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Stomatal acclimation to increased CO_2 concentration in a Florida scrub oak species *Quercus myrtifolia* Willd

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ABSTRACT

Native scrub-oak communities in Florida were exposed for three seasons in open top chambers to present atmospheric [CO_2] (approx. 350 μmol mol^{-1}) and to high [CO_2] (increased by 350 μmol mol^{-1}). Stomatal and photosynthetic acclimation to high [CO_2] of the dominant species *Quercus myrtifolia* was examined by leaf gas exchange of excised shoots. Stomatal conductance (g_s) was approximately 40% lower in the high- compared to low-[CO_2]-grown plants when measured at their respective growth concentrations. Reciprocal measurements of g_s in both high- and low-[CO_2]-grown plants showed that there was negative acclimation in the high-[CO_2]-grown plants (9–16% reduction in g_s when measured at 700 μmol mol^{-1}), but these were small compared to those for net CO_2 assimilation rate (A, 21–36%). Stomatal acclimation was more clearly evident in the curve of stomatal response to intercellular [CO_2] (c_i) which showed a reduction in stomatal sensitivity at low c_i in the high-[CO_2]-grown plants. Stomatal density showed no change in response to growth in high growth [CO_2]. Long-term stomatal and photosynthetic acclimation to growth in high [CO_2] did not markedly change the 2·5- to 3-fold increase in gas-exchange-derived water use efficiency caused by high [CO_2].

Key-words: acclimation; increased atmospheric CO_2; scrub oak; stomata; water use efficiency.

Abbreviations: A, net CO_2 assimilation rate c_a, c_i, ambient and intercellular space CO_2 concentrations, respectively; g_s, stomatal conductance; g', relative stomatal conductance; G_A, photosynthetic open feedback loop gain; G_g, stomatal open feedback loop gain; G, closed-loop gain; l, stomatal limitation; $V_{c,max}$ maximum carboxylation velocity of Rubisco; VPD, leaf–air vapour pressure difference; WUE, water use efficiency (ratio of A to transpiration rate).

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INTRODUCTION

Recent research into the acclimation of plants to growth in high atmospheric CO_2 concentration ([CO_2]) has focused on the photosynthetic mechanism (Sage 1994; Drake, Gonzales-Meier & Long 1997). When acclimation (defined as a physiological change that occurs with growth at high [CO_2], Drake *et al*. 1997) of net CO_2 assimilation rate (A) occurs it has been widely shown to involve down-regulation or loss of photosynthetic capacity (see review by Stitt & Krapp 1999), hence the term 'negative acclimation' (Arp 1991). The stimulation of A by, for example, a doubling of ambient [CO_2] (c_a) can be reduced after medium- and long-term growth in high [CO_2] from typical values around 50–60% to only 20–30% (Drake *et al*. 1997). In surprising contrast there have been relatively few studies on the long-term consequences of high [CO_2] to stomatal behaviour (see reviews by Morison 1998; Assmann 1999). There are three possibilities for the response of stomata to long-term growth in high [CO_2] (Sage 1994; Šantrůček & Sage 1996; Morison 1998). Stomata may:

1 acclimate to match any mesophyll photosynthetic acclimation, or
2 maintain the same CO_2-sensitivity as those in plants in normal [CO_2], or
3 acclimate independently of any mesophyll photosynthetic acclimation.

In addition, although anatomical and morphological changes are outside the above definition of acclimation, there may be a stomatal *adjustment* in the long term through changes in stomatal number and/or in size, although there is little consistency in effect (Woodward & Kelly 1995; Drake *et al*. 1997). Clearly, both acclimation and adjustment may have significant impact on conductance and hence on gas exchange; thus affecting plant water status, the efficiency of plant water use and with potentially profound effects on plant productivity (e.g. Morison 1993; Drake *et al*. 1997).

There are good reasons to expect response 1, as there is usually a close correlation of stomatal conductance (g_s) and A. This was first pointed out by Wong, Cowan & Farquhar (1979), and it implies a coupling that has subsequently been widely observed in many species across a range of light conditions, and with nutrient- and water supply-induced variations in A. Various suggestions for the link mechanism have been made, including metabolite transfer from mesophyll

to guard cell, but these remain unresolved (Assmann 1999; Jarvis *et al*. 1999). One of the corollaries of a close coupling of A and g_s is that the ratio of $[CO_2]$ in the intercellular space to that around the leaf (c_i/c_a) remains constant, which stems from the simplified leaf gas exchange equation: $A = (c_a - c_i).g_c$ (where g_c is leaf conductance for CO_2). Rearranging this to $A/(g_c.c_a) = 1 - c_i/c_a$ shows that for an x% increase in A with a doubling of c_a there must be $^1/_2x$% reduction in g_c for there to be no effect on c_i/c_a. However, a short-term doubling of c_a typically increases A by 50% (e.g. Drake *et al*. 1997) and reduces g_s by 40% (e.g. Morison 1987) which should lead to a readily observable change in $1 - c_i/c_a$ of 25%, or a drop in c_i/c_a from the typical value for C_3 plants of 0·70 to 0·63. However, the careful review of 33 long-term experiments with 26 species by Drake *et al*. (1997) found that c_i/c_a was *not* significantly affected by growth at doubled $[CO_2]$. Using an A stimulation figure more typical of cases of negative photosynthetic acclimation of only 25% does result in an approximately constant calculated c_i/c_a, but perplexingly the review by Drake *et al*. (1997) indicated that the mean reduction of g_s in these long-term experiments was only 20%. The calculated c_i/c_a should therefore have increased to 0·77, not what was found. Clearly, these averaged results are not internally consistent, and whether c_i/c_a remains constant with increased c_a and how the relationship between A and g_s changes after growth in high $[CO_2]$ should be examined carefully.

If stomata retained the same sensitivity to CO_2 (response 2 above) in high $[CO_2]$ as in present atmospheric $[CO_2]$ when there was negative photosynthetic acclimation, then this would lead to an increase in c_i/c_a, which is not usually reported (see above). The response of g_s to changes in c_i has been found to vary within and between species (e.g. Bunce 1992; Drake *et al*. 1997; Saxe, Ellsworth & Heath 1998), presumably reflecting the different requirements in the control of optimal plant water relations, and different photosynthetic capacities (Mansfield, Hetherington & Atkinson 1990). Several studies have suggested that g_s may not simply be responding in parallel to the acclimation of the photosynthetic biochemistry (Šantrůček & Sage 1996). Long-term high $[CO_2]$ has resulted in a wide range (large and small, closing and opening, or no response) of stomatal responses (Kerstiens *et al*. 1995; Curtis & Wang 1998). It is likely that there are different mechanisms involved in the short-term guard cell sensitivity to CO_2 and the medium- and long-term correlation of A and g_s (Assmann 1999). Therefore, the third of the outcomes for stomatal acclimation given above is a distinct possibility.

