

# Elevated CO<sub>2</sub> effects on mesophyll conductance and its consequences for interpreting photosynthetic physiology

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## ABSTRACT

**Mesophyll conductance ( $g_m$ ) generally correlates with photosynthetic capacity, although the causal relationship between the two is unclear. The response of  $g_m$  to various CO<sub>2</sub> regimes was measured to determine its relationship to environmental changes that affect photosynthesis. The overall effect of CO<sub>2</sub> growth environment on  $g_m$  was species and experiment dependent. The data did not statistically differ from the previously shown  $A$ – $g_m$  relationship and was unaffected by CO<sub>2</sub> treatment. The consequences of the CO<sub>2</sub> effect on  $g_m$  for interpreting photosynthesis in individual cases were investigated. Substantial effects of assumed versus calculated  $g_m$  on leaf properties estimated from gas-exchange measurements were found. This differential error resulted in an underestimation in ratio of maximum carboxylation to electron transport, especially in plants with high photosynthetic capacity. Including  $g_m$  in the calculations also improved the agreement between maximum carboxylation rates and *in vitro* Rubisco measurements. It is concluded that  $g_m$  is finite and varies with photosynthetic capacity. Including  $g_m$  when calculating photosynthesis parameters from gas-exchange data will avoid systematic errors.**

**Key-words:** acclimation; diffusion; climate change; Rubisco;  $J_{\max}$ ;  $V_{c\max}$ .

**Abbreviations:**  $A$ , net photosynthetic CO<sub>2</sub> assimilation;  $C_c$ , [CO<sub>2</sub>] at the site of carboxylation inside the chloroplast;  $C_i$ , [CO<sub>2</sub>] inside the leaf airspaces; FACE, Free Air CO<sub>2</sub> Enrichment;  $g_m$ , mesophyll conductance;  $J_{\max}$ , maximum potential rate of RuBP regeneration; LHC, light harvesting complexes; RuBP, Ribulose-1,5-bisphosphate;  $V_{c\max}$ , maximum potential rate of RuBP carboxylation.

## INTRODUCTION

A better understanding of the mechanism by which elevated atmospheric CO<sub>2</sub> affects photosynthesis is necessary

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to predict plant responses to future environments (Long 1998). Growth at elevated CO<sub>2</sub> frequently leads to a reduction in photosynthetic capacity (e.g. Curtis 1996; Drake, González-Meler & Long 1997). This reduction is often quantified by monitoring changes in the maximum rates of RuBP carboxylation ( $V_{c\max}$ ) and regeneration ( $J_{\max}$ ) through leaf gas-exchange measurements (e.g. Sage 1994) and is considered an acclimation response (Gunderson, Norby & Wullschleger 2000). These changes are part of a cascade of acclimation responses to growth at elevated CO<sub>2</sub> affecting the biochemistry, morphology, and phenology of the plant (Curtis & Wang 1998). Measurements of the effect of elevated CO<sub>2</sub> on mesophyll conductance ( $g_m$ ) are scarce. Mesophyll conductance (Harley *et al.* 1992; Loreto *et al.* 1992) is a measure of the transfer capacity of CO<sub>2</sub> between the leaf internal airspaces and the site of carboxylation in the chloroplast and is a fundamental property of leaves that may influence photosynthetic capacity (Epron *et al.* 1995; Evans & Loreto 2000). Changes in  $g_m$  may contribute to the acclimation of photosynthesis to elevated CO<sub>2</sub>. The primary goal of this study was to investigate the effects of growth at different ambient CO<sub>2</sub> levels on  $g_m$  and its relationship with photosynthetic capacity.

The secondary goal of this study was to investigate the relationship between *in vitro* biochemical and *in vivo* gas-exchange measurements of photosynthetic CO<sub>2</sub> acclimation, and to determine any possible differential effects of a change in  $g_m$  on the estimations of photosynthetic acclimation. This potential for error results from the common use of  $C_i$  as a basis for calculations of photosynthetic parameters such as  $V_{c\max}$  and  $J_{\max}$  even though these parameters are defined based on the CO<sub>2</sub> at the site of RuBP carboxylation ( $C_c$ ; Farquhar, von Caemmerer & Berry 1980). This interchangeable use of  $C_i$  and  $C_c$  carries with it the implicit assumption that  $g_m$  is effectively infinite; any effect of growth CO<sub>2</sub> on  $g_m$  may alter the interpretation of CO<sub>2</sub> acclimation studies. For example, if two leaves differed only in mesophyll conductance, the difference between  $C_i$  and  $C_c$  will be greater for the leaf with the lower  $g_m$ . Thus the initial slope of the photosynthesis– $C_i$  relationship would be lower in this plant even when the photosynthesis– $C_c$  relationship is identical between the two. This would make the calculated apparent  $V_{c\max}$  erroneously lower for the plant with lower  $g_m$ . Conversely, if a leaf has a lower  $V_{c\max}$  than its counterpart, a compensating decrease in  $g_m$  would erro-

neously increase its apparent  $V_{c\max}$  (measured based on  $C_i$ ), and mask changes in the underlying biochemistry.

These experiments were designed to determine whether growth  $CO_2$  substantially affects  $g_m$ , and whether using  $C_c$  rather than  $C_i$  influences the calculation of photosynthetic parameters and the interpretation of their response to elevated  $CO_2$ . We made gas-exchange measurements on trees growing in two different elevated  $CO_2$  experiments. These included a complete set of gas-exchange and chlorophyll fluorescence measurements to quantify  $g_m$  using the *constant J* method (Loreto *et al.* 1994). Further measurements were made on herbaceous plants grown in environmental chambers at elevated  $CO_2$ . We analysed the  $CO_2$  effects on  $g_m$  and on the relationship between photosynthetic capacity (assessed as both net photosynthesis rate at a standard  $C_i$  as well as by Rubisco content) and  $g_m$ . We used  $g_m$  measurements to re-analyse gas-exchange derived measurements of photosynthesis,  $V_{c\max}$  and  $J_{\max}$ , to compare the estimates based on  $C_i$  and  $C_c$ . We also investigated the relationship between acclimation to  $CO_2$  treatments as calculated from gas-exchange measurements compared with those measured by biochemical methods.

