O$ value of 21.2 ±0.3 ‰, (n=8), which is slightly lower than reported by others and also lower compared to the NBS-120c that was measured for the calibration of TU-1 and TU-2 (21.7 ±0.3 ‰, e.g., Vennemann et al., 2002; Halas et al., 2011). Because there is no internationally accepted $\delta^{18}O$ value for this material and as it is a sedimentary rock, the mammal teeth data were not corrected further. The carbon isotope compositions are expressed relative to VPDB standard (Vienna Pee Dee Belemnite), while the oxygen either to VPDB or VSMOW (Vienna Standard Mean Ocean Water).

4. Results

The results of the carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O_{CO3}$ and $\delta^{18}O_{PO4}$) isotope measurements along with the calculated $\delta^{13}C$ values of vegetation, $\delta^{18}O$ values of environmental water ($\delta^{18}O_w$) (see equations in Table 2) are presented in Table A1. The raw data, along with a step-by-step set of calculations will allow a possible future verification and recalculation of the results (recommendation of Skrzypek et al. 2016). Fig. 2 illustrates the $\delta^{13}C$ and $\delta^{18}O_{PO4}$ values of the samples clustered in time bins with vegetation estimates and relative MAT scales.

Most of the collected samples for this study have a $\delta^{18}O_{CO3} - \delta^{18}O_{PO4}$ offset between 7.2 ‰ and 10.6 ‰ (see in Table A1), which is typical for samples that have not been subjected to diagenesis. Only two enamel samples have a lower carbonate-phosphate difference than 7.2 ‰, that is 6.8 and 6.9 ‰, which could indicate some degree of diagenetic alteration, but these samples have similar phosphate $\delta^{18}O$ and carbonate $\delta^{13}C$ values as other samples from the sites, so using these results does not change the site averages. It is possible that in these samples the $\delta^{18}O_{CO3}$ values were slightly affected by late diagenetic processes (e.g., exchange with pore fluids).

To detect differences between species, MN biozones and areas, both parametric (F-tests for variances and two-tailed homo- or heteroscedastic t-tests for central values) and non-parametric (Levene tests for variances and two-tailed Mann-Whitney tests for central values) statistical tests were made. One way ANOVA and non-parametric Kruskal-Wallis tests were performed to compare variances of more than two groups. Statistical significance is based on $p < 0.05$.

4.1. Carbon isotope results

The $\delta^{13}C$ values for the whole fauna range from –15.9 ‰ to –9.2 ‰ (VPDB). The range of values is from –15.9 to –10.8 ‰ for Central Italy and from –15.1 to –9.2 ‰ for North Italy. The three samples from South Italy from MNQ19 and MNQ20 vary between –12.1 and –11.5 ‰ (VPDB).

Two tailed homoscedastic t-test ($p = 0.92$) and Mann-Whitney test ($p = 0.75$) indicate that there is no significant difference if all the values from Central and North Italy are compared.

The average values for the different time periods in Central and in North Italy are represented in Fig. 2. There are significant differences between some time periods. In the case of Central

Italy t-tests indicate a change between MNQ18 and MNQ19 (p = 0.044) and between MNQ19 and MNQ19-20 (p=0.014). However, according to the Mann-Whitney test these differences are not significant (p = 0.125 and p = 0.092). The average $\delta^{13}C$ values decrease by 1 ‰ from MNQ18 to MNQ19 and increase by 2.3 ‰ from MNQ19 to MNQ19-20.

In the case of North Italy both t-test (p=0.002) and Mann-Whitney test (p = 0.02) support changes between MN16a and MNQ16b. The change in average values is 2.1 ‰ in this transition.

The four species have average $\delta^{13}C$ values between –14 ‰ and –13 ‰ with no difference between them (ANOVA test, p = 0.501, Kruskal Wallis test, p = 0.21). The average values are –13.0 ±1.1 ‰ (n = 21) for *S. etruscus*; –13.9 ±0.9 ‰ (n = 9) for *"D." megarhinus*; –13.6 ±1.3 ‰, (n = 6) for *S. jeanvireti*; and –13.9 ±1.7 ‰ (n=7) for *A. arvernensis.*

4.2. $\delta^{18}O_{PO4}$ *value*

The $\delta^{18}O_{PO4}$ values for the whole fauna have a range from 12.0 to 18.7 ‰ (V-SMOW). Regarding the regions, the values vary from 14.1 ‰ to 18.4 ‰ in Central Italy and from 12.0 ‰ to 15.7 ‰ in North Italy. The three samples from South Italy from MNQ19 and MNQ20 have values between 16.6 ‰ and 18.7 ‰. Comparing the average $\delta^{18}O_{PO4}$ values from Central and North Italy the difference is significant (t-test, $p=5.5\times10^{-6}$, Mann-Whitney test, $p = 2.1\times10^{-5}$). In Central Italy the averages are higher than values of North Italy in all of the time intervals. The differences are 1 – 3 ‰ with the highest difference in the MN14-15 biozone. There are also differences in values between Central and South Italy in MNQ19-20. Samples from South Italy have about 3 ‰ higher average $\delta^{18}O_{PO4}$ values than samples from Central Italy.

The average values for the different time periods in Central and in North Italy are represented in Fig. 2. In Central Italy the average $\delta^{18}O_{PO4}$ values decrease significantly from MN14 to MN16 (t-test, p = 0.004, Mann-Whitney test, p = 0.006). In North Italy there are some changes in the average values between the different biozones but these changes are not significant.

The different species have the following average values: 15.5 ±1.5 ‰ (n=21) for *S. etruscus*; 15.9 ±1.9 ‰ (n=9) for *"D." megarhinus*; 15.7 ±0.7 ‰ (n=7) for *A. arvernensis* and 14.7 ±0.9 ‰ (n=6) for *S. jeanvireti*. The three species have similar average values, while *S. jeanvireti* has an average value about 1 ‰ lower. Differences among the species are not significant.

