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Physiological advantages of C_4 grasses in the field: a comparative experiment demonstrating the importance of drought

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Abstract

Global climate change is expected to shift regional rainfall patterns, influencing species distributions where they depend on water availability. Comparative studies have demonstrated that C_4 grasses inhabit drier habitats than C_3 relatives, but that both C_3 and C_4 photosynthesis are susceptible to drought. However, C_4 plants may show advantages in hydraulic performance in dry environments. We investigated the effects of seasonal variation in water availability on leaf physiology, using a common garden experiment in the Eastern Cape of South Africa to compare 12 locally occurring grass species from C_4 and C_3 sister lineages. Photosynthesis was always higher in the C_4 than C_3 grasses across every month, but the difference was not statistically significant during the wettest months. Surprisingly, stomatal conductance was typically lower in the C_3 than C_4 grasses, with the peak monthly average for C_3 species being similar to that of C_4 leaves. In water-limited, rain-fed plots, the photosynthesis of C_4 leaves was between 2.0 and 7.4 μmol m^{-2} s^{-1} higher, stomatal conductance almost double, and transpiration 60% higher than for C_3 plants. Although C_4 average instantaneous water-use efficiencies were higher (2.4–8.1 mmol mol^{-1}) than C_3 averages (0.7–6.8 mmol mol^{-1}), differences were not as great as we expected and were statistically significant only as drought became established. Photosynthesis declined earlier during drought among C_3 than C_4 species, coincident with decreases in stomatal conductance and transpiration. Eventual decreases in photosynthesis among C_4 plants were linked with declining midday leaf water potentials. However, during the same phase of drought, C_3 species showed significant decreases in hydrodynamic gradients that suggested hydraulic failure. Thus, our results indicate that stomatal and hydraulic behaviour during drought enhances the differences in photosynthesis between C_4 and C_3 species. We suggest that these drought responses are important for understanding the advantages of C_4 photosynthesis under field conditions.

Keywords: C_3 photosynthesis, C_4 photosynthesis, drought, gas exchange, PACMAD, Poaceae, stomatal conductance, water potential

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Introduction

C_4 photosynthesis is a fascinating example of a complex phenotype that has evolved repeatedly (Sage *et al.*, 2011), and influences a suite of ecophysiological traits that determine plant performance in natural settings (Long, 1999). Today, C_4 grasses are vital as agricultural crops (e.g., maize and sugarcane) and dominate the ground cover over large areas of Africa, Australia, South Asia and the Americas (Edwards *et al.*, 2010). The role of climate in determining the relative performance of C_4 and C_3 species from both monocot and eudicot lineages is, therefore, a key question in studies of global change (Epstein *et al.*, 1997; Murphy & Bowman, 2006; Arnone *et al.*, 2011; Morgan *et al.*, 2011).

The principal physiological innovation common to C_4 lineages is the development of a biochemical CO_2 pump that operates as an extension of the dark reactions of photosynthesis (Hatch & Osmond, 1976). The C_4 pump elevates CO_2 concentrations in photosynthesising chloroplasts, virtually eliminating O_2 competition for the active site of Rubisco and therefore photorespiration, while in C_3 plants, photorespiration limits net CO_2 assimilation (*A*) at higher temperatures and low partial pressures of CO_2 (Osmond *et al.*, 1982). The efficient delivery of CO_2 to Rubisco in C_4 plants improves photosynthetic efficiency at high temperatures, but bears an energetic cost that limits the maximum efficiency of photosynthesis in C_4 species at low temperatures (Ehleringer & Pearcy, 1983). The initial CO_2 assimilation step in C_4 plants, which is catalyzed by PEP-carboxylase in combination with carbonic anhydrase, also has a higher affinity for its substrate than that

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of C_3 plants. This generates the CO_2 concentrating effect of the C_4 pump and, in combination with the increased assimilation rates driven by the pump, means that C_4 leaves are able to maintain higher A at lower internal CO_2 concentrations (Collatz *et al.*, 1992). The rates of supply of CO_2 to the intercellular spaces of leaves and the loss of water through transpiration (E) are intrinsically linked, and water use can be limited by reducing stomatal conductance (g_s; Raschke, 1975). C_4 photosynthesis therefore has important consequences for leaf water-use efficiency, i.e. net CO_2 assimilation per unit of water loss, a key observation noted in the earliest studies of C_4 ecophysiology (Black *et al.*, 1969; Bjorkman, 1971).

Despite the important consequences of C_4 physiology for water use, until recently the primary ecophysiological explanation for C_4 grass species distributions was considered to be growing season temperature (Teeri & Stowe, 1976). For C_4 eudicots, however, adaptation to arid environments has long been accepted as important in shaping species distributions (Ehleringer & Monson, 1993; Ehleringer *et al.*, 1997). For the grass family (Poaceae), within which the majority of C_4 species and 30% or more of C_4 evolutionary origins occur (Sage, 2009; Sage *et al.*, 2011), habitat water availability and plant hydraulics are receiving renewed attention for their role determining the evolutionary success and distribution of C_4 species (Edwards & Still, 2008; Osborne & Freckleton, 2009; Edwards & Smith, 2010; Osborne & Sack, 2012; Pau *et al.*, 2012; Taylor *et al.*, 2012; Griffiths *et al.*, 2013). Recent use of molecular phylogenies has provided new insights into how evolutionary processes have shaped species distributions with respect to climate. Occupation of cooler habitats by C_3 species is now known to be associated with a preference for cooler climates in two (Edwards & Smith, 2010; Visser *et al.*, 2014) of the nine monophyletic subfamilies of Poaceae (Grass Phylogeny Working Group II, 2012): Pooideae (Vigeland *et al.*, 2013) and Danthonioideae (Humphreys & Linder, 2013), species of which are all C_3 (Edwards & Smith, 2010). In contrast, comparisons between C_3 and C_4 grasses within the PACMAD clade are most appropriate to studies of the adaptive advantages of C_4 photosynthesis among grasses (Edwards *et al.*, 2007; Edwards & Still, 2008). The PACMAD clade includes the monophyletic subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micrairoideae, Aristidoideae and Danthonioideae, excludes the Pooideae, and encompasses the evolutionary origins of all contemporary C_4 grass species (Christin *et al.*, 2009; Grass Phylogeny Working Group II, 2012). Within the PACMAD clade, the evolution of C_4 photosynthesis has resulted in preferences for drier habitats by C_4 lineages (Edwards & Still, 2008; Osborne & Freckleton, 2009; Edwards & Smith, 2010; Pau *et al.*, 2012), and divergence in water-use traits between C_3 and C_4 grasses (Taylor *et al.*, 2012; Griffiths *et al.*, 2013).

