**Late-Quaternary variation in C₃ and C₄ grass abundance in southeastern Australia as
inferred from $\delta^{13}\text{C}$ analysis: Assessing the roles of climate, $p\text{CO}_2$, and fire**

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Abstract

Climate, atmospheric $p\text{CO}_2$, and fire all may exert major influences on the relative abundance of C_3 and C_4 grasses in the present-day vegetation. However, the relative role of these factors in driving variation in C_3 and C_4 grass abundances in the paleorecord is uncertain, and C_4 abundance is often interpreted narrowly as a proxy indicator of aridity or $p\text{CO}_2$. We measured $\delta^{13}\text{C}$ values of individual grains of grass (Poaceae) pollen in the sediments of two sites in southeastern Australia to assess changes in the proportions of C_3 and C_4 grasses during the past 25,000 years. These data were compared with shifts in $p\text{CO}_2$, temperature, moisture balance, and fire to assess how these factors were related to long-term variation of C_4 grass abundance during the late Quaternary. At Caledonia Fen, a high-elevation site in the Snowy Mountains, C_4 grass abundance decreased from an average of 66% during the glacial period to 11% during the Holocene, primarily in response to increased $p\text{CO}_2$ and temperature. In contrast, this pattern did not exist in low-elevation savannah woodlands around Tower Hill Northwest Crater, where C_4 grass abundance instead varied in response to shifts in regional aridity. Fire did not appear to have strongly influenced the proportions of C_3 and C_4 grasses on the landscape at millennial timescales at either site. These patterns are similar to those of a recent study in East Africa, suggesting that elevation-related climatic differences influence how the abundance of C_3 and C_4 grasses responds to shifts in climate and $p\text{CO}_2$. These results caution against using C_4 plant abundance as a proxy indicator of either climate or $p\text{CO}_2$ without an adequate understanding of key controlling factors.

Keywords: Australia; atmospheric CO_2 ; C_3 grasses; C_4 grasses; carbon isotopes; climate change; fire.

1. Introduction

Grass-dominated ecosystems (e.g. grasslands, woodlands, and shrublands) cover ~70% of the Australian continent. These ecosystems span a wide range of climatic conditions, from the arid continental interior to mesic environments in coastal regions, from a winter-dominated precipitation regime in the south to a summer-dominated precipitation regime in the north, and from relatively cool and moist conditions at high elevations to warm and dry conditions at lower elevations. These variations in climate have pronounced effects on the productivity and composition of the regional vegetation. At low elevations, vegetation productivity is generally greater where precipitation is higher, whereas cold temperatures tend to limit production at high elevations. C₃ grasses dominate in far southern Australia, and C₄ grasses become dominant toward the north as warm-season precipitation increases (Murphy and Bowman, 2007; Winslow et al., 2003). Within this overall distribution, the abundance of C₃ grasses tends to increase with elevation (Hattersley, 1983), consistent with the well-known effect of elevation-induced changes in climate on the distribution of C₃ and C₄ grasses throughout the world (e.g. Cabido et al., 1997; Livingstone and Clayton, 1980). In addition to climate, the relative productivity of C₃ and C₄ grasses is also responsive to changes in factors such as atmospheric CO₂ concentrations (e.g. Higgins and Scheiter, 2012; Hovenden et al., 2014), temperature (e.g. Hattersley, 1983), and fire (e.g. Lehmann et al., 2014; Ripley et al., 2010). Thus, these ecosystems are projected to be highly sensitive to future environmental changes (IPCC, 2014). Disentangling the relative importance of different potential controls requires long-term data on the relative abundance of C₃ and C₄ grasses and relevant environmental variables.

Existing paleoenvironmental data suggest that site-specific differences in climate have influenced the relative importance of climate and $p\text{CO}_2$ as controls of C₄ plant abundance during

Earth's history (e.g. Edwards et al., 2010; Huang et al., 2001; Huang et al., 2006; Strömberg, 2011). Increase burning, perhaps in association with changes in climate and/or $p\text{CO}_2$, is also thought to be a potential driver of shifts in C_4 grass abundance (e.g. Hoetzel et al., 2013; Osborne and Beerling 2006). However, paleorecords for assessing the response of C_3 and C_4 grasses to changes in climate, $p\text{CO}_2$, and fire during the late Quaternary remain sparse from key parts of the world, such as the Australian continent. Previous reconstructions of C_3 and C_4 plant abundance in southern Australia were based on $\delta^{13}\text{C}$ data from avian eggshells (Johnson et al., 1999; Miller et al., 2005) and leaf waxes (Lopes dos Santos et al., 2013). However, the C_3 and C_4 photosynthetic pathways are used by taxa besides grasses, making it difficult to use such data to assess the abundance of C_3 and C_4 grasses on past landscapes. For example, dietary preference may influence reconstructions from $\delta^{13}\text{C}$ of eggshells, and leaf-wax $\delta^{13}\text{C}$ data may underestimate C_4 grass abundance (relative to C_3 grass abundance) because C_3 woody species have $\delta^{13}\text{C}$ values that are indistinguishable from those of C_3 grasses. Furthermore, standard palynological approaches cannot distinguish pollen of C_3 versus C_4 grasses (Fægri et al., 1989). $\delta^{13}\text{C}$ analysis of grass pollen via Single Pollen Isotope Ratio AnaLysis (SPIRAL) overcomes these challenges by distinguishing C_3 and C_4 grasses in the paleorecord (Nelson et al., 2008; Urban et al., 2013).

