Photosynthesis of $C_3$, $C_{3-4}$, and $C_4$ grasses at glacial CO$_2$

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Received 9 December 2013; Revised 2 March 2014; Accepted 5 March 2014

Abstract

Most physiology comparisons of $C_3$ and $C_4$ plants are made under current or elevated concentrations of atmospheric CO$_2$ which do not reflect the low CO$_2$ environment under which $C_4$ photosynthesis has evolved. Accordingly, photosynthetic nitrogen (PNUE) and water (PWUE) use efficiency, and the activity of the photosynthetic carboxylases [Rubisco and phosphoenolpyruvate carboxylase (PEPC)] and decarboxylases [NADP-malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PEP-CK)] were compared in eight $C_4$ grasses with NAD-ME, PCK, and NADP-ME subtypes, one $C_3$ grass, and one $C_{3-4}$ grass grown under ambient (400 μl l$^{-1}$) and glacial (180 μl l$^{-1}$) CO$_2$. Glacial CO$_2$ caused a smaller reduction of photosynthesis and a greater increase of stomatal conductance in $C_4$ relative to $C_3$ and $C_{3-4}$ species. $Panicum bisulcatum$ ($C_3$) acclimated to glacial [CO$_2$] by doubling Rubisco activity, while Rubisco was unchanged in $Panicum milioides$ ($C_{3-4}$), possibly due to its high leaf N and Rubisco contents. Glacial CO$_2$ up-regulated Rubisco and PEPC activities in concert for several $C_4$ grasses, while NADP-ME and PEP-CK activities were unchanged, reflecting the high control exerted by the carboxylases relative to the decarboxylases on the efficiency of $C_4$ metabolism. Despite having larger stomatal conductance at glacial CO$_2$, $C_4$ species maintained greater PWUE and PNUE relative to $C_{3-4}$ and $C_3$ species due to higher photosynthetic rates. Relative to other $C_4$ subtypes, NAD-ME and PEP-CK grasses had the highest PWUE and PNUE at glacial CO$_2$. Biomass accumulation was reduced by glacial CO$_2$ in the $C_3$ grass relative to the $C_{3-4}$ grass, while biomass was less reduced in NAD-ME grasses compared with NADP-ME and PCK grasses. Under glacial CO$_2$, high resource use efficiency offers a key evolutionary advantage for the transition from $C_3$ to $C_4$ photosynthesis in water- and nutrient-limited environments.

Key words: $C_3$, $C_{3-4}$, and $C_4$ photosynthesis, glacial CO$_2$, NAD-ME, NADP-ME, PEPC, PEP-CK, Rubisco, water and nitrogen use efficiency.

Introduction

The decline in atmospheric CO$_2$ concentration ([CO$_2$]) in the late Oligocene (30 million years ago) is considered to be the primary driver for the evolution of the $C_4$ photosynthetic pathway (Christin et al., 2008; Ehleringer et al., 1997; Sage et al., 2012). Geological fluctuations in atmospheric [CO$_2$] have shaped the Earth's vegetation, yet relatively little is known about the responses of $C_4$ plants to the low [CO$_2$] levels that dominated during their evolution, and that are close to the atmospheric [CO$_2$] of the recent glaciation (Pagani et al., 2005). Low [CO$_2$] promotes high rates of photorespiration and reduces the carboxylation efficiency of $C_3$ photosynthesis. The key feature of $C_4$ photosynthesis is the operation of a CO$_2$-concentrating mechanism (CCM) which suppresses photorespiration by raising [CO$_2$] around Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase). During $C_4$ photosynthesis, phosphoenolpyruvate carboxykinase (PEPC) catalyses the initial carboxylation of CO$_2$ into organic $C_4$ acids in the mesophyll. Decarboxylation of $C_4$ acids in the bundle sheath releases CO$_2$ for refixation by Rubisco (Hatch, 1987). The $C_4$ photosynthetic pathway is classified into three biochemical
subtypes based on the primary C₄ decarboxylase enzyme. These enzymes are NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEP-CK, also known as PCK) (Gutierrez et al., 1974; Kanai and Edwards, 1999). There are strong anatomical and biochemical variations associated with these biochemical subtypes (Prendergast et al., 1987; Dengler et al., 1994; Edwards and Voznesenskaya, 2011).

The operation of a CCM enhances the efficiency of C₄ relative to C₃ photosynthesis (Osmond, 1982). In particular, C₄ species attain higher photosynthetic water use efficiency (PWUE) because lower stomatal conductance (gₛ) and intercellular [CO₂] (Cᵢ) are needed to saturate Rubisco carboxylation. C₄ plants achieve higher photosynthetic nitrogen use efficiency (PNUE) due to their lower leaf N requirement as a result of a higher Rubisco catalytic turnover rate (kₑcat) (Long, 1999; Taylor et al., 2010; Ghannoum et al., 2011). Variations in resource use efficiency also occur among the C₄ subtypes (Ghannoum et al., 2011). For example, NADP-ME grasses tend to have lower leaf N content than their NAD-ME counterparts (Bowman, 1991; Knapp and Medina, 1999; Taub and Lerdau, 2000), as a result of faster Rubisco kₑcat in NADP-ME species (Ghannoum et al., 2005). Furthermore, Ghannoum et al. (2002) showed that under water stress, NAD-ME grasses increased their whole-plant WUE to a greater extent than NAD-ME counterparts. These aforementioned studies were undertaken under current ambient [CO₂] which does not reflect the low CO₂ environment under which C₄ grasses have evolved. Hence, the main aim of the current study was to investigate whether previously reported physiological differences among the C₄ subtypes at ambient [CO₂] are similarly observed at glacial [CO₂].