## MATERIALS AND METHODS

### Plant growth conditions

Mesophyll conductance was estimated on sweetgum (*Liquidambar styraciflua* L.) and aspen (*Populus tremuloides* L.) trees growing under field conditions but exposed to elevated and ambient levels of  $CO_2$ . In the FACTS-1 experiment near Chapel Hill, NC, sweetgum trees had naturally sprouted in the understorey of a 17-year-old Loblolly pine (*Pinus taeda* L.) plantation. Three 30-m-diameter plots in this plantation are continuously fumigated with  $CO_2$  to raise the ambient  $CO_2$  levels to  $200 \mu\text{mol mol}^{-1}$  above atmospheric levels (to about  $560 \mu\text{mol mol}^{-1}$ ). An additional three rings were fully instrumented to serve as controls. The treatment had been applied at the site for 3 years at the time of measurements. Additional details on the site are provided elsewhere (DeLucia *et al.* 1999; Singaas, Ort & DeLucia 2000). Field measurements on aspen were made at the FACTS-II field site in Rhinelander, WI, which had an array of treatment and control fumigation rings similar to those at FACTS-I. Aspen trees were planted as 6-month-old rooted cuttings propagated from greenhouse stock. All trees were between 2 and 3 m tall and approximately 3-year-old at the time of measurement. Additional details on the FACTS-II experiment can be found in Dickson *et al.* (2000).

To study the  $CO_2$ - $g_m$  relationship under a greater range of  $CO_2$  conditions, we performed additional experiments on potted plants grown indoors. Linden bean (*Phaseolus vulgaris* L. var. Linden), cucumber (*Cucumis sativus* L.), and spinach (*Spinacia oleracea* L.) were grown in controlled-environment chambers (Model PGW36; Conviron, Winnipeg, Manitoba, Canada). Light was provided by high-intensity fluorescent lamps and averaged  $530 \mu\text{mol m}^{-2} \text{s}^{-1}$

at 1 m above the floor throughout the experiment. Ambient temperature averaged  $26.4^\circ\text{C}$  throughout the experiment. Temperature and illumination in both chambers were monitored weekly with a thermocouple (Type T; Omega Inc., Stamford, CT, USA) and quantum photometer (Model LI-189; Li-Cor Inc., Lincoln, NE, USA).

Ambient  $CO_2$  and dewpoint were monitored in the growth chambers by an automated measurement system. Air was pumped from the chamber to a valve system that allowed airflow from each chamber to be alternately sent through an infrared gas analyser (Model 6262; Li-Cor Inc.). Ambient  $CO_2$  was elevated in the one chamber by injection of 100%  $CO_2$  through a valve controlled by a feedback loop based on the sampled  $CO_2$ . A data logger (Model CR10x; Campbell Scientific, Logan, UT, USA) was used for system control. The elevated  $CO_2$  chamber was maintained at  $745 \mu\text{mol mol}^{-1}$  during the first and second experimental blocks and  $737 \mu\text{mol mol}^{-1}$  during the third. Because both cabinets were located in a small room, the lower  $CO_2$  treatment was  $478 \mu\text{mol mol}^{-1}$  in the first and second blocks and  $501 \mu\text{mol mol}^{-1}$  in the third. For simplicity in data reporting, we have labelled the treatments 750 and  $500 \mu\text{mol mol}^{-1}$ , respectively. To further minimize any chamber effects other than  $CO_2$ , plants and  $CO_2$  control systems were switched weekly between the two chambers.

Plants were germinated from seed in 3 L pots, watered daily to maintain adequate soil moisture, and fertilized weekly with approximately 500 mL full strength Hoagland's solution. Plants were grown for 4 to 6 weeks before measurements began and then all measurements were made within 1 week. The youngest fully expanded leaf was measured in all cases.

### Photosynthesis measurements

Gas-exchange measurements were made at the FACTS-I site on shaded sweetgum leaves <3 m from the ground and on fully sunlight leaves in the upper canopy accessed from canopy towers and hydraulic lifts. At the FACTS-II site, leaves were selected for measurement from the top 1 m of the canopy. On chamber-grown plants, measurements were made on the youngest fully expanded leaf during the measurement period that began 6 weeks after germination.

Measurements were made using an open gas-exchange system (Model LI-6400; Li-Cor Inc.) where the partial pressure of  $CO_2$  in the cuvette ( $C_a$ ) was controlled using a  $CO_2$  injection system controlled by the instrument. Chlorophyll fluorescence ( $\Delta F/F_m'$ ) was measured simultaneously using a portable pulse-modulated fluorometer (Model OS-500; OptiScience Corporation, Tyngsboro, MA, USA). Light was provided using a 100 W metal halide lamp attenuated to the desired PPFD with neutral-density filters. The PPFD levels were selected to provide saturating light without causing photo-inhibition, by comparison with  $A$  versus PPFD measurements made separately before each experiment cycle (data not shown). Saturating PPFD was  $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$  for leaves grown in full sunlight,  $800 \mu\text{mol m}^{-2} \text{s}^{-1}$  for leaves grown in shade, and

1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for leaves grown in controlled-environment chambers. Because of the need for an external lamp when making combined gas-exchange and chlorophyll fluorescence measurements, leaves at each of the FACTS sites were excised with the petiole submerged in distilled water and brought to the measurement apparatus inside a portable laboratory trailer. These measurements were used to calculate  $g_m$ .

Two input parameters were required to calculate  $g_m$ : the rate of mitochondrial respiration ( $R_d$ ) and the CO<sub>2</sub> compensation point in the absence of mitochondrial respiration ( $\Gamma^*$ ). These were calculated from the common intersection points of three  $A$  versus  $C_i$  response curves (Laisk 1977; Brooks & Farquhar 1985; Villar, Held & Merino 1994, 1995). Briefly, the CO<sub>2</sub> response of photosynthesis was measured at five points below a  $C_i$  of 200  $\mu\text{mol mol}^{-1}$ . Three such curves were measured at different PPF levels (150, 100, and 75  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). The parameters are determined from the co-ordinates of the intersection point of the three lines on a graph of  $A$  versus  $C_i$ . Measurements for these parameters were made *in situ* using attached leaves at both of the FACTS sites and in the environmental chambers. Average  $\Gamma^*$  values ( $\mu\text{mol mol}^{-1}$ ) determined for each species and used in subsequent calculations were:  $42.49 \pm 2.9$ ,  $46.83 \pm 3.0$ ,  $42.52 \pm 2.1$ ,  $41.38 \pm 1.2$ ,  $43.02 \pm 1.7$ , and  $36.8 \pm 0.8$  for sweetgum sun leaves, sweetgum shade, aspen, bean, cucumber, and spinach, respectively. From the same calculations, daytime respiration ( $R_d$ ) values ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) were  $-0.92 \pm 0.2$ ,  $-0.32 \pm 0.1$ ,  $-3.73 \pm 0.7$ ,  $-1.74 \pm 0.2$ ,  $-1.16 \pm 0.2$  and  $-2.07 \pm 0.1$  for each species, respectively.