Paradoxically, as evidence has mounted to support the importance of drier habitats to the evolutionary success of C_4 photosynthesis in grasses, it has become clear that photosynthesis in these species may be more susceptible to failure under declining leaf water status (reviewed by Ghannoum, 2009; Driever & Kromdijk, 2013). Following restriction of watering in pot-based experiments, g_S of C_3 grasses declines to a greater degree and C_3 water-use efficiency can increase to match that of C_4 plants (Ripley *et al.*, 2010; Taylor *et al.*, 2011). However, there is evidence that C_3 grasses commonly operate at more negative leaf water potentials (Ψ) than C_4 species (Ripley *et al.*, 2010; Taylor *et al.*, 2010, 2011). As a consequence of these observations, it has been proposed that differences in plant hydraulics may have played an important role in allowing C_4 grasses to colonize and adapt to dry and open habitats (Osborne & Sack, 2012): decreased responsiveness of Ψ, g_s and E to water availability may result in photosynthesis among C_4 grasses showing greater resistance to the effects of drought.

To date, observations of susceptibility to drought among C_4 species have been made primarily in pot-based studies, which have several potential limitations (Poorter *et al.*, 2012). There is, therefore, only limited evidence that can be used to compare the impacts of drought on the leaf physiology of closely related C_3 and C_4 species under natural growing conditions (Ripley *et al.*, 2007; Frole, 2008; Ibrahim *et al.*, 2008). Crucially, all of these studies have focused on comparisons within the Panicoideae subfamily, and there is no evidence addressing contrasts across other key PACMAD lineages. We therefore established an outdoor common garden experiment using twelve C_3 and C_4 grass species, sampled from four closely related PACMAD lineages. All of the species used in the experiment are found within 60 km of the study site, in a region of the Eastern Cape of South Africa where climate, according to the Koppen–Geiger classification, is warm temperate, fully humid, with warm summers (Peel *et al.*, 2007). Our goal was to compare physiological responses of C_3 and C_4 grasses to an experimental manipulation of water availability, testing whether responses of leaf gas exchange and water potential previously observed under more controlled conditions are important under natural climatic conditions.

Based on our previous experiments (Ripley *et al.*, 2007, 2010; Taylor *et al.*, 2010, 2011), we hypothesized that C_4 grasses would show higher A, lower $g_{s,}$ and higher water-use efficiency when well watered. During periods of progressive drought, we expected that g_s in

C_3 grasses would decrease to a greater extent and that differences in leaf water-use efficiency might also diminish between C_3 and C_4 grasses (Frole, 2008; Ripley *et al.*, 2010; Taylor *et al.*, 2011). We further hypothesized that limitation of photosynthesis observed during drought in C_3 species would be principally driven by decreased g_s, but in C_4 species would instead be associated with decreased midday leaf water potential (Ψ_{midday}; Ghannoum *et al.*, 2003; Ripley *et al.*, 2007). We also predicted that C_4 grasses would show less negative Ψ_{midday} and maintain smaller hydrodynamic gradients from soil to leaf ($\Delta\Psi = \Psi_{\mathrm{predawn}} - \Psi_{\mathrm{midday}}$) when well watered, differences that we expected to be reduced under drought (Taylor *et al.*, 2010, 2011). Finally, we aimed to test whether differences in leaf Ψ were associated with greater plant hydraulic conductance in C_4 grasses ($K_{\mathrm{plant}} = E/-\Delta\Psi$; Osborne & Sack, 2012).

Materials and methods

Experimental design and plant species

Twelve grass species of open habitats were drawn from four lineages found in the regional species pool of the Eastern Cape of South Africa (Gibbs Russell *et al.*, 1990), based on a random sample of three species per lineage (Table 1). The two C_4 groups were the genus *Aristida* and the tribe Andropogoneae, which share a biochemical subtype (NADP-me) but have independent origins of their C_4 syndrome (Christin *et al.*, 2009; Grass Phylogeny Working Group II, 2012). The C_3 subfamily Danthonioideae and C_3 species from the tribe Paniceae were used in comparison; both are important components of grassland ecosystems in southern Africa.

Plants were collected from field locations (Table 1) between January 2007 and January 2008 and established in the outdoor common garden. The common garden had a blocked design, in which individual plots were separated by 2 m of short lawn, and paired 2 × 2 m plots within each of eight blocks were either watered or allowed to receive natural rainfall. Plants were regularly spaced and species locations were randomized within each plot but matched between watered and natural-rainfall plots in each block. All plots were watered on a regular basis until October 2008. After this time, only the plots in the watered treatment received additional water; approximately 28 l (equivalent to approximately 7 mm rainfall) was added to each plot every 2–3 days during the growing season. Following rainfall greater than 10 mm in 48 h, watering was halted for 2 weeks. Plots were weeded and the surrounding lawn mown on a regular basis.

Weather

Air temperature, humidity, wind-speed and direction, precipitation and photosynthetic photon flux density (PPFD) were recorded using a weather station. This comprised a datalogger (DL2e Delta T, Cambridge, UK); two relative humidity and temperature sensors (RHT2 nl, Delta T) positioned at 0.5 and

Table 1 Details of species used, collection locations and commonly inhabited biome types

Clade (photosynthetic type)	Species	Collection location (S:E; deg,min,sec)	Altitude (m)	Biome description (Gibbs Russell *et al.*, 1990)	Number of plants surviving, by treatment, in May 2009	
					Watered	Rain-fed
Panicoideae, Paniceae (C_3)	*Alloteropsis semialata* ssp. *eckloniana*	33,19,44.54: 26,28,44.21	726	Savanna, Grassland	7	8
	Panicum aequinerve	33,19,38.09: 26,31,14.44	660	Grassland, Forest	8	8
	Panicum ecklonii	33,19,46.38: 26,28,35.82	714	Grassland	2	4
Panicoideae, Andropogoneae (C_4)	*Heteropogon contortus*	33,19,11.87: 26,30,29.61	631	Savanna, Grassland, Fynbos, Nama-Karoo	8	7
	Hyparrhenia hirta	39,19,06.94: 26,30,37.57	618	Savanna, Grassland, Fynbos, Nama-Karoo	8	8
	Themeda triandra	33,17,05.23: 26,29,21.15	639	Savanna, Grassland, Fynbos, Nama-Karoo	7	8
Danthonioideae (C_3)	*Karoochloa curva*	33,14,54.27: 26,21,26.78	492	Grassland, Fynbos, Nama-Karoo	5	7
	Merxmuellera disticha	33,14,54.27: 26,21,26.77	492	Grassland, Fynbos, Nama-Karoo, Afro-Montane	8	8
	Pentaschistis curvifolia	33,19,46.38: 26,28,35.83	714	Fynbos	6	6
Aristidoideae (C_4)	*Aristida congesta* ssp. *barbicollis*	33,13,09.22: 26,37,40.04	487	Savanna, Grassland	7	7
	Aristida diffusa ssp. *burkei*	33,14,54.27: 26,21,26.76	492	Savanna, Grassland, Nama-Karoo	8	8
	Aristida junciformis ssp. *junciformis*	33,33,44.46: 26,53,39.36	80	Savanna, Grassland, Fynbos	8	8

2 m; an anemometer (AN4, Delta T) positioned at 2 m; a rain gauge (RG2, Delta T) and a quantum sensor (QS2, Delta T).