Growth at low [CO₂] reduces growth and photosynthesis of C₃ plants. C₃ plants respond to low [CO₂] by increasing gₛ to improve CO₂ supply and by up-regulating photosynthetic enzymes to improve CO₂ capture (Polley et al., 1992; Dippery et al., 1995; Tissue et al., 1995; Gesch et al., 2000; Anderson et al., 2001). The occurrence of a CCM in C₄ leaves makes the C₄ pathway less limited by CO₂ supply and, hence, less likely to respond and acclimate to growth at low [CO₂] relative to C₃ photosynthesis (Hatch, 1987; Gerhart and Ward, 2010). Nevertheless, increased leaf N content and gₛ have been observed under low [CO₂] in some C₄ species (Anderson et al., 2001; Maherali et al., 2002). To the authors’ knowledge there are no published studies comparing the impact of low [CO₂] on the photosynthetic gas exchange or biochemistry of C₄ grasses with different biochemical subtypes. The current study aims at addressing this knowledge gap.

A hypothesized intermediate stage during C₄ evolution, known as C₃-C₄ intermediate, restricts the activity of glycine decarboxylase to the bundle sheath (Sage et al., 2012), thus improving Rubisco efficiency by facilitating the recapture of photorespired CO₂ (Monson and Moore, 1989; Monson and Rawsthorne, 2000). The operation of a photorespiratory pump in C₃-C₄ photosynthesis is expected to elicit a response to [CO₂] that is intermediate between C₃ and C₄ photosynthesis (Monson and Rawsthorne, 2000; Sage et al., 2012). Under low [CO₂], C₃-C₄ plants have been reported to maintain greater photosynthetic rates, PWUE, and PNUE relative to C₃ species (Ku and Edwards, 1978; Bolton and Brown, 1980; Ku et al., 1991; Monson and Rawsthorne, 2000; Vogan et al., 2007; Pinto et al., 2011; Vogan and Sage, 2012). The current study seeks to determine how C₃-C₄ species perform relative to the various C₄ subtypes at low [CO₂].

Comparing the sensitivity to glacial [CO₂] of the different pathways of photosynthesis and subtypes of C₄ photosynthesis among closely related grass species may provide critical insight into the physiology of C₄ plants under conditions that led to their evolution. Consequently, this study compared the photosynthetic physiology (PWUE and PNUE) and biochemistry (activity of the photosynthetic carboxylase and decarboxylase enzymes) in C₄ grasses with different biochemical subtypes grown under ambient (400 μl l⁻¹) or glacial (180 μl l⁻¹) [CO₂]. Closely related C₃ and C₃-C₄ grass species were included for comparison.

### Materials and methods

#### Plant culture

Two matched growth chambers (1.8 m³ each; BioChambers, Winnipeg, Manitoba, Canada) were used in this study. The chambers were maintained at either glacial (180 μl l⁻¹) or ambient (400 μl l⁻¹) CO₂. Low CO₂ was achieved by passing incoming air over a CO₂ absorbent (Grace SodaSorb, WR Grace and Co.-Conn., Chicago, USA) and controlled by CO₂ gas analysers (LI-820, LI-COR, Lincoln, NE, USA). The average growth conditions during the experiment are shown in Table 1.

Locally collected soil (Ghannoum et al., 2010) was air-dried, coarsely sieved, and added (3.7 kg) to 3.5 l cylindrical pots, which were watered to 100% capacity, then transferred to the two growth chambers. Seeds for the grass species used in this study (Table 2) were obtained from the Australian Plant Genetic Resources Information System (ACT, Australia) and Queensland Agricultural Seeds Pty. Ltd (Toowoomba, Australia). Seeds were sown in trays containing a common germination mix. Three to four weeks after germination, three seedlings were transplanted into each of the soil-filled pots. Within a week of transplanting, one healthy seedling was left in the pot while the other seedlings were removed; there were four pots per species and CO₂ treatment. Two environmentally controlled growth chambers were used to generate the CO₂ treatments. In order to minimize the impact of having a single growth chamber per CO₂ treatment, pots and CO₂ treatments were switched between chambers on two occasions. In addition, pots were randomly rotated within each chamber on a weekly basis throughout the experiment.

#### Table 1. Average growth conditions in the glacial and ambient CO₂ growth chambers during the experimental period

<table>
<thead>
<tr>
<th></th>
<th>Glacial CO₂</th>
<th>Ambient CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Night</td>
</tr>
<tr>
<td>Light (μmol m⁻² s⁻¹)</td>
<td>900 ± 2</td>
<td>900 ± 3</td>
</tr>
<tr>
<td>[CO₂] (μl l⁻¹)</td>
<td>181 ± 4</td>
<td>182 ± 2</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>27 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>70 ± 1</td>
<td>70 ± 1</td>
</tr>
</tbody>
</table>

Light intensity was measured at the pot level. The photoperiod was 12h. Values are averages (± standard deviation) over the growing period.
Table 2. List of grass species used in the current study

<table>
<thead>
<tr>
<th>Species</th>
<th>Photosynthetic type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panicum bisulcatum Thunb.</td>
<td>C₃</td>
</tr>
<tr>
<td>Panicum milloides Nees</td>
<td>C₃–C₄</td>
</tr>
<tr>
<td>Astrakia lappacea (Lindl.) Domin.</td>
<td>C₃, NAD-ME</td>
</tr>
<tr>
<td>Panix coloratum L.</td>
<td>C₃, NAD-ME</td>
</tr>
<tr>
<td>Heteropogon contortus (L.) P. Beauv.</td>
<td>C₄, PCK</td>
</tr>
<tr>
<td>Ex Poem. &amp; Schult.</td>
<td>C₄, NAD-ME</td>
</tr>
<tr>
<td>Panicum monticola Hook. F.</td>
<td>C₄, PCK</td>
</tr>
<tr>
<td>Panicum maximum Jacq.</td>
<td>C₄, PCK</td>
</tr>
<tr>
<td>Chloris gayana Kunth.</td>
<td>C₄, PCK</td>
</tr>
<tr>
<td>Zea mays L.</td>
<td>C₄, NAD-ME</td>
</tr>
<tr>
<td>Echinocloa frumentaceae L.</td>
<td>C₄, NAD-ME</td>
</tr>
</tbody>
</table>

Leaf gas exchange measurements
Gas exchange measurements were made using a portable open gas exchange system (LI-6400XT, LI-COR). At 7–8 weeks after transplanting, gas exchange measurements were made at a photosynthetic photon flux density of 1800 μmol m⁻² s⁻¹ between 10:00h and 14:00h on attached, last fully expanded leaves (LFELs) of the main stems. Spot measurements of the light-saturated photosynthetic rate (Aₚₜₜ) and gₑ were made at target growth [CO₂] (180 μl l⁻¹ or 400 μl l⁻¹) and leaf temperature of 27°C. Leaf-to-air vapour pressure deficit ranged between 1.7 kPa and 2.4 kPa during the measurements. Before each measurement, the leaf was allowed to stabilize for 10–20 min until it reached a steady state of CO₂ uptake.