Leaf gas-exchange parameters were calculated using the equations of von Caemmerer & Farquhar (1981). The maximum rate of RuBP carboxylation ( $V_{c,\text{max}}$ ) and RuBP regeneration ( $J_{\text{max}}$ ) were calculated from CO<sub>2</sub> response curves with 11 points measured at ambient CO<sub>2</sub> levels between 1000 and 20  $\mu\text{mol mol}^{-1}$ . These data were fit to the Farquhar *et al.* (1980) model by non-linear least squares regression as described in Harley & Tenhunen (1991). We used *in vitro* model constants from Harley & Baldocchi (1995). We made a further comparison of approaches to determine the correct value of  $V_{c,\text{max}}$  by re-fitting the  $A$ - $C_i$  data using *in vivo* Rubisco parameters (Bernacchi *et al.* 2001). To compare photosynthesis rates among blocks on an equal basis, we standardized the photosynthesis measurements by choosing light-saturated photosynthesis measured at a  $C_i$  of approximately 400  $\mu\text{mol mol}^{-1}$ .

Mesophyll conductance was calculated using the *constant J* method (Loreto *et al.* 1992). Data were selected from CO<sub>2</sub>-response measurements in the region where  $\Delta F/F_m'$  was constant with increasing CO<sub>2</sub>. The gas-exchange data ( $A$ ,  $C_i$ ) and constants ( $\Gamma^*$  and  $R_d$ ; determined separately for each experimental block and species) were used to calculate the rate of electron transport needed to support CO<sub>2</sub> assimilation and photorespiration,  $J_p$ , for each point (Loreto *et al.* 1992). Electron transport through PSII was monitored independently from  $J_p$  using chlorophyll fluorescence measurements  $\Delta F/F_m'$  and is referred to as  $J_f$ . The variance in  $J_p$  across all the points of known constant  $J_f$  was

calculated as described in Harley *et al.* (1992), using the  $\Gamma^*$  and  $R_d$  values calculated previously for that experimental block. Conductance values were determined by least-squares regressions, minimizing the variance across the selected data points by substituting values of  $g_m$  into the  $J_p$  calculations. We report  $g_m$  in units of  $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$  to remain consistent with units in most publications (Harley *et al.* 1992; Loreto *et al.* 1992; Evans & Loreto 2000).

## Rubisco and chlorophyll

We measured Rubisco activity using a NADH-linked enzyme assay modified from Sharkey, Savitch & Butz (1991). Leaf punches (1.7 cm<sup>2</sup>) were excised with a cork borer and immediately ground in extraction buffer in a ground-glass tissue homogenizer at 0 °C. The extraction buffer contained 100 mM bicine-NaOH (pH 7.8), 100 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, 20 mM MgCl<sub>2</sub>, 1 mM ethylenediaminetetraacetic acid (EDTA), 4 mM amino-*N*-caproic acid, 0.8 mM benzamidine, 0.1% (w/w) Triton-X-100, 0.02% (w/v) bovine serum albumin, 150 mM NaHCO<sub>3</sub>, 5 mM dithiothreitol (DTT), and 30 mg poly(vinylpyrrolidone) (insoluble). The crude extract was transferred to a 1.5 mL microcentrifuge tube and spun for 30 s. Initial activity was measured using 10  $\mu\text{L}$  of supernatant assayed immediately in 1 mL of assay buffer (50 mM Bicine-NaOH (pH 8.0), 15 mM MgCl<sub>2</sub>, 1 mM EDTA, 19 mM NaCl, 9.3 mM NaHCO<sub>3</sub>, 9.3 mM DTT, 0.2 mM RuBP, 0.1 mM NADH, 4.7 mM phosphocreatine, 4.7 mM ATP, 1.4 U mL<sup>-1</sup> creatine-P-kinase, 1.4 U mL<sup>-1</sup> glyceraldehyde-3-P-dehydrogenase, and 2.9 U mL<sup>-1</sup> phosphoglycerokinase); the reaction was monitored at  $\Delta A_{340}$  for at least 3 min. A 1 mL aliquot of crude extract was incubated for 10 min with 80 mM MgCl<sub>2</sub> and 150 mM HCO<sub>3</sub><sup>-</sup> to fully activate Rubisco, and then assayed as described for the crude extract. Five aliquots of activated Rubisco extract were then incubated with CABP (concentrations of 0, 0.58, 1.1 and 1.8  $\mu\text{M}$ ) for 10 min to inhibit Rubisco activity, and assayed as above. Rubisco activity was calculated based on the slope of  $\Delta A_{340}$  versus time. Rubisco content was calculated from the y-intercept of a plot of activity versus  $[\text{CABP}]^{-1}$ .

Chlorophyll was measured spectrophotometrically. Leaf punches (1.7 cm<sup>2</sup>) were taken immediately after gas exchange measurements and ground in 96% EtOH using a chilled mortar and pestle. After centrifuging, the optical density of the supernatant was measured at 665, 649 and 654 nm. Chlorophyll concentration was calculated using the specific absorption coefficients in Wintermans & DeMots (1965).

## Experimental design and statistical analyses

The FACTS-I experiment consisted of six rings enclosing plants in paired control (ambient CO<sub>2</sub>) and treatment (ambient + 200  $\mu\text{mol mol}^{-1}$  CO<sub>2</sub>) conditions. The controls were fully instrumented. The FACTS-II experiment consisted of 12 rings in a crossed CO<sub>2</sub> × O<sub>3</sub> experiment. Measurements were only made on plants in the six rings not

receiving ozone treatment, which consisted of three ambient CO<sub>2</sub> and three ambient + 200 μmol mol<sup>-1</sup> CO<sub>2</sub> rings. Both experiments were designed with three blocks of paired (ambient + elevated) rings, and three replicate measurements were averaged within blocks. Blocked means were calculated across each species and treatment. The CO<sub>2</sub> effects on  $g_m$  were analysed for the FACE results using mixed ANOVA (JMP; SAS, Inc., Cary, NC, USA) with  $g_m$  as the main effect, treatment as a fixed factor and species and block as random factors. *Post-hoc* comparisons of treatment effects were performed within each species using the Tukey adjustment.

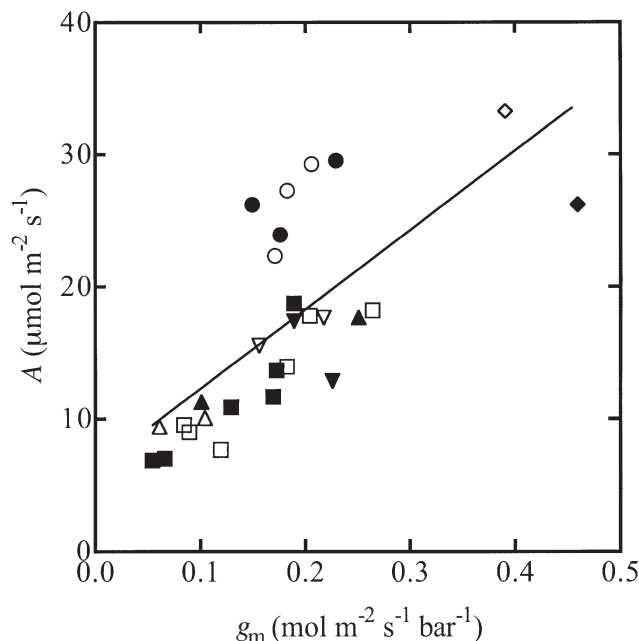
Growth chamber experiments were conducted in a pair of chambers, and blocks were replicated through time. Each block consisted of three to five individual plants of each species and ran for 6 to 8 weeks, after which the chambers were emptied, and new seedlings were started for the next experimental block. Results were analysed using ANOVA with treatment as a fixed factor and species as random. Unequal numbers made the block effects untestable. Tukey-adjusted *post-hoc* comparisons were made within species to investigate treatment effects.