5. Discussion

5.1 Palaeoenvironment, and diet of the taxa based on $\delta^{13}C$ values

In this study all the $\delta^{13}C$ values indicate that these taxa were browsers or grazers in a pure C_3 ecosystem. It is compatible with earlier suggestions that C_4 grasses were absent in Europe during the Pliocene to Late Pleistocene (Kürschner, 2010). Nonetheless, it must be mentioned that Irace et al. (2015) reported the presence of C_4 sedges in NW Italy already in the latest Pliocene. The ecology of the nearest living relatives (e.g. *Cyperus glomeratus*) suggests that the distribution of these sedges was mainly limited to the disturbed environments along the rivers, and there is no evidence permitting to reconstruct their frequency in the vegetation and their possible importance as food for vertebrates (see Mishra et al., 2015).

Several calculations were made for the interpretation of the isotope results. The tested equations are summarized in Table 2. From the $\delta^{13}C$ values the modern equivalent diet composition are calculated and for this, the changes of the isotopic composition of

atmospheric CO_2 through time have to be taken into account. Based on isotopic data from marine foraminifera, the reconstructed $\delta^{13}C$ value of the atmospheric CO_2 for the Pliocene is about –6.3 ‰, ($\delta^{13}C_{ancientatmCO2,}$ Pliocene), for the Early Pleistocene it is about –6.5 ‰ ($\delta^{13}C_{ancientatmCO2,}$ Pleistocene), while the modern value was referenced to –8 ‰ ($\delta^{13}C_{modernatm}$, year 2000, Kohn 2010), respectively (Tipple et al., 2010; Domingo et al., 2013). The modern equivalent diet composition ($\delta^{13}C_{diet,meq}$) can be calculated with the equation of Domingo et al. (2013) (Table 2.; Equation 1). Based on the expected $\delta^{13}C_{diet,\ meq}$ cut-off values for different habitats by Domingo et al. (2013) our data indicate woodland to mesic C_3 grassland (–30 ‰ to –25 ‰) as a major flora type in Italy during the Pliocene. Some samples have lower values suggesting the existence of closed-canopy forest but site averages are always higher than –30 ‰. There is only one sample that is slightly out of the above ranges (Leffe –23.7 ‰), where probably local conditions were more enhanced. The value itself would fit the category of open woodland-xeric C_3 grassland (–25 ‰ to –22 ‰) (Domingo et al., 2013).

The differences between $\delta^{13}C$ values could be the result of a complex combination of local climatic control on the vegetation, habitat and dietary differences of the species. Measuring samples from different taxa from the same location or the same species from different locations would allow a better separation of these different controls. This separation is not always possible due to sample limitations. For example, in this study all of the *S. jeanvireti* samples are from the MN16 biozone and five of the six samples are from North Italy, so different $\delta^{13}C$ values for this species could indicate dietary differences compared to the other species and / or different climatic signal. Although the results must be interpreted with caution, some implications of these differences can be considered.

Palaeontological observations of *A. arvernensis* suggest that it was a browser taxon that lived in a forest or woodland (Kahlke et al., 2011; Rivals et al., 2015). Morphologically, the bunodont molars generally imply a soft diet of leaves, fruits and twigs. Their feet were

adapted to walk on soft soil, also suggesting that it was an inhabitant of moist woodlands (Ji et al., 2002; Rivals et al., 2015). The dentition changes in the fossil record imply a trend in its diet from soft forest food towards the incorporation of grasses, requiring a grinding component to mastication (Rivals et al., 2015). In agreement with the palaeontological observations, the –13.9 ‰ average $\delta^{13}C$ values suggests that *A. arvernensis* lived in woodlands. *S. jeanvireti* seems characteristic for a humid, forest-dominated environment with relatively open areas where gramineous plants could be found (Guérin, 1972, 1980; Lacombat and Mörs, 2008). *S. etruscus* has brachyodont dentition and slender, subcursorially structured limbs (Guérin, 1980; Mazza, 1988) suggesting that this species probably inhabited environments with variable forest cover similar to those in which black rhinos live today, i.e. open scrub woodlands and the margins of small woods and feeding mainly on leaves, shrubs and twigs (Guérin, 1980; Mazza, 1988; Fortelius et al., 1993). At Leffe, palynological and faunal records of a lacustrine basin succession from approximately 1.8 to 1.1 Ma indicate several environmental changes. The rhinoceros species (*S. etruscus* or *S. hundsheimiensis*) lived in warm-temperate dense mixed or conifer forest, to open xerophytic communities and steppe with tree birch and with sparse woodland patches (Ravazzi et al., 2009).

Overall, the palaeontological and environmental observations and the –13 ‰ to –13.9 ‰ $\delta^{13}C$ values are in agreement and suggest that all the investigated taxa could live in forested and partly open environments and were browsers, feeding leaves, twigs shrubs and also grass if these were present in the area. This could also imply that changes in measured $\delta^{13}C$ values likely the effect of vegetation or climatic changes than change in food preference of the animals.

5.2. Environmental changes based on $\delta^{13}C$ data

Variation in the measured $\delta^{13}C$ values in time and space depend on several factors, including vegetation, precipitation and humidity and it is not always possible to separate these effects. For example low $\delta^{13}C$ values could represent high precipitation and can indicate close canopy forest, or both. However, temporal trends in $\delta^{13}C$ seem to reflect the environmental and climatic changes in Italy from MN14 to MNQ20. A comparison of the $\delta^{13}C$ results and the indications of palaeobotanical proxies can be seen in Table. 1.

The low $\delta^{13}C$ values in the Pliocene could represent high humidity, high precipitation values and/or more closed woodlands in North and in Central Italy. The values remain the same in Central Italy, where all the samples from MN16 biozone were averaged. In North Italy, where the samples have a better age control, this biozone can be dissected; a sharp increase in $\delta^{13}C$ values between the late Pliocene and the early Pleistocene (MN16a – MN16b) is noted. The 1.9 ‰ increase in $\delta^{13}C$ values (Figure 2) could indicate vegetation changes from more closed woodlands to more open areas and/or a large decrease in precipitation or in humidity. If only the drop in MAP had an effect on the $\delta^{13}C$ values then this drop would be 1250 ± 630 mm/year according to the equation of Kohn (2010), (table 2., Equation 2., a latitude of 45° and an altitude of 150m was substituted in the equation). The low $\delta^{13}C$ values in MN16a probably reflect warm and humid climate of the "Mid-Pliocene Warm Period", while the high values in MNQ16b are most likely linked to the Northern Hemisphere Glaciation. This result can confirm the considerable environmental changes also observed in palynological records with an increase of a cooler type forest (*Picea* and *Fagus*) in North Italy (Stirone section) and a vegetation opening in Central Italy around 2.6 Ma (Bertini, 2001, 2010).