Estimation of reference crop evapotranspiration

To assess the effects of our watering treatment, R Language and Environment version 3.0.1 (R Core Team, 2013) was used to calculate reference crop evapotranspiration (ET_0, mm day^{-1}), which was compared with rainfall and watering inputs. Daily mean values (mean of maximum and minimum) from weather station measurements were used in combination with the Penman–Monteith equation, following Allen *et al.*, (1998; Data S1). The method assumes an extensive surface of growing, green grass, completely shading the ground and not short of water. Water shortage was observed at our site and bare soil was maintained between plants, thus the calculated ET_0 is an approximate guide of true evapotranspiration.

Leaf water potential

To assess plant water deficits, Ψ_{midday} and Ψ_{predawn} were measured and $\Delta\Psi$ was estimated as the difference between them, assuming Ψ_{predawn} was equilibrated with Ψ_{soil}. Measurements of Ψ_{midday} were paired with measurements of gas exchange (below). For measurement, leaves were enclosed in polythene and immediately excised using a razor blade. The balancing pressure was determined using a Scholander-type pressure bomb. Ψ_{predawn} of leaves selected using similar criteria to those used for Ψ_{midday} was determined before sunrise within 48 h. If rainfall occurred between the collection of midday and predawn measurements, Ψ_{predawn} measurements were either discarded or repeated the following day to better represent prevailing daytime conditions.

Leaf gas exchange

Gas exchange measurements were made during the final 2 weeks of each month during the growing season. Measurements were made under all but wet and extremely overcast conditions to obtain representative snapshots of seasonal gas exchange. During each day on which leaf gas exchange was measured, measurements were taken for one block between 09:30 h and 15:00 h. The first treatment to be measured was rotated each day, and the order of sampling between species was determined by their randomized positions within each plot.

A portable open gas exchange system (LI-6400; LI-COR, Inc., Lincoln, NE, USA) was used for gas exchange measurements, equipped with a CO_2 mixer (LI-6400-01) and 30 mm × 20 mm chamber/red-blue LED light source (LI-6400-02B). The CO_2 mole fraction of air entering the chamber was maintained at 400 μmol mol^{-1}. Light levels were matched to a PPFD sensor (LI-190); attached via a 1.5 m extension lead and mounted prior to measurements in each plot, in an unshaded, north-facing position, at 45 ° from vertical and roughly 30 cm above the soil surface. Air temperature in the chamber was not controlled, but the equipment was shaded to prevent excessive heating and to allow the chamber temperature to track that of the air. Leaf temperature was estimated using an energy balance calculation. Incoming air was not scrubbed of water vapour.

As the leaves of most species were narrow (1 to 3 mm wide), multiple leaves were usually inserted into the chamber, with a minimum of 100 mm^2 total projected leaf area used for all measurements. Leaves selected for gas exchange were the youngest fully emerged leaves on their tillers, with flowering tillers being avoided wherever possible and sections of canopy where leaf blades were exposed to full sun being preferred. Leaf area was calculated based on the known dimensions of the chamber and the combined widths of the inserted leaves at either edge of the chamber, measured using a ruler. Low fluxes were encountered regularly, especially during dry periods, forcing the use of flow rates down to 100 μmol s^{-1} to obtain resolvable differences in CO_2 ($\Delta CO_2 > 10$ μmol mol^{-1}) and H_2O ($\Delta H_2O > 1$ mmol mol^{-1}) between the reference airstream and the chamber. The chamber was tested for leaks by exhaling around the seals immediately after inserting leaves. Measurements were taken as soon as the predicted intercellular CO_2 concentration (c_i) stabilized. If c_i failed to stabilize within 3 min, if $\Delta CO_2 < 10$ μmol mol^{-1}, or if leaves being measured were thick/rolled, the chamber was re-tested for leaks and, if necessary, the seal on the chamber was re-adjusted before re-commencing measurements. In all cases where ΔCO_2 was < 10 μmol mol^{-1}, reference and chamber gas analyzers were matched prior to measurement.

For the first set of measurements in November 2008, rolled leaves were routinely unrolled to take measurements. Paired measurements, taken with leaves first rolled and then unrolled, indicated that by unrolling leaves, values for c_i were elevated to an unusual degree due to increases in estimated g_s (data not shown). Thus, from December 2008 onwards, tightly rolled leaves were not unrolled during gas exchange measurements.

Estimation of leaf transpiration

To assess water use at the leaf level, a model implemented in R Language and Environment version 3.0.1 (R Core Team, 2013) was used to estimate E for individual leaves from each species in the study. The Penman–Monteith equation (Penman, 1948; Monteith, 1965) was combined with an iterative approach to modelling of leaf energy balance for a horizontal leaf suspended over a lawn (Jones, 1992; Data S2). The model was parameterized using leaf widths based on published values for each species (Data S2), mean values for climate variables (Data S3) and g_s (Data S4) from each measurement period during the growing season.

Statistical analysis

Statistical analyses were carried out using the R Language and Environment, version 3.0.1 (R Core Team, 2013). To determine the effects of the watering treatments, a Wilcoxon signed rank test was used to test for differences in weekly ET_0 − (watering+rainfall) values.

Linear mixed effect models of seasonal changes in physiological traits were fitted using maximum likelihood, and tested for significance using tools in the *lme4* package (Bates *et al.*, 2013). The data used in models were species mean values calculated for each month × treatment combination. Prior to analysis, mean values based on ≤ 2 replicates were eliminated from the dataset and, to improve balance in the dataset, species means that were unpaired across treatments in any given month were also removed. The full datasets used for analysis are plotted in Data S5. Average values for C_3 and C_4 groups in both treatments during each monthly sampling interval were predicted as fixed effects. Clade was modelled as a random effect dependent on the month of sampling. Model validation was carried out by inspection of residuals and, except for the model of instantaneous water-use efficiency *(A/E)*, log-transformation was used to improve homoscedasticity of data. Bootstrapped 95% confidence intervals for fixed effect predictions were generated using 1000 simulations of each model.

Results

Weather and effect of watering

Maximum temperatures were observed in January (mean of daily maxima during January, 28 °C), whereas rainfall and relative humidity were greatest in February (total rainfall, 139 mm; relative humidity, mean of daily minima February 74%; Fig. 1a). Relative humidity was lowest during early November (mean of daily minima November 1st–15th 53%) and late March–early April (mean of daily minima March 15th–April 15th 47%; Fig. 1b).

Supplementary watering significantly reduced the cumulative water deficit, indicated by rainfall deficit, ET_0-(watering + rainfall), on a week-by-week basis (Wilcoxon signed rank test, $P < 0.001$; windspeeds used to calculate ET_0 are shown in Fig. 1c). The total accumulated water deficit in the rain-fed plots was estimated to have been more than 3 times that in the plots receiving supplementary water (differences in water input are shown in Fig. 1d). Rainfall peaked during the week ending February 19th (Fig. 1d). In the 16 weeks prior to the peak of rainfall, total deficits in the watered treatments were 155 mm, compared with 386 mm in the rain-fed treatments. Peak rainfall in February was followed by a further period in which rainfall was low: rainfall deficits were 120 mm in the rain-fed plots and 15 mm in the watered plots over the final 10 weeks of the experiment. We note, however, that as an approximation of soil water balance, rainfall deficit calculated in this manner does not account for soil hydrology and depends on the method used to estimate ET_0.