The acclimation of A to growth at high $[CO_2]$ is often presented by a comparison of A/c_i curves which show the photosynthetic response to $[CO_2]$ in the absence of stomatal effects. These measurements are taken rapidly and as g_s responds much more slowly than A they do not allow stomatal aperture to come into equilibrium with the c_i (Šantrůček & Sage 1996; Morison 1998). The comparable method for assessing stomatal acclimation to high $[CO_2]$ is by measuring the response of steady state g_s to c_i, and examining the change in sensitivity of g_s over a range of measurement c_i. The data can also be used to determine the stomatal limitation to A over a range of c_i values (Raschke 1979; Farquhar & Sharkey 1982), and to investigate the linkage of g_s and A.

Another approach to investigating the linkage of A, g_s and c_i caused by changing c_a is to apply a feedback loop analysis. The method was first proposed by Farquhar, Dubbe & Raschke (1978) and recently applied by Šantrůček & Sage (1996) to study g_s acclimation to growth in high $[CO_2]$ in *Chenopodium album*. Feedback loop analysis assesses the change in g_s with a change in c_a and determines the strength of the stomatal and photosynthetic feedback loops controlling c_i following a perturbation of c_a, assuming other environmental factors are kept constant. In a closed system of feedback loops the amplification or attenuation (gain, G) of the initial perturbation of c_i involves interactions between the feedback loops. In an open system the loops and their physical (properties of the stomatal pore) and physiological (response of guard cell and mesophyll biochemistry) components are examined in isolation, to determine their relative contribution to the overall gain (Farquhar *et al*. 1978).

There have been several suggestions that g_s is less responsive to c_i in woody than herbaceous species, due to the perennial nature and large stature of woody species but this may be dependent on root or water restriction (Gunderson & Wullschleger 1994; Saxe *et al*. 1998). However, within all groups the available data suggest a wide range of responses (Bunce 1992), and for some measurements insensitivity to changes in c_i. Using a meta-analysis of 38 studies, Curtis (1996) suggested that the wide range of g_s responses to high growth $[CO_2]$ in tree species can be, in part, attributed to limited replication (Jasienski, Thomas & Bazzar 1998), and in part to short growth periods in high $[CO_2]$, compared to the life span of the tree. The scrub oak community under investigation at the Smithsonian Environmental Research Centre site at Cape Canaveral in Florida (Hungate *et al.* 1999; Li *et al*. 1999; Dijkstra *et al*. 2001), had been grown in high $[CO_2]$ for 2·5 years using open top chambers, and provided an opportunity to investigate the stomatal response of a woody species to growth in high $[CO_2]$. This scrub oak community has a rapid successional cycle, relative to the length of any CO_2-enrichment experiment, and as the site was burnt prior to installation of the chambers, the aerial parts of the plants have re-grown entirely in the treatment $[CO_2]$ (Li *et al*. 1999). Further, the oak species (*Quercus myrtifolia*) is hypostomatous, so the question of the differing response of the respective leaf surfaces does not apply (Pearson, Davies & Mansfield 1995; Morison 1998), simplifying analysis.

The aim of this investigation was to determine the long-term response of g_s to growth in high $[CO_2]$ and the effect of these growth conditions on the short-term stomatal sensitivity to c_i. The main objectives of the work were therefore to: (a) determine the g_s/c_i responses of the scrub oaks grown in low and high $[CO_2]$; (b) relate these to any changes in A; and (c) to examine if changes in g_s were related to any changes in stomatal density. The impacts of

changes in $[CO_2]$ on the coupling of A and g_s and on gas-exchange-derived water use efficiency (WUE) are also discussed.

MATERIALS AND METHODS

Site

The field site was situated on Merritt Island Wildlife Refuge, Cape Canaveral, Florida (28°38′N, 80°42′W) with an average annual rainfall of 1310 mm per year and a vapour pressure deficit varying from 1·6 to 2·3 kPa in summer. The soil is a moderately well drained sandy soil with low nutrients and low pH, and vegetation is a scrub oak community, principally *Q. myrtifolia* and palmetto along with two other oak species, *Quercus geminata and Quercus chapmanii* (Day *et al*. 1996; Li *et al*. 1999). The area has been periodically burned to maintain the vegetation at this scrub stage of succession. The 16 octagonal open top chambers (3·5 m diameter with 2·10 m high panels, enclosing 9·65 m^2 of ground area) were installed in February 1996 after a burn, with eight chambers maintained at high and eight at low $[CO_2]$, paired and grouped in a blocked experimental design, according to the initial similarity of the vegetation, which then regrew (Hungate *et al*. 1999; Dijkstra *et al*. 1996; 2001). The 'low $[CO_2]$' treatment was ambient concentration (approx. 377 μmol mol^{-1}) and the 'high $[CO_2]$' treatment was ambient increased by 347 μmol mol^{-1}. The main effects of the open top chambers on the microenvironment of the plants within were: increased daytime air temperature of 2–4 °C; reduced visible light by 22%; increased leaf–air vapour pressure difference (VPD) by approximately 1 kPa, and reduced soil water content in the top 10 cm of soil by about 2% (Dijkstra *et al*. 2001).