We made several comparisons of relationships common to all experiments. Because Evans & Loreto (2000) found a consistent relationship between net photosynthesis rate and  $g_m$  we made the same comparison across our different experiments. We also compared the  $V_{c\max} : J_{\max}$  ratio across our experiments. The slopes and intercepts of regression lines were compared using analysis of covariance (ANCOVA) as described by Underwood (1997).

## RESULTS

Photosynthesis increased with increasing  $g_m$  consistently across all species and observations (Fig. 1). We used a standardized measurement (the light saturated rate of net CO<sub>2</sub> assimilation at  $C_i \approx 400 \mu\text{mol mol}^{-1}$ ) as a consistent metric of the leaf capacity for photosynthesis. The linear regressions between  $g_m$  and photosynthetic capacity on the elevated and ambient CO<sub>2</sub> data differed neither in slopes (ANCOVA;  $F = 0.76$ ,  $P = 0.47$ ) nor intercepts (ANCOVA;  $F = 0.13$ ,  $P = 0.88$ ), so we show only a single regression for the combined data set. There were similar, apparently linear, relationships between both leaf Rubisco and chlorophyll content and  $g_m$  in the growth chamber experiment (Fig. 2). The difference in CO<sub>2</sub> from the intercellular airspaces to the site of carboxylation did not vary in a systematic fashion with net CO<sub>2</sub> assimilation rate (Fig. 3). This relationship also showed no discernable dependence on growth CO<sub>2</sub> or plant species.

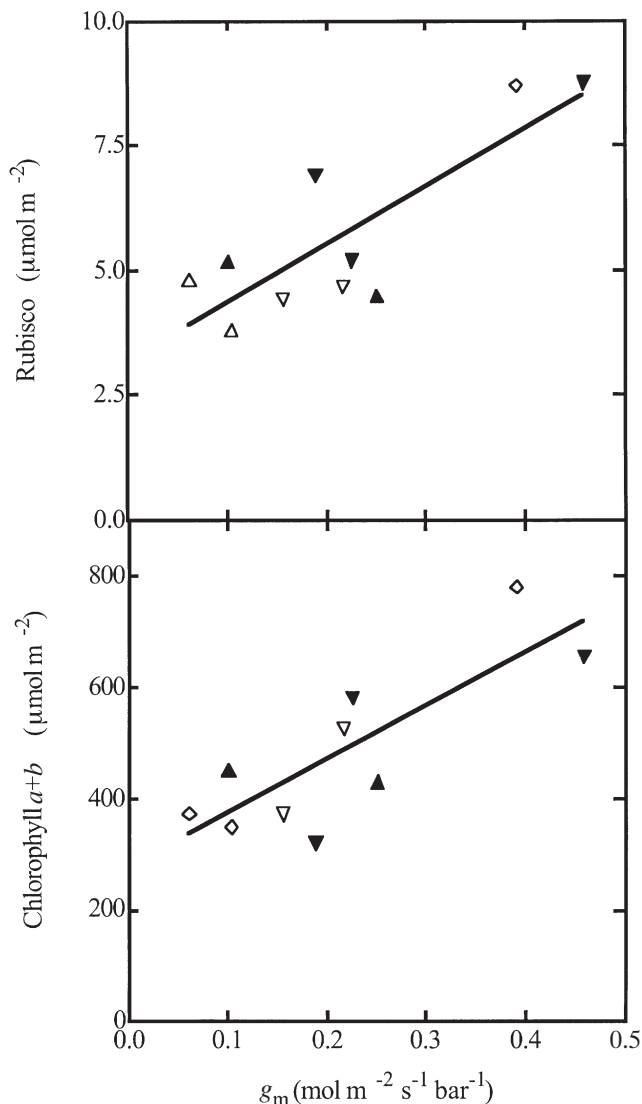
The overall effect of the CO<sub>2</sub> treatment on photosynthesis generally co-varied with the change in  $g_m$  except in spinach and linden bean (Table 1). The largest CO<sub>2</sub> effects were seen in cucumber and sweetgum (sun leaves) followed by spinach. An analysis of the growth chamber experiment data (top three rows of Table 1) showed significant CO<sub>2</sub> [ $F = 5.59$ ,  $P = 0.0248$ , degrees of freedom (d.f.) = 1] and species ( $F = 39.63$ ,  $P < 0.001$ , d.f. = 3) effects. In the FACE



**Figure 1.** The relationship between net CO<sub>2</sub> assimilation ( $A$ ) and mesophyll conductance ( $g_m$ ). Mesophyll conductance was estimated using the *constant J* method. Photosynthesis was measured at  $C_i$  between 390 and 450 in all cases. Symbol shapes represent species; sweetgum (■, □) aspen (●, ○) cucumber (▲, △) bean (▼, ▽) and spinach (◆, ◇). Open symbols represent plants grown at elevated CO<sub>2</sub>, and closed symbols represent plants grown at ambient CO<sub>2</sub>. The solid line represents a linear regression of the combined (all species and treatments) data ( $y = A + Bx$ ;  $A = 6.4$ ,  $B = 60$ ,  $r^2 = 0.51$ ).

experiments,  $g_m$  was significantly affected by species ( $F = 31.22$ ,  $P < 0.001$ , d.f. = 3) and block ( $F = 3.87$ ,  $P = 0.03$ , d.f. = 3). Mean CO<sub>2</sub> effects were significant only at the 10% level ( $F = 1.28$ ,  $P = 0.09$ , d.f. = 1). Pair wise comparisons of CO<sub>2</sub> effects within each species were significant at the 5% level in cucumber and at the 10% level in sunlit sweetgum leaves.

