CO₂ availability influences hydraulic function of C3 and C4 grass leaves

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Abstract

Atmospheric CO₂ (cₐ) has increased since the last glacial period, increasing photosynthetic water use efficiency and improving plant productivity. Evolution of C₄ photosynthesis at low cₐ led to decreased stomatal conductance (gₛ), which provided an advantage over C₃ plants that may be reduced by rising cₐ. Using controlled environments, we determined how increasing cₐ affects C₄ water use relative to C₃ plants. Leaf gas exchange and mass per area (LMA) were measured for four C₃ and four C₄ annual, crop-related grasses at glacial (200 µmol mol⁻¹), ambient (400 µmol mol⁻¹), and super-ambient (640 µmol mol⁻¹) cₐ. C₄ plants had lower gₛ, which resulted in a water use efficiency advantage at all cₐ and was broadly consistent with slower stomatal responses to shade, indicating less pressure on leaf water status. At glacial cₐ, net CO₂ assimilation and LMA were lower for C₃ than for C₄ leaves, and C₃ and C₄ grasses decreased leaf hydraulic conductance (Kₗₑᵃftype) similarly, but only C₄ leaves decreased osmotic potential at turgor loss. Greater carbon availability in C₄ leaves at glacial cₐ generated a different hydraulic adjustment relative to C₃ plants. At current and future cₐ, C₄ grasses have advantages over C₃ grasses due to lower gₛ, lower stomatal sensitivity, and higher absolute water use efficiency.

Keywords: C₄ photosynthesis, glacial CO₂, grasses, leaf gas exchange, leaf hydraulic conductance, osmotic adjustment, pressure–volume curve, stomatal conductance, turgor loss point.