Gas exchange measurements

The measurements detailed here were taken in September and October 1997 and repeated in August and September 1998. Of the possible eight blocks of paired high and low $[CO_2]$ chambers six were chosen (block numbers 1,2,4,5,7,8) in 1997 and seven in 1998 (additionally block 6), because of the uniformity of growth and species composition within the chambers. A single suitable shoot of *Q. myrtifolia* (approx. 10 leaves) was randomly selected from each of the high and low chambers of the selected block. These were shoots produced in the first flush of that year (March–April) and taken from the top of the canopy. The shoot to be used each day was excised before dawn and recut under water prior to measurement of gas exchange in an open system consisting of an infra-red gas analyser (Li 6262; Li-Cor, Lincoln, NB, USA) and gas-mixing, humidity and control unit (MPH-1000; Campbell Scientific, Logan, UT, USA). The fourth or fifth leaf attached to the stem was used under a predetermined VPD, temperature (30 ± 0·4 °C) and photon flux density (1400 ± 100 μmol m^{-2} s^{-1}) regime. The VPD (1·6 ± 0·1 kPa in 1997 and 1·7 ± 0·2 kPa in 1998) was chosen on the basis of preliminary work to find the value which closely approximated the ambient conditions and enabled steady g_s values to be maintained in the cuvette. The first readings were taken in the growth $[CO_2]$ for that shoot, then for high-$[CO_2]$-grown plants in the order 450, 150, 250, 350, 600, 700 and 800 μmol mol^{-1} and in the order 150, 250, 450, 600, 700, 800 and 350 μmol mol^{-1} for the low-$[CO_2]$-grown plants. The readings were taken after allowing g_s to reach a steady state with the altered leaf chamber conditions, typically after 30–50 min. In 1998 after the leaf gas exchange had stabilized with the cuvette at the growth $[CO_2]$ rapid readings were taken in low c_a (150 and 250 μmol mol^{-1}) for use in the A/c_i curve, the measurements were then repeated as in the previous year for the g_s/c_i response curve. In 1998, the leaf used for gas exchange measurements was then frozen and subsequently dried for the determination of leaf nitrogen content using an autoanalyser (model 2400 II CHNS/0 Analyser; Perkin Elmer, Norwalk, CT, USA).

Stomatal density

The other leaves from the shoots used for gas exchange analysis were also used to determine the stomatal density. The undersides of the leaves were first cleaned with water, dried with cloth and one or more thin layers of transparent nail varnish applied. Once dry the varnish impression was peeled away from the leaf and mounted on a microscope slide. For each leaf the number of stomata were measured under 2000 × magnification at six sites along the leaf, avoiding heavily veined areas (Poole *et al*. 1996). In 1997, nine fields of view were measured at each site and in 1998 six fields were used to provide the necessary number of replicates to detect significant differences between treatments as determined using the power calculation of Sokal & Rohlf (1995). All slides were anonymized prior to counting in order to minimize bias.

Analysis of results

The difference in response of g_s to c_i between growth $[CO_2]$ treatments was analysed using analysis of covariance, separately for each year. The linear regression model (r^2 = 0·686, 0·557 for 1997 and 1998, respectively) included effects of blocks and growth $[CO_2]$. Block effects and their interactions with growth $[CO_2]$ were significant in both years ($P < 0·001$) (Table 1). To avoid the assumption of a linear response of g_s to c_i used in the analysis of Fig. 1a paired two tailed t-test was also used at each c_a to detect differences between the g_s of high- and low-$[CO_2]$-grown plants in each block (Fig. 2a & b). For the data in Table 2, comparing key gas exchange parameters (g_s, A, c_i/c_a, and instantaneous WUE (ratio of A to transpiration rate E) for high- and low-$[CO_2]$-grown plants in either high or low c_a an analysis of variance was used for each year, using block, measurement $[CO_2]$ and growth $[CO_2]$ as factors. The same test was also used to compare g_s between treatments expressed relative to the maximum g_s (g') of that leaf using an arcsine transformation. A paired two-tailed t-test was

Table 1. The effect of growth at present atmospheric [CO_2] (approx. 350 μmol mol^{-1}, 'low') and with +350 μmol mol^{-1} increase ('high') on stomatal sensitivity to c_i, stomatal density and $V_{c,max}$ in *Q. myrtifolia* measured in the summer of 1997 (n = 6) and 1998 (n = 7). Stomatal sensitivity was assessed as the slope and intercept of linear regressions for each growth [CO_2], using analysis of covariance (see methods). Maximum carboxylation velocity of Rubisco, $V_{c,max}$, is from Fig. 4 data using the model of McMurtrie & Wang (1993). P indicates probability of difference between growth [CO_2] treatments assessed with analysis of covariance for regressions or a paired t-test for stomatal density and $V_{c,max}$ to reflect block effects. Figures in brackets are SEM; NS indicates $P > 0{\cdot}05$

		Growth [CO_2]		
Parameter	Year	Low	High	P
g_s/c_i linear regression slope (mol m^{-2} s^{-1})	1997	– 0·436 (0·047)	– 0·228 (0·047)	0·029
	1998	– 0·434 (0·065)	– 0·090 (0·065)	0·009
g_s/c_i linear regression intercept (mmol m^{-2} s^{-1})	1997	449 (19)	305 (19)	< 0·001
	1998	413 (24)	223 (24)	< 0·001
Stomatal density (mm^{-2})	1997	730 (20)	675 (32)	NS
	1998	723 (22)	708 (47)	NS
Nitrogen content (% leaf dry weight)	1998	0·97 (0·04)	0·91 (0·07)	NS
$V_{c,max}$ (μmol m^{-2} s^{-1})	1997	68 (11)	38 (12)	NS
	1998	91 (10)	34 (18)	NS