To examine the potential error in estimating photosynthetic parameters we calculated  $V_{c\max}$  and  $J_{\max}$  based on  $C_i$  and (using our values of  $g_m$ ) based on  $C_c$ . Calculated values of  $V_{c\max}$  were affected more than  $J_{\max}$  by the  $g_m$  correction, increasing between 20 and 70% when calculated based on  $C_c$  rather than  $C_i$  (Table 2). The  $g_m$  effects often were numerically uneven, affecting the ambient and elevated CO<sub>2</sub> treatments differently, thus revealing that the CO<sub>2</sub> effect on  $V_{c\max}$  could be over- or under-estimated depending on the direction of change in  $g_m$ . In sweetgum, aspen, and cucumber this meant that the change in  $V_{c\max}$  was always less than predicted based on  $C_i$ . In the case of sweetgum shade leaves, a 5% increase at elevated CO<sub>2</sub> became a 9% decrease when recalculated based on  $C_c$ . Spinach and bean behaved differently, increasing the CO<sub>2</sub> effect slightly. Sensitivity of  $J_{\max}$  to  $g_m$  was generally smaller than the sensitivity of  $V_{c\max}$  (Table 3); values of  $J_{\max}$  varied by approximately 10% with the inclusion of  $g_m$  in the calculations, with



**Figure 2.** The relationship between mesophyll conductance and leaf Rubisco content (top panel) and chlorophyll (bottom panel). Mesophyll conductance was estimated using the *constant J* method, Rubisco was measured using CABP binding, and chlorophyll was measured spectrophotometrically after extraction in 96% ethanol. Symbols represent species and treatments as in Fig. 1. Linear regressions were calculated with data from all species and treatments ( $y = A + Bx$ , top panel:  $A = 3.2$ ,  $B = 11$ ,  $r^2 = 0.67$ ; bottom panel  $A = 963$ ,  $B = 279$ ,  $r^2 = 0.67$ )

the exception of shade-grown sweetgum. Using  $C_i$  caused a slight underestimate in  $J_{\max}$  for aspen

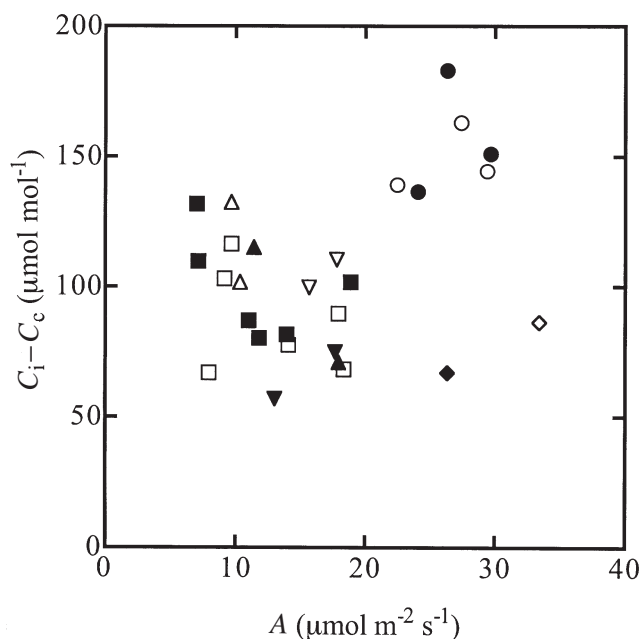
Because the choice of using  $C_i$  versus  $C_c$  in photosynthesis calculations affected  $V_{c\max}$  more than  $J_{\max}$ , the relationship between the two parameters was sensitive to  $g_m$  as seen by the change in the slope of the  $J_{\max} : V_{c\max}$  relationship (Fig. 4). The regression lines for the values calculated from  $C_i$  and  $C_c$  had significantly different slopes (ANCOVA;  $F = 27.9$ ,  $P < 0.001$ , d.f. = 52). There was no systematic effect of CO<sub>2</sub> treatment on the  $J_{\max} : V_{c\max}$  ratio using either calculation, so data from both treatments were grouped for

the regressions. We took an alternative approach to 'correcting'  $C_i$ -based calculations of  $V_{c\max}$  and  $J_{\max}$  by calculating  $V_{c\max}$  and  $J_{\max}$  using Rubisco constants determined from *in vivo* measurements (Bernacchi *et al.* 2001). This approach gave similar results to the  $C_c$ -based calculations at low photosynthesis capacity, but deviated from those data at higher values (Fig. 4).

In the growth chamber experiments, we used measurements of Rubisco activity and chlorophyll content as secondary indicators of photosynthetic capacity. Using  $C_c$  in calculations of  $V_{c\max}$  somewhat changed the relationship between *in vivo* and *in vitro* carboxylation capacity (Fig. 5; a 1 : 1 line is shown for comparison). RuBP regeneration capacity increased with total leaf chlorophyll content in a seemingly linear fashion (Fig. 6). As the  $J_{\max}$  calculations were not strongly affected by the  $g_m$ , the differences between the two data sets are relatively small.

## DISCUSSION

There was a consistent relationship between  $g_m$  and photosynthetic capacity across species, growth location, and CO<sub>2</sub>. This apparently linear relationship (Fig. 1) is similar to that reported by Evans & Loreto (2000), where they summarized the results of several earlier  $g_m$  studies. We calculated a regression line to determine the slope of the  $A$ - $g_m$  relationship from those data (not shown) and tested it against the regression of our data. The slopes were not significantly different at the 5% level (ANCOVA:  $F = 3.5$ ,  $P = 0.06$ , d.f. = 80) although they were different at the 10% level. The aspen data were the main outliers in our data that affected the slope, and without these data the two data-sets were



**Figure 3.** The difference in pCO<sub>2</sub> from the leaf internal airspaces to the sites of carboxylation,  $C_i - C_c$  versus net CO<sub>2</sub> assimilation rate,  $A$ . Measurements and symbols are as in Fig. 1.

**Table 1.** The effect of growth CO<sub>2</sub> on mesophyll conductance and photosynthesis

Species	CO <sub>2</sub> treatment ( $\mu\text{mol mol}^{-1}$ )	<i>A</i> ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	<i>C<sub>i</sub></i> ( $\mu\text{mol mol}^{-1}$ )	<i>g<sub>m</sub></i> ( $\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$ )	<i>P</i>	<i>n</i>
Cucumber	500	14.6 (3.2)	400 (11)	0.18 (0.08)	0.06	6
	750	9.9 (0.3)	407 (8)	0.08 (0.02)		6
Spinach	500	26.3	403	0.46	0.19	5
	750	33.3	413	0.39		5
Linden bean	500	15.2 (2.3)	421 (11)	0.21 (0.02)	0.47	6
	750	16.7 (1.1)	411 (5)	0.19 (0.03)		8
Aspen	360	26.6 (1.6)	415 (5)	0.18 (0.02)	0.95	9
	560	26.4 (2.0)	412 (3)	0.18 (0.01)		9
Sweetgum <sup>a</sup>	360	14.8 (2.1)	417 (5)	0.17 (0.01)	0.03	9
	560	16.7 (1.4)	415 (6)	0.32 (0.12)		9
Sweetgum <sup>b</sup>	360	8.3 (1.3)	414 (4)	0.08 (0.02)	0.49	8
	560	8.8 (0.5)	415 (5)	0.10 (0.01)		9

To compare measurements on an equal basis, all photosynthesis measurements were compared at a common *C<sub>i</sub>*. Data are means of all observations (SE). Probability (*P*) values represent the Tukey-adjusted pairwise comparisons between the CO<sub>2</sub> treatments for each species, and *n* designates the sample size. <sup>a</sup>Sun leaves; <sup>b</sup>shade leaves.

indistinguishable. Excluding these data had a minimal effect on the slope while reducing the *y*-intercept to 4.3 and increasing the *r*<sup>2</sup> to 0.84.

