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Research

Carbon isotopic composition of *Ambrosia* and *Artemisia* pollen: assessment of a C_3-plant paleophysiological indicator

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Summary

- There is limited evidence on how shifts in plant physiological performance influence vegetation variations in the paleorecord.
- To evaluate $\delta^{13}C$ of pollen from C_3 plants as an indicator of community-level physiology, small quantities (10–30 grains) of untreated pollen and sporopollenin from herbarium specimens of *Ambrosia* (*A. tomentosa* and *A. psilostachya*) and *Artemisia* (*A. frigida*, *A. ludoviciana* and *A. dracunculus*), genera abundant in grassland pollen profiles, were isolated by micromanipulation. Their $\delta^{13}C$ values were measured using a spooling-wire microcombustion device interfaced with an isotope-ratio mass spectrometer. Leaf $\delta^{13}C$ was also measured. Carbon isotope discrimination (Δ) for untreated pollen, sporopollenin and leaves was compared with historic records of seasonal precipitation amount, vapor pressure deficit and the Palmer Drought Severity Index (PDSI).
- Each species showed positive correlations between Δ of untreated pollen and sporopollenin. Sporopollenin Δ was most strongly correlated with PDSI. Correlations among leaf Δ and moisture indicators were stronger for *Ambrosia* than *Artemisia*.
- These results suggest that sporopollenin Δ indicates the level of moisture stress in C_3 plants. Therefore, $\delta^{13}C$ analysis of pollen promises to help address important paleoecological questions, such as how community-level physiology contributes to shifts in vegetation composition.

Introduction

Paleobotanical indicators, such as fossil pollen assemblages, are invaluable for the assessment of spatiotemporal variation in the composition and structure of plant communities in Earth's history (e.g. Williams *et al.*, 2004). Physiology is an important interface between plants and their environment, and vegetation variations in the paleorecord are often explicitly or implicitly hypothesized to result from the influence of shifting environmental conditions on plant physiological performance (e.g. Levis *et al.*, 1999; Bond *et al.*, 2003; Crucifix *et al.*, 2005; Prentice & Harrison, 2009; Gerhart & Ward, 2010). Paleophysiological inferences have been made from morphological (e.g. Woodward, 1987; DiMichele & Gastaldo, 2008; Boyce *et al.*, 2009) and carbon isotope (e.g. Van de Water *et al.*, 1994; Beerling, 1996; Turney *et al.*, 2002; Gerhart *et al.*, 2012) measurements of macrofossils, such as leaves and wood. However, there is relatively limited pollen-based evidence to draw inferences about plant physiological processes, which is unfortunate because plant macrofossils are often less common than pollen in the paleorecord.

Previous studies have suggested that $\delta^{13}C$ analysis of pollen, which is typically abundant and well preserved in sediments (Fægri *et al.*, 1989), holds promise for assessment of the physiological response of C_3 plants to paleoenvironmental change (Loader & Hemming, 2001, 2004; Jahren, 2004; Descolas-Gros & Scholzel, 2007). This approach is based on the observation that carbon isotope discrimination (Δ) during photosynthesis is influenced by the ratio of intercellular to atmospheric CO_2 concentrations (C_i/C_a), which is a function of the proportion of net photosynthetic assimilation and stomatal conductance (Farquhar *et al.*, 1982, 1989). In C_3 plant tissues, Δ typically declines with decreased water availability (e.g. Ehleringer & Cooper, 1988; Dawson *et al.*, 2002; Kohn, 2010), because dry conditions lead to reduced stomatal conductance, a proportionately greater decrease in transpiration than photosynthesis and relatively greater fixation of ^{13}C than ^{12}C (Cowan, 1982; Farquhar *et al.*, 1982). The response of leaf Δ to water availability is typically similar at the species and community level (e.g. Wittmer *et al.*, 2008; Prentice *et al.*, 2011).