Samples with younger ages are more common from the Central Italian area. Higher $\delta^{13}C$ for the MNQ18 could indicate drier climate, less precipitation and / or more open vegetation compared to previous biozones. This is in good agreement with pollen records, showing decreased humidity at that time. After 2.6 Ma, the next indication of the vegetation change

may be noted for MNQ18 from 1.9 to 1.7 Ma in Central Italy (Bertini, 2001, 2010). During MNQ19 (1.7 – 1.3 Ma) the $\delta^{13}C$ values decrease, which could represent wetter interglacial periods and a more closed vegetation in the area. The youngest samples from MNQ19-20 (~1.2 Ma) from Madonna della Strada site show a more marked increase in $\delta^{13}C$ values. Floral and faunal data suggest environments still characteristic of an interglacial between 1.3 and 1.1 Ma at this site (Magri et al., 2009). The recorded forest phase showed a clear development, starting with an expansion of conifers, progressively replaced by a mixed oak forest, and then showing an increase in Mediterranean vegetation. An appreciable diffusion of open vegetation is also recorded, as indicated by pollen of grasses and other herbs. The finds of *Mammuthus meridionalis* and "*Arvernoceros"* or *Eucladoceros giulii* also suggest the presence of open environments. Overall the climate of the site could be similar to present day climate, which is a semi-continental climate, with a transitional Mediterranean-temperate character with about 700 mm precipitation (Magri et al., 2009).

From North Italy only three samples are presented from the younger biozones. These samples from MNQ18-19-20 have higher average $\delta^{13}C$ values than samples from the Pliocene. The sample from Leffe has the highest $\delta^{13}C$ value in the database (–9.2 ‰) indicating open woodland-xeric grassland. As stated by Ravazzi et al. (2009), the observed variable forest cover at the Leffe record documents the occurrence of the rhinoceros (*S. etruscus* or *S. hundsheimiensis)* in cold-temperate, partially open environments, which is the coldest extreme of Early Pleistocene climate cycles registered in the Biogenic Unit of the Leffe Formation. According to this and the high $\delta^{13}C$ value, the sampled rhinoceros are presumably from the younger part of the site and could live in a more open environment than the other sampled taxa. However, as only one sample was analysed, the results are merely a suggestion as seasonal effects can also be present and there could be individual differences between the animals.

544 The average $\delta^{13}C$ values of the three samples from South Italy from MNQ19-20 (–13.8 ± 0.3
545 ‰) are similar to the values in Central and Northern Italy at that time and indicate partly open
546 vegetation.
547 Overall the temporal changes in $\delta^{13}C$ values in Italy are in good agreement with the pollen
548 data indicating that the changes in $\delta^{13}C$ reflect vegetation changes and / or changes in
549 precipitation or in humidity.
550
551 *5.3. Palaeotemperature changes derived from the $\delta^{18}O_{PO4}$ data*
552
553 Estimating MAT from $\delta^{18}O_{PO4}$ records require two data conversion steps that are based on
554 well-demonstrated correlations. The first is the correlation between the $\delta^{18}O_{PO4}$ and the $\delta^{18}O$
555 values of consumed water (environmental water – $\delta^{18}O_w$), and the second is the correlation
556 between this $\delta^{18}O_w$ and the MAT of the site. Several uncertainties and assumptions are
557 inherent to both conversion steps. Different types of equations exist to calculate drinking
558 water of the animal from the $\delta^{18}O_{PO4}$ data. General equations based on very large dataset often
559 show that the $\delta^{18}O_{PO4}$ values of different species can have very similar offsets from the
560 drinking water. The species-specific equations, however, can be more reliable for the given
561 species with the given physiology.
562 There are more uncertainties in the second step regarding MAT. An assumption is needed for
563 the calculations, such that as the $\delta^{18}O$ of drinking water represents the $\delta^{18}O$ of the local mean
564 precipitation, which is not always the case (see effects of evaporation or meltwaters in
565 background section and below in the discussion) and that the estimate of past MAT can be
566 based on present day correlation between MAT and the isotope composition of precipitation.
567 There are local, regional and global equations for the datasets of one IAEA meteorological
568 station or for several stations worldwide. The advantage of using global equations is that they

represent an average mid-latitude climate regime and that they are not so sensitive to changes in the local climates with time. Using site-specific precipitation-temperature relationships can, however, give more precise estimates for a local area, notably if it is a mountainous area where local climate variability may be important.

Amongst other influencing factors (different moisture source and air mass trajectories, latitude, altitude, continental and amount effects) $\delta^{18}O$ of the local precipitation correlates with the MAT. Due to this complex relationship, the correlation between the $\delta^{18}O$ and the MAT is changing in time and space, and the correlation may be weak for non-continental or monsoon-influenced stations. In these cases the type of the regression method and the type of the dataset (monthly or annual average precipitation data) becomes an important factor and could influence the palaeoclimatic interpretations (Pryor et al., 2014; Skrzypek et al., 2016). There is currently a debate regarding the regression type to be used for palaeotemperature reconstructions (see Pryor et al., 2014 vs. Skrzypek et al., 2016). Given the above uncertainties, the relative changes in MAT were calculated in this study, instead of absolute MAT values. Results of several equations were compared to observe the possible range of MAT changes linked to the changes in $\delta^{18}O_{PO4.}$ The results were calculated using the equations with the highest and the lowest slopes in both conversion steps. The equations used are summarized in Table 2.

In the first step ($\delta^{18}O_{PO4}$ to $\delta^{18}O_w$), different general equations (Kohn, 1996; Kohn and Cerling 2002; Amiot et al., 2004) and one species-specific equation (Ayliffe, 1992; for proboscidea) were used. In the second step ($\delta^{18}O_w$ to MAT) several regional and global equations were compared: two equations for Europe (Pryor et al., 2014; Skrzypek et al., 2016), and two global equations (Dansgaard, 1964; Amiot et al., 2004). Additionally a new equation for Italy was produced based on the dataset of the Italian GNIP stations following the work of Skrzypek et al. (2016). The R^2 of this regression is low, but the slope of the

equation is very similar to that of the regional and global equations. Equations with similar slopes can be obtained for North and Central Italy as well.