Introduction

C₄ photosynthetic pathways have evolved as solutions to photosynthetic inefficiencies linked with the oxygenation reaction of Rubisco (Sage, 2004; Sage et al., 2012). Because of its potential for greater efficiency, engineered C₄ photosynthesis has been proposed as a potential solution for improving global food security (von Caemmerer et al., 2012), and C₄ crops are leading contenders as sources of renewable biomass energy (Byrt et al., 2011). Our understanding of C₄ photosynthesis as an ecological adaptation is continuing to develop rapidly (Edwards and Smith, 2010; Lundgren et al., 2015; Atkinson et al., 2016; Watcharamongkol et al., 2018). New insights into the timing and sequence of C₄ evolution from phylogenetic studies have renewed debate about its expected physiological advantages (Edwards and Still, 2008; Edwards et al., 2010; Christin et al., 2011b, 2014; Sage et al., 2011). Some C₄ lineages probably arose during the Oligocene (~30 million years ago).
but most arose over the last 20 million years, during the sub-
sequent Miocene (Christin et al., 2008, 2011; Vicentini et al.,
2008; Besnard et al., 2009; but see Kadereit et al., 2012). During
this period, ‘icehouse’ conditions of globally cooler tempera-
tures and drier climates were linked with atmospheric CO₂
concentrations (ĉ) lower than present day (Pagani et al., 2005).
Atmospheric CO₂ has increased since the last glacial period,
and consequent increases in photosynthetic water use efficiency
have been associated with declines in water stress and improve-
ments in plant productivity (Polley et al., 1993; Mayeux et al.,
1997). It has been speculated that in addition to impacts on
photosynthetic performance (Ehleringer et al., 1997), hydraulic
function in C₃ and C₄ plants was differentially affected at
low atmospheric CO₂ (Osborne and Sack, 2012) because of
greater stomatal opening in C₃ plants resulting in greater water
stress (Polley et al., 1993). It is also expected that under future,
high CO₂ climates, the combination of CO₂ fertilization and
improved water use efficiency will continue to influence the
relative performance of C₃ and C₄ plants (Ghannoum et al.,
2000; Ainsworth and Long, 2005; Leakey, 2009). To establish
whether C₄ plants gain hydraulic advantages because of rela-
tively small increases in stomatal conductance (gₛ) responding
to ĉ (Osborne and Sack, 2012), it is important to verify relative
stomatal responses experimentally and investigate their impact
on physiological function, including hydraulic properties.
Photosynthesis in C₄ leaves is characterized by biochem-
ical pumps that initially combine phosphoenolpyruvate
(PEP) and CO₂ to form C₄ acids and subsequently trans-
fer those acids, release CO₂ in the presence of Rubisco,
and recycle PEP (Edwards et al., 2001; Sage, 2004). The initial
biochemical step used to form C₄ acids is highly efficient,
and a high CO₂ concentration at the site of Rubisco carbo-
oxylase minimizes photorespiration in C₄ plants. Therefore,
leaf internal CO₂ concentrations (ĉ) are lower for CO₂ com-
ensation and photosynthetic saturation, and quantum yield
can be greater (Pearcy and Ehleringer, 1984). Importantly for
plant hydraulics, photosynthetic water use efficiency is con-
sequently high (Pearcy and Ehleringer, 1984; Long, 1999).
A central question has been whether this improved water
use efficiency provides advantages for C₄ plants over C₃
plants in habitats with restricted water availability (Osmond
et al., 1982; Hattersley, 1983; Pearcy and Ehleringer, 1984).
Recent comparative studies of the numerous C₄ lineages in
the grass family have supported the idea that their evolution
and maintenance were often linked with improved perform-
ance in drier or more open habitats compared with C₃ sister
groups (Osborne and Freckleton, 2009; Edwards and Smith,
2010; Christin and Osborne, 2014). Osborne and Sack (2012)
proposed that improved hydraulic safety, afforded by the evo-
lution of lower gₛ among C₄ species (Osmond et al., 1982;
Taylor et al., 2010, 2012), might have increased the potential
of C₄ grasses to colonize drier habitats when ĉ was lower
than it is today. They also noted that gₛ is usually higher at
clinical ĉ compared with ambient ĉ, but the increase in gₛ is
less at glacial ĉ in C₄ plants than in C₃ plants. Using steady-
state models of coupled photosynthesis and plant hydraulics,
they showed that lower gₛ could have protected C₃ plants
from loss of hydraulic conductivity and allowed net CO₂
assimilation (A) to be maintained as soil dried at low ĉ.
They therefore proposed that in addition to biochemi-
ical advantages supporting higher A at low ĉ, protection of
hydraulic function was an important advantage to C₄ grasses
at low ĉ.
Importantly, the models that Osborne and Sack (2012) used
to predict hydraulic performance in C₃ and C₄ species at gla-
cial ĉ did not predict potential adjustments to co-ordination
of leaf gas exchange and hydraulic function at low ĉ. Although
evidence suggests that in non-woody species, decreased gₛ at
elevated ĉ is associated with less negative leaf water potentials,
lower hydraulic conductivity, and greater resistance to embol-
ism, little is known about the influence of ĉ on co-ordina-
tion between photosynthetic capacity and hydraulic function
(Domec et al., 2017). Changing irradiance results in parallel
changes in leaf hydraulic conductance (Kₑₐₙ) and photosyn-
thetic capacity of woody C₃ plants, optimizing leaf hydraulic
function (Brodribb and Jordan, 2011; Carins Murphy et al.,
2012). In contrast, adjustment to high vapour pressure deficit
(VPD) is linked with closure of stomata to protect hydraulic
function (Carins Murphy et al., 2014). In the case of ĉ, hydraulic
demand is influenced by changes in gₛ that compensate for car-
bon availability (Franks et al., 2013). Because the economics
of leaf structure–function relationships may depend on ĉ, it is
likely that ĉ has complex effects on co-ordination between Kₑₐₙ
and gₛ. For instance, smaller, more densely packed stomata are
sometimes observed at low c (Woodward, 1987; Woodward and
Bazzaz, 1988; Franks and Beering, 2009b), which may increase
the sensitivity of gₛ to VPD (Franks and Beering, 2009a; Drake
et al., 2013), serving a protective function. Conversely, higher
anatomical maxima for gₛ observed at low c in sunflower,
which were a result of larger, more densely packed stomata,
were linked with greater xylem-specific conductivity, but the
phloem ratio and hydraulic safety were decreased (Rico et al.,
2013). Photosynthetic type may further affect the impact of
ĉ on the relationships between hydraulic supply and demand
because the carbon assimilation advantage provided by C₄
photosynthesis may support additional flexibility in hydraulic
adjustment. At ambient CO₂, relative to C₃ species, C₄ dicots
maintain A at relatively lower gₛ, and either increase hydraulic
safety by decreasing xylem conduit diameter, or display greater
leaf area for similar investments in stem xylem supply (Kocacin-
ar and Sage, 2003, 2004; Kocacinar et al., 2008).
In grasses, leaf hydraulic performance is particularly impor-
tant: leaves contribute 50–72% of resistance along whole-plant
hydraulic pathways (Meinzer et al., 1992; Martre et al., 2001).
The relative sensitivities of Kₑₐₙ and gₛ are also crucial in determin-
ing water use strategies among grasses. Both C₃ and C₄ grasses
have been reported to show routine diurnal declines in leaf hydraulic
conductivity when stomata do not close sufficiently to protect
hydraulic function (Neufeld et al., 1992; Holloway-Phillips
and Brodribb, 2011b). Susceptibility to declines in conductivity
is variable both among species and among cultivars (Holloway-
Phillips and Brodribb, 2011a), and nocturnal root pressure and
refilling of embolized vessels facilitates recovery from diurnal
stress in some grass species (McCully, 1999; Holloway-Phillips
and Brodribb, 2011b; Cao et al., 2012; Gleason et al., 2017).
Protection against runaway declines in Kₑₐₙ can be provided by
stomatal closure (Brodribb and Holbrook, 2003), and fast sto-
matal responses are considered a key characteristic of grasses.
Faster stomatal responses to light can improve intrinsic water use efficiency (iWUE=$A/g_{\text{sw}}$, where $g_{\text{sw}}$ is $g_{s}$ for water) by producing a better match between rapid photosynthetic responses and the slower stomata, which may improve overall water use efficiency, resulting in greater conservation of soil water and thereby decreased hydraulic stress (Lawson and Blatt, 2014).

Our goal was to determine whether growth $q_{s}$ had different impacts on leaf function in selected C₃ and C₄ annual grasses comparable with crop species. We predicted that to support increased transpiration at low $q_{s}$, $K_{\text{leaf}}$ would increase and turgor loss points would decrease to compensate for increased hydraulic demand. In addition, we determined whether rates of stomatal closure, responding to low light, increased at low $q_{s}$. We anticipated that leaf mass per area (LMA) would decrease in plants with carbon limitation at low $q_{s}$, and that decreases in iWUE and the extent of carbon limitation imposed by low $q_{s}$ would be greater for C₃ than for C₄ species (Osborne and Sack, 2012). We therefore expected that leaf physiological responses to a range of $q_{s}$ would be larger in C₃ than in C₄ grasses. Plants were grown in CO₂ concentrations that represented: some of the glacial period during which C₄ grass lineages diversified (Biochambers, Winnipeg, Manitoba) equipped with additive CO₂, and within PACMAD two C₄ crop species have been domesticated from wild relatives in the Chloridoideae (teff and finger millet), and several from the Paniceoideae subfamily (Christin et al., 2009). Grasses with C₃ photosynthesis used as grain crops originate in the subfamilies Pooidae and Oryzoideae, which belong to a separate clade currently referred to as BEP (Kellogg, 1998; Aliscioni et al., 2012). Relevant Chloridoideae species could not be obtained, so we used only C₃ grasses from the Paniceoideae. Sorghum bicolor (great millet), Setaria italica (foxtail millet), and Digitaria exilis (fonio millet) represent independent evolutionary origins of the NADP-malate (NADP-ME) subtype of C₄ photosynthesis (Aliscioni et al., 2012); Panicum miliaceum (proso millet) represents the NAD-ME C₄ subtype (Giussani et al., 2001; Aliscioni et al., 2012). C₃ species were Panicum bisulcatum and Steinchisma laxa (two wild relatives from Paniceoideae), Triticum turgidum (durum wheat, Pooidae), and Oryza sativa ssp. japonica (rice, Ehrhartoideae; Table 1).