Using SPIRAL, Urban et al. (2015) observed striking spatial and temporal variability in the relative influence of temperature, moisture balance, and $p\text{CO}_2$ on C_3 and C_4 grass abundance at high and low elevation sites in equatorial East Africa during the past 25 kyr, with little evidence that fire influenced long-term shifts in C_4 grass abundance. These results imply that in addition to shaping the present-day spatial distribution of C_3 and C_4 grasses, elevation-related climatic variation can also shape the temporal trajectory of shifts in vegetation composition in response to environmental change. That is, generally warm and dry conditions at low elevations

may cause variation in moisture to have greater effect on C₄ grass abundance than at high elevations, where generally cool and wet conditions cause changes in temperature and/or $p\text{CO}_2$ to have greater influence. This hypothesis has implications for projecting shifts in the abundances of C₃ and C₄ grasses to ongoing environmental change, as well as understanding the drivers of past vegetation changes, but its generality remains untested.

Here we use SPIRAL to reconstruct variation in C₃ and C₄ grass abundance at high and low elevation sites in southeastern Australia during the last 25 kyr. Our evaluation of the environmental controls of C₄ grass abundance is placed within the context of charcoal data from each site, along with records of $p\text{CO}_2$ (Monnin et al., 2001) and air temperature from Antarctica (Petit et al., 1999), the latter recording broad-scale temporal changes in temperature in the Southern Hemisphere (Fletcher et al., 2010; Petherick et al., 2013; Lopes dos Santos et al., 2013; Reeves et al., 2013). We also compared our data with interpretations of shifts in moisture availability based on ostracode and diatom assemblages at Tower Hill (D'Costa et al., 1989) and other vegetation-independent proxies from the region (e.g. Kemp et al., 2012; Marx et al., 2011; McGowan et al., 2008). Our results provide a context for assessing the extent to which elevation-induced environmental variation influences temporal variability of C₃ and C₄ grass abundance.

2. Materials and Methods

2.1. Study sites

Caledonia Fen (37°20'S, 146°44'E; 1280 m a.s.l.) is situated in the Snowy Plains region of the Australian Alps. The fen has a surface area of ~15 ha and is presently drained by a small, intermittent outlet (Fig. 1). The region experiences a temperate climate with a mean annual

temperature (MAT) of ~7°C and mean annual precipitation (MAP) of ~1530 mm/yr (Kershaw et al., 2007). The majority of precipitation falls during the winter from predominant westerly winds, whereas southward expansion of high pressure during the summer results in hot and dry conditions. The site is located at the upper limit of montane forest dominated by *Eucalyptus delegatensis* in moist areas and other eucalypts (e.g., *E. dalrympleana*, *E. rubida*) in drier areas. The understory of these forests consists of small trees, woody shrubs, grasses, herbs, and ferns. On higher slopes the forests become more open and are replaced by sub-alpine woodland with a predominantly grassy ground cover. At present, <10% of the grass species in the mountainous region of southeastern Australia use C₄ photosynthesis, in terms of species richness and biomass (Hattersley, 1983). Aquatic grasses do not occur at the site or within the region today.

Northwest Crater (NWC; 38°19'S, 142°22'E; 20 m a.s.l.) is a Cyperaceae-dominated swamp with a surface area of <1 ha located within a steep sided cinder cone of the inactive Tower Hill volcano. The local MAT is 13°C and MAP is 720 mm/yr (D'Costa et al., 1989). Seasonal precipitation patterns in the region are similar to those at Caledonia Fen. The regional vegetation consists mostly of open savannah woodlands dominated by *Allocasuarina verticillata* and various *Eucalyptus* species with scattered grasslands in the drier areas. Presently the slopes of NWC are dominated by *Pteridium* and introduced grasses along with a variety of herbaceous species, while on the crater rim *Allocasuarina verticillata*, *Acacia melanoxylon*, *Eucalyptus ovata* and *E. viminalis* dominate (D'Costa et al., 1989). In the western plains of Victoria today, 10-30% of grasses are C₄ (Hattersley, 1983). Unlike high elevation sites, such as Caledonia Fen, *Phragmites* does occur in the lowlands of southeastern Australia today. However, there is no *Phragmites* or other aquatic or wetland grasses on Tower Hill today, and even if *Phragmites* had occurred during the past its pollen is not thought to preserve well (Kershaw, 1979).