The spatial distribution of the isotopic compositions of precipitation in present day Italy is quite complex. Air masses from different origins have different $\delta^{18}O$ values and the Apennines and the Alps also have an influence. Longinelli and Selmo (2002) found that the relationship between the isotopic composition of precipitation and the mean monthly temperature values are, on average, very poor. They found no latitudinal gradient along the Tyrrhenian coast from Sicily to the Italian–French border, despite the considerable range of latitude. However, there are 4–5 ‰ spatial differences in the isotopic composition of precipitations and about 6–7 °C differences in MAT, as a result of the complex topography and climate of Italy, there is not a simple south-north gradient.

The calculated MAT changes between MN14 and MNQ20 in Central Italy compared to other proxies are shown in Table 1 and illustrated in Figure 2. The 1.4 ‰ decrease in $\delta^{18}O_{PO4}$ values from MN14 to MN16 can represent a 1.5 – 1.8 ‰ change in $\delta^{18}O_w$ and a 2 – 3.6 °C decrease in MAT. The cooling trend from Early- to Late Pliocene –to Early Pleistocene is in good agreement with indications of other proxies. Another decrease from MNQ18 to MNQ19-20 is not significant, but with the average shift of –1.4 ‰ in $\delta^{18}O_{PO4}$ could imply a similar 2 – 3.6 °C decrease in MAT. Because of the climate in MN19-20 (1.3 – 1.1 Ma) the Madonna della Strada site was described as an interglacial with thermophilous taxa (Magri et al., 2009), it can be assumed that during the glacial peaks in Early Pleistocene, temperature changes with higher amplitude could have occurred.

In arid climates high evaporation of water reservoirs could raise the $\delta^{18}O$ values of the animals' drinking water and thus the $\delta^{18}O_{PO4}$ values. If the humidity changed with time, calculations from the $\delta^{18}O_{PO4}$ values would lead to over- or underestimation of MAT changes. In this study for most of the time intervals the $\delta^{18}O_{PO4}$ and $\delta^{13}C$ values appear to co-vary,

619 likely reflecting the warmer / wetter and cooler / more arid climates. However, some
620 exceptions, for example the MNQ19 zone in Central Italy, could be more humid and slightly
621 cooler than the previous mammal zone, which could imply that samples from that biozone are
622 from a different phase of a glacial-interglacial cycle. Low $\delta^{13}C$ values in most of the time
623 periods are in agreement with palaeobotanical proxies, indicating humid climates with dense
624 woodlands. In most of these conditions it is unlikely that strong evaporation could have led to
625 unusual ^{18}O enrichment in water and also phosphate. Some influence is only conceivable in
626 the case of the relatively cooler and more arid time periods such as MNQ18 in Central and
627 MNQ18-19-20 in North or in South Italy but in these cases the $\delta^{13}C$ values still indicate
628 woodlands so this effect should not be strong.
629 For Central Italy the decreasing temperatures from Early Pliocene to Late Pliocene – Early
630 Pleistocene followed by fluctuations but a long-term cooling trend towards the glacial-
631 interglacial cycles in Early Pleistocene are supported by the $\delta^{18}O_{PO4}$ in the results and are in
632 agreement with other proxies.
633 In North Italy there are no large temporal changes in MAT. Whereas, the small increase in
634 $\delta^{18}O_{PO4}$ values between MN14-MN15 and MN16a could be linked to the MPWP, while the
635 following decrease for the MNQ16b can refer to the NHG global cooling event. After this the
636 values stayed relatively constant for the younger time periods. The average $\delta^{18}O_{PO4}$ values are
637 all lower than the values in Central Italy. The measured low $\delta^{18}O_{PO4}$ values could suggest
638 colder MAT, especially during MN14-15, but would contradict other reported proxy data
639 (Fauquette et al., 1999, 2006, Bertini, 2001, 2010). In contrast, while samples from the end of
640 the Zanclean may well have lower $\delta^{18}O_{PO4}$ compared to values from the warmest part of the
641 MPPW, the values being similar to MNQ18-19-20 are considered unlikely.
642 One explanation for the low $\delta^{18}O_{PO4}$ values could be the influence of glacial meltwaters and
643 river waters draining from the Alps. This would have lower isotopic composition compared to

644 the local precipitation given an altitude effect on the catchment and/or a rain-shadow effect
645 with air mass transport dominated from the north. Therefore, drinking from water source
646 influenced by meltwater could have also lowered the $\delta^{18}O_{PO4}$ values in the teeth. Isotope
647 measurements of waters from the upper part of the Po river support this assumption for a
648 present-day situation. The average composition of the river water is characterized by $\delta^{18}O$ of
649 –12.5 ‰, while the $\delta^{18}O$ values of the precipitation are –7 to –9 ‰ in the same area (e. g.,
650 Marchina et al., 2014, Longinelli and Selmo, 2002). As in Central Italy trends in $\delta^{18}O_{PO4}$
651 values are in agreement with other proxies, these local source-water effects, are considered
652 more likely compared to a regional-scale cooler climate. If it is the case, the $\delta^{18}O$ values in
653 North Italy could not be used for MAT calculations unless appropriate equations can be
654 deduced. Nonetheless, samples from more locations and broader time segments could
655 corroborate these hypotheses.
656 Palynological proxies indicate that the Zanclean MAT and precipitation were higher than the
657 Piacenzian, and the climate in North Italy was comparable to that of the other sites of the
658 north-western Mediterranean region (Table 1.). From the Piacenzian climatic and vegetation
659 characteristics of the middle European region have been suggested (Fauquette and Bertini,
660 2003). Mediterranean plants are poorly presented in the pollen records and totally lack in
661 plant macrofossil records (Martinetto, 2015 and unpublished data), while the persistence of
662 forests indicates constant humidity. Stable isotope measurements from the Carpathian Basin
663 also support similar results to those for North Italy in the given time period (e. g., Kovács et
664 al., 2015). For a present-day situation, an approximate 1 to 2 ‰ difference in $\delta^{18}O$ values in
665 precipitation and about 1 to 3 °C differences in MAT are given between areas near Torino and
666 Central Italy (e.g., Longinelli and Selmo, 2002, Combourieu-Nebout et al., 2015). It can be
667 concluded that about 1 to 2 ‰ lower $\delta^{18}O_{ppt}$ values in North Italy can be realistic compared to