Existing evidence reveals a generally close (± 3‰) relationship between $\delta^{13}C$ of pollen and leaves from a range of taxa grown in arboreta, botanical gardens and field settings (Amundson *et al.*, 1997; Jahren, 2004). Previous studies have also demonstrated that $\delta^{13}C$ values of raw untreated pollen correlate well with those of pollen chemically treated to isolate sporopollenin (the highly

resistant outer wall of pollen preserved in sediments), but with an offset related to the removal of non-sporopollenin material from chemically treated samples (Loader & Hemming, 2000; Nelson *et al.*, 2006). Beyond these initial investigations, there has been limited assessment of pollen $\delta^{13}C$ as an indicator of the paleophysiology of C_3 plants. In particular, the existence of a consistent relationship between moisture availability and intra- and inter-species variability of pollen $\delta^{13}C$ has not been investigated, but if such a relationship exists, then $\delta^{13}C$ of pollen from C_3 plants would be a reliable indicator of community-level paleophysiology. One constraint on progress has been sample-size limitations related to the isolation of sufficient quantities of pollen for isotopic analysis. However, recent advances in the separation and $\delta^{13}C$ analysis of pollen (Nelson *et al.*, 2006, 2007, 2008) have greatly reduced the amount of carbon required for analysis, and thus pollen isolation time.

Here, I report the results of a study to determine the potential of $\delta^{13}C$ of pollen from *Ambrosia* and *Artemisia* to record paleophysiological information. These C_3 genera are two of the most abundant taxa in pollen records from North American grasslands (Grimm, 2001). Previous studies have demonstrated that stomatal conductance and Δ in leaves of these genera are influenced by intra- (Smedley *et al.*, 1991; Letts *et al.*, 2010) and inter-year (Laundre, 1999) variability in moisture availability, although exceptions exist (e.g. Evans & Black, 1993). Furthermore, both genera possess photosynthetic inflorescences (Bazzaz & Carlson, 1979; Evans *et al.*, 1991; Aschan & Pfanz, 2003), which may cause pollen Δ to be particularly responsive to variation in moisture availability during the flowering period, which is typically mid–late summer. The present study used herbarium specimens of these taxa to address three objectives: to evaluate the feasibility, as well as the obtainable isotopic precision and accuracy, of compositing multiple pollen grains into single samples for $\delta^{13}C$ analysis via a spooling-wire microcombustion device (Sessions *et al.*, 2005; Eek *et al.*, 2007) interfaced with an isotope-ratio mass spectrometer (SWiM-IRMS); to assess the intra- and inter-species relationships among Δ of untreated pollen, sporopollenin and leaves; and to determine the relationship between sporopollenin Δ and moisture availability using historic climate data.

Materials and Methods

Leaves and flowers were obtained from 43 specimens of *Ambrosia tomentosa* Nutt., *Ambrosia psilostachya* DC., *Artemisia frigida* Willd., *Artemisia ludoviciana* Nutt. and *Artemisia dracunculus* L. housed in the Rocky Mountain Herbarium (www.rmh.uwyo.edu/). To my knowledge, no carbon-containing chemicals were used for the preservation of these specimens. These species are perennial forbs and subshrubs common in the northern Great Plains of North America (Grimm, 2001). The specimens selected for analysis were preferentially chosen (using information on their labels) from upland grassland habitats. Most of the specimens came from Wyoming, with the remainder from western South Dakota. Two specimens were collected during June, 16 during July, 24 during August and one during September; the years of collection span the period 1900–1995 (Supporting Information Table S1).

Untreated pollen from each specimen was first subjected to $\delta^{13}C$ analysis. The remaining unanalyzed pollen, when available, was treated to isolate the chemically resistant sporopollenin exine (Loader & Hemming, 2000). Treatment followed standard pollen preparation techniques modified to exclude carbon-containing chemicals (Nelson *et al.*, 2006), except that HF and HCl were not used because these chemicals have little influence on pollen $\delta^{13}C$ (Jahren, 2004) and the pollen was not being extracted from sediments.