2.2. Coring, chronology, and sources of environmental data

A 19.06 m sediment core was obtained from a *Sphagnum* hummock on Caledonia Fen in 2001 using a portable percussion-drilling rig (Kershaw et al., 2007). The top 280 cm represented the past ~25,000 years. However, the upper meter of the 2001 core was poorly recovered because of its highly organic nature. Therefore, an additional one-meter core was taken in 2009 using a Russian peat corer. The 2001 core and upper portion of the 2009 core were stratigraphically aligned based on laminations and an abrupt (within ~2 cm) transition from silty mud to peat. Terrestrial plant macrofossils (Table 1) were isolated from six subsamples from the 2009 core and one subsample from the 2001 core and treated with an acid-base-acid procedure (Oswald et al., 2005) prior to AMS ^{14}C dating. All radiocarbon ages (including four that were previously obtained from the 2001 core; Table 1) were converted to calibrated years before 1950 (kyr BP) using CALIB 6.0.1 (Stuiver and Reimer, 2011) with the IntCal09 data set (Reimer et al., 2009). An age–depth model was created with a weighted cubic smoothing spline derived from 1000 bootstrapped samples from the calibrated age distributions using the program MCAgeDepth 0.1 in MATLAB (Higuera et al., 2009). We compared our new C_4 -grass abundance and charcoal data from Caledonia Fen with previously published pollen assemblage data from this site (Kershaw et al., 2007).

A sediment core of ~15.5 m total length was obtained from Tower Hill NWC in 2008 using a modified Livingstone piston sampler. Terrestrial plant macrofossils (Table 1) were isolated from twelve subsamples for AMS ^{14}C dating, and an age-depth model was constructed using the same procedures as for Caledonia Fen. We compared our estimates of C_4 -grass abundance and charcoal data from the 2008 Tower Hill NWC cores using the chronology we

developed as part of the present study with previously published pollen assemblage data from different cores from this site that have their own chronology (D'Costa et al., 1989). The shape of our age-depth model for Tower Hill NWC is highly similar to that of D'Costa et al. (1989) for this site, as are trends in the accumulation rates of microscopic charcoal from the different cores, and thus we assume that there is little offset between the data we present from the 2008 cores and from the cores used in D'Costa et al. (1989).

2.3. Treatment of samples for $\delta^{13}C$ analysis

Carbon-isotope analysis of individual grass-pollen grains followed the protocol described by Nelson et al. (2007, 2008). Briefly, we extracted pollen from sediment samples (~1 cm³ each) from Caledonia Fen (30 samples) and Tower Hill NWC (25 samples) following standard techniques (Fægri et al., 1989), with modifications to exclude carbon-containing compounds (Nelson et al., 2006). The protocol involved successive treatments with 10% HCl, 10% KOH, concentrated HF, and 10% HCl. We washed the residue onto a 74 µm sieve and treated what passed through the sieve with 50% H₂SO₄. We then washed that residue onto a 12 µm sieve, and treated what remained on the sieve with 5% Chlorox. Following each chemical treatment, the residue was washed thoroughly with nano-pure water.

Grass (Poaceae) pollen was isolated from a pollen-water slurry at 200x magnification using an Eppendorf Transferrman micromanipulation device. Sedge (Cyperaceae) pollen is morphologically distinct from Poaceae pollen, and we did not isolate or analyze Cyperaceae pollen in the present study. Individual grass pollen grains were rinsed in nano-pure water and transferred to ~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

ThermoFinnigan Delta V isotope ratio mass spectrometer (IRMS) using a glass syringe. The total number of individual grains of grass pollen applied to the SWiM device ranged from 95 to 142 per sample. We also analyzed blanks, which consisted of ~0.4 μL drops of nano-pure water to which single grains of grass pollen were added and then removed. The carbon content of blanks was assessed as CO_2 yields in volt-second (Vs, peak area) units, and the $\delta^{13}\text{C}$ value of blanks was determined as described in Nelson et al. (2007).

The mean plus 2 standard deviations (2σ) of blank CO_2 yields was used as a minimum size threshold; grains below this threshold were excluded from further analysis. The final $\delta^{13}\text{C}$ data were blank-corrected using isotopic mass balance and normalized to VPDB (Urban et al., 2013). These $\delta^{13}\text{C}$ 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. 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 and 95% confidence intervals of C_3 and C_4 grass pollen in a sample of unknown composition from the measured $\delta^{13}\text{C}$ values of grass pollen in each sample, as described in Urban et al. (2013). We attempted to obtain ~50 grains above the blank size threshold from each sample, as prior results indicate that the Bayesian model reliably predicts the relative abundance of C_4 grass pollen from $\delta^{13}\text{C}$ values of ≥ 50 grains (Urban et al. 2013). Because of uncertainty (i.e. imprecision) associated with estimating the relative abundance of C_3 - and C_4 -grass pollen in individual samples (Table S1, Urban et al., 2013), we focus our interpretation on the long-term trends in C_4 -grass pollen abundance, as represented by a 3-point running average of the respective % C_4 -grass time series.