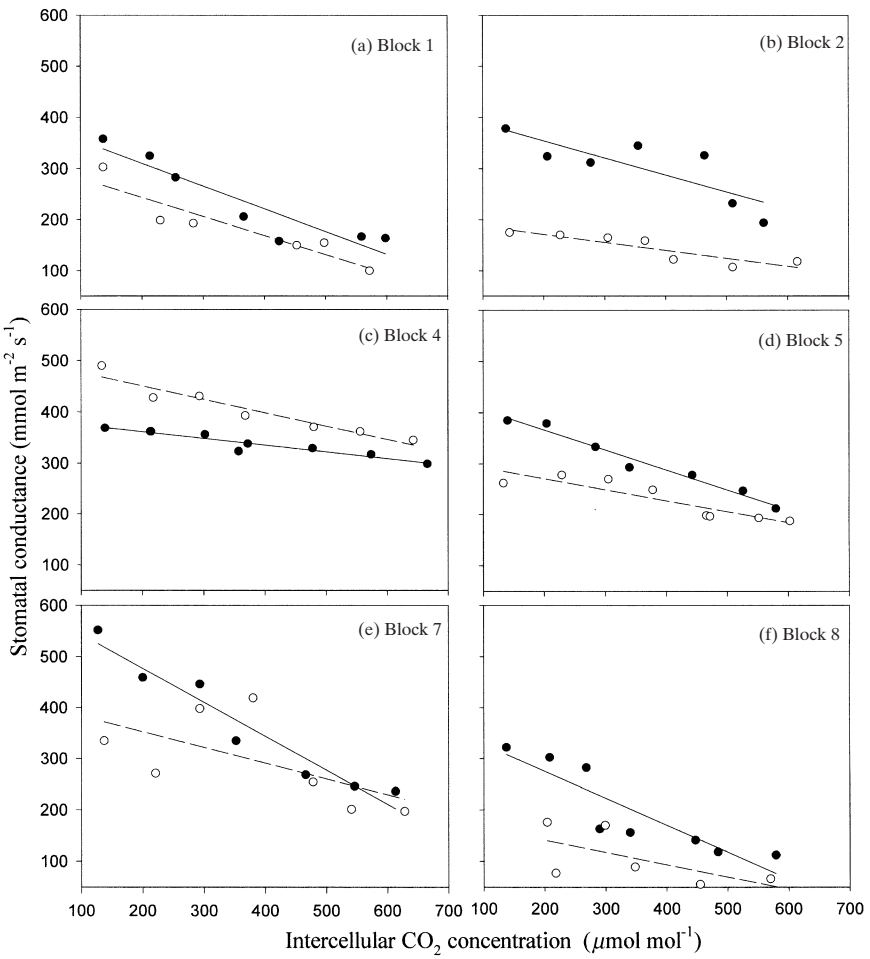

Figure 1. The response of stomatal conductance to intercellular [CO_2] in *Q. myrtifolia* in the late summer of 1997. Each figure shows results for a leaf in each of a pair of open top chambers, arranged in six blocks. Lines shown are linear regressions. (●) indicates plants grown in present atmospheric [CO_2] (approx. 350 μmol mol^{-1}) and (○) indicates plants grown with +350 μmol mol^{-1} increase.

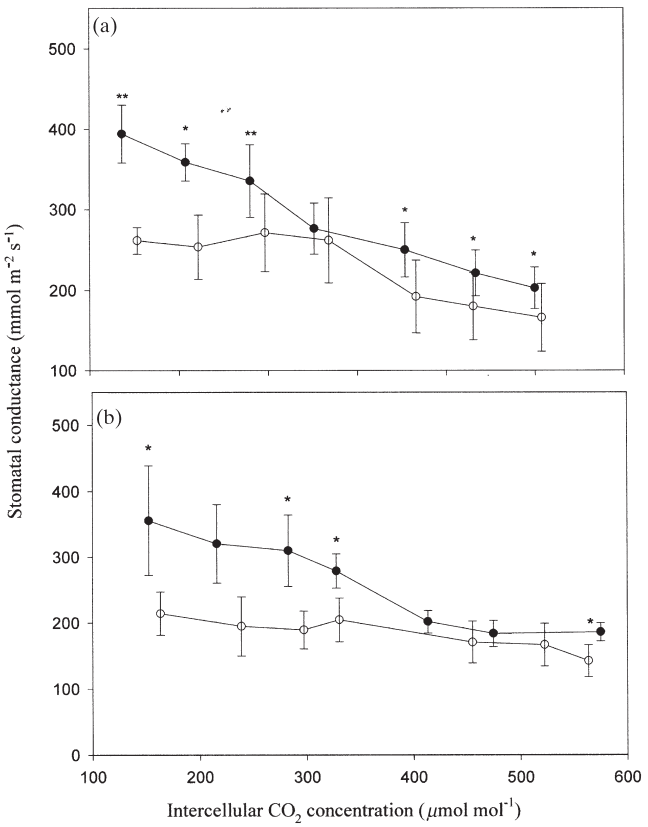

Figure 2. The mean response of stomatal conductance to intercellular [CO_2] in *Q. myrtifolia* in (a) 1997 ($n = 6$) and (b) 1998 ($n = 7$). (●) indicates plants grown in present atmospheric [CO_2] (approx. 350 μmol mol^{-1}) and (○) indicates plants grown with +350 μmol mol^{-1} increase. Statistical significance was assessed with a paired *t*-test; ** indicates $P < 0{\cdot}05$, and * indicates P between 0·05 and 0·1. Error bars indicate the standard error of the mean.

also used to test the significance of the difference between growth [CO_2] for stomatal density, nitrogen content and maximum carboxylation velocity of Rubisco ($V_{c,max}$) (Table 1).

The A/c_i curves were fitted using the SigmaPlot v.4 software package (SPSS Science Software UK, Ltd, Birmingham, UK) to fit a rectangular hyperbola, and $V_{c,max}$ estimated using the model of McMurtrie & Wang (1993). The stomatal limitation of photosynthesis was calculated according to the method of Farquhar & Sharkey (1982). The gain analysis theory of Farquhar *et al.* (1978) was applied according to the methods of Šantrůček & Sage (1996). Stomatal gain analysis involves a system of two feedback loops (stomatal and photosynthetic), both interacting to maintain c_i following a change in c_a. These loops each consist of physical and physiological components. First, the physiological components of both the photosynthetic and stomatal feedback loops were calculated from A/c_i and g_s/c_i data, respectively, by determining (dg_s/dc_i) and (dA/dc_i) as the tangents of fitted curves at a range of c_a values (250, 350, 450, 600, 700 μmol mol^{-1}). The physical components of the feedback loops were derived from the properties of [CO_2] diffusion into the stomatal pore ($[\delta c_i/\delta g_s]_{A,c} = 1{\cdot}6/g_s^2$ and $[\delta c_i/\delta A]_{c,g} = -1{\cdot}6/g_s$) over the same range of c_a values. The physiological and physical components were then combined to give values for the open feedback loop gains ($G_A = (\partial c_i/\partial A)_{c,g}$ and $G_g = (\partial c_i/\partial g_s)_{c,A}$) and the overall closed-loop gain [$G = 1/(1 - G_g - G_A)$] (Šantrůček & Sage 1996).