Pollen grains were isolated in nanopure water by micromanipulation at 200× magnification on a microscope slide, as described previously for $\delta^{13}C$ analysis of individual grains of grass pollen for the assessment of C_3/C_4 grass abundance (Nelson *et al.*, 2006, 2007, 2008). However, rather than analyzing single grains, in the present study, *c.* 10 grains of untreated pollen per sample and *c.* 20–30 grains of sporopollenin per sample were rinsed in nanopure water and transferred to a 0.4-μl drop of water. The drop was then applied to an SWiM-IRMS using a steel and glass syringe. Multiple samples were typically analyzed for each specimen (Table S1). The configuration and operation of the SWiM-IRMS at the Central Appalachians Stable Isotope Facility, which was used in the present study, was the same as that at Harvard University, which has been described previously for the analysis of grass pollen (e.g. Urban *et al.*, 2010), with the following modifications: in the present study, a ThermoFinnigan Delta V+ IRMS and Conflo IV interface were used (ThermoFinnigan, Bremen, Germany), and the helium carrier gas was set to 3.5 psi. Sample yields (as CO_2 gas) are reported in volt-second units (Vs, peak area) of the mass 44 ion current. Blanks (nanopure water to which grains of pollen were added and then removed) were analyzed concomitantly with each sample. Yields of blanks were, on average, 14 times less than the yields of samples (Table S1). To assess the accuracy and precision of the SWiM-IRMS data, two dissolved organic standards, leucine and sorbitol, which contained varying amounts of carbon and have $\delta^{13}C$ values that encompass the range of those anticipated for C_3 plants, were analyzed.

Approximately 1 mg of homogenized leaf material from each specimen was analyzed for $\delta^{13}C$ using a Carlo Erba NC2500 elemental analyzer (CE Instruments, Milano, Italy) interfaced with a ThermoFinnigan Delta V+ IRMS. The analytical precision for leaf $\delta^{13}C$ was 0.12‰ based on internal laboratory standards. Carbon isotope discrimination was calculated from the untreated pollen, sporopollenin and leaf $\delta^{13}C$ data using plant collection dates and records of the post-industrial depletion of $\delta^{13}C$ of atmospheric CO_2 (Friedli *et al.*, 1986; Francey *et al.*, 1999) with the equation: $\Delta = (\delta^{13}C_{air} - \delta^{13}C_{plant})/(1 + \delta^{13}C_{plant})$.

Measures of plant moisture availability, which previous studies have shown often correlate with Δ for C_3 plant tissues (e.g. Turney *et al.*, 1999; Leavitt, 2007; Kohn, 2010; Prentice *et al.*, 2011), were obtained or calculated for each specimen using the specimen location and collection year. These measures included precipitation amount, vapor pressure deficit (VPD) and the Palmer Drought Severity Index (PDSI). Monthly precipitation

data were obtained from PRISM (Daly *et al.*, 2008). The spatial resolution of these data is *c.* 2 km. The range of total summer (June–August) precipitation represented by the specimens was 330–41 mm. VPD, a climatic parameter closely related to evapotranspiration, was calculated from average monthly maximum, minimum and dewpoint temperatures (also obtained from PRISM) as in Gerhart *et al.* (2012). Monthly values of the PDSI, an indicator of the degree of dryness and wetness derived from precipitation, air temperature and soil moisture data (Palmer, 1965), were obtained from the closest climate divisions to the location at which each specimen was obtained (http://www.ncdc.noaa.gov/climate-monitoring/index.php). Negative PDSI values indicate drier conditions. Seasonal averages were calculated for the precipitation, VPD and PDSI data associated with each specimen for (1) the summer in which the specimen was collected (defined here as the average of June–August data), (2) the preceding spring (average of March–May data), and (3) the preceding winter (average of December–February data).

The significance of the correlations among Δ of untreated pollen, sporopollenin and leaves was assessed using major-axis regression because of symmetry in the variables on the *x* and *y* axes (Smith, 2009). The significance of correlations between Δ (of sporopollenin and leaves) and seasonal indicators of moisture availability was assessed using ordinary least-squares regression because of asymmetry in the variables on the *x* and *y* axes (Smith, 2009). These analyses were performed in PAST (Hammer *et al.*, 2001).

Results

There is a general decline in precision and accuracy of $\delta^{13}C$ with decreasing mass of sample carbon for leucine and sorbitol standards (Fig. 1). To help to ensure that the accuracy and precision of the obtained pollen $\delta^{13}C$ data were no worse than *c.* 1‰, on average, a combustion yield threshold of 1.2 Vs was set as the minimum acceptable sample yield. Data from leucine and sorbitol indicate that this threshold equates to *c.* 0.8 nmol C. Approximately 10 grains of untreated pollen and 20–30 grains of sporopollenin per analysis were usually sufficient to produce yields exceeding this threshold. Any samples with yields below this threshold were excluded from further analysis.