668 Central Italy but larger differences may have an additional effect indicated, such as surface
669 waters from the Alpine catchments.
670 In South Italy the three samples from MNQ19-20 have higher $\delta^{18}O_{PO4}$ values than samples
671 from Central Italy from this period. Two of the three samples are from the Pirro Nord site,
672 with very high values. This site is close to the Adriatic shore, closer than the other
673 investigated sites. Recent annual average $\delta^{18}O_{ppt}$ values in that area are about –5 ‰, higher
674 than the –6 to –9 ‰ for other areas further from the sea, so the measured difference could be
675 the result of the shore-inland gradient in $\delta^{18}O_{ppt}$ values rather than a south-north temperature
676 gradient at that time (Longinelli and Selmo, 2003).
677
678 **6. Conclusions**
679
680 Stable isotope analyses of 51 enamel samples from fossil megaherbivores from North, Central
681 and South Italy (from 5.2 to about 1 Ma) provides information about the Pliocene and Early
682 Pleistocene climate in Italy. Most of the samples have a $\delta^{18}O_{CO3} - \delta^{18}O_{PO4}$ offsets between 7.2
683 to 10.6 ‰, which is compatible with a range typical for non-altered samples. All of the $\delta^{13}C$
684 values indicate that the investigated taxa lived in C_3 ecosystem. The $\delta^{13}C$ values support a
685 humid climate with woodlands during the Early Pliocene in North and in Central Italy. The
686 Northern Hemisphere Glaciation at 2.7 Ma is indicated as a sharp increase in $\delta^{13}C$ values
687 between MN16a and MNQ16b in North Italy. After MNQ16b the fluctuations probably
688 represent the moderate glacial-interglacial cycles with a long term increasing trend in $\delta^{13}C$
689 values. Higher values during MNQ18 and MNQ19-20 are in good agreement with pollen data
690 indicating more open vegetation. In Central Italy both stable isotope systems change in
691 tandem indicating warmer and more humid or cooler and more arid climates, respectively.
692 The $\delta^{18}O$ values support the warmest climate during the Early Pliocene followed by cooling

with fluctuations toward the end of the Early Pleistocene. In North Italy higher $\delta^{18}O_{PO4}$ values in MN16a are apparent – probably linked to the MPWP – but no other trends can be observed. Attributing the low $\delta^{18}O_{PO4}$ values in North Italy solely to MAT changes would result in low MAT values during the Early Pliocene, in contrast to temperature changes inferred from other proxies (Fauquette et al., 1999, 2006, Bertini, 2001, 2010). A possible explanation could be that the drinking water was influenced by an Alpine catchment with typically lower mean $\delta^{18}O$ values because of altitude and / or air mass origin differences for the northern parts of Italy. With the exception of the low $\delta^{18}O$ values in North Italy, the Early Pliocene vegetation and temperature changes fit well within the trends based on palynological records and changes in mammal fossil assemblages.

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Figures and table captions

Figure 1. Location of paleontological sites cited in Table A1 (the map shows the present day locations.)

Figure 2. Average $\delta^{13}C$ (‰, VPDB) and $\delta^{18}O_{PO4}$ (‰, VSMOW) values obtained from the teeth with calculated environmental water values, relative MAT scales and vegetation estimation. A) North Italy B) Central Italy,

Vegetation information is from Domingo et al. (2013), environmental water values were calculated by applying Equation 3. (after Kohn and Cerling, 2002), relative MAT scales were calculated using the minimum and maximum slopes of the equations from Table 2.

Chronology according to Hilgen et al (2012). The onset of the Quaternary according to the chronology confirmed in 2009 by the International Union of Geological Sciences. NHG = Northern Hemisphere Glaciation, MPWP = "Mid-Pliocene Warm Period", VMT = Villafranchian mammal turnover, EEQ = Elephant-Equus event, the * sign represent an outlier in Central Italy in MNQ19.

Table 1. Calculated MAT changes based on $\delta^{18}O_{PO4}$ and vegetation estimates based on the $\delta^{13}C_{CO3}$ isotope results compared with palaeobotanical proxies, (1) Bertini, 2001; (2) Bertini, 2010; (3) Fauquette et al., 1999; (4) Combourieu-Nebout et al., 2015; (5) Fauquette and Bertini, 2003; (6) Magri et al., 2009; (7) Klotz et al., 2006; (8) Fauquette et al., 2006; MLV: most likely value, NHG: Northern Hemisphere Glaciation.

Table 2. The equations used for the calculations of water $\delta^{18}O$ values from those measured for the phosphates.

Appendix

Table A1. Locations and age estimates for the fossil localities, measured carbon and oxygen isotopic compositions, calculated isotopic composition of diet and environmental water. Selected references give information about the palaeontology and the geological settings of the fossils. Institutional abbreviations are explained in the text.