Plants were grown from seed in Osmocote Professional Seed Raising & Cutting Mix (Scots Australia Pty Ltd, Bella Vista, NSW) in 0.55 litre plastic square tubes (Garden City Plastics, Somersby NSW: top dimension 70 × 70 mm, 160 mm deep). Seeds were sown directly into six pots and germinated under the different CO₂ treatments; the number of plants per pot and the size of plants varied depending on the species. To allow for balanced sampling and to account for within-cabinet variability, at germination, pots were arranged into a fully randomized block design with one pot from every species in each block. The pots and germinated under the different CO₂ treatments; the number of plants per pot and the size of plants varied depending on the species. To allow for balanced sampling and to account for within-cabinet variability, at germination, pots were arranged into a fully randomized block design with one pot from every species in each block. The pots were checked daily and watered as necessary to prevent surface drying. To minimize root binding, roots were allowed to grow into a layer of wetted Scoria. To minimize nutrient limitation, plants were fed with a complete fertilizer (Thrive All Purpose Soluble Plant Food, Yates, Auckland, New Zealand) every 2–3 weeks during the course of the experiment.

### Materials and methods

#### Growth conditions

Plants were grown in walk-in climate-controlled growth chambers (Biochambers, Winnipeg, Manitoba) equipped with additive CO₂ and CO₂ scrubber equipment. Three $q_{s}$ treatments were imposed: glacial ($q_{\text{GLA}}$, 204 ± 27 µmol mol⁻¹); ambient ($q_{\text{AMB}}$, 408 ± 11 µmol mol⁻¹); and super-ambient ($q_{\text{SUP}}$, 640 ± 2 µmol mol⁻¹) (mean ±SD; 72 daily means). The $q_{\text{AMB}}$ and $q_{\text{SUP}}$ treatments were rotated between cabinets 1 week prior to the first measurements, during the fourth week after sowing. The $q_{\text{GLA}}$ treatment was maintained in a single cabinet throughout the experiment because of the technical demands of obtaining a stable CO₂ concentration at glacial $q_{s}$. Growing conditions were set to a night-time temperature of 18 °C and a daytime temperature of 26 °C, resulting in a daily mean temperature of 22 °C (mean ±SD for 72 daily means: $q_{\text{GLA}}$, 21.9 ± 0.23; $q_{\text{AMB}}$, 22.1 ± 0.21; and $q_{\text{SUP}}$, 22.1 ± 0.28).

#### Table 1. Sources and phylogenetic placement of study species

| Species                  | Photosynthetic type | Phylogenetic placement | Accession (source)                      |
|--------------------------|---------------------|------------------------|----------------------------------------|
| Triticum turgidum L. ssp. durum | C₃                  | Pooidae                | AUS-26564 /PERSIA1128 (Tony Condon, CSIRO Agriculture, ACT) |
| Oryza sativa L. ssp. japonica | C₃                  | Ehrhartoideae          | IAC1131 (Brian Atwell, Macquarie University, Sydney NSW) |
| Panicum bisulcatum Thunb. | C₃                  | Panicoideae: Paniceae  | (Ghannoum laboratory)                  |
| Steinchisma laxa (Sw.) Zuloaga | C₃                  | Panicoideae: Paspalaeae | (Ghannoum laboratory)                  |
| Sorghum bicolor (L.) Moench | C₄                  | Panicoideae: Andropogoneae | Tx623 (Alan Cruickshank, Department of Agriculture and Fisheries, Hermitage Research Facility, Warwick QLD) |
| Setaria italica (L.) P.Beauv.  | C₄                  | Panicoideae: Paniceae: Cenchrinae | AusTRCF 108040 (AusPGRIS: Tropical Crops and Forages Collection) |
| Digitaria exilis (Kippist) Stapf | C₄                  | Panicoideae: Paniceae: Anephorineae | AusTRCF 108024/PDE7 (AusPGRIS: Tropical Crops and Forages Collection) |
| Panicum miliaceum L.        | C₄                  | Panicoideae: Paniceae  | (Ghannoum laboratory)                  |

* Aliscioni et al. (2012).*
Steady-state gas exchange and stomatal response to PPFD

We measured gas exchange using six LI-6400XT photosynthesis systems (LI-COR Inc., Lincoln NE, USA) equipped with CO₂ mixers (LI-6400-01) and 2 × 3 cm red-blue LED light sources (LI-6400-02B). Pairs of LI-6400XT machines were randomly allocated to the three treatments and were rotated every 2 d: each pair of machines was used to measure two of the six blocks in every cabinet over the course of the experiment. Measurements were made under the growth conditions. To minimize disruption of treatments, the cuvette and integrated gas analysers of the LI-6400XT were placed inside the growth chambers and consoles outside the growth chambers (growth chambers were opened briefly before and after switching leaves). Measurements were conducted on the mid-section of individual, recently expanded leaves inserted parallel to the long axis of the 2 × 3 cm chamber, and leaf areas were calculated as cuvette length×average leaf width, measured to the nearest 0.5 mm with a ruler. Leaves were allowed to come to steady state [showing no systematic trends with a coefficient of variation (CV) <0.1 over a 5 min period] at a PPFD of 500 μmol m⁻² s⁻¹ (growth light levels) and cuvette CO₂ concentrations matched to \( c_a \) at the time of measurement: \( c_{GLA} = 184 ± 4 \mu mol mol⁻¹; c_{AMB} = 406 ± 5 \mu mol mol⁻¹; \) and \( c_{SUP} = 647 ± 6 \mu mol mol⁻¹; \) mean ±SD ≥43 leaves, CV for individual leaves <5%). Relative humidity was maintained at ~70% and block temperature at 26 °C, resulting in leafVPDs of 1 ± 0.07 kPa (mean ±SD, n=137 leaves; CV for individual leaves <8.1%). An auto-program (logging every 10 s) was used to record initial steady-state values for gas exchange (\( A, c_{i}, g_{sw} \), and iWUE), followed by the response of \( c_{i} \) to a step-change decrease in light availability from 500 μmol m⁻² s⁻¹ to 100 μmol m⁻² s⁻¹ PPFD. The rate of stomatal response to PPFD (\( \Delta \psi \psi_a \) characterised on the magnitude \( \Delta \psi_a \) and duration \( \Delta t \)) of the initial decrease in \( c_{sw} \).