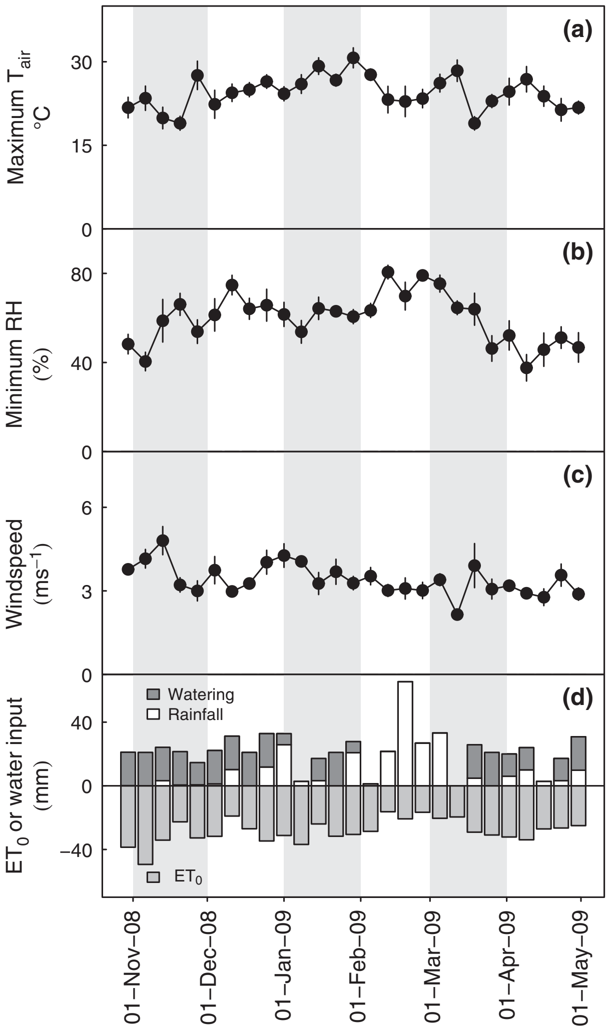

Fig. 1 Climate conditions relevant for midday photosynthesis in the common garden experiment carried out in Grahamstown, Eastern Cape of South Africa, during November 2008–April 2009. Weekly values (mean ± SEM) for: (a) daily maximum temperature; (b) daily minimum relative humidity; (c) daily mean windspeed. Weekly totals (d) for water added to the supplementary water treatment, rainfall, and reference crop evapotranspiration calculated using micrometerological data (ET_0, shown as negative values). Months in the experiment are highlighted by grey-filled areas.

Plant survival

A number of plants died during the 2008–2009 growing season (Table 1). The small sample size meant that there was no clear evidence that mortality for any species differed between the watered and rain-fed plots (Table 1). Compared with nine deaths in the rain-fed plots, 14 plants died in the watered plots, but six of the dead plants in watered plots were of a single species, *P. ecklonii*. This was one of three species for which more than two of the 16 planted individuals died; *P. ecklonii* (ten dead), *K. curva* (four dead), and *P. curvifolia* (four dead), are all C_3 plants. Overall, therefore, 19 C_3 plants died, compared with four C_4 plants.

Leaf water potentials

Leaf water potentials in rain-fed plots were significantly more negative than in watered plots. Watering caused significant increases in average Ψ_{predawn}, of 0.26–1.07 MPa, for both C_3 and C_4 photosynthetic types during January and April (Fig. 2a,b). In December and March, watering led to significant improvements in average Ψ_{predawn} for C_3 grasses (increase by 0.18–0.29 MPa), but smaller improvements for C_4 grasses (0–0.05 MPa) were not statistically significant (Fig. 2a,b).

Mean Ψ values were always more negative for the C_3 than C_4 groups (Fig. 2). Except during the wettest month, February, differences in Ψ_{predawn} between the photosynthetic types in rain-fed plots (0.19–0.42 MPa) were statistically significant (Fig. 2a). These differences were eliminated in the watered plots, except when drought was most acute during January (0.16 MPa difference) and April (0.19 MPa difference, Fig. 2b).

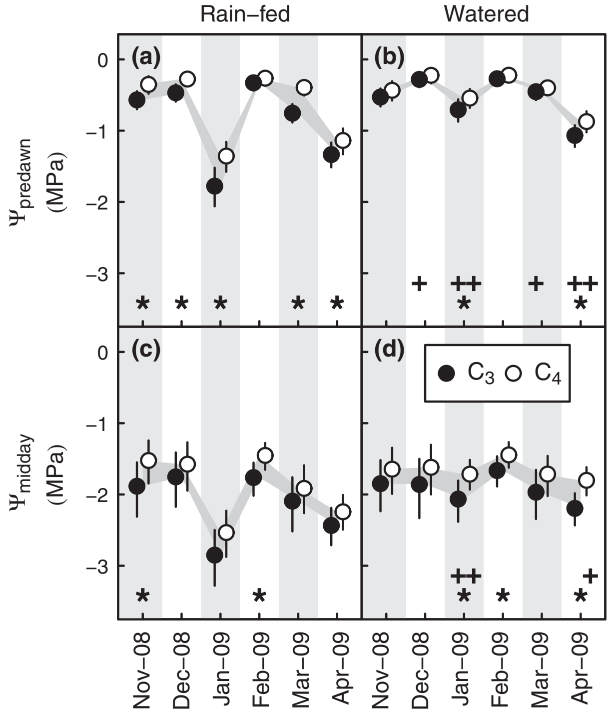

Fig. 2 Seasonal contrasts in leaf water potential between C_3 and C_4 PACMAD grasses in a common garden experiment at Grahamstown, Eastern Cape of South Africa, between November 2008–April 2009. (a,b) Predawn water potential, Ψ_{predawn}; (c,d) midday water potential, Ψ_{midday}. Points represent pooled means and 95% confidence intervals for 4–6 species in plots that were rain-fed (a,c) or given supplemental water (b,d). Values are back-transformed from the log-transformed scale used for statistical analysis. Differences between photosynthetic types and months in the experiment are highlighted by grey-filled areas. Photosynthetic type comparisons for which confidence intervals indicate significance at the $P < 0.05$ level are highlighted by * within each pane. Significant differences within photosynthetic types that resulted from watering are indicated by + below the relevant means in (b) and (d).