The responses of CO₂ assimilation rates (A) to step increases of CO₂ were measured under conditions similar to spot measurements by raising the cuvette [CO₂] in 10 steps between 50 μl l⁻¹ and 1500 μl l⁻¹. There were 3–4 replicates per treatment. The A–Cᵣ curves were fitted using the C₄ photosynthesis model (von Caemmerer, 2000) to estimate maximal PEPC (in vivo Vₑₑₑ) and Rubisco (in vivo Vₑₑₑ) activities. The biochemical model of C₄ photosynthesis was used to estimate Vₑₑₑ (apparent, maximal RuBP-carboxylation limited rate) for the C₃ grass (Farquhar et al., 1980), using Rubisco catalytic parameters obtained for Panicum bisulcatum (RE Sharwood, O Ghannoum, and SM Whitney, unpublished).

Growth and nitrogen analyses
Plants were harvested 12–13 weeks after transplanting. At harvest, the area of the LFELs and total leaf area were measured using a leaf area meter (LI-3100AA, LI-COR). Shoots were separated into stems and leaves. Roots were washed free of soil. Plant materials were oven-dried at 80 °C for 48h before dry mass was measured. Leaf mass per area (LMA, g m⁻²) was calculated as total leaf dry mass/total leaf area. For each treatment, three dried LFELs of each species were milled to a fine powder. Tissue N was determined on a leaf mass per area (LMA, g m⁻²) was calculated as total leaf dry mass/total leaf area. Roots were washed free of soil. Plant materials

Subsamples (75 μl) were taken from the total extract for SDS–PAGE analysis of total leaf protein. The remaining extract was centrifuged at 16 100 g for 1 min and the supernatant used for enzyme activity, Rubisco content, and soluble protein assays. Rubisco content was estimated by the irreversible binding of [¹⁴C]carboxyarabinitol bisphosphate (CABP) to the fully carboxylated enzyme (Ruuska et al., 1998). Rubisco activity (in vitro Vₑₑₑₑ) was estimated by multiplying the concentration of active sites determined using the [¹⁴C]CABP assay by the in vitro turnover rate (kₑₑₑₑ) at 25 °C of the various C₃ grasses (Supplementary Table S1 available at JXB online). Activities of PEPC and NADP-ME enzymes were determined at 25 °C using a UV-VIS spectrophotometer (model 8453, Agilent Technologies Australia, Mulgrave, Victoria) as previously described by Pengelly et al. (2012) and Ashton et al. (1990). Soluble proteins were measured using the Pierce Coomassie Plus (Bradford) protein assay kit (Thermo Scientific, Rockford, IL, USA).

P-EP-C activity was assayed at 25 °C in the carboxylase direction (Walker et al., 2002). Each leaf disc was extracted in 1 ml of ice-cold extraction buffer [50 mM HEPES pH 7.2, 5 mM DTT, 2 mM EDTA, 2 mM MnCl₂, 0.05% Triton, 4% (v/v) protease inhibitor cocktail (Sigma), and 1% (v/v) PVPP] using a 2 ml Potter–Elvehjem glass homogenizer kept on ice. The extract was centrifuged at 16 100 g for 1 min and the supernatant used. PEP-C activity was measured in assay buffer containing 100 mM HEPES, pH 7.0, 4% mercaptoethanol (v/v), 100 mM KCI, 90 mM NaHCO₃, 1 mM ADP, 2 mM MnCl₂, 0.14 mM NADH, and malate dehydrogenase after the addition of phosphoenolpyruvate (PEP) to 5 mM. It was not possible to assay reliably for NAD-ME activity in this study.

Immunoblot analysis
To confirm the presence or absence of assayed enzyme activities, especially the decarboxylases in the C₃ species and PEPC in C₃ and C₃–C₄ species, immunoblot analysis of the proteins in question was carried out. Subsamples of total leaf extracts (used for enzyme assays) were mixed with 0.25 vol of 4× LDS buffer (Invitrogen) containing 100 mM DTT, snap-frozen in liquid nitrogen, and stored at −20 °C until analysed. Protein samples were separated by SDS–PAGE at 200 V using TGX Any kD (Bio-Rad Laboratories, Hercules, CA, USA) pre-cast polyacrylamide gels in the Mini-Protean apparatus buffered with TRIS-glycine SDS buffer (Bio-Rad). Separated proteins were transferred at 4 °C to nitrocellulose membranes (0.45 μm; Bio-Rad) using the Xcell Surelock western transfer module (Invitrogen) buffered with 1× Transfer buffer [20×; 25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 20% (v/v) methanol]. After 1 h of transfer at 30V, the membrane was placed in blocking solution [3% (w/v) skim milk powder in TRIS-buffed saline (TBS); 50 mM TRIS-HCl pH 8, 150 mM NaCl] for 1 h at room temperature with gentle agitation.