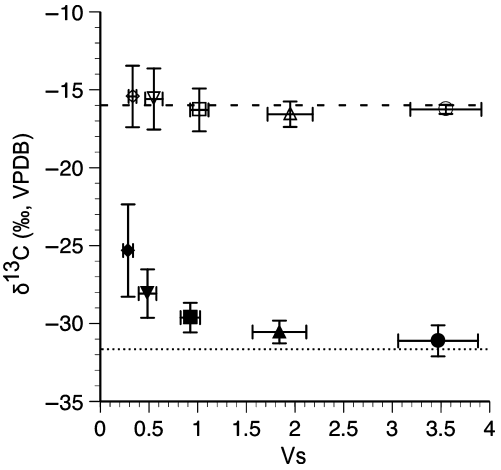

Fig. 1 Data for organic standards. Open symbols, sorbitol (known $\delta^{13}C = -15.96‰$; dashed horizontal line); closed symbols, leucine (known $\delta^{13}C = -31.70‰$; dotted horizontal line). Data for each standard are shown for samples of mass 2.50 nmol C (circles), 1.25 nmol C (triangles), 0.625 nmol C (squares), 0.312 nmol C (inverted triangles) and 0.156 nmol C (diamonds); $n = 7$ replicates for each standard. CO_2 area data are in volt-second units (Vs). Means and 1σ error bars are shown. VPDB, Vienna Pee Dee Belemnite.

A total of 332 isotopic measurements were made on samples of untreated pollen and sporopollenin from *Ambrosia* and *Artemisia*. $\delta^{13}C$ values range from −22.2‰ to −29.2‰ for untreated pollen, from −23.8‰ to −31.4‰ for sporopollenin and from −24.1‰ to −30.0‰ for leaves (Table S1). These values are within the typical ranges of $\delta^{13}C$ values for tissues of C_3 plants. The average yield for samples of untreated pollen (2.5 Vs) is similar to that of sporopollenin samples (2.6 Vs). Approximately two to three times as many pollen grains were applied to the SWiM device for samples of sporopollenin relative to untreated pollen, so these results are at the lower end of reports that sporopollenin comprises *c.* 55–85% of the mass of pollen (Shaw & Yeadon, 1964; Brooks & Shaw, 1968). The average precision values (1σ) of $\delta^{13}C$ for samples of untreated pollen and sporopollenin are 0.5‰ and 0.8‰, respectively.

The $\delta^{13}C$ values of sporopollenin are lower (Δ more positive) than those of untreated pollen with one exception (Fig. 2 and Table S1). The average difference between $\delta^{13}C$ of sporopollenin and untreated pollen for each specimen is 1.45‰ (range: −0.15‰ to 2.71‰). A similar offset was observed in previous studies (Loader & Hemming, 2000; Nelson *et al.*, 2006) and is likely caused by the removal of isotopically heavy cellulose from the pollen intine during chemical treatment. Strong positive relationships exist between Δ of untreated pollen and sporopollenin for each species (Fig. 2). Therefore, I refer to sporopollenin Δ for the remainder of this article unless otherwise indicated.

A strong positive correlation exists between Δ of sporopollenin and leaves for *A. psilostachya* ($r = 0.88$, $P = 0.009$, $n = 7$; Fig. 3). There is also a moderately significant correlation between Δ of sporopollenin and leaves for *A. tomentosa* ($r = 0.7$, $P = 0.08$, $n = 7$; Fig. 3). By contrast, correlations between Δ of sporopollenin and leaves for each of the three species of *Artemisia* are not statistically significant. Sporopollenin Δ values are generally lower for *A. frigida* than for the other species (Fig. 3).

Sporopollenin Δ is positively correlated with PDSI for each species of *Ambrosia* and *Artemisia* (Fig. 4, Table S2). These relationships are strongest during the spring and summer (Table S2). The slopes of the linear regressions between sporopollenin Δ and summer PDSI have confidence intervals that overlap with each other (Fig. 4). These correlations are significant at $P < 0.1$ for four of the five species and $P < 0.05$ for three of the five species. The only measure of moisture availability that is strongly correlated ($P < 0.05$) with sporopollenin Δ, beside PDSI, is spring and summer VPD for *A. tomentosa* (Table S2).