Leaf hydraulic conductance and LMA

Because \( C_3 \) and \( C_4 \) species differed in the response of \( A \) to \( c_a \) between \( c_{GLA} \) and \( c_{AMB} \), we determined \( K_{dev} \) in those treatments using the evaporative flux method (Sack et al., 2002). Cut stems were transported to the lab in water, where flag or second-leaf laminas were excised and, using LI-6400XT machines were randomly allocated to the three treatments. Measurements were made under the growth conditions. To minimize disruption of treatments, the cuvette and integrated gas analysers of the LI-6400XT were placed inside the growth chambers and consoles outside the growth chambers (growth chambers were opened briefly before and after switching leaves). Measurements were conducted on the mid-section of individual, recently expanded leaves inserted parallel to the long axis of the 2 × 3 cm chamber, and leaf areas were calculated as cuvette length×average leaf width, measured to the nearest 0.5 mm with a ruler. Leaves were allowed to come to steady state [showing no systematic trends with a coefficient of variation (CV) <0.1 over a 5 min period] at a PPFD of 500 μmol m⁻² s⁻¹ (growth light levels) and cuvette CO₂ concentrations matched to \( c_a \) at the time of measurement: \( c_{GLA} = 184 ± 4 \mu mol mol⁻¹; c_{AMB} = 406 ± 5 \mu mol mol⁻¹; \) and \( c_{SUP} = 647 ± 6 \mu mol mol⁻¹; \) mean ±SD ≥43 leaves, CV for individual leaves <5%). Relative humidity was maintained at ~70% and block temperature at 26 °C, resulting in leafVPDs of 1 ± 0.07 kPa (mean ±SD, n=137 leaves; CV for individual leaves <8.1%). An auto-program (logging every 10 s) was used to record initial steady-state values for gas exchange (\( A, c_{i}, g_{sw} \), and iWUE), followed by the response of \( c_{i} \) to a step-change decrease in light availability from 500 μmol m⁻² s⁻¹ to 100 μmol m⁻² s⁻¹ PPFD. The rate of stomatal response to PPFD (\( \Delta \psi \psi_a \) characterised on the magnitude \( \Delta \psi_a \) and duration \( \Delta t \)) of the initial decrease in \( c_{sw} \).

Pressure–volume relationships

Pressure–volume (P–V) relationships were determined using bench-dry-drying. On the morning of measurement, attached flag leaves were sealed into plastic bags containing exhaled breath and were allowed to equilibrate for a minimum of 40 min to quench transpiration and ensure high turgor. Leaves sheathed in this manner were subsequently excised at the base of the lamina and moved to the laboratory. Initially, leaves remained sealed in plastic between measurements of fresh mass (FM, g) and water potential (\( \psi \psi \), Scholander pressure bomb). As water potential declined, leaves were occasionally removed from the plastic for short periods to increase the rate of drying. A minimum of 20 min equilibration was ensured between pressure bomb measurements. At the conclusion of \( FM \) and \( \psi \psi \) measurements, leaves were dried for a minimum of 48 h at 65 °C to determine dry mass (DM, g). The turgid mass (TM, g) was estimated by extrapolation of the initial linear FM–\( \psi \psi \) relationship (Kubiske and Abrams, 1990) and used to calculate relative water contents (RWC, %) for entire leaves as \( FM–DM)/(TM–DM) \), and leaf dry matter content (LDMC=DM/TM).

We optimized parameter selection for P–V relationships of individual leaves by minimizing the absolute difference between estimates of osmotic potential at full turgor (\( \psi \psi_{a} \), MPa) obtained below \( \psi \psi_{a} \) and above \( \psi \psi_{a} \) turgor loss, comparing all possible combinations that could be fit for each leaf within our data set. First, below-turgor loss fits for \( 1/\psi \psi = 1/(\psi \psi_{a} + \psi \psi_{1}) \) (linear regression with slope \( a \) and \( y \)-intercept \( \psi \psi_{1} \)) were obtained from all sequences representing at least three of the smallest RWC values and excluding two or more of the highest RWC values. Next, the \( a \)-intercept of the below–turgor loss relationship (apoplastic fraction, \( a \)) was used to establish the RWC of the symplasm \( \psi \psi_{2} = \psi \psi_{a} - \psi \psi_{1} \). Then the osmotic potential, \( \psi \psi_{o} = -e(1-\psi \psi_{2} - \psi \psi_{3}) \). Turgor loss point characteristics were calculated for the pair of linear relationships where \( \psi \psi_{a} = 1 \) and \( \psi \psi_{a} = 1 \) was smallest, and \( \psi \psi_{a} < 1 \). Using \( \psi \psi_{a} \) to estimate \( \psi \psi_{a} \) at turgor loss \( \psi \psi_{c} \) was established by determining the RWC at which \( \psi \psi_{a} = 0 \), and was used to predict osmotic potential at turgor loss \( \psi \psi_{a} \) from the equation for \( \pi \).
Table 2. Impact of photosynthetic type on leaf gas exchange relative to ambient CO2 ($c_{\text{AMB}}$ ~400 μmol mol$^{-1}$), at glacial CO2 ($c_{\text{GLA}}$ ~200 μmol mol$^{-1}$), and at super-ambient CO2 ($c_{\text{SUP}}$ ~640 μmol mol$^{-1}$)