For immunoblot analysis, primary antisera raised in rabbit against tobacco Rubisco (prepared by SM Whitney) were diluted 1:4000 in TBS before incubation at 1 h with membranes at room temperature with gentle agitation. Antiserum raised against PEPC (Cat. AS09 458) was obtained from Agrisera (Agrisera AB, Vännäs, Sweden) and diluted 1:2000 with TBS. For immunoblot analysis of NADP-ME and P-EP-C, synthetic peptides based on monocot amino acid sequences for each were synthesized by GL Biochem [GL Biochem (Shanghai) Ltd., Shanghai, China] and antiserum was raised to each peptide in rabbits. The reactive antisera were antigen purified and used for immunobots (GL Biochem). The NADP-ME assay (Product ID A-003198) and P-EP-C assay (Product ID A-003200) antisera were diluted in TBS 1:1000 and 1:500, respectively. All primary antisera were incubated with membranes at room temperature for 1 h with gentle agitation before washing three times with TBS. Secondary goat anti-rabbit antisera conjugated to horseradish peroxidase (HRP; Cat. NEF 812001EA, Perkin Elmer) were diluted 1:2000 with TBS. For immunoblot analysis, primary antisera raised in rabbit against tobacco Rubisco (prepared by SM Whitney) were diluted 1:4000 in TBS before incubation at 1 h with membranes at room temperature with gentle agitation. Antiserum raised against PEPC (Cat. AS09 458) was obtained from Agrisera (Agrisera AB, Vännäs, Sweden) and diluted 1:2000 with TBS. For immunoblot analysis of NADP-ME and P-EP-C, synthetic peptides based on monocot amino acid sequences for each were synthesized by GL Biochem [GL Biochem (Shanghai) Ltd., Shanghai, China] and antiserum was raised to each peptide in rabbits. The reactive antisera were antigen purified and used for immunobots (GL Biochem). The NADP-ME assay (Product ID A-003198) and P-EP-C assay (Product ID A-003200) antisera were diluted in TBS 1:1000 and 1:500, respectively. All primary antisera were incubated with membranes at room temperature for 1 h with gentle agitation before washing three times with TBS. Secondary goat anti-rabbit antisera conjugated to horseradish peroxidase (HRP; Cat. NEF 812001EA, Perkin Elmer) were diluted 1:3000 in TBS and incubated with the membranes for 1 h at room temperature followed by three washes with TBS. Immunoreactive peptides were detected using the Immun-Star Western C kit (Cat. Downloaded from at 12:00 PM October 19, 2016 http://jxb.oxfordjournals.org/.
Statistical and data analysis

PWUE was calculated as $A_{sat} \frac{(\mu mol \text{ m}^{-2} \text{ s}^{-1})}{g_{s} \text{ (mol m}^{-2} \text{ s}^{-1})}$. PNUE was calculated as $A_{sat} \frac{(\mu mol \text{ m}^{-2} \text{ s}^{-1})}{\text{leaf } [N]_{mass} \text{ (mmol m}^{-2})}$. The proportion of leaf N invested in Rubisco (Rubisco-N) was calculated by assuming that Rubisco contained 16% N on a mass basis (Evans and Seemann, 1989).

There were four replicates per treatment for growth, gas exchange, and enzyme assay measurements. There were three replicate measurements for the leaf N analysis and the $A-C_{i}$ curves. The relationship between the various response variables and the main effects (species, photosynthetic type, and CO₂ treatment) and their interactions were fitted using a linear model in R (V. 3.0.2; R Foundation for statistical computing, Vienna, Austria). Analysis of variance (ANOVA; summarized in Table 2) was conducted for each fitted model. Multiple comparisons (shown in Table 4 and Supplementary Table S1 at JXB online) of species means were made using the Tukey test.

Results

Photosynthetic rates and WUE

Under both [CO₂] treatments, photosynthetic rates measured at high light and growth [CO₂] ($A_{sat}$) were higher in the C₄ species relative to the C₃–C₄ and C₃ species. Amongst the C₄ species, variation in $A_{sat}$ was unrelated to their subtype. Relative to ambient [CO₂], glacial [CO₂] decreased $A_{sat}$ to a greater extent in the C₃–C₄ (65%) and C₃ (60%) species relative to the C₄ species (26%) (Figs 1A, 2A; Table 3; Supplementary Table S1 at JXB online).

![Fig. 1. Gas exchange and growth parameters. Light-saturated photosynthesis, $A_{sat}$ (A), stomatal conductance, $g_{s}$ (B), photosynthetic water use efficiency, PWUE (C), leaf N per unit dry mass, $[N]_{mass}$ (D), photosynthetic nitrogen use efficiency, PNUE (E), and plant dry mass, PDM (F) of 10 grass species belonging to C₃, C₃–C₄, NAD-ME, PCK, NADP-ME photosynthetic types grown at glacial (180 µL L⁻¹, open columns) or ambient (400 µL L⁻¹, filled columns) [CO₂]. Values are means ±SE of species within each photosynthetic type.](http://jxb.oxfordjournals.org/)

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At ambient [CO₂], variation in \( g_s \) was unrelated to the photosynthetic type or subtype of the grasses. At glacial [CO₂], the C₄ species had higher \( g_s \) relative to the C₃ and C₃–C₄ counterparts. Glacial [CO₂] increased \( g_s \) to a greater extent in the C₄ relative to the C₃ (1.1-fold) and C₃–C₄ (1.3-fold) species, with NADP-ME (1.5-fold) grasses showing the greatest increase in \( g_s \) relative to the other C₄ species (1.35-fold) (Figs 1B, 2B; Table 3; Supplementary Table S1 at JXB online).

At ambient [CO₂], PWUE was higher in the C₄ relative to the two C₃–C₄ and C₃ species. At glacial [CO₂], PWUE was highest in NAD-ME and PCK species, intermediate in NADP-ME and C₃–C₄, and lowest in C₃ species. Amongst the C₄ species, the two NAD-ME grasses had higher PWUE relative to their PCK and NADP-ME counterparts. Glacial [CO₂] decreased PWUE in all species by an average of 55% (Figs 1C, 2C; Table 3; Supplementary Table S1 at JXB online).