RESULTS

The response of steady state g_s to c_i was measured between 150 and 800 μmol mol^{-1} c_a (Fig. 1) for single shoots, one from each high and low [CO_2] chamber for each of the six blocks in the summer of 1997. Stomatal conductance

Table 2. Mean gas exchange parameters of *Q. myrtifolia* leaves grown and measured at both present atmospheric [CO_2] (approx. 350 μmol mol^{-1}, 'low') and with +350 μmol mol^{-1} increase ('high') in the late summer of 1997 ($n = 6$) and 1998 ($n = 7$). g_s = stomatal conductance, g' = relative stomatal conductance, A = net CO_2 assimilation rate, c_i/c_a = the ratio of intercellular to external [CO_2], and WUE = water use efficiency (mmol CO_2 [mol H_2O]$^{-1}$). Means followed by the same letter in the same row are not significantly different ($P > 0{\cdot}05$). Figures in brackets are SEM

		Growth[CO_2]			
		Low		High	
Measurement c_a ($m$mol mol^{-1})	Year	350	700	350	700
g_s (mmol m^{-2} s^{-1})	1997	297 (45)^{a}	221 (28)^{bc}	271 (48)^{ac}	185 (42)^{b}
	1998	310 (54)^{a}	184 (20)^{b}	201 (29)^{b}	167 (33)^{b}
g' (dimensionless)	1997	0·86 (0·03)^{a}	0·57 (0·04)^{b}	0·91 (0·08)^{ab}	0·57 (0·06)^{ab}
	1998	0·90 (0·05)^{a}	0·55 (0·05)^{b}	0·90 (0·07)^{ab}	0·65 (0·04)^{ab}
A ($m$mol m^{-2} s^{-1})	1997	9·1 (1·3)^{a}	20·2 (1·9)^{b}	6·7 (1·4)^{a}	16·0 (2·4)^{b}
	1998	9·8 (6·0)^{a}	24·5 (12·0)^{b}	5·2 (1·7)^{a}	15·7 (1·9)^{c}
c_i/c_a (dimensionless)	1997	0·80 (0·02)^{ab}	0·76 (0·02)^{a}	0·85 (0·01)^{b}	0·76 (0·03)^{a}
	1998	0·81 (0·02)^{ab}	0·68 (0·02)^{c}	0·85 (0·04)^{a}	0·75 (0·02)^{b}
WUE (mmol mol^{-1})	1997	2·00 (0·12)^{a}	5·82 (0·32)^{b}	1·57 (0·06)^{a}	5·36 (0·23)^{b}
	1998	1·96 (0·20)^{a}	7·21 (0·39)^{b}	1·38 (0·34)^{a}	5·99 (0·68)^{c}

declined with increasing c_i in all cases, although the degree of reduction varied between leaves and blocks. The response of g_s to c_i was approximately linear (Fig. 1). In all cases except Block 4, g_s at any particular c_i was higher for plants grown in low [CO_2] conditions than for those in high [CO_2], with this difference being most evident at low c_i in both years.

The slope (dg_s/dc_i, indicating the stomatal sensitivity to c_i) and intercept (extrapolated maximum g_s at $c_i = 0$) of the linear regressions, shown in Fig. 1, and equivalent regressions for the 1998 results were compared between growth [CO_2], using analysis of covariance (Table 1). There were significant block effects ($P < 0{\cdot}001$) and interactions between block and growth [CO_2] ($P = 0{\cdot}033$ in 1997, $P < 0{\cdot}001$ in 1998) in both years. Even including Block 4, the mean stomatal sensitivity of high-[CO_2]-grown plants was lower than that of the low-[CO_2]-grown plants (48 and 79% lower in 1997 and 1998, respectively) and the maximum g_s was 32 and 46% lower in 1997 and 1998, respectively (Table 1).

The mean g_s/c_i response of the leaves from the low and high [CO_2] chambers (Fig. 2a & b) showed that g_s did not increase at c_i lower than 350 μmol mol^{-1} in high-[CO_2]-grown plants. When measured in c_a equal to growth [CO_2], g_s was substantially lower in high- compared to low-[CO_2]-grown plants (38% in 1997, 46% in 1998) (Table 2). However, g_s of the high-[CO_2]-grown plants was only 16% lower in 1997 and 9% in 1998 than the low-[CO_2]-grown plants when both were measured in high c_a. When both treatments were measured at 350 μmol mol^{-1} g_s was 9 and 35% lower in 1997 and 1998, respectively, in the high-[CO_2]-grown plants.

To examine if the lower g_s of high-[CO_2]-grown plants (Fig. 2a & b), was the cause of the reduced sensitivity of g_s to c_i, the data for each leaf were expressed as g_s relative to the maximum g_s for that leaf ($g' = g_s/g_{smax}$) and averaged within growth [CO_2] treatments (Table 2). Relative stomatal conductance was very similar in both growth treatments at high and low c_a, confirming that reduced stomatal opening at low measurement c_a in high-[CO_2]-grown plants was the key effect.

Figure 3 shows A/c_i curves derived from the measured assimilation rates corresponding to the g_s data in Fig. 1. Usually A/c_i data are collected over a short time interval to avoid changes in Rubisco activation state, but more than 20 min were required to ensure steady-state g_s values