Leaf Δ is not significantly correlated with seasonal precipitation, VPD or PDSI for *Artemisia* (Fig. 5, Table S3). By contrast, leaf Δ for *A. psilostachya* is significantly correlated with spring and summer PDSI, as well as with summer precipitation (Fig. 5,

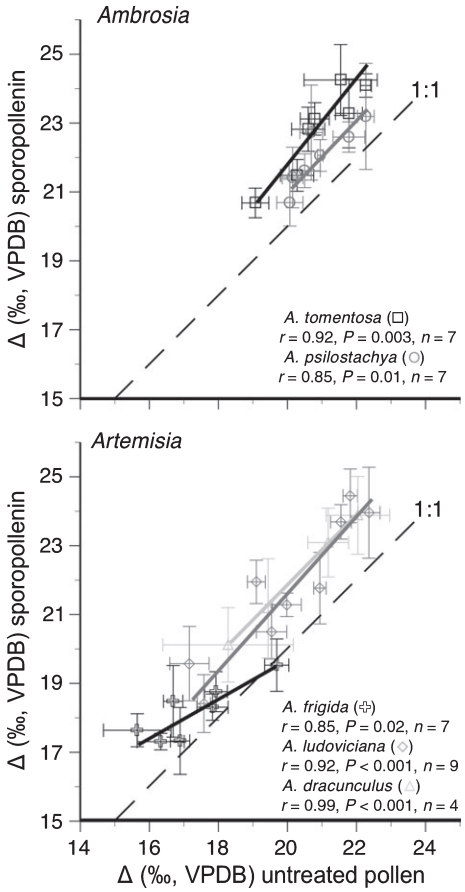

Fig. 2 Relationship between mean Δ values (with 1σ error bars) of untreated pollen and sporopollenin (treated pollen) for *Ambrosia* and *Artemisia* species. The thin dashed lines represent 1 : 1 relationships. The solid lines of different color represent linear regressions through the data for each species. The major axis slopes and 95% confidence intervals of the slopes are as follows: *Ambrosia tomentosa* (1.25, 0.55–1.70), *Ambrosia psilostachya* (1.05, − 0.7 to 1.48), *Artemisia frigida* (0.57, 0–0.83), *Artemisia ludoviciana* (1.12, 0.75–1.43) and *Artemisia dracunculus* (1.02, 0.63–1.11). VPDB, Vienna Pee Dee Belemnite.

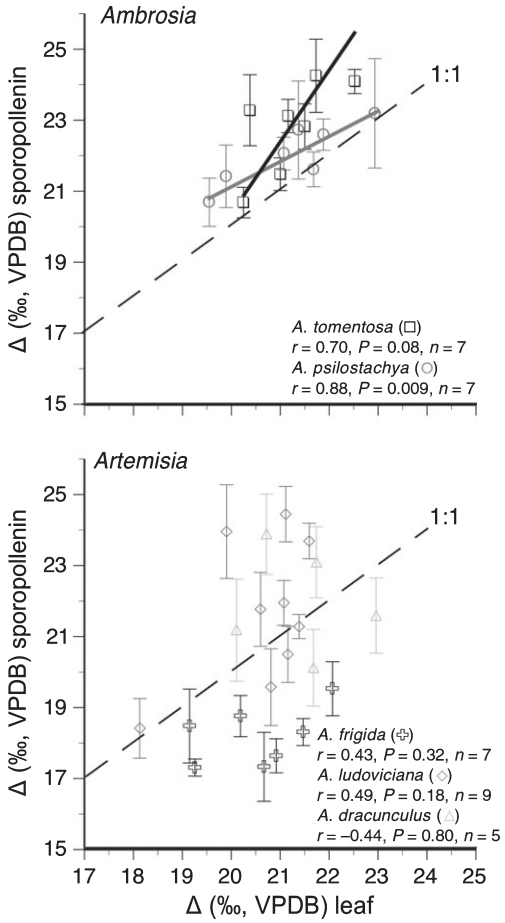

Fig. 3 Relationship between Δ of leaves and sporopollenin (treated pollen) for *Ambrosia* and *Artemisia* species. The thin dashed lines represent 1 : 1 relationships. The solid lines of different color represent linear regressions through the data for the *Ambrosia* species. The major axis slopes and 95% confidence intervals of the slopes with $P < 0.1$ are as follows: *Ambrosia tomentosa* (2.02, − 2.3 to 3.67) and *Ambrosia psilostachya* (0.71, 0.31–1.01). Means and 1σ error bars are shown for the sporopollenin Δ data. VPDB, Vienna Pee Dee Belemnite.