**Leaf N use efficiency and plant dry mass**

Under both [CO₂] treatments, leaf \([N]_{\text{mass}}\) was highest in *P. milioides* (C₃–C₄) and lowest in *Heteropogon contortus* (PCK). Glacial [CO₂] enhanced leaf \([N]_{\text{mass}}\) in all grasses except for *Panicum monticola* and *Chloris gayana* (PCK). The largest enhancement was observed in the C₃ (51%) and NADP-ME (29%) species (Figs 1D, 2D; Table 3; Supplementary Table S1 at JXB online).

![Fig. 2. CO₂ sensitivity of photosynthetic and growth parameters. Glacial to ambient CO₂ ratios of light-saturated photosynthesis, \( A_{\text{sat}} \) (A), stomatal conductance, \( g_s \) (B), photosynthetic water use efficiency, PWUE (C), leaf N per unit dry mass, \([N]_{\text{mass}}\) (D), photosynthetic nitrogen use efficiency, PNUE (E), Rubisco activity (F), plant dry mass, PDM (G), and PEPC activity (H). Original data are shown in Supplementary Table S1 at JXB online.](http://jxb.oxfordjournals.org/online)
Table 3. Statistical summary

Summary of statistical analysis using three-way ANOVA for the effects of [CO$_2$], species, and the photosynthetic type on various parameters collected for 10 grass species grown at glacial (180 l l$^{-1}$) and ambient (400 l l$^{-1}$) [CO$_2$].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Main effects (P)</th>
<th>Interactions (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Species</td>
<td>Type</td>
</tr>
<tr>
<td>Photosynthesis, $A_{\text{cell}}$ (μmol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Conductance, g, (mol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Intercellular [CO$_2$], C, (μ l l$^{-1}$)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>PWUE [μmol (mol H$_2$O)$^{-1}$]</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>LMA (g m$^{-2}$)</td>
<td>0.000</td>
<td>0.028</td>
</tr>
<tr>
<td>Leaf [N]$_{\text{max}}$ (mg g$^{-1}$)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Leaf [N]$_{\text{cell}}$ (mmol m$^{-2}$)</td>
<td>0.000</td>
<td>0.036</td>
</tr>
<tr>
<td>PNUE [μmol (mol N)$^{-1}$ s$^{-1}$]</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Plant dry mass, PDM (g per plant)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Soluble protein (g m$^{-2}$)</td>
<td>0.004</td>
<td>0.448</td>
</tr>
<tr>
<td>Rubisco sites (mmol m$^{-2}$)</td>
<td>0.594</td>
<td>0.000</td>
</tr>
<tr>
<td>Rubisco/soluble protein</td>
<td>0.001</td>
<td>0.000</td>
</tr>
<tr>
<td>Rubisco-N (% leaf N)</td>
<td>0.006</td>
<td>0.407</td>
</tr>
<tr>
<td>Rubisco activity (μmol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>PEPC activity (μmol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>NADP-ME activity (μmol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.340</td>
</tr>
<tr>
<td>PEP-CK activity (μmol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.133</td>
</tr>
<tr>
<td>PEPC/Rubisco</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>In vivo $V_{\text{pmax}}$ (μmol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.010</td>
</tr>
<tr>
<td>In vivo $V_{\text{p}}/V_{\text{c}}$</td>
<td>0.000</td>
<td>0.010</td>
</tr>
</tbody>
</table>

At ambient [CO$_2$], PNUE varied 3-fold amongst the species in a manner unrelated to their photosynthetic type. Glacial [CO$_2$] reduced PNUE to a lesser extent in the C$_4$ (30%) relative to the C$_3$ (58%) and C$_3$–C$_4$ (79%) species. At glacial [CO$_2$], PNUE was highest in C$_4$ plants (PCK >NADP-ME and NAD-ME) and lowest in C$_3$ and C$_3$–C$_4$ plants (Figs 1E, 2F; Table 3; Supplementary Table S1 at JXB online).

At ambient [CO$_2$], plant dry mass (PDM) was lower in the C$_3$–C$_4$ and NAD-ME species relative to the C$_4$ and other C$_4$ species. At glacial [CO$_2$], the C$_4$ species accumulated more biomass than their C$_3$ and C$_3$–C$_4$ counterparts, which had similar PDM. Glacial [CO$_2$] reduced PDM to a greater extent in the C$_3$ (70%) and C$_3$–C$_4$ (42%) species relative to the C$_4$ species. Amongst the C$_4$ species, PDM was least and most inhibited by glacial [CO$_2$] in the NAD-ME and NAD-ME grasses, respectively (Figs 1F, 2H; Table 3; Supplementary Table S1 at JXB online).

Rubisco and soluble protein content

Under both [CO$_2$] treatments, leaf Rubisco content was higher in Panicum milioides (C$_4$–C$_4$) relative to the other species, and in the two NAD-ME species relative to the other C$_4$ grasses. At ambient [CO$_2$], P. bisulcatum (C$_3$) and NAD-ME grasses had similar Rubisco contents. Glacial [CO$_2$] increased Rubisco content in P. bisulcatum (2.3-fold) and in three (Astrebla lappacea, Panicum coloratum, and H. contortus; 1.2- to 1.7-fold) of the eight C$_4$ species (Tables 3, 4).

The ratio of Rubisco to soluble proteins and the proportion of leaf N invested in Rubisco (Rubisco-N) were higher in the C$_4$ and C$_3$–C$_4$ species relative to the C$_3$ species. Amongst the C$_4$ species, the NADP-ME grasses tended to have the lowest leaf N or soluble protein investment in Rubisco. Glacial [CO$_2$] increased Rubisco-N in the C$_3$ species, reduced it in the C$_3$–C$_4$ species, and had little effect in the C$_4$ species (Tables 3, 4).

At ambient [CO$_2$], the C$_4$ species accumulated more bio-

mass than their C$_3$ and C$_3$–C$_4$ counterparts, which had similar PDM. Glacial [CO$_2$] reduced PDM to a greater extent in the C$_3$ species, and C$_3$–C$_4$ species relative to the C$_4$ species only (Tables 3, 4).