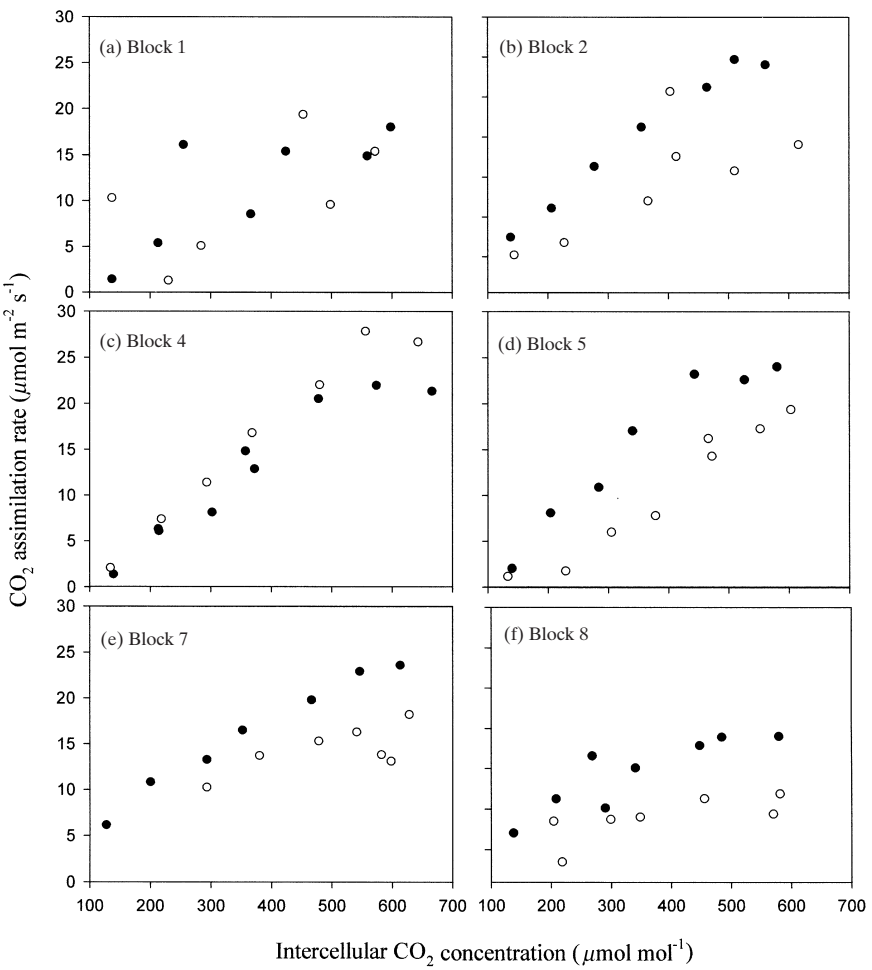

Figure 3. The response of CO_2 assimilation rate to intercellular [CO_2] in single leaves of *Q. myrtifolia* in the late summer of 1997. Each figure shows results for a leaf in each of a pair of open top chambers, arranged in six blocks. (●) indicates plants grown in present atmospheric [CO_2] (approx. 350 μmol mol^{-1}) and (○) indicates plants grown with +350 μmol mol^{-1} increase.

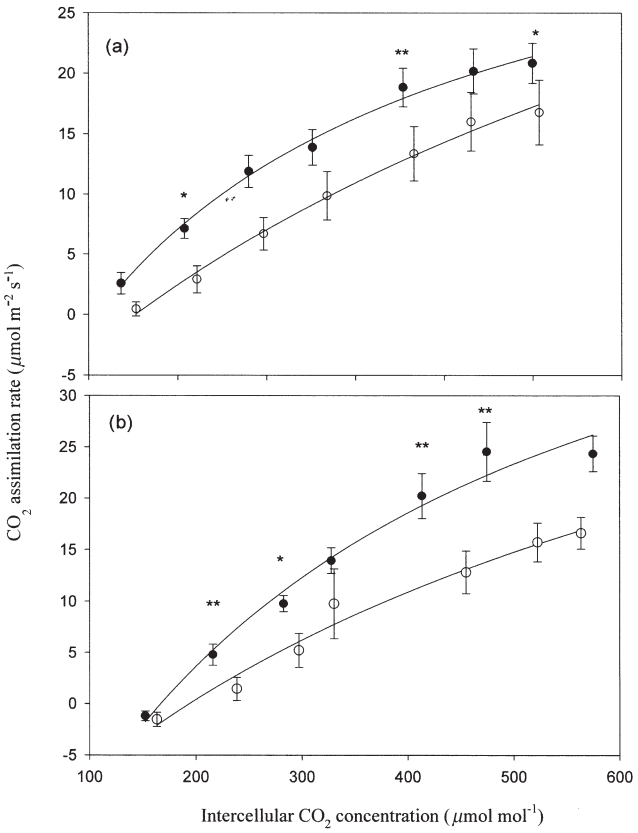

Figure 4. The mean response of CO_2 assimilation rate to intercellular [CO_2] of *Q. myrtifolia* leaves (●) in (a) 1997 (n = 6) and (b) 1998 (n = 7). (l) indicates plants grown in present atmospheric [CO_2] (approx. 350 μmol mol^{-1}) and (○) indicates plants grown with + 350 μmol mol^{-1} increase. Statistical significance shown as in Figure 2.

during our measurements. There was considerable variation in A between blocks and years (Fig. 3) but there was evidence of negative photosynthetic acclimation to growth [CO_2]. As with g_s measurements (Fig. 1), block 4 leaves responded differently, with increased A in the high-[CO_2]-grown plants, compared with low-[CO_2]-grown plants at any c_i. Mean A values (Fig. 4) show negative photosynthetic acclimation in high-[CO_2]-grown plants, with lower A at high c_i values and lower calculated $V_{c,max}$ values in both years (Tables 1 & 2). The high-[CO_2]-grown plants had a higher A compared with those grown in low [CO_2] when measured at growth c_a (76% higher in 1997 and 61% higher in 1998). When A was measured in 700 μmol mol^{-1} c_a to examine the difference between the short- and long-term increases in c_a the high-[CO_2]-grown plants showed 21 and 36% lower A in 1997 and 1998, respectively, compared with those grown in low [CO_2] (Table 2).

Clearly, there was substantial leaf-to-leaf variation in A and g_s (Figs 1 & 3). However, there was a linear correlation of A with g_s for each shoot within a [CO_2] treatment (Fig. 5). The slope of the A/g_s relationship for the low-[CO_2]-grown and measured plants in both years was much lower than that of the high-[CO_2]-grown and measured, because A was smaller and g_s higher over the range of c_a values. Even with the large difference in A/g_s slope ($\approx c_a[1 - c_i/c_a]$) between high and low c_a measurement plants, intercepts for both treatments approached the origin in 1998. The c_i/c_a ratios for both growth [CO_2] treatments (Table 2) were higher than the generally accepted value for C_3 plants of 0·7. When measured in growth [CO_2] or at 350 μmol mol^{-1} the c_i/c_a ratio was not significantly different between growth treatments. However, when high-[CO_2]-grown plants were measured at low c_a the c_i/c_a value increased substantially (Table 2).

The instantaneous WUE (Table 2) in high-[CO_2]-grown and measured plants was 2·6–3 times higher than that of low-[CO_2]-grown and measured plants ($P < 0.001$ in both years). When measured at low c_a the WUE of the high-[CO_2]-grown plants was 21 and 29% lower than that of low-[CO_2]-grown plants in 1997 and 1998, respectively ($P = 0.02$ and 0·08). As these measurements were taken under identical VPD conditions, the increases in WUE with [CO_2] were equivalent to increases in the so-called 'intrinsic WUE', or A/g_s as shown in Fig. 5.