Significant differences in Ψ_{midday} (Fig. 2c,d) did not entirely mirror the pattern of drought response shown by Ψ_{predawn}. Significant positive effects of watering on Ψ_{midday} were observed for C_4 grasses in January (0.82 MPa) and April (0.43 MPa; Fig. 2d). However, watering significantly increased Ψ_{midday} among C_3 plants only in January (0.79 MPa), not in April (0.24 MPa; Fig. 2d); a contrast with Ψ_{predawn} which was affected consistently across the photosynthetic types at the two timepoints. During the wetter month of February, differences between the average Ψ_{midday} of C_3 and C_4 grass leaves were 0.22–0.31 MPa and were significant in both rain-fed and watered plots (Fig. 2c,d): these differences were particularly notable, given the lack of differences in Ψ_{predawn} in February (Fig. 2a,b). In addition, Ψ_{midday} differed significantly between the photosynthetic types in the rain-fed plots during November. In the watered plots during January and April (Fig. 2c,d), significant differences in Ψ_{midday} between the photosynthetic types were coincident with significant effects of watering on Ψ_{midday} of one or both types (Fig. 2c,d).

Gas exchange

In each month, average *A* was always higher among C_4 grasses (range of means 5.1–14.7 μmol m^{-2} s^{-1}) than among C_3 grasses (range of means 0.6–11.5 μmol m^{-2} s^{-1}). In the rain-fed treatment, differences between the photosynthetic types during the drought periods, December–January and March–April, ranged between 3.1 and 8.2 μmol m^{-2} s^{-1}, and confidence limits indicated that they were statistically significant (Fig. 3a). In the wettest month, February, differences between the photosynthetic types were smaller in the rain-fed plots and were not significant (1.7 μmol m^{-2} s^{-1}). This was also true at the start of the growing season in November (2.4 μmol m^{-2} s^{-1}; Fig. 3a). During this first month of the experiment water deficits in the rain-fed plots may still have been establishing, as watering ceased during October.

In the watered plots, there were also significant differences in *A* between the two photosynthetic types during December, January and March (Fig. 3b), which ranged from 4.9 to 6.2 μmol m^{-2} s^{-1}. Both photosynthetic types showed significant increases in *A* (4.8–6.0 μmol m^{-2} s^{-1}) in response to watering during the most severe drought periods (January and April, Fig. 3b), but only the C_3 group increased *A* significantly

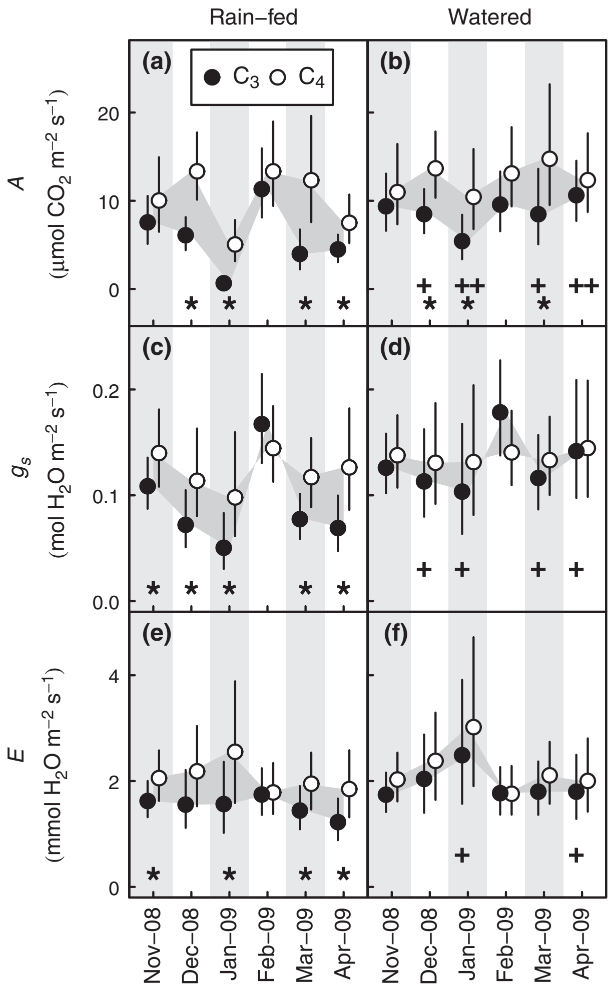

Fig. 3 Seasonal contrasts in leaf gas exchange between C_3 and C_4 PACMAD grasses in a common garden experiment at Grahamstown, Eastern Cape of South Africa, between November 2008 and April 2009. (a,b) Net CO_2 assimilation, A; (c,d) stomatal conductance to H_2O, g_s; (e,f) transpiration, E. Experimental plots were rain-fed (a,c,e) or given supplemental water (b,d,f). Points represent pooled means and lines 95% confidence intervals for 4–6 species, values are back-transformed from the log-transformed scale used for statistical analysis. Differences between photosynthetic types and months in the experiment are highlighted by the grey-filled areas. Photosynthetic type comparisons for which confidence intervals indicate significance at the $P < 0.05$ level are highlighted by * within each pane. Significant differences within photosynthetic types that resulted from watering are indicated by + below the relevant means in (b), (d) and (f).

in response to watering under less severe drought during December (increase of 2.3 μmol m^{-2} s^{-1}) and March (increase of 4.5 μmol m^{-2} s^{-1}; Fig. 3b). Watering had no significant effect on A in the wettest month, February, or in November, at the start of the growing season (Fig. 3b).

Unexpectedly, in the natural-rainfall treatment, average g_s among C_3 species was often significantly lower, by 0.031–0.056 mol m^{-2} s^{-1}, than among C_4 species (Fig. 3c); the exception was the wettest month, February, during which average g_s among C_3 species was slightly, but not significantly greater than among C_4 species (0.022 mol m^{-2} s^{-1}, Fig. 3c). Differences in average g_s between the photosynthetic types in the watered treatment were never significant, but showed a similar pattern to those in the rain-fed plots (Fig. 3d); the average g_s of C_4 species was greater by 0.001–0.028 mol m^{-2} s^{-1}, except during February when the C_3 value was 0.038 mol m^{-2} s^{-1} greater than for C_4 species. Similar values for g_s between the photosynthetic types in the watered plots were a result of significant increases in mean g_s among C_3 species in December, January, March and April, relative to rain-fed plots, of 0.039–0.075 mol m^{-2} s^{-1} (Fig. 3d); watering did not significantly influence the mean g_s among C_4 species (Fig. 3d). These results contrasted with our expectation that well-watered C_3 plants would show significantly higher g_s than their C_4 relatives.

As expected, patterns in modelled E were broadly consistent with the patterns seen for g_s (Fig. 3e,f). Average values of E were 0.04–0.64 mmol m^{-2} s^{-1} higher for C_4 species when compared with C_3 species in rain-fed plots, and significantly so in November, January, March and April (Fig. 3e). Watering eliminated these differences in February, and the maximum difference between photosynthetic types in the watered plots was 0.54 mmol m^{-2} s^{-1} (Fig. 3f). The smaller difference in E between C_3 and C_4 species in the watered plots resulted from watering-induced increases of 2–59% in E among C_3 species. Differences in average E in the rain-fed plots were also eliminated during the wettest month, February (Fig. 3e). However, in contrast with patterns in g_s, where watering had a significant influence on values in four of six months, watering had a significant effect on E among C_3 species only in the driest months, January and April (Fig. 3d,f).

