Carbon isotope analyses reveal relatively high abundance of C₄ grasses during early-middle Miocene in southwestern Europe

Michael A. Urban¹, David M. Nelson², Gonzalo Jiménez-Moreno³, Feng Sheng Hu^{1,4,*}

¹Program in Ecology, Evolution, and Conservation Biology, University of Illinois, Urbana, IL 61801, USA

²University of Maryland Center for Environmental Science, Appalachian Laboratory, Frostburg, MD 21532, USA

³Department of Stratigraphy and Paleontology, University of Granada, Granada, Spain

⁴Department of Plant Biology and Department of Geology, University of Illinois, Urbana, IL 61801, USA

*** Corresponding author. Tel.: +1 217 244 2982**

E-mail address: fshu@life.illinois.edu (F. S. Hu)

Abstract

Before their rapid expansion during the late Miocene, C₄ plants were generally uncommon or absent from most grasslands. Recent studies have furthered our understanding of shifts in C₄ abundance prior to their late-Miocene expansion; however, the early history of C₄ grasses remains poorly constrained. Distinguishing C₄ grasses from C₃ taxa (i.e., most trees, shrubs, herbs, and about half of all grasses) using isotopic data in the paleorecord is challenging, especially when C₄ plants are in relatively low abundance. We used SPIRAL (Single Pollen Isotope Ratio AnaLysis) to measure $\delta^{13}\text{C}$ values of individual grass-pollen grains in early-middle Miocene sediments from three sites in Spain where grass pollen represents 5-20% of the pollen assemblages, including 25 samples from Rubielos de Mora, (20.3 - 15.5 Ma), seven from Gor (15.2 - 13.7 Ma) and seven from Andalucía A1 (13.7 - 10.1 Ma). Results indicate that C₄-grass abundance varied between 21 and 72% of total grasses during the early-middle Miocene. C₄ grasses are uncommon (10-20% of the grass flora) in this region today, and thus C₄-grass abundance likely decreased after the early-middle Miocene as the region became cooler and precipitation shifted to the winter months. This pattern contrasts with that of a late-Miocene expansion of C₄ grasses in many other regions of the world. Our results thus help elucidate the history of grasslands in Europe and the spatiotemporal dynamics of C₄ grasses worldwide.

1. Introduction

C₄ grasses account for ~25% of global terrestrial primary productivity and represent important crops and biofuels, despite comprising <3% of the number of species of angiosperms (Sage, 2004; Tipple et al., 2010). Recent syntheses of paleorecords indicate that grasses originated during the Paleogene and that C₄ grass-dominated grasslands expanded subsequently during the late Miocene-early Pliocene at the expense of C₃ grass-dominated grasslands in many parts of the world (Edwards et al., 2010; Strömberg, 2011; Chen et al., 2015). Prior to this expansion, C₄ plants were present but in low proportions during the early-middle Miocene in various regions, such as the Great Plains of North America (Strömberg and McInerney, 2011; Cotton and Sheldon, 2012), Kenya (e.g., Levin et al., 2004; Feakins et al., 2005) and Pakistan (e.g., Behrensmeyer et al., 2007; Sanyal et al., 2010). In other places, such as Argentina (Cotton et al., 2014) and central China (Passey et al., 2009), there is little evidence of C₄ plants prior to the late Miocene. A general consensus has emerged that C₄ plants were uncommon or absent from grasslands prior to their rapid expansion during the late Miocene-early Pliocene (e.g., Edwards et al., 2010; Strömberg, 2011). However, data are inadequate to decipher the geographic patterns of early C₄-grass distribution, and the history of C₄ grasses prior to the late Miocene remains poorly constrained.

A primary challenge is the difficulty of assessing the presence and abundance of C₄ grasses in the paleorecord, particularly when they are at low abundance. For example, standard palynological approaches cannot be used to distinguish pollen of C₃ *versus* C₄ grasses (Fægri et al., 1989). Furthermore, because the C₃ and C₄ photosynthetic pathways are used by taxa besides grasses, it is difficult to use $\delta^{13}\text{C}$ data from substrates such carbonates, teeth, and leaf waxes to assess the abundance of C₃ *versus* C₄ grasses, especially when grasses are uncommon on the

71 landscape (Cerling et al., 1999).

72 One region where the early history of C₄ grasses is particularly uncertain is western
73 Eurasia. Grassland ecosystems have been a component of the regional landscape since the early
74 Eocene (Jacobs et al., 1999; Kovar-eder et al., 2008), but C₄ grasses are thought to have never
75 been abundant in the region. Today only 10-20% of the grasses in the region use C₄
76 photosynthesis (Sage, 1999), and existing $\delta^{13}\text{C}$ analyses of fossil soils and teeth from that region
77 suggest that C₃ plants were dominant throughout the Miocene (Strömberg, 2011). However, the
78 closest modern analogs for the early-middle Miocene pollen assemblages from some areas of
79 western Eurasia, such as Spain, are from regions of the Eastern Mediterranean, Arabian
80 Peninsula, and North Africa (e.g., Jiménez-Moreno and Suc, 2007; Jiménez-Moreno et al., 2010)
81 where today C₄ grasses dominate over C₃ grasses. Thus, climate conditions may have been
82 suitable for C₄ grasses during that period, although if $p\text{CO}_2$ (Pagani et al., 2005; Royer et al.,
83 2006; Beerling and Royer, 2011) were higher than at present then C₄ grasses may not have been
84 favored. A recent study (Urban et al., 2010) provided grass-pollen $\delta^{13}\text{C}$ evidence from four
85 samples at a single site in northeastern Spain that C₄ grasses were present in the region during
86 the early-middle Miocene. However, the limited temporal span (19-15 Ma) and small sample
87 size of that study hamper understanding of the early history of C₄ grasses in the region.