| Photosynthetic type (C4 subtype) | Species            | iWUE (μmol mol$^{-1}$) | Net CO2 assimilation (μmol m$^{-2}$ s$^{-1}$) | Stomatal conductance (mol m$^{-2}$ s$^{-1}$) |
|----------------------------------|--------------------|-------------------------|---------------------------------------------|---------------------------------------------|
|                                  |                    | $c_{\text{GLA}}/c_{\text{AMB}}$ | $c_{\text{SUP}}/c_{\text{AMB}}$ | $c_{\text{GLA}}/c_{\text{AMB}}$ | $c_{\text{SUP}}/c_{\text{AMB}}$ | $c_{\text{GLA}}/c_{\text{AMB}}$ | $c_{\text{SUP}}/c_{\text{AMB}}$ |
| C4 (NADP-ME)                    | T. turgidum        | 0.66                    | 1.35                                        | 0.51                                        | 1.07                                        | 0.77                                        | 0.79                                        |
| C3 (NAD-PME)                    | S. bicolor         | 0.37                    | 1.1                                         | 0.63                                        | 1.2                                         | 1.72                                        | 0.93                                        |
| C4 (NADP-ME)                    | D. exilis          | 0.53                    | 1.04                                        | 0.87                                        | 0.92                                        | 1.65                                        | 0.88                                        |
| C4 (NADP-ME)                    | S. italica         | 0.42                    | 1.13                                        | 0.93                                        | 0.94                                        | 2.23                                        | 0.83                                        |
| C4 (NADP-ME)                    | P. miliaceum       | 0.51                    | 1.56                                        | 0.93                                        | 1.11                                        | 2.43                                        | 0.71                                        |

Kruskal–Wallis P C3/C4 (df=1)

*P<0.05.

Impact of $c_a$ on LMA

At $c_{\text{SUP}}$, LMA values for flag leaves of C3 and C4 species were similar (Fig. 3); however, the response of LMA to $c_a$ differed among the eight species (Fig. 3; $F_{1,6,42}=0.0004$). None of the C4 species exhibited significant changes in LMA in response to $c_a$ (Tukey’s HSD, $P>0.72$). In the C3 species, LMA was similar across the three $c_a$ treatments for S. laxa, but T. turgidum, P. bisulcatum, and O. sativa all showed significant reductions in LMA from either $c_{\text{GLA}}$ to $c_{\text{AMB}}$ (T. turgidum and P. bisulcatum, Tukey’s HSD $P<0.05$) or from $c_{\text{AMB}}$ to $c_{\text{GLA}}$ (O. sativa, $P=0.039$; Fig. 3). The contrasts term for photosynthetic type $\times c_a$, which was statistically significant (t-test $P=0.014$), was therefore broadly associated with less sensitivity of LMA to $c_a$ among the C4 species. Conservation of LMA across CO2 treatments in most C4 species was linked with proportionate decreases in mass and area of the flag leaves as $c_a$ was reduced. Among C3 species, decreases in LMA arose because flag leaf mass decreased with $c_a$ from $c_{\text{SUP}}$ to $c_{\text{GLA}}$ and flag leaf area decreased from $c_{\text{AMB}}$ to $c_{\text{GLA}}$, but leaf areas were often similar at $c_{\text{GLA}}$ and $c_{\text{AMB}}$ (Fig. 4).

Response of $K_{\text{leaf}}$ and P–V characteristics to decreases in $c_a$ from $c_{\text{AMB}}$ to $c_{\text{GLA}}$

There were no significant species $\times c_a$ effects on $K_{\text{leaf}}$ (species $\times c_a$, $F_{1,6,41}=0.814$); however, on average, $K_{\text{leaf}}$ was lower in plants grown at $c_{\text{GLA}}$ ($F_{1,6,41}=0.005$). The exception was D. exilis, a C4 species with small leaves, for which measurement errors were large (Fig. 5A).

In the P–V analysis, the response of LDMC to $c_a$ was consistent with that of LMA measured during determination of $K_{\text{leaf}}$. LDMC was not significantly different between the photosynthetic types at either $c_a$, but was lower at $c_{\text{GLA}}$ among C3 and not C4 leaves (Table 3). LDMC decreased by 4–11% in C3 grasses grown at $c_{\text{GLA}}$, but C4 species showed no adjustment to $c_{\text{GLA}}$ or increased LDMC by ≤3% at $c_{\text{GLA}}$. This difference in average LDMC responses to $c_a$ was statistically significant when comparing C3 and C4 species (Table 3). Despite these differences in LDMC responses between C3 and C4 species, we found no evidence for significant effects of photosynthetic type on the response of ε or RWC$_{\text{TLT}}$ to $c_a$. In contrast, the median π$_{\text{TLT}}$ differed between C3 and C4 grasses at $c_{\text{AMB}}$ but not at $c_{\text{GLA}}$, linked...
with a significant effect of photosynthetic type (Table 3). At \( \epsilon_{\text{AMB}} \), \( \pi_{\text{LTP}} \) was less negative among C4 species (C4, −0.72 MPa to −0.87 MPa; C3, −0.94 MPa to −1.36 MPa). This difference was eliminated at \( \epsilon_{\text{GLA}} \) because only C4 grasses decreased \( \pi_{\text{LTP}} \) to more negative values (C4, −0.79 MPa to −1.27 MPa; C3, −0.91 MPa to −1.21 MPa; Table 2).