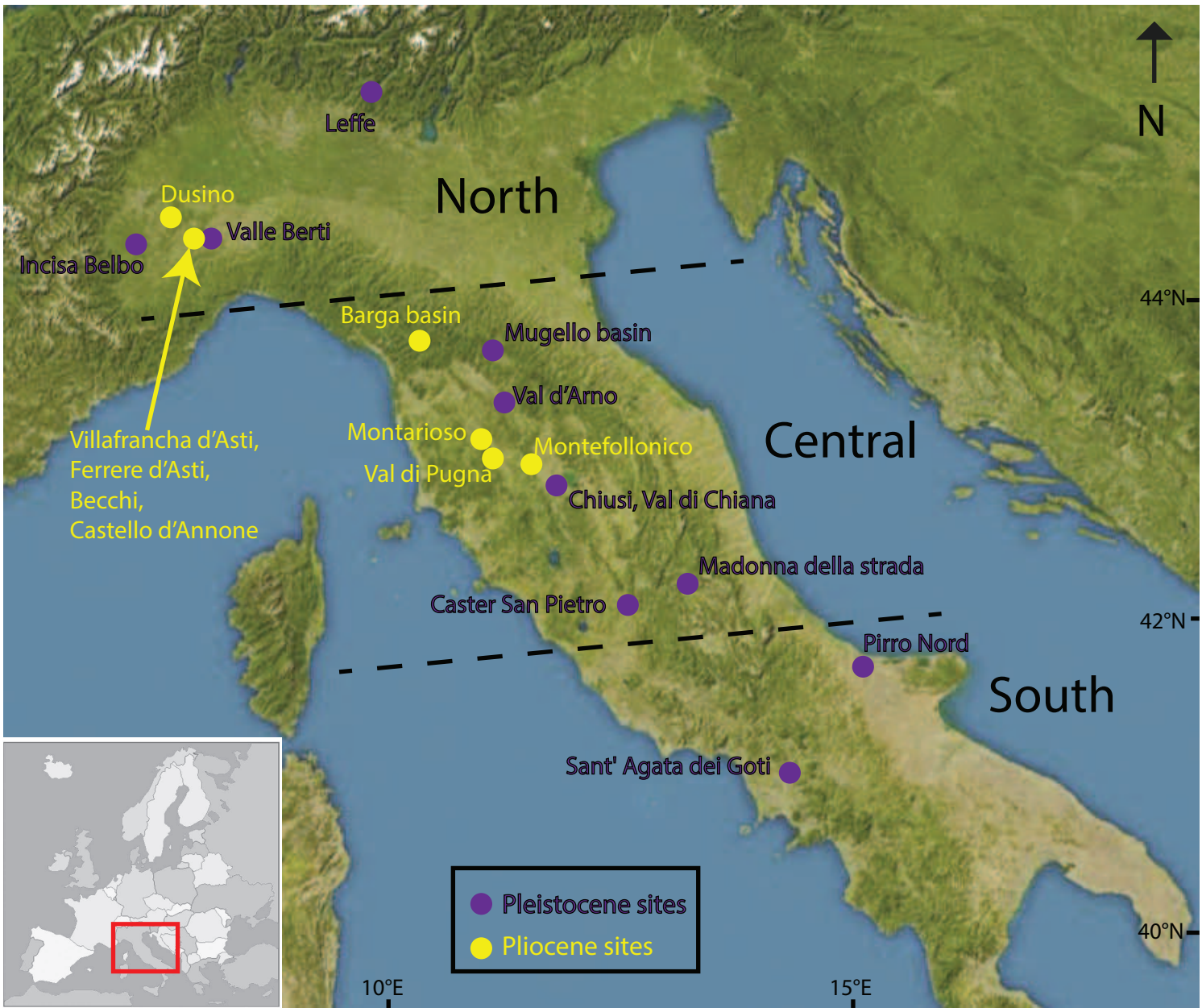
Table

	age (Ma)	~5.2 - 3.5	~3.3 - 2.8	~2.8-2.5	~2.5-1.9	~1.9-1.7	~1.7-1.3	~1.3-1	recent
	MN zone	MN14-15	MN16a	MN16b	MN17	MNQ18	MNQ19	MNQ19-20	
Central Italy	MAT changes (^{o}C) indications (this study)		2 - 3.6 ^{o}C decrease of MAT from MN14			2 - 3.6 ^{o}C decrease of MAT from MNQ18 to MNQ19-20			
	estimated vegetation (this study)	woodland-mesic grassland	woodland-mesic grassland			woodland-mesic grassland, more open vegetation / less humid / less precitipation than in the former biozones	woodland-mesic grassland	woodland-mesic grassland, more open vegetation / less humid / less precitipation than in the former biozones	
	palaeobotanical indications			open vegetation at 2.7, NHG (1)	Interglacials: higher MAT and MAP than present day. Glacials: similar MAT and MAP to present day (2), vegetation openings at 2.5Ma and from 1.9 to 1.7Ma (MNQ18) (1)				MAT 14-16 °C, MAP 760-970mm (4), 730mm (6)
North Italy	estimated vegetation (this study)	woodland-mesic grassland	woodland-mesic grassland	woodland-mesic grassland, more open vegetation / less humid / less precitipation than in MN6a		woodland-mesic grassland, (one sample from Leffe indicate open wooland-xeric grassland)			
	palaeobotanical indications	MAT 16-20 °C (MLV 17-19°C) (1;2), 12-20 °C (3), 1-4 °C higher than present day (8), 1100-1600mm (MLV 1200-1300mm) (1,2), 1100-1400mm (3), 400-700mm higher than present day (8)			Interglacials: similar MAP and MAT to Early Pliocene, Glacials: similar MAT to present day, and 500-600mm higher MAP than present, (5) (Stirone site), alternations of different types of forests with no open vegetation (2)				MAT, 13-15 °C, MAP 930-1100mm (4)
South Italy	estimated						woodland-mesic grassland		
	palaeobotanical indications	>22 ^{o}C, equal to or 5 °C higher than present day (8), 600mm (3), drier or equal humidity than present day (8), some subdesertic plants (2)			Interglacials: MAT 2.8 °C higher than today , MAP 500mm higher than today, Glacials: MAT lower than today, MAP equal to present day, (7) (Semaforo site)			open vegetation and forest alternations (2)	MAT, 15-18 °C, MAP 440-830mm (4)