To summarize, although watering always eliminated the differences in g_s and E between photosynthetic types under rain-fed conditions (Fig. 3d,f), the differences in A between the photosynthetic types persisted in both watered and rain-fed treatments during drought (Fig. 3b). Thus, C_4 grasses held a photosynthetic advantage over their C_3 relatives when operating at similar E and g_s. Furthermore, while decreased photosynthesis among C_3 species was associated with significant declines in g_s and $\Psi_{predawn}$ due to drought, the same was not true for their C_4 counterparts. Among C_4 species, g_s and E were not significantly affected by water supply, and decreases in A coincided instead with significant decreases in $\Psi_{predawn}$.

Water-use efficiency

Although leaf-level water-use efficiency tended to be higher, on average, among C_4 grasses, differences between the photosynthetic types during each monthly sampling interval were rarely significant (Fig. 4). At the beginning (November) and end (April) of the growing season, differences in intrinsic water-use efficiency (A/g_s) between the photosynthetic types in rain-fed plots were small (6–7 mmol mol^{-1}) and were not significant (Fig. 4a). Throughout the remainder of the growing season, average A/g_s of C_4 leaves in rain-fed plots was 64–125 mmol mol^{-1}, greater than the values for C_3 leaves (26–91 mmol mol^{-1}) and significantly so during periods of intermediate drought stress in December and March (Fig. 4a). This A/g_s advantage to C_4 grasses in rain-fed plots was therefore at its maximum when A, g_s and Ψ_{predawn} were water limited (i.e. significantly affected by the watering treatment) among C_3 but not C_4 grasses (Fig. 3). When drought was most severe during January, however, although the difference in A/g_s between the photosynthetic types in the rain-fed plots was large (38 mmol mol^{-1}) it was not significant. In contrast, during the wettest month, February, differences of a similar size to that seen in January were significant in both watered (33 mmol mol^{-1}) and rain-fed (37 mmol mol^{-1}) plots (Fig. 4a). To summarize, advantages to the C_4 grasses in A/g_s were largest in well-watered soil and during mild drought, but were lost under severe drought and were also small at the beginning and end of the growing season (Fig. 4a,b).

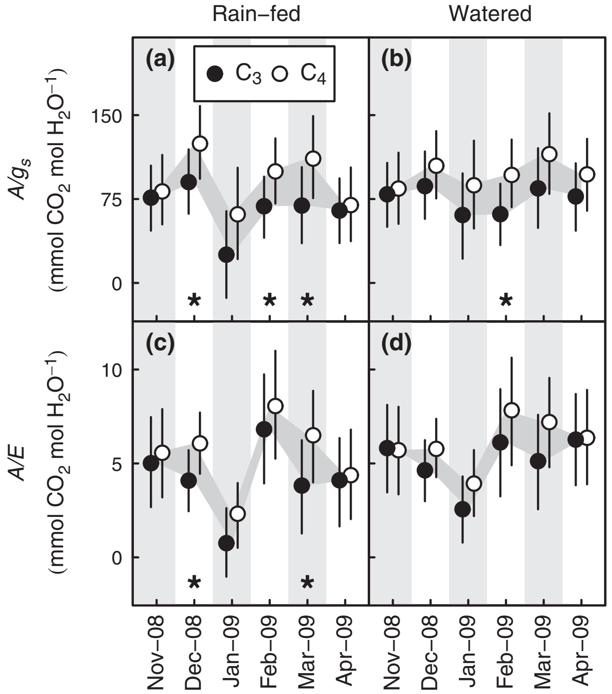

Fig. 4 Seasonal contrasts in leaf-level water-use efficiency between C_3 and C_4 PACMAD grasses in a common garden experiment at Grahamstown, Eastern Cape of South Africa, between November 2008 and April 2009. (a,b) Intrinsic water-use efficiency, A/g_s; (c and d) instantaneous water-use efficiency, A/E. Points represent pooled means and 95% confidence intervals for 4–6 species in plots that were rain-fed (a,c) or given supplemental water (b,d). Values are back-transformed from the log-transformed scale used for statistical analysis (a,b), analysis was carried out on untransformed data (c,d). Differences between photosynthetic types and months in the experiment are highlighted by the grey-filled areas. Photosynthetic type comparisons for which confidence intervals indicate significance at the $P < 0.05$ level are highlighted by * within each pane. There were no significant differences due to watering.

Variation over the growing season and across treatments meant that average instantaneous water-use efficiency (A/E) ranged from 0.72 to 6.89 mmol mol^{-1} for C_3 and 2.29 to 7.99 mmol mol^{-1} for C_4 plants, and was 1.10–2.61 mmol mol^{-1} greater among C_4 grasses from December to March. Differences in A/E were similar to A/g_s in that they usually favoured C_4 grasses and that in the rain-fed treatment they were smallest in November and April (0.14–0.5 mmol mol^{-1}). Indeed, C_3 grasses showed very similar A/E to their C_4 relatives in the watered plots (−0.1–0.06 mmol mol^{-1} difference; Fig. 4c,d) in November and April. However, statistically significant advantages to C_4 grasses were observed for A/E in the rain-fed plots in December and March (Fig. 4c), consistent with significant differences in A/g_s. We were surprised to find that, in contrast with A/g_s, having accounted for the effects of leaf energy budget by calculating A/E, differences in leaf instantaneous water-use efficiency between the photosynthetic types were not significant in either treatment during February (Fig. 4c,d), the wettest month in the study.

Plant hydraulics

The size of $\Delta\Psi$ reflects the hydraulic balance between water loss from the leaves and supply from the roots and soil, with more negative values for individual plants indicating greater strain. Average values by photosynthetic type ranged between −0.73 and −1.59 MPa. In watered plots, although differences in $\Delta\Psi$ between C_3 and C_4 species were never significant (Fig. 5b), average values for C_4 species were consistently smaller (−0.91 to −1.38 MPa) than those for C_3 species (−1.10 to −1.59 MPa; Fig. 5a,b). In rain-fed plots, average values for C_3 species were similar to or smaller than those for C_4 plants during drought in December (C_3 −1.27 MPa, C_4 −1.29 MPa), March (C_3 −1.30 MPa, C_4 −1.51 MPa) and April (C_3 −1.04 MPa, C_4 −1.08 MPa),