At glacial [CO$_2$], PEPC activity was highest in A. lappacea (NAD-ME) and C. gayana (PCK), and lowest in P. maximum (PCK). At glacial [CO$_2$], PEPC activity was highest in A. lappacea and lowest in P. monticola (PCK). Glacial [CO$_2$] stimulated PEPC activity in five out of the eight C$_4$ species (Figs 2H, 3B; Table 3; Supplementary Table S1 at JXB online). Variations in the ratio of PEPC to Rubisco activity reflected changes in PEPC activity (Fig. 3H; Table 3; Supplementary Table S1).

Activity of $C_4$ cycle enzymes in $C_4$ grasses

At ambient [CO$_2$], PEPC activity was highest in A. lappacea (NAD-ME) and C. gayana (PCK), and lowest in P. maximum (PCK). At glacial [CO$_2$], PEPC activity was highest in A. lappacea and lowest in P. monticola (PCK). Glacial [CO$_2$] stimulated PEPC activity in five out of the eight C$_4$ species (Figs 2H, 3B; Table 3; Supplementary Table S1 at JXB online). Variations in the ratio of PEPC to Rubisco activity reflected changes in PEPC activity (Fig. 3H; Table 3; Supplementary Table S1).

In this study, only the activities of the decarboxylases NADP-ME and PEP-CK were measured. Significant activity of NADP-ME was measured in the two NADP-ME species, while marginal NADP-ME activity was detected in the two NAD-ME species and in one of the PCK species (Fig. 3C). In contrast, PEP-CK activity was ubiquitous among the C$_4$ species used, with C. gayana showing the highest PEP-CK activity in the C$_3$ and C$_3$–C$_4$ species relative to the C$_4$ species. Amongst the C$_4$ species, the NADP-ME grasses tended to have the lowest leaf N or soluble protein investment in Rubisco. Glacial [CO$_2$] increased Rubisco-N in the C$_3$ species, reduced it in the C$_3$–C$_4$ species, and had little effect in the C$_4$ species (Tables 3, 4).

The C$_3$, C$_3$–C$_4$, and NAD-ME species had similar Rubisco activities, which were higher relative to the PCK and NAD-ME species. Glacial [CO$_2$] significantly up-regulated Rubisco activity in the C$_3$ and NAD-ME grasses only (Figs 2F, 3A, Table 3; Supplementary Table S1 at JXB online).

Activity of $C_4$ cycle enzymes in $C_4$ grasses

At ambient [CO$_2$], PEPC activity was highest in A. lappacea (NAD-ME) and C. gayana (PCK), and lowest in P. maximum (PCK). At glacial [CO$_2$], PEPC activity was highest in A. lappacea and lowest in P. monticola (PCK). Glacial [CO$_2$] stimulated PEPC activity in five out of the eight C$_4$ species (Figs 2H, 3B; Table 3; Supplementary Table S1 at JXB online). Variations in the ratio of PEPC to Rubisco activity reflected changes in PEPC activity (Fig. 3H; Table 3; Supplementary Table S1).
activity. Overall, growth [CO₂] had no significant effect on the activity of either decarboxylase (Fig. 3C–D, Table 3; Supplementary Table S1 at JXB online).

The detectability of the activity of both carboxylases and decarboxylases was corroborated by immunodetection of the corresponding protein (Fig. 6). PEPC activity and protein were lacking from the C₃ and C₃–C₄ species and present in all C₄ grasses. NADP-ME activity and protein were found in two C₄ species only. PEP-Cr activity was measured in all C₄ grasses, and the protein was readily detected in six grasses, with A. lappacea and H. contortus exhibiting weak immunoreaction with the available antibody, possibly due to divergent amino acid sequences of PEP-Cr in these two species (Fig. 6).

In vivo estimates of maximal Rubisco (Nₐmax) and PEPC activity (Nₚmax) in C₄ Grasses

In vivo estimates of Vₐmax and Vₚmax were calculated using the C₄ photosynthesis model (von Caemmerer, 2000) from A–C curves measured at high light and 27 °C (Fig. 5). The variation of gas exchange-derived Vₐmax between the C₄ species was unrelated to their biochemical subtype. In contrast to its effect on in vitro Vₐmax (Rubisco activity), glacial [CO₂] reduced gas exchange Vₐmax in two out of the eight C₄ species (Fig. 3E; Table 3; Supplementary Table S1 at JXB online). Consequently, in vivo and in vitro estimates of Vₐmax were unrelated among the C₄ grasses (Fig. 6B). In contrast, PEPC activity was positively correlated with that of Rubisco across the C₄ species and [CO₂] treatments (Fig. 6A).

On average, NAD-ME species tended to have higher Vₚmax and Vₚmax/Vₐmax relative to the other C₄ grasses, especially at glacial [CO₂]. Glacial [CO₂] increased Vₚmax and the Vₚmax/Vₐmax ratio in all C₄ species, except for C. gayana, by an average of 25% and 19%, respectively (Fig. 3F, G; Table 3; Supplementary Table S1 at JXB online). Within the C₄ species, Vₚmax showed significant positive correlations with in vitro PEPC and Rubisco activities (Fig. 6C, D).

Discussion

Photosynthetic efficiency under glacial CO₂: C₃, C₃–C₄, and C₄ pathways

In accordance with theoretical understanding, the current study revealed that photosynthetic rates (Amax) were most responsive to decreased [CO₂] from ambient to glacial levels in C₃ followed by C₃–C₄ and then C₄ species. In addition, the C₄ grasses had higher photosynthesis under ambient and glacial [CO₂] relative to their C₃ and C₃–C₄ counterparts (Figs 1A, 2A). Similar responses were observed for other C₃, C₃–C₄, and C₄ species exposed to 180 μL CO₂ L⁻¹ (Ward et al., 1999; Cunniff et al., 2010; Pinto et al., 2011; Vogan and Sage, 2012).