88 We measured the carbon isotopic composition of individual grass pollen grains from 39
89 samples from three sites in Spain. These samples together span the early-middle Miocene (20.3
90 to 10.1 Ma). The $\delta^{13}\text{C}$ data were analyzed using a hierarchical Bayesian model, which provides a
91 statistically rigorous assessment of both the presence and abundance of C₄ grasses on the
92 landscape (Urban et al., 2013). The study sites have previously published age control and pollen-

assemblage data, providing a context for evaluating the environmental conditions under which C_4 grasses existed during the early-middle Miocene.

2. Materials and methods

2.1 Study Sites and environmental context

Sediment samples were collected from three sites for this study (Table 1). We obtained 25 samples from a 320 m-long core in the Rubielos de Mora basin (Figure 1; 940 m.a.s.l.; 40.1830078° N, -0.657257° W) in northeastern Spain. Biostratigraphic (micromammals) and magnetostratigraphic markers indicate that these samples date to the Burdigalian-Langhian (22.2 - 16.4 Ma; Jiménez-Moreno et al., 2007a; 2007b). During the early Miocene, the basin contained a relatively deep lake with anoxic hypolimnetic waters (Anadón et al., 1988). The precise elevation of Rubielos de Mora during the Miocene is uncertain, but was probably slightly lower than at present. We also obtained seven sediment samples deposited in an ancient lagoon from an exposed stratigraphic section near the town of Gor, Granada (Fig. 1; 1180 m.a.s.l.; 37.425879° N, -3.054695° W) in southeastern Spain. Calcareous nannofossil markers indicate the Gor samples date to the Langhian (15.9 – 13.6 Ma; Marín-Pérez and Viseras, 1994). Finally, we obtained seven samples from the ~3000-m-long Andalucía A1 marine core in the Alborán basin of the Mediterranean Sea off the coast of southern Spain, ~95 km southwest of Gor (Figure 1; marine core; 36.6011119° N, -2.840311° W). Calcareous nannofossils and planktonic foraminifera data indicate that these samples date to the Serravallian-Tortonian (13.8 - 7.2 Ma; Rodríguez-Fernández et al., 1999). The paleoelevation for our samples from Gor and Andalucía A1 was sea level, as these were lagoon and nearshore marine environments.

The modern distribution of vegetation in western Europe is primarily controlled by

climatic variations associated with latitude, altitude, and proximity to coastal areas (Quézel and Médail, 2003). The vegetation in the north and northwestern portion of the Iberian Peninsula consists of a mosaic of mixed deciduous and coniferous forests interspaced with open steppe-grassy environments. Presently, C₄ grasses consist of 10-20% of total grasses on the Iberian Peninsula (Sage et al., 1999). In Spain, Mediterranean open sclerophyllous-pine forest, shrublands, and grasslands are mostly found along the coast and interior and eastern regions (Rivas-Martínez, 1987). The region exhibits a distinct latitudinal gradient of climate with higher temperatures and more severe summer droughts in the south transitioning to cooler and wetter conditions in the north.

At Rubielos de Mora (Fig. 1), pollen-assemblage data suggest strong control of local water availability on vegetation composition during the Burdigalian-Langhian (22.2 – 16.4 Ma). Hygrophilous or riparian taxa suggest the presence of wetlands around the lake. In the uplands, conditions were dry, with a dry season of approximately 7–9 months, as inferred from the relatively high abundance of xerophytes. Grasses comprised 5-18% of terrestrial pollen abundance. The pollen data from this site suggest an open landscape similar to modern warm-steppe or grass-dominated woodland (Fig. 1, Jiménez-Moreno et al., 2007b; 2010). The combination of different taxa, ranging from subtropical trees to subdesertic herbaceous species in the pollen assemblages at Rubielos de Mora also suggests that local environments were patchy, which makes a quantitative reconstruction of paleoclimatic conditions difficult. For example, the presence of thermophilous taxa suggests that mean-annual air temperatures may have been 17-21 °C and mean annual precipitation ~900-1700 mm. Yet, subdesertic plants suggest a mean annual precipitation of <500 mm (Jiménez-Moreno et al., 2007b; Jiménez-Moreno and Suc, 2007). Paleosol data from northeastern Spain provide an independent constraint and suggest that mean-

annual air temperatures in the region were 10-14 °C and mean annual precipitation was 400-1200 mm (Hamer et al., 2007a; Hamer et al., 2007b).