2.4. Charcoal analysis

Low-resolution microscopic charcoal data were previously reported for Caledonia Fen (~540 yr sample resolution; Kershaw et al. 2007) and Tower Hill NWC (~390 yr sample resolution; D'Costa et al. 1989). We performed higher-resolution analyses of microscopic charcoal, as well as of macroscopic charcoal, in the present study. Sediment subsamples for macroscopic (>180 μm) and microscopic (11-180 μm) charcoal analysis were prepared as previously described (Nelson et al., 2012). The sampling intervals corresponded to ~50-60 and ~100-200 year resolution for macroscopic and microscopic charcoal, respectively, for Caledonia Fen and ~10-20 and ~250 year resolution, respectively, for Tower Hill NWC. Charcoal pieces were counted with either a dissecting (macroscopic) or a compound (microscopic) microscope. The microscopic charcoal data were standardized using a *Lycopodium* pollen tracer of known concentration. Raw microscopic and macroscopic charcoal counts were converted to charcoal accumulation rates (CHAR; particles $\text{cm}^{-2} \text{yr}^{-1}$).

2.5. Statistical analysis

Regression analysis was used to evaluate relationships between C₄ grass abundance (%) and variations in climate, $p\text{CO}_2$, and fire. Prior to regression, all datasets were interpolated and re-sampled to the dataset with the lowest temporal resolution (% C₄ grass pollen at both sites). Regression diagnostics were used to evaluate model assumptions and to identify outlier data points. We used a block bootstrap to account for temporal autocorrelation in the relationships between C₄ grass abundance, climate, and fire (Tian et al., 2011). These analyses were performed in R (R Development Core Team, 2013).

3. Results

The chronology used for Caledonia Fen in the present study is based on the stratigraphically aligned portions of the 2001 and 2009 cores obtained from this site. This chronology includes five AMS ^{14}C ages on organic sediment or plant remains from the 2001 core (one of which was obtained as part of the present study) and six AMS ^{14}C ages that we obtained on plant remains from the 2009 core (Table 1; Fig. 2). All dates are in stratigraphic order, with the exception of two dates from overlapping depths (77-78.5 cm and 77-81 cm; the former date was obtained as part of the present study from the 2009 core and the latter date obtained as part of the Kershaw et al. 2007 study from the 2001 core). All dates were included in the age-depth model. Sedimentation rates were low and stable (average: 0.005 cm/yr) between 25 and ~14 kyr BP. Between 14 and 12 kyr BP there was a relatively rapid deposition of ~50 cm of sediment. After this period, sedimentation rates decreased (average: 0.02 cm/yr), with the exception of a rapid increase near present.

The chronology for Tower Hill NWC is based on 12 AMS ^{14}C ages on terrestrial plant macrofossils (Table 1; Fig. 2). All dates are in stratigraphic order. Sedimentation rates were relatively constant (average: 0.07 cm/yr) between 25 and ~6 kyr BP, after which they generally increased (average: 0.13 cm/yr).

The number of grass-pollen grains applied to the spooling wire with peak areas exceeding the 2σ threshold of blank varies between 45 and 71 (Fig. S1 and Table S1). On average, 38% of applications yield a peak area above the blank threshold, consistent with previous studies using SPIRAL (e.g., Nelson et al., 2007; Nelson et al., 2008). Samples below the blank threshold may indicate that the pollen grain fell off the wire before reaching the combustion oven or that pollen grains in these samples were smaller than the 2σ threshold and thus indistinguishable from

blanks. The majority (82%) of the 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 (Cerling, 1999). Values more negative than -34‰ or more positive than -10‰ likely indicate inadequate analytical precision and uncertainty in the isotopic composition and variability of blanks (Nelson et al. 2007). Samples with such values in the present study were included in calculations of the relative abundance of C_3 and C_4 grass pollen in samples of unknown composition because such values also occur in our training dataset (Urban et al. 2013).

C_4 grass abundance at Caledonia Fen, as indicated by the SPIRAL data, declines from an average of ~90% at 24.0 kyr BP to <50% at 20.5 kyr BP. It then increases to >70% at 16.0 kyr BP before declining to <10% at 9.0 kyr BP, with the exception of a short increase centered on 12.5 kyr BP in the raw data. C_4 grass abundance remains <15% during the remainder of the Holocene (Fig. 3a) with the exception of a peak of ~40% centered on ~5.8 kyr BP. During the last 25 kyr, C_4 -grass abundance at Caledonia Fen is negatively correlated with air temperature in Antarctica ($r = -0.88$, $p < .001$, $n = 30$) and $p\text{CO}_2$ ($r = -0.86$, $p < 0.001$, $n = 30$; Fig. 4).

Before 16.0 kyr BP, macro-CHAR at Caledonia Fen remains generally low with a small peak at ~19.5 kyr BP. Macro-CHAR values are high and variable between ~16.0 and 12.0 kyr BP, low between 12.0 and 9.0 kyr BP, and then high and variable between 9.0 and 0.0 kyr BP. During the glacial period, micro-CHAR displays peak values around ~23.0-22.0 kyr BP and ~18.0-17.0 kyr BP. It exhibits a gradual increasing trend to peak values around 7.0 kyr BP. It is then high and variable before declining after 1.5 kyr BP (Fig. 3a). C_4 -grass abundance is negatively correlated with macro-CHAR ($r = -0.45$, $p = 0.012$, $n = 30$) and micro-CHAR ($r = -0.70$, $p < 0.001$, $n = 27$) at Caledonia Fen (Fig. 4).