Stomatal limitation (l) increased at low c_i (Fig. 6) but was rarely higher than 30%. The lowest measurement c_a was not included as the l calculation is invalid. The low stomatal limitation of A reflected the high c_i/c_a ratio for all measurements. In 1997, l was larger in the high-[CO_2]-grown plants at high c_i, compared with those grown in low [CO_2], whereas there was only a small difference in the 1998 data. It should be noted that the differences in l between seasons were comparable to the changes in l with c_i and these large seasonal effects may be the result of an unusually long drought period in 1998 in the months prior to the meas-

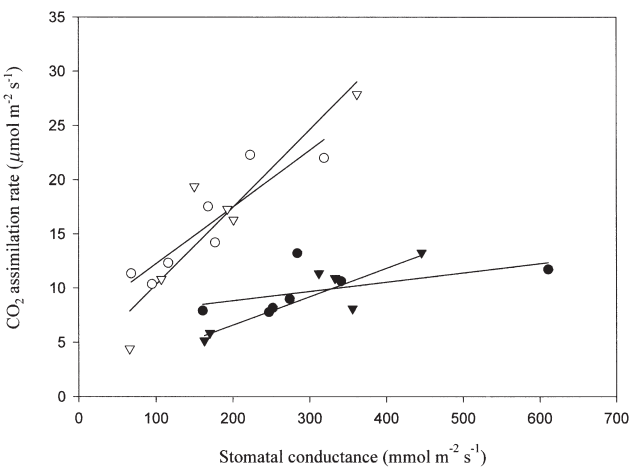

Figure 5. The relationship between CO_2 assimilation rate and stomatal conductance for *Q. myrtifolia* leaves grown in present atmospheric [CO_2] (approx. 350 μmol mol^{-1}, ● ▼) and with + 350 μmol mol^{-1} increase (○ ▽). Symbols ● ○ for 1997, n = 6 and ○ ▽ for 1998 n = 7, all measured at growth [CO_2]. Lines shown are linear regressions, r^2 = 0·79 and 0·86 for low and high [CO_2] in 1997 and 0·33 and 0·82 for low and high [CO_2] in 1998.

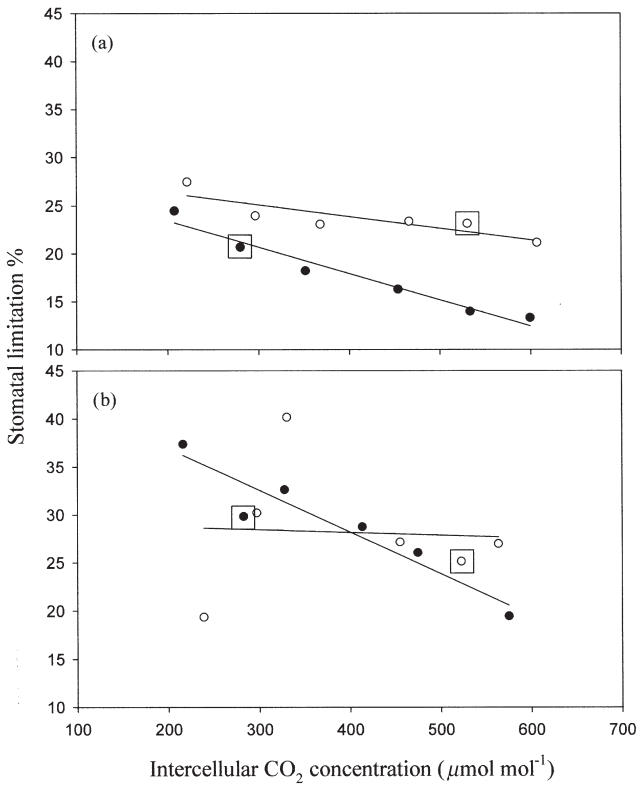

Figure 6. The change of stomatal limitation of CO_2 assimilation rate with changing intercellular $[CO_2]$ for *Q. myrtifolia* leaves grown in present atmospheric $[CO_2]$ (approx. 350 μmol mol^{-1}, ●) or with + 350 μmol mol^{-1} increase (○) in (a) 1997 (n = 6) and (b) 1998 (n = 7). Points with □ superimposed are values calculated at growth $[CO_2]$. Lines shown are linear regressions, r^2 = 0·96 and 0·74 for low and high growth $[CO_2]$ in 1997 and 0·89 and 0·71 for low and high growth $[CO_2]$ in 1998, respectively.

urements. When measured in growth $[CO_2]$ l in the high-$[CO_2]$-grown plants was similar in both years but for the low-$[CO_2]$-grown plants l was higher in 1998 than in 1997, perhaps reflecting a slower recovery from drought.

The feedback loop parameters (Table 3) reflect the shape of the curves fitted to the A and g_s responses to c_i. The higher A values of plants in 1998 compared to 1997 gave larger assimilation loop physiological and open-loop (G_A) gains, whereas the equivalent conductance loop gains were similar between years. With increasing c_a, the values of G_A became less negative as the photosynthetic biochemistry became saturated. Whereas in 1998 the photosynthetic acclimation of high-$[CO_2]$-grown plants (Fig. 4) was reflected in lower G_A values, the slightly more saturating A/c_i of the low-$[CO_2]$-grown plants in 1997 resulted in similar values of G_A in both growth treatments (Table 3). In the low-$[CO_2]$-grown plants in both years the nearly linear response of g_s to c_i resulted in similar physiological gains (dg_s/dc_i) across the range of c_a, although the larger gain (more negative value) around 350 μmol mol^{-1} c_a is noticeable. In marked contrast, the lack of response of g_s to low c_i in high-$[CO_2]$-grown plants resulted in physiological and open-loop gains (G_g) near zero when $c_a \leq 350$ μmol mol^{-1}, thus providing little attenuation of perturbations of c_i (see Table 2). When compared at growth $[CO_2]$ G_g was similar in both years in the high- and low-$[CO_2]$-grown plants. In contrast G_A was less negative in high- compared to the low-$[CO_2]$-grown plants in 1998, with only a small difference between growth $[CO_2]$ treatments in 1997. The closed-loop gain G combines both open feedback loops to quantify the degree to which a change in c_a is reflected in a change of c_i. There was little consistent difference in the closed-loop value between growth $[CO_2]$ treatments and measurement c_a in 1997, but G was lower in the low- compared to high-$[CO_2]$-grown plants in 1998.