Stomatal conductance was greater at glacial [CO₂] compared with ambient [CO₂] in all species, but in particular was higher in C₃ species relative to the C₃ and C₃–C₄ species (Figs 1B, 2B). Huxman and Monson (2003) found that gₛ was more sensitive to changing Cᵢ in C₄ relative to C₃ and C₃–C₄
Flaveria species. Recently, Vogan and Sage (2011) presented evidence of changed $C_3$ sensitivity for $g_s$ in Flaveria species during their evolutionary transition from $C_3$ to $C_4$ photosynthesis. In contrast, Morison and Gifford (1983) observed little difference in stomatal sensitivity to short-term changes of $[CO_2]$ or vapour pressure deficit between two $C_3$ and two $C_4$ grasses. Growth at low $[CO_2]$ may cause acclimation of the stomatal response that is not necessarily captured during short-term gas exchange measurements. However, a number of studies found no evidence of differential stomatal acclimation between $C_3$ and $C_4$ plants (Cunniff et al., 2010; Vogan and Sage, 2012). Hence, there does not seem to be a consensus regarding the relative stomatal sensitivity to short- or long-term changes in $[CO_2]$ between $C_3$ and $C_4$ plants, which remains an area worthy of further investigation.

Despite having larger $g_s$ at glacial $[CO_2]$, $C_4$ species maintained greater PWUE than $C_3-C_4$ and $C_3$ species as a result of higher photosynthetic rates in $C_4$ plants (Fig. 1). Improved
PWUE is one of the most consistently reported advantages of C₄ species (Long, 1999; Taylor et al., 2010). Higher PWUE in the C₃–C₄ species relative to the C₃ species under both growth [CO₂] confirmed that the photorespiratory pump of the intermediate pathway confers greater water use efficiency relative to the C₃ pathway (Pinto et al., 2011; Vogan and Sage, 2011), thereby achieving PWUE similar to the C₄, NADP-ME pathway under glacial [CO₂] (Fig. 1C).

Higher PNUE in C₄ relative to C₃ plants under ambient [CO₂] is well established (Brown, 1978; Long, 1999; Taylor et al., 2010). In this study, these differences were maintained under glacial [CO₂] as a result of higher photosynthetic rates and lower leaf [N] in the C₄ relative to the C₃ and C₃–C₄ species (Fig. 1). The C₃–C₄ species had no PNUE advantage over the C₃ species, mainly due to the higher leaf [N] and Rubisco-N of the intermediate species (Table 4). In contrast, intermediate Flaveria species maintained higher photosynthesis and PNUE relative to C₃ congeners at ambient and glacial [CO₂] (Vogan and Sage, 2012).

Growth of P. bisulcatum (C₃) at glacial [CO₂] increased Rubisco activity and gs to improve photosynthetic capacity and CO₂ supply, respectively (Tissue et al., 1995; Gesch et al., 2000; Anderson et al., 2001). These commonly reported responses represent significant N and water costs for C₃ plants at glacial [CO₂], thus reducing their PWUE and PNUE. The additional resource requirements at low [CO₂] may have contributed to the more pronounced reduction in plant biomass in C₃ relative to C₄ plants observed in this study (Fig. 2F) as in others (Ward et al., 1999; Cunniff et al., 2010; Ripley et al., 2013). Consequently, low WUE and NUE of C₃ photosynthesis at low [CO₂] may have favoured the evolution of C₄ photosynthesis.

Photosynthetic efficiency under glacial CO₂: the C₄ subtypes

Results obtained in this study at glacial [CO₂] largely confirmed previously reported differences in photosynthetic efficiency among the C₄ subtypes at ambient [CO₂], and revealed a number of insights into the physiology of C₄ subtypes, as discussed below.

First, there were no subtype differences in photosynthetic rates or their sensitivity to decreased growth [CO₂]. These results constitute new evidence that there are no discernible differences in the efficiency of the CCM operating in the three C₄ subtypes, despite their diverse leaf biochemistry and anatomy. This conclusion is supported by the findings that CO₂ leakiness out of the bundle sheath (a surrogate measure of CCM efficiency) is similar among C₄ grasses with different subtypes (Henderson et al., 1992; Cousins et al., 2008).

Secondly, NAD-ME species had lower gs and higher PWUE relative to NADP-ME and PCK counterparts at glacial [CO₂]. Moreover, gs was less affected by glacial [CO₂] in NAD-ME than in NADP-ME and PCK grasses (Fig. 2). Previous studies demonstrated that photosynthetic activity was less sensitive to water deficit, and leaf traits were better suited for arid habitats in an NAD-ME relative to an NADP-ME and a PCK grass (Carmo-Silva et al., 2007, 2009). In another study, Ghannoum et al. (2002) showed that NAD-ME grasses increased their whole-plant WUE to a greater extent than their NADP-ME and PCK grasses under water stress. Taken together, these findings are consistent with the observation that grasses with the NAD-ME subtype predominate in more arid regions relative to the other two C₄ subtypes (Hattersley, 1992; Taub, 2000).

Thirdly, NADP-ME grasses showed the greatest increase of leaf [N]mass, which may be linked to their stomatal response in that the correlation between N uptake (proxy leaf [N]) and mass flow of soil water through the transpiration stream (proxy gs) is commonly reported in plants grown under different atmospheric [CO₂] (Conroy and Hocking, 1993; McDonald et al., 2002; Sherwin et al., 2013).

Fourthly, NAD-ME grasses showed the lowest biomass reduction in response to decreased growth [CO₂] relative...
to the PCK and NADP-ME species. NAD-ME grasses also had lower plant biomass relative to the other C4 species at both growth [CO2]. Studies conducted at elevated [CO2] have shown that growth response to high [CO2] decreases with decreasing growth potential (Poorter, 1993; Ziska and Bunce, 1997). Extrapolating these findings to low [CO2] suggests that the lower growth response to glacial [CO2] in NAD-ME plants may be related to their smaller biomass accumulation relative to the other, larger C4 species.