C₄ grass abundance at Tower Hill NWC varies between an average of 35 and 59% before 16.0 kyr BP, but with high sample-to-sample variability in the raw data, including a peak centered on 16.0 kyr BP. There is a peak of >70% at 12.0 kyr BP followed by a decline to <10% at 8.0 kyr BP. C₄ grass abundance then increases to values of >60% at ~5.0 kyr BP, followed by a variable but decreasing trend, reaching ~20% at present (Fig. 3b). C₄-grass abundance at Tower Hill NWC is not correlated with air temperature in Antarctica or *p*CO₂ (Fig. 4).

At Tower Hill NWC, macro-CHAR and micro-CHAR display similar stratigraphic patterns. They exhibit low values prior to ~10.0 kyr BP and then gradually increase until ~6.0 kyr BP, after which they both increase more quickly. Macro-CHAR reaches a peak at ~2.0 kyr BP and then declines, whereas micro-CHAR continues to increase steadily until the present. C₄ grass abundance is negatively correlated with macro-CHAR (*r* = -0.71, *p* < 0.001, *n* = 25), but is not correlated with micro-CHAR (*r* = -0.36, *p* = 0.13, *n* = 25; Fig. 4).

4. Discussion

The SPIRAL $\delta^{13}\text{C}$ values of grass pollen from the youngest sediments analyzed from Caledonia Fen (510 years BP; 95% confidence interval = 4-32%) and Tower Hill NWC (68 years BP; 95% confidence interval = 0-45%; Table S1) are broadly consistent with the ranges the C₄ grass abundance expected on the landscape at these sites today (0-10% at Caledonia Fen and 10-30% at Tower Hill NWC; Table S1). These results suggest that SPIRAL $\delta^{13}\text{C}$ data reflect the abundance of C₄ grasses on the landscape, and that excluding $\delta^{13}\text{C}$ values from samples below the blank size threshold does not appear to introduce bias into estimates of the relative abundance of C₄ grasses, as previously demonstrated with data from the surface sediments of 24 lakes in Africa, North America, and Australia (Urban et al., 2013). The significant positive

relationship (that did not differ from a 1:1 relationship) between the Bayesian estimates of % C₄ grass pollen in the surface-sediment samples and C₄-grass abundance around the sites analyzed in Urban et al. (2013) also indicates no consistent bias in the representation of C₃ and C₄ grasses that could otherwise be associated with factors such as differences in the size, productivity or preservation of pollen from C₃ and C₄ grasses.

The SPIRAL $\delta^{13}\text{C}$ data from both of our study sites suggest large variation in C₄ grass abundance during the past 25 kyr and thus that C₃ grasses have not consistently dominated over C₄ grasses in southeastern Australia to the extent that they do today. Although a portion of the high sample-to-sample variability can be attributed to uncertainty in estimating C₄ grass abundance based on grass-pollen $\delta^{13}\text{C}$ distributions in individual samples (Table S1, Urban et al. 2013), long-term patterns are robust (Nelson et al., 2014; Urban et al., 2010; Urban et al., 2015). At Caledonia Fen, the relatively high proportions of C₄ grasses prior to ~15.0 kyr BP were followed by a declining trend and then relatively low proportions of C₄ grasses during the Holocene. In contrast, at Tower Hill NWC, C₄ grass abundance varied greatly during the past 25 kyr BP but did not decrease from the glacial period to the Holocene. These results suggest that climate and atmospheric $p\text{CO}_2$ have been the primary drivers of fluctuations in the proportions of C₃ and C₄ grasses during the last 25 kyr in southeastern Australia. However, the relative importance of these factors differed between low and high elevations and through time. At high elevation on the Snowy Mountains, C₄ grass abundance was high during the glacial period followed by steady decrease towards the Holocene as air temperature in Antarctica and $p\text{CO}_2$ increased (Figs. 3-4). The ability of C₄ grasses to concentrate CO₂ and use water more efficiently than C₃ grasses (Ehleringer, 1978) likely provided C₄ grasses with a competitive advantage during the glacial period when $p\text{CO}_2$ was low and increased aridity occurred across much of

Australia (Ehleringer, 1978; McGowan et al., 2008). Although C₄ grasses are typically more common than C₃ grasses at higher temperatures, low $p\text{CO}_2$ during the glacial period likely allowed C₄ grasses to remain competitive at lower temperatures than today (Boom et al., 2002). Thus, to the extent that the air temperature data from Antarctica faithfully record the broad temporal trends in temperature in our study region (Fletcher et al, 2010; Petherick et al., 2013; Lopes dos Santos et al., 2013; Reeve et al. 2013), the negative correlation of C₄ grass abundance with temperature likely occurs because temperature co-varied with $p\text{CO}_2$, and changes in $p\text{CO}_2$ had greater relative influence on C₄ grass abundance. The specific cause of the short-term fluctuations in C₄ grass abundance at Caledonia Fen is uncertain, although some variation could be related to shifts in moisture balance.