Table S3). Leaf Δ for *A. tomentosa* is not significantly correlated with seasonal precipitation, VPD or PDSI (Fig. 5, Table S3). However, exclusion of the outlier leaf Δ datapoint for *A. tomentosa* (Fig. 5) leads to significant relationships between leaf Δ and spring PDSI (r^2 = 0.71, P = 0.04, n = 6) and spring and summer VPD (r^2 = 0.59, P = 0.04, n = 6 and r^2 = 0.8, P = 0.01, n = 6, respectively) for this species (Table S3). The relatively high leaf (as well as sporopollenin, Fig. 4) Δ value of this outlying plant suggests that it occupied a location on the landscape that was wetter than the larger region.

Discussion

The observed relationships provide confidence for the use of Δ, as measured from $\delta^{13}C$ of pollen from the C_3 taxa *Ambrosia* and *Artemisia*, as a quantitative indicator of community-level moisture stress for sediment-based paleoecological studies. The moisture indicator most strongly correlated with sporopollenin Δ was PDSI (Fig. 4, Table S2). Therefore, the level of soil moisture availability during the growing season may be reconstructed using Δ of fossil *Ambrosia* and *Artemisia* pollen. Because only 20–30 grains of *Ambrosia* and *Artemisia* sporopollenin are required per analysis, and *c.* 60 pollen grains per hour can be isolated from a sediment sample using micromanipulation (Nelson *et al.*, 2006), it is feasible to assess paleophysiology at relatively high temporal resolution.

Fossil pollen data represent time-averaged assemblages of different pollen types and this integration also affects inferences of water availability from Δ of pollen. For example, Δ for one pollen type in a sediment sample is a spatially and temporally mixed signal of water availability on the landscape for that taxon, which may represent multiple species from a given genus or family. In the present study, Δ of sporopollenin for *A. frigida* was generally lower than that of the other *Artemisia* species (Fig. 3), which suggests that *A. frigida* occupies portions of the landscape that are relatively drier during the growing season. Such local-scale differences in water availability, as inferred from Δ of sporopollenin, are likely to be minimized in sediment records that receive pollen from throughout the region around a site.

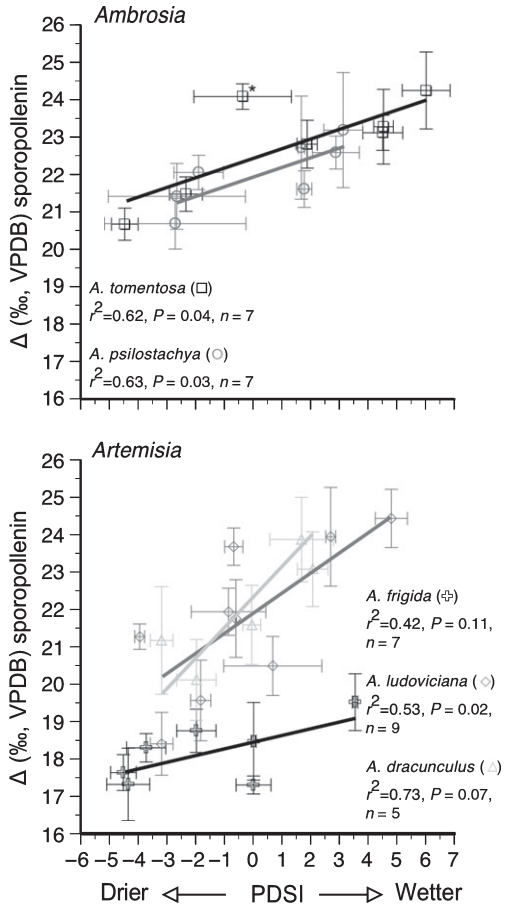

Fig. 4 Relationship between average summer (June–August) Palmer Drought Severity Index (PDSI) and Δ of sporopollenin for *Ambrosia* and *Artemisia* species. Means and 1σ error bars are shown. The solid lines of different color represent linear regressions through the data for each species. The ordinary least-squares slopes and 95% confidence intervals of the slopes are as follows: *Ambrosia tomentosa* (0.26, 0.17–0.58), *Ambrosia psilostachya* (0.26, 0.11–0.44), *Artemisia frigida* (0.18, 0.05–0.46), *Artemisia ludoviciana* (0.54, 0.13–0.96) and *Artemisia dracunculus* (0.82, 0.07–1.06). The outlier in the *A. tomentosa* data that is discussed in the text is labeled with an asterisk. VPDB, Vienna Pee Dee Belemnite.