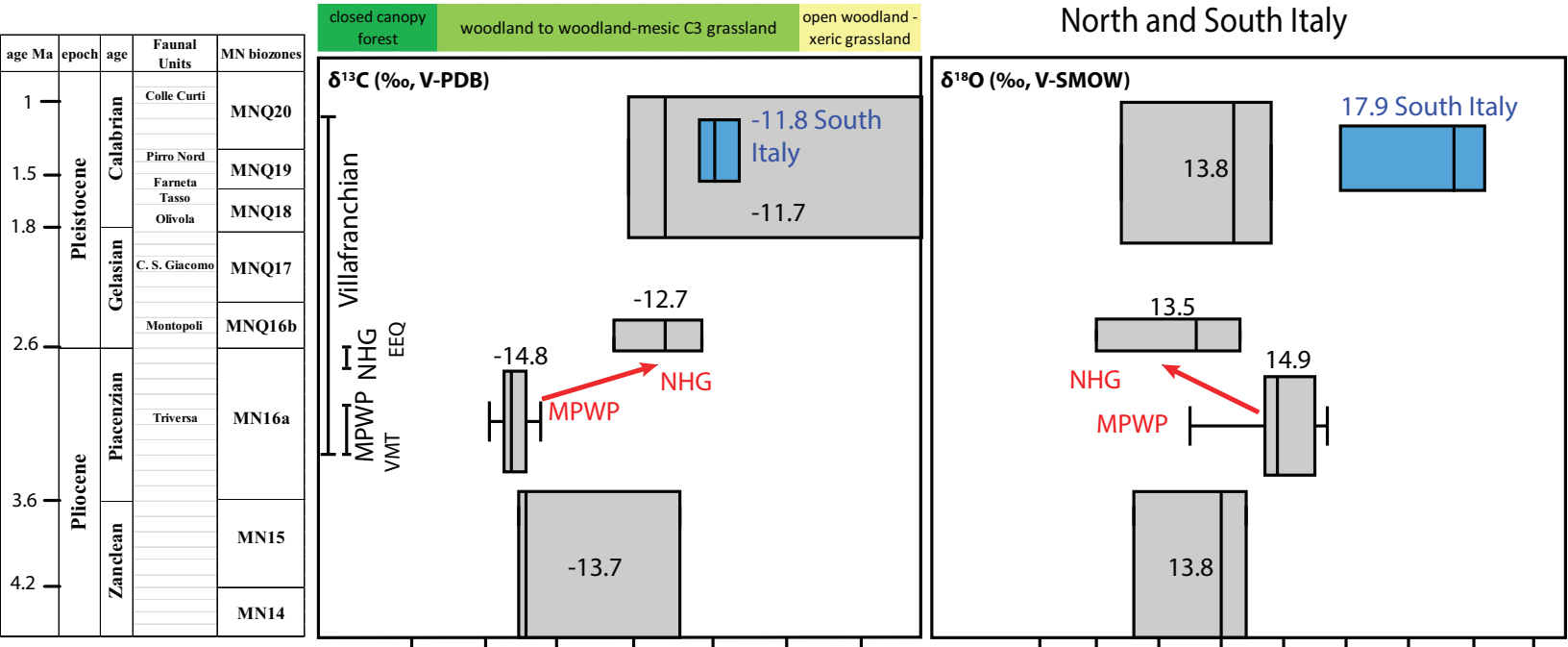
Table

Number of equation	Relationship	Equation	type of regression	R^2, slope	References
1	$\delta^{13}C_{diet,\ meq}$; $\delta^{13}C_{leaf}$	$\delta^{13}C_{diet,\ meq} = \delta^{13}C_{leaf} + (\delta^{13}C_{modernatmCO2} - \delta^{13}C_{ancientatmCO2})$			Domingo et al., (2013)
2	$\delta^{13}C_{diet,\ meq}$; MA_P	$\delta^{13}C_{diet,\ meq}$(‰, VPDB) = –10.29 + 1.9 × 10–4 altitude (m)–5.61 log10(MAP+300, mm/yr) – 0.0124 Abs (latitude, o)			Kohn, (2010)
3	$\delta^{18}O_w$; $\delta^{18}O_{PO4}$	$\delta^{18}O_W = (\delta^{18}O_{PO4}-23)/0.90$	inverted forward regression	slope = 1.11	Kohn and Cerling, (2002)
4	$\delta^{18}O_w$; $\delta^{18}O_{PO4}$	$\delta^{18}O_w = (\delta^{18}O_{PO4} - 26.8 + 8.9h) / 0.76$; (where h is the relative humidity)	inverted forward regression	slope = 1.32	Kohn, (1996)
5	$\delta^{18}O_w$; $\delta^{18}O_{PO4}$	$\delta^{18}O_w = 1.1128\ (\pm 0{,}0029)\ \delta^{18}O_{pO4} - 26.4414\ (\pm 0.0508)$	transposed regression	$R^2 = 0.998$; slope = 1.11	Amiot et al., (2004)
6	$\delta^{18}O_w$; $\delta^{18}O_{PO4}$	$\delta^{18}O_w=(\delta^{18}O_{PO4} - 23.3\ (+/-0.7)) / 0.94\ (+/-0.10)$	inverted forward regression	$R^2 = 0.85$; slope = 1.06	Ayliffe et al., (1992)
7	MAT; $\delta^{18}O_w$; for Europe	$MAT = (\delta^{18}O_w + 13.74) / 0.53$	inverted forward regression	$R^2 = 0.6$, slope = 1.89	Pryor et al., (2014)
8	MAT; $\delta^{18}O_w$; for Europe	$MAT = 1.41^{18}O_w + 21.64$	transposed regression	$R^2 = 0.49$, slope = 1.41	Skrzypek et al., (2016)
9	MAT; $\delta^{18}O_w$; for Italy (excluded the islands)	$MAT = 1.56\delta^{18}O_w + 23.31$	transposed regression	$R^2 = 0.3$, slope = 1.56	data of GNIP stations from Skrzypek et al., (2016)
10	MAT; $\delta^{18}O_w$; global equation	$MAT = (\delta^{18}O_W + 13.6) / 0.69$	inverted forward regression	slope = 1.45	Dansgaard, (1964)
11	MAT; $\delta^{18}O_w$; global equation	$MAT = (\delta^{18}O_W + 14.18 \pm 0.52) / (0.49 \pm 0.03)$	inverted forward regression	$R^2 = 0.81$; slope = 2.04	Amiot et al., (2004)

Figure


N
Leffe
North
Dusino
Valle Berti
Incisa Belbo
44°N
Barga basin
Mugello basin
Val d'Arno
Villafrancha d'Asti,
Ferrere d'Asti,
Becchi,
Castello d'Annone
Montarioso
Montefollonico
Central
Val di Pugna
Chiusi, Val di Chiana
Madonna della strada
Caster San Pietro
42°N
Pirro Nord
South
Sant' Agata dei Goti
Pleistocene sites
Pliocene sites
40°N
10°E
15°E