Photosynthetic enzymes under glacial CO2

Generally, growth at low [CO2] leads to increased photosynthetic capacity, g, and leaf [N] in C3 plants (Dippery et al., 1995; Ward et al., 1999; Anderson et al., 2001; Cunniff et al., 2010; Gerhart and Ward, 2010; Ripley et al., 2013). Accordingly, P. bisulcatum (C3) exhibited increased leaf proteins, including Rubisco at glacial [CO2] (Fig. 2; Table 4). Panicum milioides (C3–C4) did not up-regulate Rubisco content at glacial [CO2], possibly due to the high leaf [N] and Rubisco-N in this species; a consequence of the high N costs of operating two Calvin cycles in the mesophyll and bundle sheath cells (Monson, 1989; Monson and Rawsthorne, 2000).

The operation of Rubisco under elevated [CO2] in the bundle sheath, the multiplicity of metabolic cycles and cells involved in C4 photosynthesis, and the complexity of its regulation thwart the task of predicting how C4 photosynthesis will acclimate to growth at low [CO2]. Measurements of photosynthetic rates under growth [CO2] (A sat) indicated that photosynthesis in the C4 grasses was CO2 limited at glacial [CO2], albeit to a lesser extent than C3 and C3–C4 counterparts (Fig. 2A). This may explain the significant up-regulation of the two carboxylases, Rubisco and PEPC, which was observed in a number of the C4 grasses (Figs 3–6). Generally, the activities of Rubisco and PEPC changed in concert, a reflection of the fine balance operating between these two enzymes which modulate the pace of the C3 and C4 cycles during C4 photosynthesis, respectively (von Caemmerer and Furbank, 2003). There is strong evidence showing that CO2 delivery into the bundle sheath and fixation in the mesophyll are tightly regulated, as indicated by the constancy of leakiness (a measure of CO2 fixed by PEPC but not Rubisco, subsequently leaking back from the bundle sheath) under a wide range of environmental conditions (Henderson et al., 1992; Cousins et al., 2008). Nevertheless, the PEPC/Rubisco ratio increased at glacial [CO2] in two C4 species (Fig. 3H). Increasing PEPC/Rubisco via transgenic transformation in Flaveria bidentis led to increased leakiness, an indication of reduced efficiency of the C4 mechanism (von Caemmerer et al., 1997b). In the current study, Vpmax and PEPC activity were linearly correlated, while Vcmax and Rubisco activity showed no correlation (Fig. 5). Reconciling the in vivo and in vitro estimates of Rubisco and PEPC activity will require greater knowledge about bundle sheath cell wall conductance and [CO2] than is currently available (von Caemmerer et al., 1997a; von Caemmerer and Furbank, 2003).

The activities of the two measured decarboxylases were not affected by growth [CO2], possibly reflecting the low control that decarboxylases exert on the photosynthetic flux. Pengelly et al. (2012) reported that NADP-ME activity in transgenic F. bidentis can be halved without affecting photosynthetic rates or growth. Accordingly, the rate of the decarboxylases measured at ambient [CO2] may be sufficient under glacial [CO2], where Rubisco and PEPC activities were up-regulated in a number of C4 species. Although PEPC and NADP-ME have significant effects on the efficiency of the C4 pathway as evidenced by changes in leakiness, Rubisco retains a high
control of metabolic flux in C4 leaves (Furbank et al., 1997; von Caemmerer et al., 1997b; Pengelly et al., 2012).

It is worth noting that PEP-CK activity and, to a lesser extent, PEP-CK protein were ubiquitously detected in the C4 species used in this study. Significant PEP-CK activity in C4 grasses and eudicots of the NADP-ME and NAD-ME subtypes has been previously reported (Walker et al., 1997; Wingler et al., 1999; Carmo-Silva et al., 2008; Muhaidat and McKown, 2013). These findings challenge the classical view of the C4 subtypes, where a single decarboxylase dominates (Hatch, 1987; Furbank, 2011). Recent studies have postulated a role for PEP-CK as a second decarboxylase in maize that serves to match ATP and NADPH demand in bundle sheath and mesophyll cells under different light environments (Bellasio and Griffiths, 2013). The full physiological significance of PEP-CK in a wider range of C4 grasses and environments is yet to be elucidated.

**Conclusions**

Various photosynthetic responses, including increased leaf Rubisco, nitrogen, and gs, were observed in response to growth at glacial [CO2]. Nevertheless, the operation of a CCM ensured that PWUE and PNUE remained higher in C4 species relative to C3 and C3–C4 species, while the photorespiration pump ensured higher PWUE in the C3–C4 relative to the C3 species. Greater resource use efficiency promotes cheaper biomass construction costs, and hence reduces productivity losses at low [CO2]. Accordingly, high resource use efficiency may have constituted a key evolutionary advantage for the transition from C3 to C4 photosynthesis under low [CO2] (Cerling et al., 1998; Sage, 2004). Results obtained in this study support the notion that Rubisco and PEPC, rather than the decarboxylases, modulate the response to glacial [CO2] for C4 grasses with different biochemical subtypes.

**Fig. 6.** Relationships between the *in vitro* and *in vivo* estimates of Rubisco and PEPC activities in eight C4 grass species. Values are means for each species grown at glacial (180 μl l⁻¹, inverted open triangles) or ambient (400 μl l⁻¹, filled triangles) [CO2]. Solid lines represent linear regressions of all data points. Original data are shown in Supplementary Table S1 at *JXB* online.
Supplementary data

Supplementary data are available at JXB online

Table S1. Summary of leaf gas exchange, resource use efficiency, and activity of photosynthetic enzymes.

Acknowledgements

We thank Balasheb Sonawane for assistance with biochemical analysis. This research was partially funded by the Hawkesbury Institute for the Environment at UWS through the award of a PhD scholarship to HP and a research fellowship to RES. This research was also supported by a Discovery Project awarded to OG from the Australian Research Council (DP120101603).

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