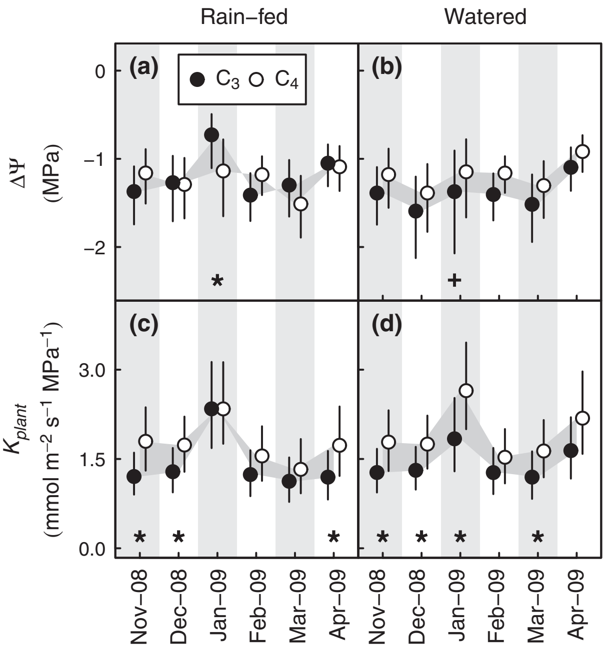

Fig. 5 Seasonal contrasts in plant hydraulics between C_3 and C_4 PACMAD grasses in a common garden experiment at Grahamstown, Eastern Cape of South Africa, between November 2008 and April 2009. (a,b) Hydrodynamic gradient, $\Delta\Psi = \Psi_{\mathrm{midday}} - \Psi_{\mathrm{predawn}}$; (c,d) hydraulic conductance, $K_{\mathrm{plant}} = E/-\Delta\Psi$. Points represent pooled means and 95% confidence intervals for 4–6 species exposed to natural rainfall (a,c) or given supplemental water (b,d). Values are back-transformed from the log-transformed scale used for statistical analysis. Differences between photosynthetic types and months in the experiment are highlighted by the grey-filled areas. Photosynthetic type comparisons for which confidence intervals indicate significance at the $P < 0.05$ level are highlighted by * within each pane. The significant difference within the C_3 photosynthetic type that resulted from watering is indicated by + below the relevant mean in (b); there were no significant differences due to watering in (d).

differences that were not statistically significant (Fig. 5a). The only significant differences in $\Delta\Psi$ were observed under the most severe drought in January, when the C_3 $\Delta\Psi$ (−0.73 MPa) was significantly smaller than both C_3 grasses in the watered plots (−1.39 MPa) and C_4 grasses in the rain-fed plots (−1.14 MPa; Fig. 5a). This significantly smaller value of average $\Delta\Psi$ among C_3 grasses was observed during a period of acute leaf water deficit (Fig. 2). Therefore, the nonsignificant changes in a similar direction, which were observed in December, March and April, might also be interpreted as indicative of reduced or more variable hydraulic performance among C_3 species.

A measure of whole-plant leaf-specific hydraulic conductance (K_{plant}, mmol m^{-2} s^{-1} MPa^{-1}) is provided by the flux of water due to E (mmol m^{-2} s^{-1}) normalized by $\Delta\Psi$ (MPa). K_{plant} was almost always greater among C_4 species (1.32–2.69 mmol m^{-2} s^{-1} MPa^{-1}) than C_3 species (1.12–2.35 mmol m^{-2} s^{-1} MPa^{-1}; Fig. 5c,d), a difference of 0.2–0.88 mmol m^{-2} s^{-1} MPa^{-1}. The exception to the general rule that K_{plant} was greater among C_4 species was in the rain-fed plots during January (Fig. 5c), when the difference was almost zero (Fig. 5a), coincident with the significant decline in average $\Delta\Psi$ for C_3 species. Differences in K_{plant} between C_3 and C_4 species were significant in November, December and April in the rain-fed plots (Fig. 5c) and in November, December, January and March in the watered plots (Fig. 5d), but watering had no significant effects on K_{plant} (Fig. 5d).

Discussion

Our results demonstrate that plant water relations play a key role in maintaining the physiological advantages of C_4 over C_3 PACMAD grasses under field conditions. We found that A, E and g_s among C_3 grasses declined significantly in response to drought, in concert with reductions in $\Delta\Psi$. In contrast, C_4 grasses maintained $\Delta\Psi$, E and g_s throughout the growing season, and A was limited by water supply only under the most extreme drought conditions when both Ψ_{predawn} and Ψ_{midday} decreased. The findings that g_s is more sensitive to drought among C_3 grasses and that A is more obviously associated with Ψ_{midday} than with g_s in C_4 grasses are consistent with evidence from previous experiments (Ghannoum *et al.*, 2003; Ripley *et al.*, 2010; Taylor *et al.*, 2011). That C_4 gas exchange was relatively independent of water supply and that g_s among C_3 species was commonly lower than among C_4 relatives are novel findings that highlight the importance of both taking a field-based approach and monitoring performance throughout a growing season. While our measurements of $\Delta\Psi$ and estimates of K_{plant} provide some support for the hypothesis that K_{plant} is often higher among C_4 grasses, these differences were not clear-cut, and their physiological basis remains unclear. Therefore, important questions remain about the causes of C_4 resistance to drought.

We found that g_s among C_4 plants was independent of our watering treatment, but A decreased in conjunction with Ψ_{midday} under more severe drought. Among C_3 species, decreases in photosynthesis were paired with decreases in Ψ_{predawn} and g_s, but among C_4 species, decreases in Ψ_{predawn} occurred later and g_s never decreased significantly. Although they represent average responses and summarize the performance of species with sometimes distinct behaviours, these results are consistent with previous demonstrations that drought sensitivity of C_4 photosynthesis in grasses depends on metabolic rather than stomatal limitations

(Ghannoum *et al.*, 2003; Ripley *et al.*, 2007, 2010). Ultimately, photosynthesis in both C_3 and C_4 grasses was limited by drought in our experiment, but our results suggest that the cause differed and show that significant effects on C_4 photosynthesis occurred later. Consistent with our expectations, the greatest photosynthetic advantages for C_4 grasses occurred during the development of drought in the warmest parts of the growing season.

We have previously shown that drought can narrow the gap in water-use efficiency between C_3 and C_4 grasses (Ripley *et al.*, 2010; Taylor *et al.*, 2011), which is large in well-watered, controlled conditions (e.g., Taylor *et al.*, 2010 found that C_4 A/g_s was double that of C_3 grasses). Here we show that, under natural conditions, with a relatively diverse group of PACMAD species, the intrinsic water-use efficiency advantage to C_4 species was much smaller when well watered (ca. 40% greater than C_3). It was often difficult to distinguish C_3 and C_4 species based on differences in A/g_s, which, outside of the wettest periods in the experiment were significant only during periods of intermediate drought. Assuming that water deficits were generally greater in the field environment than controlled growth conditions, this result is consistent with our previous finding that drought reduces the difference in intrinsic water-use efficiency between C_3 and C_4 grasses (Taylor *et al.*, 2011), but implies that advantages may be regained as water availability continues to decline. The response of A to g_s is expected to saturate more quickly in C_3 than C_4 grasses (Osborne & Sack, 2012), and C_3 species under well-watered conditions often operate above the point where increasing g_s results in diminishing returns for A (this study, data not shown). When faced with a need to reduce g_s, the leaves of well-watered C_3 plants initially face a relatively small penalty in A, and A/g_s increases, but C_4 leaves retain a clear photosynthetic advantage at low g_s (Osborne & Sack, 2012). Importantly, we found that, when we accounted for leaf energy balance, the C_4 advantage in A/g_s translated into significant differences in A/E only during periods of intermediate drought.