In the lowland western Victoria Plains around Tower Hill NWC, C₄ grass abundance was not related to glacial-to-interglacial changes in air temperature in Antarctica and $p\text{CO}_2$. Instead, C₄ grass abundance appeared to have been responsive to shifts in regional aridity. C₄ grass abundance was moderately high (but with high sample-to-sample variability) during the generally dry conditions of the late glacial period in southeastern Australia (Fig. 3). Aridity further increased to a maximum between 14.0 and 10.0 kyr BP (D'Costa et al., 1989; Kemp et al., 2012; Marx et al., 2011) coincident with increased C₄ grass abundance. C₄ grass abundance declined to its lowest values of the record between 10.0 and 6.0 kyr BP, coincident with much wetter conditions. It then increased with decreased moisture availability between 6.0 and 2.0 kyr BP and finally declined toward the present as moisture availability in the region again increased. In contrast to Tower Hill NWC, the lack of shifts in C₄ grass abundance in response to changes in aridity during the Holocene at Caledonia Fen when $p\text{CO}_2$ was relatively stable may occur because moisture availability remained high and less limiting at higher elevations. In addition to

the potential elevation-related differences in the direct influences of climate and $p\text{CO}_2$ on the proportions of C_3 and C_4 grasses, C_4 grasses have higher light requirements than C_3 grasses, which causes C_4 grasses to become less common than C_3 grasses in shaded environments (Sage et al., 1999). Thus, increased shading caused by the relative high abundance of woody species at Caledonia Fen during the Holocene (Fig. 3) may have also helped C_3 grasses outcompete C_4 grasses at high elevation.

SPIRAL $\delta^{13}\text{C}$ -based estimates of the abundances C_3 and C_4 grasses in the regional vegetation may be compromised if grasses are a major component of the local aquatic or wetland vegetation. At our sites, we interpreted the SPIRAL $\delta^{13}\text{C}$ records as primarily reflecting shifts in the abundances of terrestrial grasses for three reasons. First, Poaceae pollen in late-Quaternary pollen records from southeastern Australia is thought to derive predominantly from regional vegetation (Kershaw 1998). Second, there is no evidence of wetland C_3 grasses at either site today. Third, if C_3 wetland grasses were important at either site during the past they would likely have expanded in abundance in association with the transition of each site from a lake to a fen or swamp. Such a transition occurred at Caledonia Fen around 11.5 kyr BP (Kershaw et al. 2007), but the overall abundance of grasses (C_3 and C_4 combined) generally declined from values of >40% before the glacial-interglacial transition to <10% during the Holocene as *Eucalyptus* increased in abundance (Fig. 3; Kershaw et al., 2007). Pollen assemblages suggest that the major shift to a sedge-dominated environment at Tower Hill NWC did not occur until between 8 and 5 kyr BP (Fig. 3), which is coincident with increased C_4 grass abundance at Tower Hill NWC and opposite of the expectation if C_3 wetland grasses expanded in association with the development of the swamp.

$\delta^{13}\text{C}$ data from emu (*Dromaius novaehollandiae*) eggshells from the Lake Eyre Basin

(Johnson et al., 1999) and leaf waxes in sediments offshore the Murray River Basin (Lopes dos Santos et al., 2013) suggest that C₄ plant abundance was slightly higher during the Holocene than glacial period, and thus that the summer monsoon may have been more effective during the Holocene than glacial period in southeastern Australia (Petherick et al., 2013). These temporal patterns differ from, and are more muted than, those at Caledonia Fen and Tower Hill NWC (Fig. 3). There are at least two likely reasons for such discrepancies. First, $\delta^{13}\text{C}$ data from leaf waxes from marine sediments, and to some extent $\delta^{13}\text{C}$ data from the eggshells of emus, which move across the landscape, likely reflect an integration of C₄ plant abundance over a larger area than do $\delta^{13}\text{C}$ data from grass pollen from small deposition basins, such as our sites. Second, the $\delta^{13}\text{C}$ data from eggshells and leaf waxes record the overall abundance of C₃ and C₄ plants in emu diets and sedimentary leaf waxes, whereas $\delta^{13}\text{C}$ data from grass pollen specifically record the proportions of C₃ and C₄ grasses on the landscape.