**Discussion**

We exposed C3 and C4 grasses to atmospheric CO2 concentrations ranging from levels that occurred during the last 30 million years, when C4 lineages evolved and diversified, to those that could be experienced in the coming centuries. Across the range of \( \epsilon \), we expected that C4 species would maintain an iWUE advantage and show smaller physiological adjustments. Our results broadly support this expectation: the absolute response of \( g_{\text{sw}} \) to \( \epsilon \) was greater among C3 than among C4 grasses; and, as \( \epsilon \) decreased from \( \epsilon_{\text{AMB}} \) to \( \epsilon_{\text{GLA}} \), A, LDMC, and LMA declined more among C3 than among C4 species. Investigation of leaf hydraulic function at \( \epsilon_{\text{AMB}} \) and \( \epsilon_{\text{GLA}} \) showed that at \( \epsilon_{\text{GLA}} \), \( K_{\text{leaf}} \) decreased in both C3 and C4 species;
and \( \pi_{\text{TLP}} \) of \( \text{C}_4 \) leaves became more negative, hence more similar to \( \pi_{\text{TLP}} \) of \( \text{C}_3 \) leaves, which did not adjust. Assaying the stomatal response to shade showed that higher steady-state \( g_{\text{sw}} \) of \( \text{C}_3 \) species was linked with more rapid adjustment of \( g_{\text{sw}} \) to match \( A \). Rates of stomatal closure were slightly more similar for \( \text{C}_3 \) and \( \text{C}_4 \) species at low \( c_a \), driven by strong responses of species that achieved high iWUE at elevated \( c_a \). These new findings are consistent with the hypothesis that carbon limitation is an important factor influencing leaf hydraulic function at different atmospheric [CO2]. Although there was substantial variation among species, photosynthetic type affected how leaf dry matter was deployed and how leaf turgor characteristics responded to \( c_{\text{GLA}} \).

Gas exchange responses to \( c_a \)

Steady-state gas exchange measurements provided the expected outcomes: \( g_s \) usually increased as \( c_a \) decreased (Osborne and Sack, 2012; Franks et al., 2013); high \( g_s \) of \( \text{C}_3 \) grasses was associated with greater \( g_s \) responses to \( c_a \); and low \( g_{\text{sw}} \) of \( \text{C}_4 \) leaves resulted in higher iWUE at all levels of \( c_a \). Importantly, \( A \) declined for \( \text{C}_3 \) but not \( \text{C}_4 \) grasses at \( c_{\text{GLA}} \). Greater \( A \) among some \( \text{C}_3 \) species compared with \( \text{C}_4 \) species at \( c_{\text{AMB}} \) and \( c_{\text{SUP}} \) suggested that \( \text{C}_4 \) photosynthetic performance may have been limited by PPFD, so the iWUE advantage to \( \text{C}_4 \) species may underestimate advantages to \( \text{C}_4 \) species that could arise at higher irradiances (Osmond et al., 1982).

\( \text{C}_3 \) grass leaves generally closed their stomata more quickly than \( \text{C}_4 \) leaves in response to shade. The higher steady-state \( g_{\text{sw}} \) of \( \text{C}_3 \) leaves may partially explain this difference between the photosynthetic types, but closer inspection of the data shows that \( \Delta g_{\text{sw}} / \Delta t \) did not parallel the steady-state \( g_{\text{sw}} \) for species within each photosynthetic type. Interestingly, among \( \text{C}_4 \) species, the rate of \( g_{\text{sw}} \) responses to light was slightly, but consistently, greater at \( c_{\text{GLA}} \) compared with \( c_{\text{AMB}} \) and \( c_{\text{SUP}} \). This decreased the difference in \( \Delta g_{\text{sw}} / \Delta t \) between \( \text{C}_3 \) and \( \text{C}_4 \) species. However, a more striking trend, that probably underpinned the subtle difference in relative performance based on photosynthetic type, was that species with higher iWUEs showed greater changes in \( \Delta g_{\text{sw}} / \Delta t \) in response to decreasing \( c_a \). At \( c_{\text{GLA}} \), species with high iWUE showed some of the slowest stomatal responses to shade. Because faster stomatal responses are consistent with improved water use efficiency (Lawson and Blatt, 2014), this suggests that transpiration is regulated less tightly at high \( c_a \), supporting the overarching hypothesis that increasing \( c_a \) minimizes the costs associated with hydraulic stress (Polley et al., 1993). It also suggests that characteristics producing high iWUE in the steady state may be costly in low-\( c_a \)-like scenarios that increase transpiration. For example, high iWUE is likely to be facilitated by
Table 3. Impact of growth $c_3$ on pressure–volume curve characteristics and leaf dry matter content (medians, n=3–5; $c_{GLA}$, glacial CO2 ~200 μmol mol$^{-1}$; $c_{AMB}$, ambient CO2 ~400 μmol mol$^{-1}$)