The non-zero intercept of our regression differs from Evans & Loreto (2000), which probably results different methods of determining photosynthesis for the *y*-axis. We defined this as the light-saturated photosynthesis rate at *C<sub>i</sub>* ≈ 400  $\mu\text{mol mol}^{-1}$  to make consistent comparisons among all treatments. Although the conditions are not specified in Evans & Loreto (2000) most authors use light-saturated photosynthesis rates measured at ambient CO<sub>2</sub> for photosynthetic capacity. Thus all our measurements were made at CO<sub>2</sub> levels between 600 and 1000  $\mu\text{mol CO}_2 \text{mol}^{-1}$ , resulting in higher net *A*. To demonstrate this we re-fit our data using photosynthesis measured at the growth CO<sub>2</sub> in each case, and calculated an intercept of 2.83  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (data not shown). This approach also improved the agreement

between aspen and all other species. This strongly suggests that the intercept of this relationship depends solely on the conditions under which photosynthetic capacity is defined, and has no physiological interpretation.

By similar comparison with the data in Evans & Loreto (2000), the calculated difference between *C<sub>i</sub>* and *C<sub>c</sub>* determined from *g<sub>m</sub>* and *A* values averaged  $106 \pm 6 \mu\text{mol mol}^{-1}$ , but by excluding the aspen data were indistinguishable from the 78  $\mu\text{mol mol}^{-1}$  value of the data summarized by Evans & Loreto (2000). The relationship between *A* and *g<sub>m</sub>*, and the consistency of the *C<sub>i</sub>*–*C<sub>c</sub>* draw-down, appear to be general features of leaves that are empirically predictable based on photosynthetic capacity measurements. This is especially notable considering the values from different studies used different methods to measure *g<sub>m</sub>*, seemingly confirming the consistency of *g<sub>m</sub>* measurement regardless of measurement technique (Loreto *et al.* 1992). Thus, this

	Analysis	<i>V<sub>cmax</sub></i> ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )		
		Ambient CO <sub>2</sub>	Elevated CO <sub>2</sub>	CO <sub>2</sub> effect (%)
Cucumber	<i>C<sub>i</sub></i>	36.3	26.0	–28
	<i>C<sub>c</sub></i>	48.7	40.1	–18
Spinach	<i>C<sub>i</sub></i>	64.8	85.0	31
	<i>C<sub>c</sub></i>	83.0	112	35
Linden bean	<i>C<sub>i</sub></i>	44.0	39.9	–9
	<i>C<sub>c</sub></i>	57.8	54.2	–6
Aspen	<i>C<sub>i</sub></i>	79.9	78.1	–2
	<i>C<sub>c</sub></i>	102	92.9	–9
Sweetgum <sup>a</sup>	<i>C<sub>i</sub></i>	38.8	46.1	19
	<i>C<sub>c</sub></i>	55.3	62.1	12
Sweetgum <sup>b</sup>	<i>C<sub>i</sub></i>	24.8	26.1	5
	<i>C<sub>c</sub></i>	43.0	39.0	–9

**Table 2.** The effect of growth CO<sub>2</sub> on *V<sub>cmax</sub>* from gas-exchange measurements

Calculations based on *C<sub>i</sub>* were done with standard gas-exchange equations. *C<sub>c</sub>* was calculated from mesophyll conductance and *C<sub>i</sub>*. All parameters were calculated separately for each leaf.

<sup>a</sup>Sun leaves; <sup>b</sup>shade leaves.

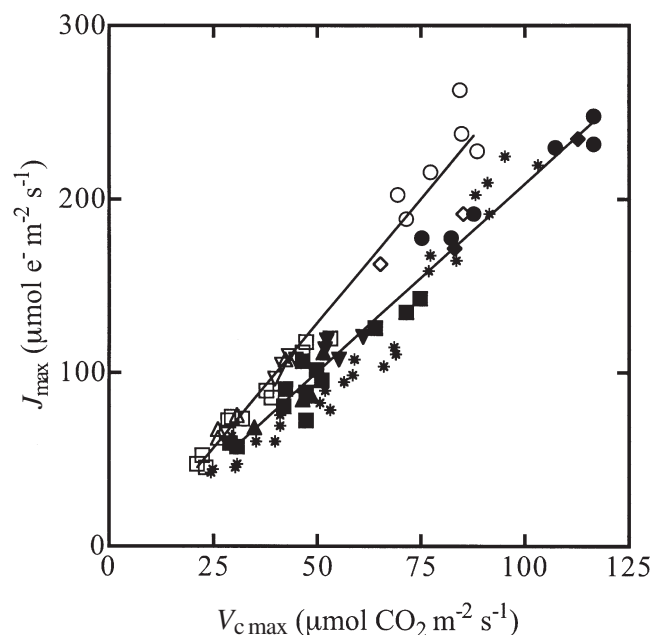
		$J_{\max}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )		
	Analysis	Ambient CO <sub>2</sub>	Elevated CO <sub>2</sub>	CO <sub>2</sub> effect (%)
Cucumber	C <sub>i</sub>	91.8	65.1	-29
	C <sub>c</sub>	98.4	76.5	-22
Spinach	C <sub>i</sub>	163	192	18
	C <sub>c</sub>	173	235	36
Aspen	C <sub>i</sub>	230	216	-6
	C <sub>c</sub>	217	200	-8
Linden bean	C <sub>i</sub>	109	88.8	-19
	C <sub>c</sub>	120	99.2	-13
Sweetgum <sup>a</sup>	C <sub>i</sub>	94.0	106	13
	C <sub>c</sub>	109	119	9
Sweetgum <sup>b</sup>	C <sub>i</sub>	57.5	64.2	12
	C <sub>c</sub>	75.5	86.2	14