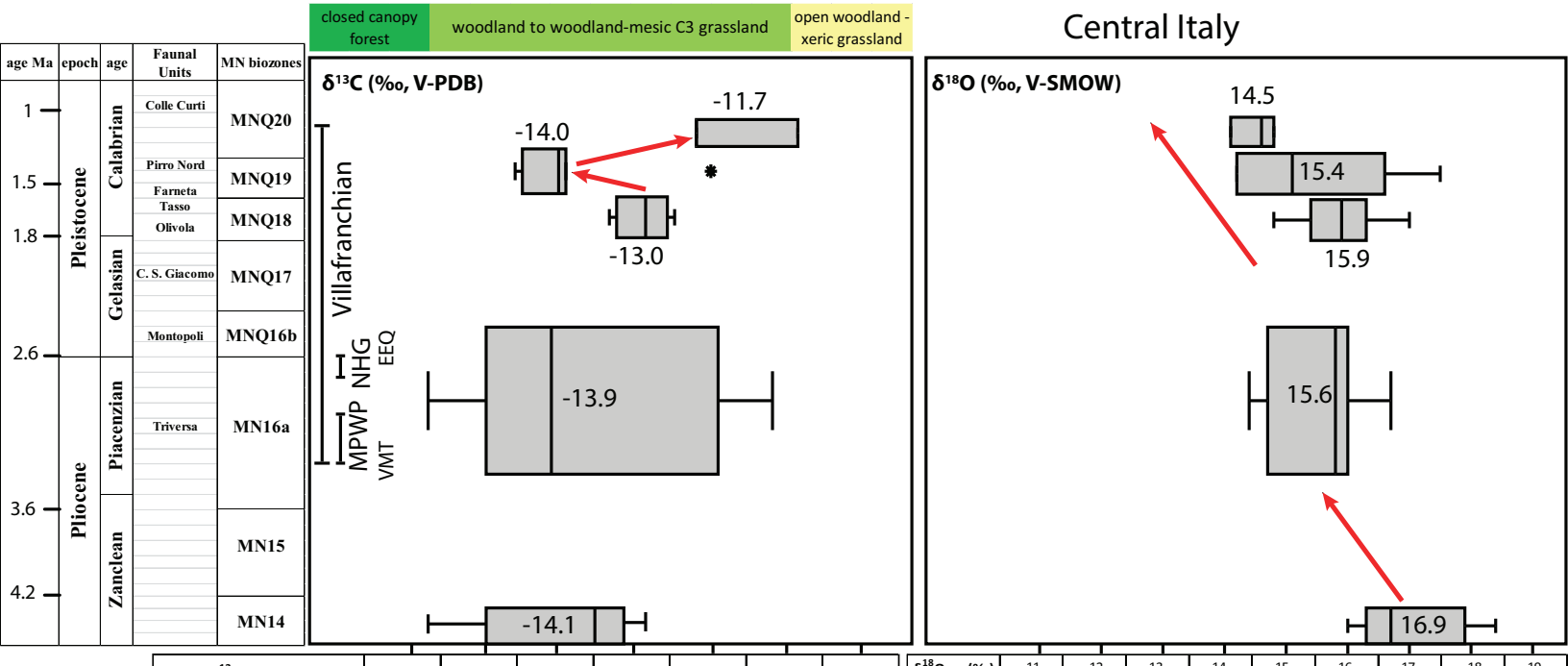
Figure



A)

$\delta^{13}C_{enamel}$(‰)	-16	-15	-14	-13	-12	-11	-10
$\delta^{13}C_{diet,\ meq}$ (‰) Peistocene	-30.4	-29.4	-28.4	-27.5	-26.5	-25.5	-24.5
$\delta^{13}C_{diet,\ meq}$ (‰) Pliocene	-30.6	-29.6	-28.6	-27.7	-26.7	-25.7	-24.7

$\delta^{18}O_{PO4}$ (‰)	11	12	13	14	15	16	17	18	19
$\delta^{18}O_{water}$ (‰)	-13.3	-12.2	-11.1	-10	-8.9	-7.8	-6.7	-5.6	-4.4
relative MAT scale (°C)	-5.6 - -8.2	-4.2 - -6.1	-2.8 - -4.1	-1.4 - -2	0	1.4 - 2	2.8 - 4.1	4.2 - 6.1	5.6 - 8.2



B)

$\delta^{13}C_{enamel}$(‰)	-16	-15	-14	-13	-12	-11	-10
$\delta^{13}C_{diet,\ meq}$ (‰) Peistocene	-30.4	-29.4	-28.4	-27.5	-26.5	-25.5	-24.5
$\delta^{13}C_{diet,\ meq}$ (‰) Pliocene	-30.6	-29.6	-28.6	-27.7	-26.7	-25.7	-24.7

$\delta^{18}O_{PO4}$ (‰)	11	12	13	14	15	16	17	18	19
$\delta^{18}O_{water}$ (‰)	-13.3	-12.2	-11.1	-10	-8.9	-7.8	-6.7	-5.6	-4.4
relative MAT scale (°C)	-5.6 - -8.2	-4.2 - -6.1	-2.8 - -4.1	-1.4 - -2	0	1.4 - 2	2.8 - 4.1	4.2 - 6.1	5.6 - 8.2

Highlights

- $\delta^{13}C_{CO3}$ results indicate that the investigated taxa were grazers-browsers in a pure C_3 vegetation.
- According to the $\delta^{18}O_{PO4}$ results Pliocene was characterized by higher temperatures than Early Pleistocene in Central Italy
- In North Italy the $\delta^{13}C$ values show a sharp increase between MN16a and MNQ16b biozones that is most likely linked to the Northern Hemisphere Glaciation at 2.7 Ma.
- Higher $\delta^{13}C$ values in MNQ18 and MNQ19-20 biozones in Central Italy are in agreement with pollen data indicating more open vegetation than in the Pliocene.

Supplementary Data

Click here to download Supplementary Data: table_A1.xlsx