Increased burning is hypothesized to have caused the expansion of C₄ grasses in Earth's history (e.g. Hoetzel et al., 2013). Fires also influence the abundance of C₄ grasses on the landscape today (Bond et al., 2005; Ripley et al., 2010). We observed no positive relationships between C₄ grass abundance and micro-CHAR or macro-CHAR at either of our study sites at millennial timescales, suggesting that there is not a direct linkage between long term increases in burning and increased proportions of C₄ grasses at our sites. Rather, micro-CHAR at Caledonia Fen and macro-CHAR at both sites displayed a negative relationship with C₄ grass abundance (Fig. 4). We interpret these negative relationships to result from the effect of the expansion of fire-tolerant and highly flammable *Eucalyptus* trees, as indicated by pollen data (Kershaw et al., 2007), on fire regimes at both sites. Charcoal accumulation rates were typically low at both sites during the glacial period, probably because the landscape was sparsely vegetated as the result of

cold and generally dry climatic conditions that limited fuel production (Fig. 3; Kershaw et al., 2007; Mooney et al., 2011). The increase in the abundance of *Eucalyptus* at Caledonia Fen after ~11.5 kyr BP is associated with higher micro-CHAR and macro-CHAR despite decreased abundance of C₄ grasses (Fig. 3). At Tower Hill NWC, macro-CHAR increased and *Eucalyptus* began to replace the fire-sensitive *Casuarina* (D'Costa et al. 1995) around ~9.0 kyr BP, but C₃ grasses were relatively abundant (Fig. 3). These results suggest that fire is not the main direct control of the long-term (i.e. millennial-scale) balance between C₃ and C₄ grasses in southwestern Australia during the late Quaternary. Similarly, a recent comparative experiment concluded that evolutionary and environmental history have a greater influence than photosynthetic pathway on the response of grasses to fire (Ripley et al., 2015), and thus that a direct relationship between increased burning and expansion of C₄ grasses may not always occur.

Our results reveal spatiotemporal variation in the relative influence of moisture balance, temperature, and *p*CO₂ on C₄ grass abundance during the late Quaternary, resulting from elevation-related spatial variation in the regional climate of southeastern Australia. These results are strikingly similar to those from equatorial East Africa during the past 25 kyr (Urban et al., 2015). Specifically, Urban et al. (2015) also found that moisture exerted a greater influence than temperature and *p*CO₂ on the proportions of C₄ grasses at a low elevation site and that long-term shifts in C₄ grass abundance were more strongly controlled by changes in *p*CO₂ and/or temperature than moisture balance at a high elevation site. Low elevation sites in southeastern Australia and East Africa exhibit largely anti-phased shifts in the proportions of C₄ grasses (Fig. 5), probably because these regions experienced generally opposite patterns of changes in moisture balance during the past 25 kyr.

Overall, our results reinforce the notion that fire did not exert a strong direct influence on spatiotemporal variation in the relative abundance of C₃ and C₄ grasses at multi-centennial and millennial timescales (Urban et al., 2015). Furthermore, they challenge the idea that *p*CO₂ was the main driver of C₃ and C₄ grass abundance during the late Quaternary (Ehleringer et al., 1997; Street-Perrott et al., 1997). Instead, our data support studies from the late Quaternary (e.g. Huang et al., 2001; Huang et al., 2006) and Miocene (Edwards et al., 2010; Strömberg, 2011) that suggest that site-specific differences in climate have influenced the relative importance of climate and *p*CO₂ as controls of C₄ plant abundance during Earth's history. Our results, in conjunction with those of Urban et al. (2015) from East Africa, illustrate how elevation-associated climatic differences have an overarching influence on the relative importance of different drivers of C₄ grass abundance through time. These results suggest that spatial heterogeneity in the patterns of shifts of C₄ grass abundance in Earth's history occurred because of variation in environmental drivers across space and through time (Strömberg, 2011). They also caution against using C₄ plant abundance as a proxy indicator of climate or *p*CO₂ in paleoenvironmental studies without adequate knowledge of the environmental controls of C₃/C₄ dynamics at any particular study site.

C₃ grasses currently dominate over C₄ grasses at both high and low elevation in southeastern Australia. However, their abundance has fluctuated greatly throughout the past 25 kyr as the result of spatiotemporal variability in different environmental factors. This region has experienced significant drying during the past century, and this trend is expected to continue in the coming decades as *p*CO₂ increases and anthropogenic warming continues (CSIRO, 2010). Our results suggest that increased regional aridity will more likely increase C₄ grass abundance at low than high elevations, whereas rising *p*CO₂ will more likely favor C₃ grasses at high

elevations. Shifts in vegetation composition may result from changes in climate and $p\text{CO}_2$, but the effects of such environmental changes on C_4 grass abundance are likely to vary across locations and through time.

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Figure Legends

Figure 1. (A) Map of the distribution of major pre-European (i.e., pre-1750) vegetation types (<http://www.environment.gov.au/land/native-vegetation/national-vegetation-information-system>), seasonal precipitation (bold lines; Magee et al., 2004) and C_4 grass abundance in Australia (dashed lines; Hattersley, 1983). (B) Southeastern Australia showing the locations of Tower Hill and Caledonia Fen.

Figure 2. Age-depth models for (A) Caledonia Fen and (B) Tower Hill NWC calculated using a cubic spline. Diamonds represent ^{14}C ages and the error bars on each age represent the 95%

ranges of the calibrated dates. The thick lines represent the mean ages and the thin lines the 95% confidence intervals based on the bootstrapping procedure described in the text.