Pollen-assemblage data from Andalucía A1 and Gor (Fig. 1) indicate that herbs and shrubs dominated the middle-Miocene plant communities on the southern Iberian Peninsula, with grasses comprising 8-20% of terrestrial pollen abundance. Xerophytic subdesertic plants are more common at both localities than at Rubielos de Mora, indicating warm and dry conditions similar to the hyper-arid conditions that occur today in North Africa (Jiménez-Moreno et al., 2010). As in the northern Iberian Peninsula, the high percentages of xerophytes and herbs are representative of warm-steppe or grass-dominated woodlands. Similar xerophytic pollen assemblages occur in Africa today around the Red Sea, on the Arabian Peninsula, and in Northern Africa where >60% of grasses can be C₄ (Fig. 1, Jiménez-Moreno et al., 2007b; 2010; Marsahi et al. 2012). Based on thermophilous taxa, temperatures in southern Spain during the middle Miocene were inferred to be 17-25 °C. Mean annual precipitation as inferred from the pollen spectra was ~400-900 mm. However, as at Rubielos de Mora, the unusual combination of taxa at Andalucía A1 and Gor makes these inferred paleoclimatic conditions somewhat uncertain.

2.2 Sample treatment, $\delta^{13}C$ analysis, and statistical model

Carbon-isotope analysis of individual grass-pollen grains followed the protocol described in Nelson et al. (2006, 2007). We extracted pollen from sediment samples (~10-30 cm³ each) from Rubielos de Mora, Gor, and Andalucía A1, following standard protocols (Fægri et al., 1989) modified to exclude carbon-containing compounds (Nelson et al., 2006; 2008). The protocol involved successive treatments with 10% HCl, 10% KOH, concentrated HF, and 10%

HCl. The residue was then washed through a 74 μm sieve, treated with 50% H_2SO_4 , washed onto a 12 μm sieve, and treated with 5% Chlorox. Following each chemical treatment, the residue was washed thoroughly with nano-pure water.

Grass pollen was identified at 200x magnification in the pollen-water slurries of each sample based on its distinct single pore, annulus, and psilate/scabrate ornamentation (Fægri et al., 1989). The grass pollen grains were suctioned into a borosilicate glass capillary tube with a piston pump attached to an Eppendorf Transferrman micromanipulation device. Single grains of pollen were rinsed with nano-pure water and transferred into ~ 0.4 μL drops of nano-pure water. Each drop containing a single grain of grass pollen was then applied to a spooling wire microcombustion (SWiM) device interfaced with a Thermo-Finnigan Delta V isotope ratio mass spectrometer using a glass syringe. The total number of individual grains of grass pollen applied to the SWiM device ranged from 109 to 222 per sample.

A 2σ threshold of blank (each blank consisted of a ~ 0.4 μL drop of nano-pure water to which a single grain of grass pollen was added and then removed) CO_2 yields was used as a minimum size threshold; samples below this threshold were considered unreliable and excluded from further analysis. The final $\delta^{13}\text{C}$ data were blank-corrected using isotopic mass balance and normalized to VPDB using a two-point normalization curve with in-house carbon standards that were calibrated against USGS40 and USGS41 (Urban et al. 2013).

The $\delta^{13}\text{C}$ of atmospheric CO_2 ($\delta^{13}\text{C}_{\text{CO}_2}$) was thought to vary between -6.0 and -5.2‰ during the early-middle Miocene based on benthic foraminifera $\delta^{13}\text{C}$ data (Tippie et al., 2010). We estimated $\delta^{13}\text{C}_{\text{CO}_2}$ values for each sample and then adjusted the $\delta^{13}\text{C}$ value of each grass pollen grain to a pre-industrial $\delta^{13}\text{C}_{\text{CO}_2}$ of -6.3‰ (Francey et al. 1999, Friedli et al. 1986) prior to statistical analysis since this is the $\delta^{13}\text{C}_{\text{CO}_2}$ value that our training dataset is based upon. All $\delta^{13}\text{C}$

data were incorporated into a hierarchical Bayesian model, trained with 1,921 $\delta^{13}\text{C}$ values from 31 different grass species known to be either C_3 or C_4 , to estimate the relative abundance of C_3 - and C_4 -grass pollen in each sample (Urban et al., 2013). The model uses the means and variances of $\delta^{13}\text{C}$ values for C_3 and C_4 grasses in the training dataset to determine the relative abundance of C_3 and C_4 grass pollen in a sample of unknown composition while simultaneously considering candidate models in which one or both pollen types are present. The posterior model probabilities quantify the probability that sample $\delta^{13}\text{C}$ data support each candidate model as the true model. We evaluated the null hypothesis that a sample contains pollen from only C_3 grasses in terms of the posterior probability of a C_3 -only model [$P(\text{C}_3\text{-only})$]. We reject the null if $P(\text{C}_3\text{-only})$ is < 0.05 . Finally, we used a two-sample t-test (assuming unequal variances) to compare average C_4 -grass abundance among sites.

3. Results and Discussion

The number of grass pollen grains applied to the spooling wire with peak areas exceeding the 2σ threshold of the blank CO_2 yields varies between 57 and 112 (Table 1). On average, 48% of applications yield a peak area above the blank threshold, and 75% of the grass pollen $\delta^{13}\text{C}$ values fall within or between known $\delta^{13}\text{C}$ ranges for C_3 (-34‰ to -22‰) and C_4 (-15‰ to -10‰) plants (Fig. 3; Supplemental information; Cerling, 1999). These results are comparable to previous studies using Single Pollen Isotope Ratio AnaLysis, SPIRAL (e.g., Urban et al., 2010; Nelson et al. 2014; Urban et al., 2015). Values from grass pollen that are more negative than -34‰ or more positive than -10‰ likely indicate inadequate analytical precision and uncertainty in the isotopic composition and variability of analytical blanks (Nelson et al., 2007). Such values also occur in our training dataset (Urban et al. 2013) and thus samples with such values were not

excluded from the present study.