**Table 3.** The effect of growth CO<sub>2</sub> on  $J_{\max}$  from gas-exchange measurements

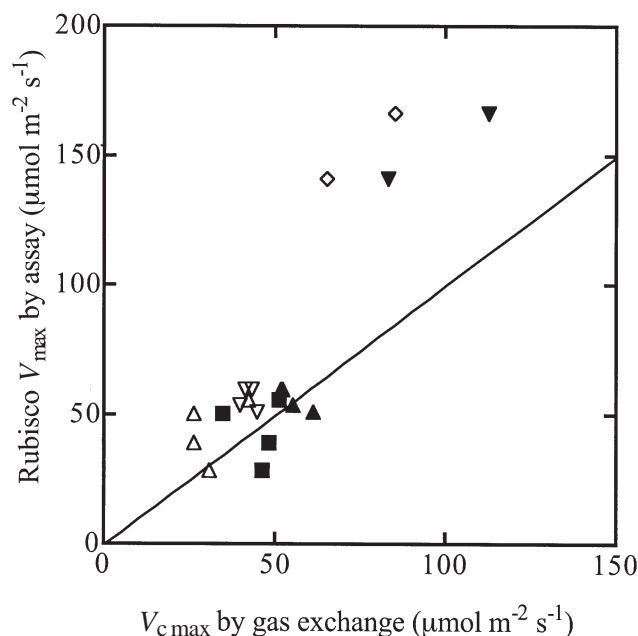
Calculations based on C<sub>i</sub> were done with standard gas-exchange equations. C<sub>c</sub> was calculated from mesophyll conductance and C<sub>i</sub>. All parameters were calculated separately for each leaf. <sup>a</sup>Sun leaves; <sup>b</sup>shade leaves.

relationship might in principle be used to improve the estimated CO<sub>2</sub> effect on  $V_{c\max}$  and  $J_{\max}$  even when  $g_m$  was not explicitly measured.

Growth at different CO<sub>2</sub> concentrations caused a small effect on  $g_m$  in almost all species studied which was statistically significant at either the 5 or 10% levels (Table 1).

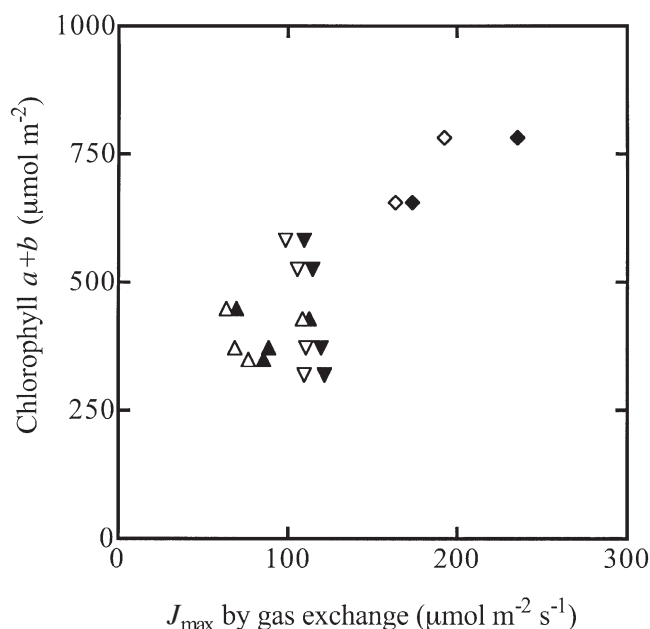


**Figure 4.** The relationship between maximal carboxylation and electron transport rates as calculated from gas-exchange measurements ( $A-C_i$  curves).  $V_{c\max}$  and  $J_{\max}$  were fitted using non-linear regression using *in vitro* Rubisco constants. Open symbols represent values calculated based on C<sub>i</sub>, and closed symbols represent values calculated based on C<sub>c</sub>. Symbol shapes represent species as in Fig. 1. The same raw data, re-fitted using *in vivo* Rubisco parameters, are included for comparison (\*). The lines were fitted to each data set by linear regression ( $y = A + Bx$ ; C<sub>i</sub>:  $A = -14.2$ ,  $B = 2.87$ ,  $r^2 = 0.96$ ; C<sub>c</sub>:  $A = 5.06$ ,  $B = 2.17$ ,  $r^2 = 0.97$ ).



**Figure 5.** Maximal Rubisco activity ( $V_{\max}$ ) determined by *in vitro* assay versus measured by gas-exchange based on C<sub>i</sub> and C<sub>c</sub>. Open symbols represent values calculated based on C<sub>i</sub>, and closed symbols represent values calculated based on C<sub>c</sub>. Symbol shapes represent species as in Fig. 1. The solid line marks a 1 : 1 relationship.

Since the magnitude and direction of photosynthetic acclimation to CO<sub>2</sub> varied considerably across treatments, and  $g_m$  was consistently related to photosynthesis, the use of block means is misleading in cases where the direction of acclimation differed between blocks. This was the case in sweetgum (one block showed a decrease in photosynthesis rates whereas the others showed an increase), linden bean (one decrease and one increase) and aspen (two decrease and one increase). Thus the general effect of CO<sub>2</sub> enrichment on  $g_m$  is to cause it to move up and down the  $A$  versus



**Figure 6.** The relationship between leaf chlorophyll content and maximum electron transport activity ( $J_{\max}$ ) estimated based on  $C_c$  and  $C_i$ . Open symbols represent values calculated based on  $C_i$ , and closed symbols represent values calculated based on  $C_c$ . Symbol shapes represent species as in Fig. 1.

$g_m$  regression line without substantially changing the relationship between the two.

There remains uncertainty about the mechanism relating  $g_m$  to any particular anatomical, biochemical or physical feature of a leaf. Substantial work has gone into relating leaf thickness and cell wall structure to  $g_m$  in various species, but the changes in  $g_m$  over the growing season and during leaf senescence (Loreto *et al.* 1994; Evans & Vellen 1996), when cell wall structure is relatively fixed, suggest wall structure may only be minimally involved. Gas diffusion within the leaf may contribute to  $g_m$  in some instances (Parkhurst & Mott 1990; von Caemmerer & Evans 1991; Syvertsen *et al.* 1995) although this effect has only been found in thick, hypostomatous leaves. A more consistent predictor of  $g_m$  is the surface area of chloroplasts appressed to the cell surface exposed to the leaf airspaces (von Caemmerer & Evans 1991; Evans *et al.* 1994). The relationship between chlorophyll content, Rubisco content and  $g_m$  (Fig. 2) may be indicative of this relationship, although it is impossible to determine a mechanistic relationship using these data.

Although no single anatomical measurement accurately predicts  $g_m$  (Evans *et al.* 1994; Syvertsen *et al.* 1995), empirical models relating several anatomical measurements to  $g_m$  have shown promise (Syvertsen *et al.* 1995). Using stable isotope discrimination analysis to partition leaf conductance into its components, Gillon & Yakir (2000) found cell wall conductance was most limiting to overall leaf conductance in oak, whereas resistances in the chloroplasts were more limiting in tobacco and soybeans. Liquid-phase

dynamics are likely to be more limiting to  $g_m$ , and thus enzymatic processes such as leaf carbonic anhydrase activity and aquaporins (Coleman 2000; Gillon & Yakir 2000, Ono & Terashima 2002) may also play important roles in determining  $g_m$ . These conclusions are consistent with the observed temperature response of  $g_m$  *in vivo* (Bernacchi *et al.* 2002). Given the substantially different conclusions of the various studies, it seems that growth  $CO_2$  may well affect the various determinants of  $g_m$  differently across species. This added complexity might mean that a mechanistic prediction of mesophyll conductance is not possible in the general case, and must be considered on a species-by-species basis.