Figure 3. C₄ grass pollen abundance (diamonds= raw data points; curves are 3-sample moving averages) in comparison with pollen percentages of dominant groups, atmospheric *p*CO₂, and air temperatures at Caledonia Fen (A) and Tower Hill NWC (B). Sources of data in (A): pollen percentages (Kershaw et al., 2007), micro-CHAR and macro-CHAR data (micro: 5-sample moving averages, macro: 350 year loess smooth; this study). Sources for data in (B): pollen percentages (D’Costa et al., 1989), micro-CHAR and macro-CHAR data (5-sample moving averages; this study). Sources of data in (A) and (B): atmospheric *p*CO₂ (Monnin et al., 2001) and Vostok dome air temperatures (Petit et al., 1999). The *p*CO₂ curves are plotted on the updated gas-age chronology of Lemieux-Dudon et al. (2010) for the period 22–10 kyr BP, and Loulergue et al. (2007) for the period 9.5–0 kyr BP. For reference, the gray shading indicates Heinrich events 2 and 1 (H2 and H1) and the Antarctica Cold Reversal (ACR) event. Hatched shading indicates periods of decreased aridity in the region as inferred from vegetation-independent proxies (e.g. Kemp et al., 2012; Marx et al., 2011; McGowan et al., 2008).

Figure 4. Relationships between % C₄-grass pollen and environmental variables at (A) Caledonia Fen and (B) Tower Hill NWC based on resampled time series. Three outlier datapoints from micro-CHAR at Caledonia Fen (red data points) were excluded from the regression. The sources of the *p*CO₂ and temperature data are provided in the legend of Figure 3.

Figure 5. Comparison of SPIRAL based C₄-grass pollen abundance records from southeastern Australia (black curves) and East Africa (gray curves). Lake Challa (840 m a.s.l) is a large crater lake southeast of Mt. Kilimanjaro (Verschuren et al., 2009) and Lake Rutundu is a high elevation (3000 m a.s.l) lake located on Mt. Kenya. The African records are from Urban et al. (2015).

Table 1. ¹⁴C dates for Caledonia Fen and Tower Hill NWC.

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Figure 1

Figure 2

Figure 3

Figure 4

A) Caledonia Fen

B) Tower Hill NWC

Figure 5

Table 1. ^{14}C dates from Tower Hill NWC and Caledonia Fen

Lab number	Depth (cm)	^{14}C date, year B.P.	Calibrated age	Material
Tower Hill NWC				
CAMS-157116	26-27	1730 $\pm$ 40	1640 (1550-1752)	wood, leaf material
CAMS-157117	208-209	4030 $\pm$ 30	4484 (4429-4605)	charcoal, leaf material
CAMS-157118	431-433	4945 $\pm$ 30	5666 (5608-5735)	charcoal, wood
CAMS-157119	506-508	5270 $\pm$ 30	6059 (5945-6174)	leaf material
CAMS-157120	649-650	6225 $\pm$ 45	7128 (7003-7250)	charcoal, leaf material
CAMS-157121	789-790	8900 $\pm$ 110	9984 (9629-10226)	wood
CAMS-157122	910-911	10895 $\pm$ 30	12757 (12641-12905)	leaf material
CAMS-157123	1138-1139	14250 $\pm$ 180	17349 (16930-17842)	seed, charcoal, wood
CAMS-157124	1240-1241	16240 $\pm$ 140	19378 (18942-19792)	charcoal, leaf material
CAMS-157125	1430-1432	18610 $\pm$ 250	22164 (21536-23027)	leaf material
CAMS-157126	1504-1505	20720 $\pm$ 1460	24835 (21341-28247)	wood, leaf material
CAMS-157127	1543-1544	20870 $\pm$ 230	24878 (24349-25597)	charcoal
Caledonia Fen				
CAMS-157180	20.5-21.5	150 $\pm$ 30	155 (6-278)	seed, leaf material
CAMS-157181	38-39.5	1630 $\pm$ 60	1523 (1394-1685)	charcoal, wood
CAMS-157182	58-59.5	5530 $\pm$ 50	6334 (6226-6414)	charcoal
CAMS-158767	63-64.5	6265 $\pm$ 35	7207 (7047-7261)	charcoal, leaf material
CAMS-157183	76-78	8500 $\pm$ 45	9504 (9446-9539)	leaf material
CAMS -158768	77-78.5	9040 $\pm$ 30	10215 (10185-10240)	charcoal, leaf material
GX6515*	77-81	8495 $\pm$ 460	9433 (8406-10600)	Organic sediment
OZ493*	114-118	10300 $\pm$ 280	12007 (112226-12655)	Organic sediment
CAMS-157115	177.5-179.5	12970 $\pm$ 160	15655 (15016-16475)	leaf material
OZ495*	216-220	20900 $\pm$ 420	24969 (23959-26069)	organic clay/silt
OZ792*	226-230	22110 $\pm$ 260	26566 (25977-27555)	plant remains

* radiocarbon dates from Kershaw et al., 2007

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