We were surprised to find that g_s was similar across the two photosynthetic types, even in watered plots. In previous comparisons of well-watered grasses from a diverse array of habitats, g_s was significantly higher among C_3 grasses, though the full range of g_s observed across C_3 and C_4 species overlapped substantially (Taylor *et al.*, 2010, 2011). However, we have previously observed similar g_s between C_3 and C_4 grasses in a subset of the species studied here (Frole, 2008; Ripley *et al.*, 2010). We have also previously demonstrated that habitat water availability is important in determining stomatal trait differences among C_3 and C_4 grasses (Taylor *et al.*, 2012), and it has been repeatedly shown that C_3 and C_4 lineages sort into distinct hydrological niches (Edwards & Still, 2008; Osborne & Freckleton, 2009; Edwards & Smith, 2010; Pau *et al.*, 2012; Visser *et al.*, 2014). It is possible that differential sensitivity to vapour pressure deficit between C_3 and C_4 leaves (Bunce, 1983; El-Sharkawy *et al.*, 1985) contributed to the smaller difference in g_s values observed in these experiments. However, we suggest that similar g_s was observed among the species in this study because they were sampled from a restricted suite of habitats within a seasonally dry climate region. It follows that the smaller differences we observed in A and A/g_s may also depend on these factors. This interpretation reinforces the importance of plant water relations in structuring species assemblages, and leads to the prediction that differences in gas exchange traits between C_3 and C_4 grasses are likely to be more extreme among species from diverse habitats (Taylor *et al.*, 2010, 2011, 2012).

The clear advantage for C_4 grasses in midday gas exchange, particularly A, during the growing season implies a disadvantage to C_3 grasses that might ultimately influence their local persistence. Of the deaths observed during the 2008–2009 growing season, the majority were among C_3 plants, but they were not clearly associated with the watering treatment, a reminder that other factors may ultimately determine the local habitat preferences of these grasses (Visser *et al.*, 2011). Seasonal differences in performance are one possibility: differences in leaf survival during winter have been demonstrated for the C_3 and C_4 subspecies of *A. semialata* when grown close to our field site (Ibrahim *et al.*, 2008; Osborne *et al.*, 2008), and a recent phylogenetic study investigating the grass flora of Hawaii demonstrated that the niche of C_3 PACMAD species is associated with winter precipitation (Pau *et al.*, 2012). Seasonal differences in productivity are also important in mixed C_3/C_4 grasslands (Ode *et al.*, 1980; Still *et al.*, 2003) and will no doubt be influenced by shifting patterns of precipitation and seasonality under global change. We found that performance of C_3 and C_4 grasses was most similar at the beginning and end of the growing season. A key question remaining to be tested, therefore, is whether performance and growth of our C_3 species in the late autumn, winter and early spring offset the physiological advantages of their C_4 relatives during the summer.

When the soil was wetter, leaf Ψ was less negative and $\Delta\Psi$ smaller among the C_4 than C_3 species, consistent with our expectations (Taylor *et al.*, 2010, 2011). During drought, we observed that $\Delta\Psi$ decreased among C_3 grasses and was maintained among C_4 grasses. Because declines in $\Delta\Psi$ were paired with decreasing E among C_3 species, whereas E among C_4

grasses increased with evaporative demand, we interpret the pattern of decreases in $\Delta\Psi$ among C_3 grasses as indicating greater vulnerability of their hydraulic systems to failure under drought. Smaller $\Delta\Psi$ was associated with more negative $\Psi_{predawn}$, not less negative Ψ_{midday}. It is plausible that night-time rehydration under severe drought was insufficient to bring $\Psi_{predawn}$ into equilibrium with soil Ψ in at least some of the C_3 species (predawn disequilibrium; Donovan *et al.*, 2001, 2003). Alternatively, increases of K_{plant}, if genuine, may act to maintain *E*, hydration and physiological function in the face of increased evaporative demand (Jones, 1992). Changes in K_{plant} can be regulated by several physiological processes, including changes in tissue conductance due to aquaporin activity (Kaldenhoff *et al.*, 2008); changes in water requirements of growing tissues (Boyer, 1985); or changes in mass allocation such that water supply via roots is enhanced relative to water demand from leaf area (Maseda & Fernández, 2006). Differences in hydraulic traits will have contributed to the overall differences in K_{plant} in this experiment, and one realistic possibility is that less negative Ψ and smaller $\Delta\Psi$ in C_4 grasses in this experiment was a result of better root system access to available soil water; we have previously observed that the C_4 lineages in this experiment have higher root mass ratios than the C_3 lineages (Taylor *et al.*, 2010). However, it is important to note that, although K_{plant} tended to be lower among C_3 species, it was not always so: although suggestive, our evidence is not sufficient to claim that a clear difference in K_{plant} between C_3 and C_4 species was the principal driver for C_4 performance advantages. Nonetheless, our results highlight a need to address mechanistic questions about the integration of hydraulic and photosynthetic performance among grasses, ideally in field experiments and especially under drought.

The characterization of ecophysiological traits associated with C_4 photosynthesis is vital for understanding the natural diversity and ecological success of C_4 species, and the differential impacts of global change on C_3 and C_4 species. The comparisons reported here, using four lineages of PACMAD grasses sampled from the same regional species pool and grown under natural climatic conditions, are a crucial complement to previous experiments that were pot-based, carried out in controlled environments, or completed using a less diverse panel of species. Our experimental manipulation of water availability influenced contrasts in leaf physiology during a growing season and we found that C_4 photosynthetic advantages were maintained when a diverse panel of grass species were exposed to natural water shortages. Under mild drought, the C_4 advantage in *A* was increased, as C_3 leaves faced stomatal limitation of photosynthesis associated with earlier decreases in Ψ. We show that, under native climatic conditions in a location where both C_3 and C_4 PACMAD grasses are naturally abundant, water availability plays a crucial role in determining the magnitude of differences in physiological performance associated with photosynthetic type. Importantly, our experimental evidence supports a need to rigorously examine the proposition that advantages of C_4 photosynthesis in dry environments are significantly modified by dynamic responses of the stomata and hydraulic system to drought (Osborne & Sack, 2012). Understanding the interplay between C_4 photosynthesis and hydraulics will be crucial as we aim to better understand the response of plant communities to global change, including the question of why the distribution of C_4 PACMAD grasses is so strongly linked with water availability (Edwards & Smith, 2010; Pau *et al.*, 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Equations used for estimation of reference crop evapotranspiration (ET_0).
Data S2. Model for estimation of leaf-level transpiration (E).
Data S3. Mean values for microclimate used in modelling of leaf-level transpiration (E).
Data S4. Tabulated values for physiological traits determined in the field, by species, month of measurement and treatment.
Data S5. Plotted species means used for analysis.