Given the complex interacting factors contributing to  $g_m$ , the consistency of the  $A$ - $g_m$  relationship is puzzling. It is likely that this consistency results from covariance of several attributes. For example, the relationship between Rubisco and  $g_m$  (Fig. 2) may result from a correlation between Rubisco and carbonic anhydrase (CA; Coleman 2000) rather than through any direct mechanism, given the relationship between CA activity and  $g_m$ . Since the relationship between Rubisco and  $g_m$  is broken in antisense-Rubisco-transformed plants (Evans *et al.* 1994), we conclude that it is coincidental and not causal.

The most common anatomical changes resulting from growth at elevated  $CO_2$  are associated with stomatal and epidermal cell density (Ferris *et al.* 1996; Masle 2000), neither of which is likely to substantially affect  $g_m$  or photosynthetic capacity, at least when measured independently of stomatal conductance. Elevated  $CO_2$  can cause changes in leaf thickness (Kürschner *et al.* 1998), increasing airspace diffusion limitations that in hypostomatous leaves may reduce  $g_m$ . Significant  $CO_2$  effects have also been noted on total mesophyll cell cross-sectional area (Ferris *et al.* 1996; Masle 2000), which may correspond to an increase in mesophyll cell surface area and cause an increase in  $g_m$  (Evans *et al.* 1994). Studies of growth  $CO_2$  effects on carbonic anhydrase activity have shown either substantial changes in Rubisco and carbonic anhydrase (Majeau & Coleman 1996), or changes in Rubisco with no changes in carbonic anhydrase (Sicher, Kremer & Rodermel 1994). The uncertainty of which leaf properties substantially affect  $g_m$  makes the use of these as a proxy for determining  $CO_2$  effects on  $g_m$  difficult.

Although mesophyll conductance defies a complete physical or mathematical description, its consideration when calculating photosynthetic parameters is necessary especially when a treatment is anticipated to affect  $g_m$ . Including  $g_m$  in photosynthesis calculations changed our interpretation of the effect of growth  $CO_2$  on photosynthesis. In one case, an apparent increase in  $V_{c,\max}$  with  $CO_2$  treatment was revealed to actually be a decrease when analysed on a  $C_c$  basis (Table 1). In most cases the apparent  $CO_2$  effect was smaller when  $g_m$  was considered. The effects of growth  $CO_2$  on  $J_{\max}$  were quite small in all cases (Table 2). As  $V_{c,\max}$  was affected more by the recalculation than  $J_{\max}$ , the relationship between the two parameters changed and the slope of  $J_{\max} : V_{c,\max}$  was reduced (Fig. 4).

In both analyses, however, there was no systematic CO<sub>2</sub> effect across species on the ratio of the parameters. A change in the relationship between these parameters was predicted by the mechanistic analysis of CO<sub>2</sub> acclimation and photosynthesis (Medlyn 1996). In the model, lower  $g_m$  at elevated CO<sub>2</sub> increased the difference between  $C_i$  and  $C_c$ , thus reducing the need to reallocate of N from carboxylation (reflected in  $V_{c,max}$ ) to RuBP regeneration (reflected in  $J_{max}$ ). This could happen if CO<sub>2</sub> affected  $g_m$  independently of any acclimation in photosynthesis, but our data indicate that this is not the case. The  $C_i$ – $C_c$  relationship remains unchanged because  $g_m$  and photosynthesis change proportionally (Figs 1 & 3). These observations support the work showing that the relationship between carboxylation and RuBP regeneration rates is not affected by growth CO<sub>2</sub> made in other gas-exchange and biochemical analyses (Maxwell, Griffiths & Young 1994; Hymus *et al.* 1999).

Mechanistic photosynthesis models are based on [CO<sub>2</sub>] in the chloroplast, yet the majority of studies report values calculated based on  $C_i$ . The implicit assumption in such cases is that  $g_m$  is infinite. When  $g_m$  is not considered, treatment effects such as growth at elevated CO<sub>2</sub> that affect diffusion within the leaf may be falsely attributed to changes in leaf biochemistry (Parkhurst & Mott 1990). This principle is illustrated by Delfine *et al.* (1998) who show apparent treatment differences in the  $A$ – $C_i$  response of photosynthesis that are not seen in the  $A$ – $C_c$  relationship. The inclusion of  $g_m$  in our calculations improved the agreement between gas exchange and biochemical measurements of carboxylation capacity (Fig. 5). This effect was relatively small for the relationship between chlorophyll and RuBP regeneration capacity (Fig. 6). This is more difficult to evaluate, however, because there is no reason to expect a linear relationship between the two values.

Bernacchi *et al.* (2001) avoid the problems associated with the assumption of infinite  $g_m$  by measuring Rubisco kinetic parameters from *in vivo* measurements. This approach simplifies gas-exchange analysis because  $g_m$  is included in the Rubisco parameters since they were determined from  $C_i$ -based measurements. We re-analysed our gas-exchange data from both experiments using these parameters in place of the Baldocchi & Harley (1995) constants, and found the  $V_{c,max} : J_{max}$  relationship matched the ratios we calculated based on  $C_c$  at low  $V_{c,max} : J_{max}$ , but the two approaches systematically deviate from one another at higher  $V_{c,max}$  (Fig. 4). The deviation between these approaches occurs because the *in vivo* calculations assume  $g_m$  is constant (although not infinite), whereas our approach of determining  $C_c$  for each set of measurements accounts for the increasing  $g_m$  as photosynthetic capacity increases (Fig. 1).

We conclude that there was a potentially important effect of growth CO<sub>2</sub> on  $g_m$  that corresponded with photosynthetic acclimation to CO<sub>2</sub> through the consistent linear relationship between photosynthetic capacity and  $g_m$ . Many gas-exchange studies calculate  $V_{c,max}$  and  $J_{max}$  based on  $A$  versus  $C_i$  measurements rather than  $A$  versus  $C_c$ , implicitly assuming that  $g_m$  is infinite. We found  $g_m$  is neither infinite nor

constant in field or growth-chamber experiments. Including  $g_m$  in the analysis of photosynthetic responses to the CO<sub>2</sub> environment significantly changed the relationship between parameters estimated by gas-exchange measurements and improved the agreement of Rubisco activity measured with gas-exchange with biochemical assays.

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