Our hierarchical Bayesian approach can reliably detect the presence and abundance of C₄-grass pollen in unknown pollen samples (Urban et al., 2013). Based on our analysis of grass-pollen $\delta^{13}\text{C}$ values with the Bayesian model, estimates of P(C₃-only) range between 0.0 and 0.015 (Table 1). These values are well below the 0.05 level required to reject the C₃-only hypothesis. All estimates of P(C₄-only) were 0.0 (data not shown), indicating that none of our samples contained exclusively C₄-grass pollen. Thus all samples unequivocally contain a mixture of C₃- and C₄-grass pollen grains. Our grass-pollen $\delta^{13}\text{C}$ data further indicate that the proportions of C₄ grasses range from 21-56% (average= 39%) at Rubielos de Mora, 50-72% at Gor (average = 60%), and 50-72% (average= 64%) at Andalucía A1 (Fig. 2b, Table 1). The averages do not differ between the two southern sites, and the values at the southern sites are significantly higher than at Rubielos de Mora ($p < 0.0001$).

We considered potential factors that could bias our estimates of C₄ grass abundance. Diagenesis could influence the measured $\delta^{13}\text{C}$ values of grass pollen. However, the sediments of our study were preserved under anoxic conditions, and the morphology of pollen grains (including grass pollen) from these sites shows no signs of strong diagenetic alteration (Jiménez-Moreno and Suc, 2007). In fact, a majority of the samples contained well-preserved plant cuticle, which is less resilient than the sporopollenin exine of pollen grains to degradation (van Bergen et al., 1993). Furthermore, a previous study by Urban et al. (2013) using lake surface-sediment samples from twenty-four sites on three continents found a significant positive relationship (that did not differ from a 1:1 relationship) between Bayesian estimates of % C₄ grass pollen in the surface-sediment samples and C₄-grass abundance around these sites. These results indicate no consistent bias in the representation of C₃ and C₄ grasses that could otherwise potentially be

associated with an affect of the initial stages of diagenesis on $\delta^{13}\text{C}$ values of grass pollen or with potential differences in pollen productivity or taphonomy between C_3 and C_4 grasses.

Under conditions of low water availability, such as may have occurred during the Miocene in southern Spain, $\delta^{13}\text{C}$ values of C_3 plants often become more positive, which could lead to the misclassification of C_3 -grass pollen as C_4 . During periods of low water availability, the $\delta^{13}\text{C}$ values of C_3 plants may become up to $\sim 3\text{‰}$ more positive (e.g., Ehleringer and Cooper, 1988). To explore the potential effect of aridity, we shifted the mean $\delta^{13}\text{C}$ value of C_3 grasses in our Bayesian model by up to $+3\text{‰}$. With this shift, the estimated proportions of C_4 grasses declined by an average of 13% (Table 1). However, the $\text{P}(\text{C}_3\text{-only})$ of 33 of our 39 samples remained <0.05 . Thus, our results indicating that C_4 -grass pollen exists in most of our samples are robust to the potential effect of aridity.

Large shifts in $p\text{CO}_2$ could also affect the difference between intercellular and atmospheric $p\text{CO}_2$ (Ci/Ca), and thus the $\delta^{13}\text{C}$ values of C_3 plants (e.g., Ehleringer et al., 1997; Schubert and Jahren, 2012). Precise $p\text{CO}_2$ values during the early-middle Miocene are uncertain, but probably exhibited only modest variations within a range of approximately 250-600 ppm (Pagani et al., 2005; Royer et al., 2006; Beerling and Royer, 2011). These $p\text{CO}_2$ values are similar to, or slightly higher than, the historical range of $p\text{CO}_2$ (about 300-350 ppm) represented by our herbarium-based training data. If $p\text{CO}_2$ values were similar during the early-middle Miocene and historically, then it is unlikely that shifts in $p\text{CO}_2$ affected our estimates of C_4 -grass abundance. To attempt to account for the maximum influence of potentially higher $p\text{CO}_2$ values during the early-middle Miocene than historically we shifted the mean $\delta^{13}\text{C}$ values of C_3 grasses in our training dataset by 3‰ in the negative direction based on a shift from 600 ppm to 350 ppm using equation 5 in Schubert and Jahren (2012). After this shift, values of $\text{P}(\text{C}_3\text{-only})$ and $\text{P}(\text{C}_4\text{-$

only) remained < 0.01 , which indicates that our conclusion that all of the samples contain a mixture of C_3 - and C_4 -grass pollen grains is not effected by potential shifts in pCO_2 .