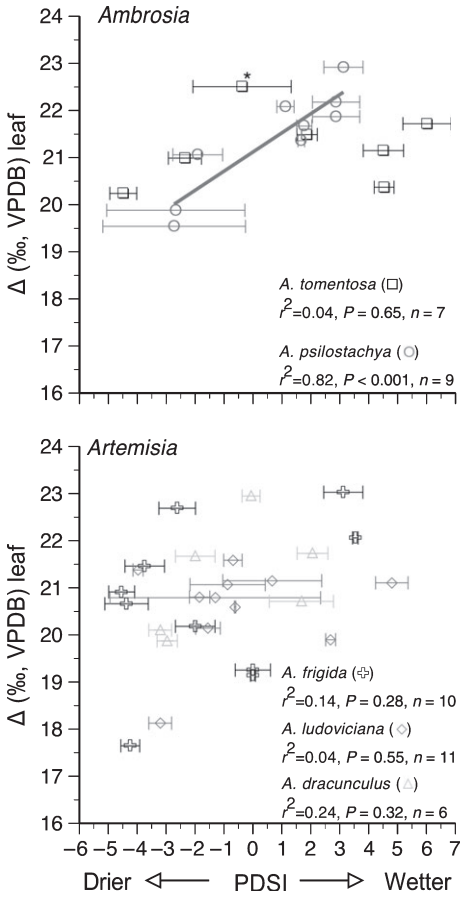

Fig. 5 Relationship between average summer (June–August) Palmer Drought Severity Index (PDSI) and Δ of leaves for *Ambrosia* and *Artemisia* species. 1σ error bars are shown for the PDSI data. The solid gray line represents a linear regression through the data for *Ambrosia psilostachya*. The ordinary least-squares slopes and 95% confidence intervals of the slopes are as follows: *Ambrosia tomentosa* (0.04, − 0.1 to 0.31), *Ambrosia psilostachya* (0.41, 0.28–0.62), *Artemisia frigida* (0.21, − 0.07 to 0.71), *Artemisia ludoviciana* (0.08, − 0.39 to 0.25) and *Artemisia dracunculus* (0.25, − 0.52 to 0.78). The outlier in the *A. tomentosa* data that is discussed in the text is labeled with an asterisk. VPDB, Vienna Pee Dee Belemnite.

Sporopollenin Δ, leaf Δ and growing-season moisture availability were positively correlated for both species of *Ambrosia* (Figs 3–5, Tables S2, S3, excluding the outlier for *A. tomentosa*). *Ambrosia*'s vegetative growth occurs throughout the spring and summer, and its reproductive growth occurs in the summer (Bazzaz & Carlson, 1979; Grimm, 2001). Therefore, these results suggest that the pollen and leaves of *Ambrosia* are formed primarily from photosynthate with Δ values that are influenced by moisture availability in the present growing season.

In contrast with *Ambrosia*, neither sporopollenin and leaf Δ, nor growing season moisture availability and leaf Δ, were strongly correlated for any of the *Artemisia* species (Figs 3, 5, Table S3). These results, together with the generally strong relationships of sporopollenin Δ and moisture availability for *Artemisia* (Fig. 4, Table S2), suggest that this taxon's pollen and leaves are produced from pools of photosynthate with Δ values that are controlled by different environmental factors. *Artemisia* species possess deep and shallow roots (Letts *et al.*, 2010). Therefore, such differences in Δ may arise if *Artemisia*'s leaves are more responsive to deep soil moisture replenished in winter and its inflorescences are more responsive to the availability of shallow soil moisture during the summer. However, strong relationships between leaf Δ and precipitation during the winter preceding the collection of each specimen were not observed (Table S3).