| Photosynthetic type (C$_4$ subtype) | Species | Modulus of elasticity ($t$, MPa) | Osmotic potential at turgor loss (mtLP, MPa) | RWC at turgor loss (RWC$_{TLP}$, %) | Leaf dry matter content (LDMC, %) |
|------------------------------------|---------|---------------------------------|------------------------------------------|----------------------------------|----------------------------------|
|                                    |         | $c_{GLA}$ | $c_{AMB}$ | Difference in median: $c_{GLA}$−$c_{AMB}$ | $c_{GLA}$ | $c_{AMB}$ | Difference in median: $c_{GLA}$−$c_{AMB}$ | $c_{GLA}$ | $c_{AMB}$ | Difference in median: $c_{GLA}$−$c_{AMB}$ | $c_{GLA}$ | $c_{AMB}$ |
| C$_1$                               | T. turgidum | 11      | 9.4     | 1.6 | −1.17 | −1.31 | 0.14 | 94.8 | 94.5 | −0 | 17 | 26 | −8$^*$ |
| C$_1$                               | O. sativa | 5.4     | 5.7     | −0.3 | −1.21 | −1.36 | 0.15 | 94.2 | 89.4 | 4.8 | 24 | 28 | −4 |
| C$_1$                               | S. laxa | 8       | 7.1     | 0.9 | −1.13 | −1.01 | 0.12 | 93.3 | 92.3 | 1 | 18 | 29 | −11$^*$ |
| C$_1$                               | P. bisulcatum | 4.1     | 6.2     | −2.1 | −0.91 | −0.94 | 0.04 | 89.4 | 93.9 | −4.5 | 26 | 32 | −6$^*$ |
| C$_1$ (NADP-ME)                     | S. bicolor | 7.7     | 5.1     | 2.6 | −1.03 | −0.82 | −0.2 | 97 | 97 | 0 | 26 | 23 | 3 |
| C$_1$ (NADP-ME)                     | D. exilis | 5.9     | 9.4     | −3.5 | −0.79 | −0.72 | −0.08 | 92.1 | 93.6 | −1.5 | 19 | 19 | 0 |
| C$_1$ (NADP-ME)                     | S. italica | 8.1     | 7.4     | 0.7 | −1.27 | −0.87 | −0.41$^{**}$ | 93.2 | 95.7 | −2.5 | 28 | 26 | 2 |
| C$_1$ (NADP-ME)                     | P. miliaceum | 6.5     | 7.3     | −0.8 | −1 | −0.85 | −0.18$^{*}$ | 94.8 | 95.8 | −1 | 31 | 29 | 2 |
| Kruskal–Wallis P species (df=7)     | NS       | NS      | NS      | NS | * | * | * | NS | NS | NS | NS | NS | * |
| Kruskal–Wallis P C$_3$/C$_4$ (df=1) | NS       | NS      | NS      | NS | * | * | * | NS | NS | NS | NS | NS | * |

$^*$P<0.05; **P<0.01; †P<0.001; $^*$0.001<P<0.01; †P<0.05 after exclusion of an extreme value >40% from $c_{GLA}$ treatment that led to a median of 22% and difference in median of 10%

high rates of internal diffusion, which are linked with decreases in cell wall dry matter (Onoda et al., 2017) and might increase vulnerability to changes in leaf water status.

Among-species variation was an important feature of our gas exchange results. This is consistent with previous studies, which have indicated that the degree to which grass stomata protect against decreases in hydraulic conductance varies even among genotypes (Neufeld et al., 1992; Holloway-Phillips and Brodribb, 2011a). Among C$_3$ species in our study, only that with the highest iWUE, P. bisulcatum, showed a clear negative association between $c_t$ and the stomatal response to shade. At the other extreme, T. turgidum showed exceptionally high steady-state $g_{sw}$ and slow stomatal responses to shade in all three $c_t$ treatments, suggesting high transpiration irrespective of leaf water status, a strategy that can maximize CO2 uptake at a cost to hydraulic conductance (Holloway-Phillips and Brodribb, 2011b). The apparent lack of stomatal regulation in T. turgidum compared with other C$_3$ species is important to note because iWUE for this species did not decrease at $c_{GLA}$, contradicting the otherwise consistent trend towards greater decreases in iWUE among C$_3$ compared with C$_4$ species.

Impact of glacial $c_3$ on LMA and hydraulic characteristics

LMA decreased at $c_{GLA}$ among C$_3$ but not C$_4$ grasses. This finding is consistent with observed differences in $A_t$, results from a meta-analysis addressing variation in LMA (Poorter et al., 2009), and more recent comparisons using species and $c_t$ treatments similar to those chosen for our experiment (Pinto et al., 2014). Further evidence is needed, however, before this result can be generalized as a photosynthetic type effect. LMA responses can, for example, be modified by temperature (Pinto et al., 2011). It is also important to note that the C$_3$ and two of the wild C$_3$ species included in our experiment were drawn from one subfamily of the Poaceae: Panicoideae, a broadly mesic-adapted clade (Taub, 2000; Osborne, 2008; Edwards and Smith, 2010; Visser et al., 2014). We expect leaf functional traits to reflect adaptations to habitat, and some major C$_4$ lineages are adapted to drier environments than those favoured by the Panicoideae (Taub, 2000; Edwards and Smith, 2010). In addition, LMA responses to $c_t$ (Pinto et al., 2016) and leaf size (Liu et al., 2012) differ between the Chloridoideae and Panicoideae grass subfamilies. While further work will be needed to establish whether the patterns we observed are general across grass lineages, our findings are directly relevant to crop and crop-related annual grass species from mesic habitats. Taken together with the gas exchange results, differences in LMA indicate that $c_{GLA}$ was linked with greater carbon limitation in C$_3$ grasses compared with their C$_4$ relatives. This is important because differences in carbon supply affecting plant size and allocation at the whole-plant level have previously been highlighted as central to functional contrasts between C$_3$ and C$_4$ plants (Long, 1999; Atkinson et al., 2016), and influence the mechanisms by which plants acclimate to hydraulic stress (Maseda and Fernández, 2006).

C$_3$ and C$_4$ grasses showed similar $K_{leaf}$ and $K_{leaf}$ decreased at $c_{GLA}$. The finding that there was no clear difference in $K_{leaf}$ between photosynthetic types is consistent with a previous comparison using the high pressure flow meter technique, applied to predominantly perennial, North American prairie grasses (Ocheltree et al., 2014b). Both of these results are surprising because the clearest anatomical differences between C$_3$ and C$_4$ grass lineages are in the ratio of bundle sheath to mesophyll (Hattersley, 1984; Dengler et al., 1994; Christin et al., 2013; Griffiths et al., 2013; Lundgren et al., 2014). Increases in this ratio should decrease hydraulic resistance external to the xylem (Buckley et al., 2015), supporting the hypothesis that differences in leaf hydraulic properties could affect responses to stress imposed by low $c_t$ and/or water availability (Osborne and Sack, 2012; Griffiths et al., 2013). It is possible that other aspects of C$_4$ leaf anatomy or function counteract positive effects of
increased bundle sheath ratios on $K_{\text{leaf}}$ in C$_4$ grasses. It is also important to note that C$_3$ and C$_4$ grasses often show similar average mesophyll cell sizes at ambient CO$_2$ (Lundgren et al., 2014), and the cross-sectional area of vascular relative to chloro-enchyma tissues does not necessarily change with photosynthetic type (Dengler et al., 1994).