Overall, our data reveal that C_4 grasses constituted substantial proportions of total grasses in Spain during the early-middle Miocene, and that C_4 -grass abundance varied across sites at different periods of time. A major pattern in our data is the contrast in C_4 -grass abundance between Rubielos de Mora in northeastern Spain (20.5-15.5 Ma) and Gor (16.3-13.7 Ma) and Andalucía A1 (14-10.1 Ma) in southern Spain (Fig. 2b). However, a limitation is that these data are not from a single location through time, posing a challenge to attribute spatiotemporal variation in C_4 -grass abundance to specific environmental factors. On the Arabian Peninsula today, C_3 grasses are favored where temperatures are lower and precipitation mainly falls during the winter, whereas C_4 grasses are favored where temperatures are higher and precipitation is more common in the summer (Masrahi et al., 2012). Thus, perhaps there was greater C_4 -grass abundance in our samples from southern than northern Spain because of generally warmer conditions (with adequate summer moisture) to the south during the middle Miocene than to the north during the early Miocene. Furthermore, our site in northern Spain was at higher elevation than our sites in southern Spain during the Miocene, and there are well-known effects of elevation on the distribution of C_3 and C_4 grasses, with C_3 favored in cooler and wetter high-elevation settings (e.g. Tieszen et al., 1979; Masrahi et al., 2012). Thus, the greater abundance of C_3 grasses at Rubielos de Mora could also occur because of the higher elevation of this site. Finally, relatively high local availability of water at Rubielos de Mora could have reduced C_4 -grass abundance at this site. The site was a large lake throughout much of the early-middle Miocene (Jiménez-Moreno et al., 2005; 2007a and 2007b), which provided extensive habitats for mesic vegetation (perhaps including C_3 grasses) around its margins, as evidenced by an

abundance of temperate taxa with high water requirements (e.g., mesothermic plants in Fig. 2a). The subdesertic plants in the same pollen assemblages were likely located outside of the riparian area. Such juxtapositions of xerophytic and mesophytic vegetation assemblages are common in subtropical and tropical basins in Africa today (e.g. Lake Tanganyika; Tierney et al., 2010).

Regardless of differences in C₄-grass abundance between Rubielos de Mora and our southern sites, the presence of significant proportions of C₄ grasses on the Iberian Peninsula during the early-middle Miocene provides new information on the development of grasslands in Europe. Grasses have been a component of the European landscape since the Eocene (Strömberg, 2011), and C₄ grasses have been present since at least the late Oligocene (Urban et al., 2010). Our data suggest that relatively warm conditions during the late Oligocene to middle Miocene (Mosbrugger et al., 2005; Jiménez-Moreno et al., 2010) likely promoted photorespiration in C₃ plants and conferred a competitive advantage to C₄ grasses in relatively open areas of southwestern Europe. After the middle-Miocene Climatic Optimum (around 17 – 15 Ma), temperatures in western Europe began to decline steadily (Bessedik, 1985; Jiménez-Moreno et al., 2005; 2010). This regional cooling led to a general decrease in thermophilous taxa and a change from mostly evergreen towards deciduous mesophilic taxa (*Quercus*, *Fagus*, *Alnus*, *Acer*), especially in northern Spain (Pinilla and Bustillo 1997, Barrón et al. 2006). Subdesertic plants still persist in southern Spain, but not in the quantities that they did during the Miocene (Jiménez-Moreno et al., 2010). By the early Pliocene almost all thermophilous taxa disappeared even in southern Spain, as a response to further cooling and a shift in seasonality toward winter precipitation. C₄ grasses generally prefer seasonal climates where precipitation falls mostly during warm weather months (Sage 1999). With regional cooling and the stabilization of Mediterranean type seasonality in Middle Pliocene (~3.3 Ma, Fauquette et al., 1999), a threshold

was likely crossed where C₃ grasses achieved a competitive advantage over C₄, leading to the low levels of C₄ grasses (10-20% of total grasses; Fig. 1; Sage, 1999) on the modern landscape.

Our results show that although grasses comprised only 5-20% in the early-middle Miocene pollen assemblages from Spain, a significant proportion of them used C₄ photosynthesis. In most other regions of the world, C₄ grasses were thought to be absent or uncommon during the early-middle Miocene and then greatly increase in abundance during the late Miocene and Pliocene (e.g., McInerney and Strömberg, 2011; Strömberg and McInerney, 2011). Although we lack a continuous record of C₄ grasses from the early Miocene-present at our sites, the proportions of C₄ grasses in the grass flora decreased sometime between 10.1 Ma (the end of our record) and today in Spain. This pattern cannot be explained by shifts in *p*CO₂ because, if anything, *p*CO₂ declined between the Miocene and present, which should have favored C₄ grasses because of their ability to concentrate CO₂ (Ehleringer et al., 1997). These results stand in contrast with the late-Miocene expansion of C₄ grasslands in other parts of the world. Thus our data suggest that regions with low modern proportions of C₄ grasses may have experienced higher proportions in the past when climatic conditions were presumably more suitable than at present. These data contribute to understanding the spatiotemporal complexity and environmental controls of C₄ grassland dynamics in earth's history.

Acknowledgements

We thank members of the Hu lab for helpful feedback on an early version of the manuscript. This research was supported by National Science Foundation grant DEB-0816610 (FSH and DMN) and a Dissertation Completion Fellowship from the University of Illinois (MAU).

Figure legends

Figure 1. Locations of sample sites (RdM: Rubielos de Mora; Gor: Gor; AA1: Andalucía A1) in Europe and surrounding regions. Shading indicates the modern abundances of C₄ grasses in grass flora (from Sage et al. 1999).