An alternative explanation is the temporal separation of vegetative and reproductive growth in *Artemisia*. For example, Evans & Black (1993) showed that *Artemisia tridentata* exhibits little control over water loss during vegetative growth, which primarily occurs in the spring and early summer. This finding is consistent with the observed poor correlation of leaf Δ and spring, as well as summer, moisture availability for *Artemisia* (Fig. 5). By contrast, *Artemisia*'s reproductive growth occurs in summer, when soil moisture is often more limiting, and via inflorescences that fix their own carbon and do not import carbon from leaves (Evans

et al., 1991). Floral leaves and heads of *A. tridentata* given supplemental water during the summer were produced with lower water-use efficiency than those not receiving such water (Evans & Black, 1993), which is consistent with the correlations of sporopollenin Δ and growing-season moisture availability observed in the present study (Fig. 4). Therefore, different strategies of water use by *Artemisia* during the development of its vegetative and reproductive structures at different times of the growing season may account for the stronger correlations between sporopollenin Δ and moisture availability than between leaf Δ and moisture availability.

There are numerous potential applications for $\delta^{13}C$ analysis of pollen from C_3 plants using SWiM-IRMS. Water availability often constrains plant growth in subhumid to semi-arid grasslands, and the determination of Δ from *Ambrosia* and *Artemisia* pollen promises to help to assess the potential influence of plant physiological performance on shifts in grassland community composition. For example, an enigma of the Holocene vegetation history of the northern Great Plains of North America is the high (> 50%) and variable abundance of *Ambrosia* during the overall dry middle-Holocene and the driest phases of the middle- and late-Holocene (e.g. Grimm, 2001; Nelson *et al.*, 2004; Grimm *et al.*, 2011). The overall high abundance of *Ambrosia* is paradoxical because, today, the common *Ambrosia* species of the Great Plains are more abundant in the eastern, wetter part of this region (Grimm, 2001). *Ambrosia* thrives in disturbed areas, and Grimm (2001) and Grimm *et al.* (2011) have proposed that the high abundance of *Ambrosia* during periods of aridity resulted from high interannual variability of summer moisture availability. Recurrent severe droughts within multi-decadal dry periods would have repeatedly disturbed the vegetation, creating favorable conditions for *Ambrosia* during periodic wet summers. Observations from historic droughts in the Great Plains lend support to this hypothesis (Weaver & Albertson, 1936), but no direct paleoecological evidence exists. $\delta^{13}C$ analysis of pollen would enable assessment of the hypothesis of Grimm (2001) and Grimm *et al.* (2011). This hypothesis suggests that Δ of *Ambrosia* pollen was more positive and less variable than that of *Artemisia* within and between multi-decadal droughts, because *Ambrosia* would have grown primarily during wet summers, whereas drought-tolerant *Artemisia* species would also have endured dry conditions.

More generally, carbon isotopic analysis of pollen may be useful for the reconstruction of variations in C_i/C_a and C_i from C_3 plants. Gerhart *et al.* (2012) used $\delta^{13}C$ of *Juniperus* wood, together with ice-core records of C_a, to show that C_i of this species was low during the last glacial period in southern California because of carbon limitation. These results support the hypothesis that C_i/C_a is a metabolic set point that is maintained within taxa (Ehleringer & Cerling, 1995), at least under low C_a. Less certain is the response of C_i/C_a to elevated C_a. $\delta^{13}C$ of C_3 pollen from periods of Earth's history when C_a was > 500 ppm (e.g. Pagani *et al.*, 2011) may be useful for assessment of potential shifts in C_i/C_a under atmospheric CO_2 concentrations expected to occur in Earth's atmosphere within the next century (IPCC, 2007).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 $\delta^{13}C$ data from untreated pollen, chemically treated pollen (sporopollenin) and leaves of specimens from the Rocky Mountain Herbarium

Table S2 r^2 values for ordinary least-squares regressions among sporopollenin Δ and seasonal vapor pressure deficit (VPD), precipitation amount and the Palmer Drought Severity Index (PDSI)

Table S3 r^2 values for ordinary least-squares regressions among leaf Δ and seasonal vapor pressure deficit (VPD), precipitation amount and the Palmer Drought Severity Index (PDSI)

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