The evidence we found for decreased $K_{\text{leaf}}$ at c$_{\text{GLA}}$ was surprising, because xylem conductivity generally increases with declining c$_l$ to support increased $g_{\text{sw}}$ (Rico et al., 2013; Domec et al., 2017). Previous in situ measurements of transpiration and leaf water potential in sunflower plants grown at c$_l$ similar to c$_{\text{GLA}}$ and c$_{\text{AMB}}$ showed the expected result: that $K_{\text{leaf}}$ increased for plants grown at c$_{\text{GLA}}$, minimizing the impact of increased $g_{\text{sw}}$ on ΔΨ (Simonin et al., 2015). A decrease in xylem conductivity, linked with smaller conduits in water-stressed tissue that would increase redundancy among conducting elements (Comstock and Sperry, 2000), might contribute to decreases in $K_{\text{leaf}}$ for leaves grown at c$_{\text{GLA}}$. However, this is not consistent with the decrease in LMA that we observed and, since transpiration was driven using moderate levels of light, we expect that the primary source of hydraulic resistance was exterior to the xylem (Ocheltree et al., 2014a).

The values of $K_{\text{leaf}}$ were low compared with other recently published estimates for similar species [S. bicolor, 19–38 mmol m$^{-2}$ s$^{-2}$ MPa$^{-1}$ (Ocheltree et al., 2014a); O. sativa cultivars, 7.1–8.7 mmol m$^{-2}$ s$^{-2}$ MPa$^{-1}$ (Xiong et al., 2015)], but are within the range reported in the literature for grasses (~0.44–51 mmol m$^{-2}$ s$^{-2}$ MPa$^{-1}$; Holloway-Phillips and Brodribb, 2011a; Ocheltree et al., 2014a; Liu and Osborne, 2015; Xiong et al., 2015) and may be a consequence of moderate PPFD during growth and measurements (Cochard et al., 2007; Ocheltree et al., 2014a). Further experimentation and comparison of methods is needed for measurements of $K_{\text{leaf}}$ in grasses.

We need to understand why measurements of $K_{\text{leaf}}$ produce similar values for C$_3$ and C$_4$ species; to establish whether $K_{\text{leaf}}$ responses to c$_l$ correspond to changes in hydraulic vulnerability; and to determine the anatomical basis of adjustments to $K_{\text{leaf}}$, especially given evidence for declining LDLC and LMA among C$_3$ species at c$_{\text{GLA}}$. It will also be important to measure $K_{\text{leaf}}$ at different [CO$_2$]; as in the study of $K_{\text{leaf}}$ responses to c$_l$ that used sunflower (Simonin et al., 2015), we measured $K_{\text{leaf}}$ at ambient [CO$_2$].

Effects of c$_l$ on P–V characteristics also provide motivation for further investigation of photosynthetic type×c$_l$ responses. As leaf size decreased at c$_{\text{GLA}}$, C$_4$ grasses maintained LDLC and C$_3$ grasses did not. In parallel, $\pi_{\text{TLP}}$ of C$_4$ grasses became more negative at c$_{\text{GLA}}$, while $\pi_{\text{TLP}}$ of C$_3$ grasses did not change. This is an important result because $\pi_{\text{TLP}}$ is a powerful indicator of physiological responses that is expected to integrate smaller changes in, for example, $\pi_s$ and ε (Bartlett et al., 2012). The decrease in LDLC shown by C$_3$ leaves grown at c$_{\text{GLA}}$ is consistent with both lower A and LMA, and previous evidence that C$_3$ leaves decrease mesophyll cell volume and total non-structural carbohydrates as c$_l$ declines (Poorter et al., 2009). Maintenance of LDLC and more negative $\pi_{\text{TLP}}$ in C$_4$ grasses therefore might be linked with solute accumulation at c$_{\text{GLA}}$. Presumably, decreases in $\pi_{\text{TLP}}$ of C$_4$ leaves at low c$_l$ would support maintenance of turgor in the presence of larger ΔΨ induced by higher $g_{\text{sw}}$ (Franks, 2006; Simonin et al., 2015); however, we do not know how leaf-level changes were integrated with adjustments in root and stem properties. The lack of an adjustment in $\pi_{\text{TLP}}$ by C$_3$ grasses grown at c$_{\text{GLA}}$ might be associated with maintenance of leaf water status if root and stem xylem hydraulic conductivity increased or xylem solute concentrations decreased.

Conclusions

We predicted that gas exchange would show greater absolute responses to c$_l$ in C$_3$ compared with C$_4$ grass leaves, especially in terms of the positive relationship between iWUE and c$_l$. We also predicted that low iWUE at c$_{\text{GLA}}$ would be linked with changes in leaf hydraulic properties. We found that while the iWUE advantage of some C$_4$ grass leaves increased in absolute terms at c$_{\text{SUP}}$, co-ordination among leaf traits was more strongly affected by c$_{\text{GLA}}$ than by c$_{\text{SUP}}$. These experimental results broadly support predicted smaller impacts of c$_{\text{GLA}}$ on performance of C$_4$ grasses (Osborne and Sack, 2012), and suggest that iWUE advantages to C$_4$ species will continue to be important in future. A finding with potential importance for crop improvement programmes is that as c$_l$ increases, pressure on plants to improve iWUE through rapid stomatal responses to shade may be reduced, particularly for species capable of achieving high iWUE. These results highlight the need for continued efforts to establish how hydractics and photosynthetic performance are co-ordinated, both within leaves and at the scale of whole plants. The mechanistic basis of these responses still needs to be better understood to predict the physiological implications of C$_4$ photosynthesis, both under past glacial climates and as they will affect performance in a future high CO$_2$ world.

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