Figure 2. (A) Simplified pollen diagrams for Rubielos de Mora, Gor, and Andalucía A1 localities. The taxa are grouped according to their ecological affinity, following Jiménez-Moreno et al. (2007a, 2010). (B) C₄-grass pollen abundance from these localities. The error bars represent 95% confidence intervals.

Figure 3. Histograms of pollen $\delta^{13}\text{C}$ values from study sites (AA1: Andalucía A1; Gor: Gor; RdM: Rubielos de Mora).

References

- Anadón, P., Cabrera, L., and Julia, R. 1988. Anoxic-oxic cyclical lacustrine sedimentation in the Miocene Rubielos de Mora Basin, Spain. Geological Society, London, Special Publications 40, 353–367.
- Barrón E, Lassaletta, L., Alcalde-Olivares C. 2006. Changes in the Early Miocene palynoflora and vegetation in the east of the Rubielos de Mora Basin (SE Iberian Ranges, Spain). Neues Jahrbuch Geologie Palaontologie-Ab. 242, 171–204.
- Beerling, D.J. and Royer, D.L. 2011. Convergent Cenozoic CO₂ history. Nature Geoscience 4, 418-420.

346 Behrensmeyer, A. K., Quade, J., Cerling, T. E., Kappelman, J., Khan, et al. 2007. The structure
 347 and rate of late Miocene expansion of C₄ plants: Evidence from lateral variation in stable
 348 isotopes in paleosols of the Siwalik Group, northern Pakistan. *Geological Society of*
 349 *America Bulletin* 119, 1486–1505.

350 Bessedik, M. 1984. The Early Aquitanian and Upper Langhian– Lower Serravalian environments
 351 in the Northwestern Mediterranean region. *Paléobiologie Continentale* 15, 153–179.

352 Bessedik, M. 1985. Reconstitution des environnements Miocenes des regions nord-ouest
 353 Mediterraneennes à partir de la palynologie. Ph. D. Thesis. University Montpellier 2,
 354 France. (162 pp.)

355 Cerling, T.E., 1999. Paleorecords of C₄ plants and ecosystems. In: Sage, R.F., Monson, R.K.
 356 (Eds.), *C₄ Plant Biology*. Academic Press, San Diego, pp. 445–469.

357 Chen, S.T., Smith, S.Y., Sheldon, N.D., and Strömberg, C.A.E. 2015. Regional-scale variability
 358 in the spread of grasslands in the late Miocene. *Palaeogeography, Palaeoclimatology,*
 359 *Palaeoecology* 437, 42-52.

360 Cotton, J.M., Hyland, E.G. , and Sheldon, N.D. 2014. Multi-proxy evidence for tectonic control
 361 on the expansion of C₄ grasses in northwest Argentina. *Earth and Planetary Science*
 362 *Letters* 395, 41-50.

363 Cotton, J. M., and Sheldon, N. D. 2012. High-resolution isotopic record of C₄ photosynthesis in a
 364 Miocene grassland. *Palaeogeography, Palaeoclimatology, Palaeoecology* 337-338, 88-98.

365 Edwards, E. J., Osborne, C.P., Strömberg, CAE, et al. 2010. The origins of C₄ grasslands:
 366 integrating evolutionary and ecosystem science. *Science (New York, N.Y.)* 328, 587–91.

367 Ehleringer, J. R., Cerling, T. E., and Helliker, B. R. 1997. C₄ photosynthesis, atmospheric CO₂,
 368 and climate. *Oecologia* 112, 285–299.

369 Ehleringer, J. R., and Cooper, T. A.. 1988. Correlations between carbon isotope ratio and
 370 microhabitat in desert plants. *Botanical Review* 76, 562–566.

371 Fauquette, S., Suc, J-P., Guiot, J., Diniz, F., Feddi, N., Zheng, Z., Bessais, E., Drivaliari, A.
 372 1999. Climate and biomes in the West Mediterranean area during the Pliocene.
 373 *Palaeogeography, Palaeoclimatology, Palaeoecology* 152, 15-36.

374 Fægri, K., Iverson, J., Kaland, P.E., Krzywinski, K., 1989. *Textbook of Pollen Analysis*. Wiley,
 375 New York. (178 pp.)

376 Feakins, S. J., deMenocal, P. B. and Eglinton, T. I. 2005. Biomarker records of late Neogene
 377 changes in northeast African vegetation. *Geology* 33, 977–980.

378 Francey, R.J. et al., 1999. A 1000-year high precision record of $\delta^{13}\text{C}$ in atmospheric CO_2 .
 379 *Tellus Series B-Chemical and Physical Meteorology* 51, 170-193.

380 Friedli, H., Lotscher, H., Oeschger, H., Siegenthaler, U. , and Stauffer, B. 1986. Ice core record
 381 of the $^{13}\text{C}/^{12}\text{C}$ ratio of atmospheric CO_2 in the past two centuries. *Nature* 324, 237-238.

382 Hamer, J.M.M., Sheldon, N.D. , and Nichols, G.J. 2007a. Global aridity during the early
 383 Miocene? A terrestrial paleoclimate record from the Ebro Basin, Spain. *Journal of*
 384 *Geology* 115, 601-608.

385 Hamer, J.M.M., Sheldon, N.D., Nichols, G.J. , and Collinson, M.E. 2007b. Late Oligocene-Early
 386 Miocene paleosols of distal fluvial systems, Ebro Basin, Spain. *Palaeogeography*
 387 *Palaeoclimatology Palaeoecology* 247, 220-235.

388 Jacobs, B. F., Kingston, J. and Jacobs, L. 1999. The origin of grass-dominated ecosystems.
 389 *Annals of the Missouri Botanical Garden* 86, 590–643.

390 Jiménez-Moreno, G., Aziz, H. A., Rodríguez-Tovar, F. J., Pardo-Igúzquiza, E. and Suc, J.-P.
 391 2007a. Palynological evidence for astronomical forcing in Early Miocene lacustrine

392 deposits from Rubielos de Mora Basin (NE Spain). *Palaeogeography, Palaeoclimatology,*
393 *Palaeoecology* 252, 601–616.

394 Jiménez-Moreno, G., Fauquette, S., Suc, J.-P. and Aziz, H. A. 2007b. Early Miocene repetitive
395 vegetation and climatic changes in the lacustrine deposits of the Rubielos de Mora Basin
396 (Teruel, NE Spain). *Palaeogeography, Palaeoclimatology, Palaeoecology* 250, 101–113.

397 Jiménez-Moreno, G., Fauquette, S., and Suc J.-P. 2010. Miocene to Pliocene vegetation
398 reconstruction and climate estimates in the Iberian Peninsula from pollen data. *Review of*
399 *Palaeobotany and Palynology* 162, 403–415.

400 Jiménez-Moreno, G., F. J. Rodríguez-Tovar, E. Pardo-Igúzquiza, S. Fauquette, J.-P. Suc, and P.
401 Müller. 2005. High-resolution palynological analysis in late early–middle Miocene core
402 from the Pannonian Basin, Hungary: climatic changes, astronomical forcing and eustatic
403 fluctuations in the Central Paratethys. *Palaeogeography, Palaeoclimatology, Palaeoecology*
404 216, 73–97.

405 Jiménez-Moreno, G., and Suc, J.-P. 2007. Middle Miocene latitudinal climatic gradient in
406 Western Europe: Evidence from pollen records. *Palaeogeography, Palaeoclimatology,*
407 *Palaeoecology* 253, 208–225.

408 Kleinert, K., and Strecker, M. R. 2001. Climate change in response to orographic barrier uplift:
409 Paleosol and stable isotope evidence from the late Neogene Santa María basin, northwestern
410 Argentina. *Geological Society of America Bulletin* 113, 728–742.

411 Kovar-eder, J., Jechorek, H. and Kvac, Z. 2008. The integrated plant record : an essential tool for
412 reconstructing neogene zonal vegetation in Europe. *Palaios* 23, 97–111.

413 Levin, N. E., Quade, J., Simpson, S. W., Semaw, S., and Rogers, M. 2004. Isotopic evidence for
414 Plio-Pleistocene environmental change. *Earth and Planetary Science Letters* 219, 93–110.

415 Martín Pérez, J.A., Viseras, C., 1994. Sobre la posición estratigráfica de las “Margas de Gor”,
 416 Sierra de Baza, Cordillera Bética. *Geogaceta* 15, 63–66.

417 Masrahi, Y.S., Al-Turki, T.A., and Sayed, O.H. 2012. Geographical distribution and chorology
 418 of grasses in the Arabian Peninsula. *Flora* 207, 250-256.

419 McInerney, F. A., and Strömberg, CAE. 2011. The Neogene transition from C₃ to C₄ grasslands
 420 in North America: stable carbon isotope ratios of fossil phytoliths. *Paleobiology* 37, 23–49.

421 Mosbrugger, V., Utescher, T., and Dilcher, D. L. 2005. Cenozoic continental climatic evolution
 422 of Central Europe. *Proceedings of the National Academy of Sciences of the United States of*
 423 *America* 102, 14964–9.

424 Nelson, D. M., Hu, F. S., and Michener, R. H. 2006. Stable-carbon isotope composition of
 425 Poaceae pollen: an assessment for reconstructing C₃ and C₄ grass abundance. *The Holocene*
 426 16, 819–825.

427 Nelson, D. M., Hu, F.S., Mikucki, J., Tian, J., and Pearson, A. 2007. Carbon isotopic analysis of
 428 individual pollen grains from C₃ and C₄ grasses using a spooling wire microcombustion
 429 interface. *Geochimica et Cosmochimica Acta* 71, 4005-4014.

430 Nelson, D. M., Hu, F. S., Scholes, D. R., Joshi, N., and Pearson, A. 2008. Using SPIRAL (Single
 431 Pollen Isotope Ratio AnaLysis) to estimate C₃- and C₄-grass abundance in the paleorecord.
 432 *Earth and Planetary Science Letters* 269, 11–16.

433 Nelson, D. M., Urban, M. A., and Hu, F.S. 2014. Spatiotemporal variation in the origin of C₄
 434 grasses: $\delta^{13}\text{C}$ analysis of grass pollen from the southeastern United States.
 435 *Palaeogeography Palaeoclimatology Palaeoecology* 396, 227-231.

436 Pagani, M., Zachos, J. C., Freeman, K. H., Tipple, B., and Bohaty. 2005. Marked decline in
 437 atmospheric carbon dioxide concentrations during the Paleogene. *Science* (New York,
 438 N.Y.) 309, 600–603.

439 Passey, B. H., Ayliffe, L. K., Kaakinen, A., Zhang, Z., Eronen, J. T., et al. 2009. Strengthened
 440 East Asian summer monsoons during a period of high-latitude warmth? Isotopic evidence
 441 from Mio-Pliocene fossil mammals and soil carbonates from northern China. *Earth and*
 442 *Planetary Science Letters* 277, 443–452.

443 Pinilla A, and Bustillo A. 1997. Sílicofitolitos en secuencias arcillosas con silcretas. Mioceno
 444 Medio, Madrid, in Pinilla, A., Juan-Tresserras, J., and Machado, M.J., eds., *The State of the*
 445 *Art of Phytoliths in Soils and Plants: Madrid*, CSIC, p. 255–65.

446 Quézel, P., Médail, F., 2003. *Ecologie et biogéographie des forêts du bassin méditerranéen*.
 447 Elsevier, France (571 pp.)

448 Rivas-Martínez, S., 1987. *Memoria del mapa de las series de vegetación de España*. Icona,
 449 Madrid (268 pp.)

450 Rodríguez-Fernández, J., Comas, M.C., Soría, J., Martín-Pérez, J.A., Soto, J.I., 1999. In: Zahn,
 451 R., Comas, M.C., Klaus, A. (Eds.), *The sedimentary record of the Alborán Basin: an*
 452 *attempt at sedimentary sequence correlation and subsidence analysis: Proceedings of the*
 453 *Ocean Drilling Program Scientific Results*, vol. 161, pp. 69–76.

454 Royer, D.L. 2006. CO₂-forced climate thresholds during the Phanerozoic. *Geochimica et*
 455 *Cosmochimica Acta* 70, 5665–5675.

456 Sage, R. F. 2004. The evolution of C₄ photosynthesis. *New Phytologist* 161, 341–370.

457 Sage, R.F., Monson, R.K. (Eds.), 1999. *C₄ Plant Biology*. Academic Press, San Diego (596 pp.).

458 Sage, R.F., Wedin, D.A., and Li, M., 1999, The biogeography of C₄ photosynthesis: Patterns and
459 controlling factors, in Sage, R.F., and Monson, R.K., eds., C₄ plant biology: New York,
460 Academic Press, p. 313–373.

461 Sanyal, P., Sarkar, A., Bhattacharya, S. K., Kumar, R., Ghosh, S. K., and Agrawal, S. 2010.
462 Intensification of monsoon, microclimate and asynchronous C₄ appearance: Isotopic
463 evidence from the Indian Siwalik sediments. *Palaeogeography, Palaeoclimatology,*
464 *Palaeoecology* 296, 165–173.

465 Sanz de Siria Catalan, A., 1993. Datos sobre la paleoclimatología y paleoecología del Neógeno
466 del Vallès-Penedès según las macrofloras halladas en la cuenca y zonas próximas.
467 *Paleontologia i Evolució* 26–27, 281–289.

468 Schubert, B. A., and Jahren, A. H. 2012. The effect of atmospheric CO₂ concentration on carbon
469 isotope fractionation in C₃ land plants. *Geochimica et Cosmochimica Acta* 96, 29–43.

470 Strömberg, CAE. 2011. Evolution of grasses and grassland ecosystems. *Annual Review of Earth*
471 *and Planetary Sciences* 39, 517–544.

472 Strömberg, CAE., and F. A. McInerney. 2011. The Neogene transition from C₃ to C₄ grasslands
473 in North America: assemblage analysis of fossil phytoliths. *Paleobiology* 37:50–71.

474 Tieszen, L.L., Senyimba, M.M., Imbamba, S.K. , and Troughton, J.H. 1979. The distribution of
475 C₃ and C₄ grasses and carbon isotope discrimination along an altitudinal and moisture
476 gradient in Kenya. *Oecologia* 37, 337–350.

477 Tipple, B. J., Meyers, S. R., and Pagani, M. 2010. Carbon isotope ratio of Cenozoic CO₂: A
478 comparative evaluation of available geochemical proxies. *Paleoceanography* 25, 1–11.

479 Urban, M. A., Nelson, D. M., Jiménez-Moreno, G., Chateauneuf, J.-J, Pearson, A., and Hu, F. S.
480 2010. Isotopic evidence of C₄ grasses in southwestern Europe during the Early Oligocene-
481 Middle Miocene. *Geology* 38, 1091–1094.

482 Urban, M. A., Nelson, D. M., Kelly, R., Ibrahim, T., Dietze, M., Pearson, A., and Hu, F. S.
483 2013. A hierarchical Bayesian approach to the classification of C₃ and C₄ grass pollen based
484 on SPIRAL $\delta^{13}\text{C}$ data. *Geochimica et Cosmochimica Acta* 121, 168–176.

485 Urban, M. A., Nelson, D. M., Street-Perrott, A. Verschuren, D. and Hu, F.S. 2015. A late-
486 Quaternary perspective on atmospheric $p\text{CO}_2$, climate, fire as alternative drivers of C₄-
487 grass abundance. *Ecology* 96, 642-653.

488 van Bergen, P., Collinson, M, and de Leeuw, J. 1993. Chemical composition and ultrastructure
489 of fossil and extant salvinialean microspore massulae and megaspores. *Grana* 32, 18–30.
490
491

Figure 1

Figure 2

Figure 3