Stomatal density was high in both treatments and years (Table 1), with approximately 700 stomata per mm^2. No significant differences were detected between leaves grown in high or low $[CO_2]$, and there was no significant relationship between stomatal density and conductance measured at growth $[CO_2]$ (data not shown).

DISCUSSION

Shoots of *Q. myrtifolia* plants grown and measured in high $[CO_2]$ (≈700 μmol mol^{-1}), had much lower g_s (approx. 40% reduction, Table 2) when compared with those grown and measured in low $[CO_2]$ (present atmospheric concentration). It has been suggested that there is a smaller response of g_s to c_a in tree than in herbaceous species (e.g. Saxe *et al.* 1998) and a recent meta-analysis of 48 studies with tree species found only an average reduction of g_s of 11% with a doubling of c_a, but the variation was such that this was not statistically significant (Curtis & Wang 1998). However, the decrease observed here is certainly consistent with many prior observations on mainly herbaceous species (e.g. Morison 1993). The observed sensitivity of g_s to c_i (dg_s/dc_i) for the low-$[CO_2]$-grown plants (Table 1) was low, but similar to that published for *Eucalyptus tetrodonta* (600 mol m^{-2} s^{-1} estimated from figure) at present atmospheric concentrations (Thomas & Eamus 1999). Although lower than the value of dg_s/dc_i found in the work of Šantrůček & Sage (1996) on *C. album* it should be noted that low conductances produce low stomatal sensitivities. Indeed, the dg_s/dc_i values for all these species measured in very different conditions lie close to the linear relationship between dg_s/dc_i and g_s previously found in four grass species (Morison & Gifford 1983).

Stomatal acclimation to $[CO_2]$ has been assessed previously by comparisons of g_s in plants grown and measured in high c_a to that of low-$[CO_2]$-grown plants measured in high c_a, and more rarely the reciprocal comparison. In these oaks, high-$[CO_2]$-grown plants had 16 and 9% lower g_s (1997 and 1998, respectively) when measured at c_a = 700 μmol mol^{-1} than those grown in low $[CO_2]$ (Table 2). When plants were measured in present atmospheric c_a the high-$[CO_2]$-grown plants showed lower g_s than those grown in low $[CO_2]$ (9 and 33% lower, respectively). Both of these results suggest a degree of negative stomatal

Table 3. Feedback loop analysis of mean stomatal conductance and net assimilation rate in *Q. myrtifolia* grown at present atmospheric $[CO_2]$ (approx. 350 μmol mol^{-1}, 'low') and with + 350 μmol mol^{-1} increase ('high') and measured at four different $[CO_2]$, c_a μmol mol^{-1} in the late summer of 1997 and 1998. Loop gains were calculated as proposed by Farquhar *et al.* (1978)

Year:	1997		1998		1997		1998	
Growth CO_2	Low	High	Low	High	Low	High	Low	High
Stomatal conductance loop								
c_a	Physical gains $(\partial c_i/\partial g_s)_{c,A}$ (m^2 s mol^{-1} × 10^{-6})				Physiological gains (dg_s/dc_i) (mol m^{-2} s^{-1})			
250	94	87	81	118	– 417	74	– 421	– 204
350	164	150	183	270	– 577	48	– 587	110
450	305	228	292	295	– 491	– 390	– 504	– 37
600	459	573	758	721	– 306	– 401	– 314	– 230
700	653	754	1056	896	– 322	– 178	– 324	– 139
Net CO_2 assimilation rate loop								
c_a	Physical gains $(\partial c_i/\partial A)_{c,g}$ (m^2 s mol^{-1})				Physiological gains (dA/dc_i) (mmol m^{-2} s^{-1})			
250	– 4·77	– 5·90	– 5·16	– 8·45	35·3	33·1	67·8	37·4
350	– 5·79	– 6·11	– 5·73	– 7·82	23·1	24·8	48·5	29·0
450	– 6·40	– 8·33	– 7·93	– 9·38	16·2	19·2	39·6	25·3
600	– 7·23	– 8·90	– 8·72	– 9·61	10·6	13·9	28·2	16·1
700	– 7·89	– 9·64	– 8·61	– 11·25	8·1	11·5	22·7	13·0
Open-loop gains								
c_a	G_g				G_A			
250	– 0·04	0·01	– 0·03	– 0·02	– 0·16	– 0·21	– 0·34	– 0·31
350	– 0·09	0·01	– 0·11	0·03	– 0·11	– 0·15	– 0·25	– 0·24
450	– 0·15	– 0·09	– 0·15	– 0·01	– 0·09	– 0·12	– 0·23	– 0·20
600	– 0·14	– 0·23	– 0·24	– 0·17	– 0·07	– 0·12	– 0·22	– 0·15
700	– 0·21	– 0·13	– 0·34	– 0·12	– 0·06	– 0·10	– 0·20	– 0·12
Closed-loop gain								
c_a	$G = 1/(1 - G_g - G_A)$							
250	0·84	0·83	0·73	0·75				
350	0·83	0·88	0·74	0·82				
450	0·80	0·83	0·73	0·83				
600	0·83	0·74	0·68	0·76				
700	0·79	0·81	0·65	0·80				

acclimation. However, because complete g_s/c_i response curves were measured it is clear that when plants had been grown in high $[CO_2]$ there was stomatal acclimation in the form of a reduction in stomatal sensitivity to c_i (Fig. 2, Table 1). Stomatal insensitivity to low c_i after growth in high $[CO_2]$ was similar to that found in *Ginkgo biloba* saplings (Beerling, McElwain & Osborne 1998) and in seedlings of *E. tetrodonta* and *Maranthes coryembosa* when measured in a reciprocal transfer experiment (Berryman, Eamus & Duff 1994). In both of those studies the insensitivity to low c_i was attributed to reductions in stomatal density, whereas we found no changes (Table 1). Although only a few leaves were sampled (those used for gas exchange), the sampling strategy we used should have detected changes of 5% in stomatal density on 95% of occasions. We therefore conclude that the reduction in sensitivity of conductance to $[CO_2]$ demonstrates a change in guard cell function. However, this acclimation will have limited impact unless environmental conditions force c_i below approximately 400 μmol mol^{-1}. In another recent study of stomatal acclimation to $[CO_2]$ in which there was no observed change in stomatal density Šantrůček & Sage (1996) found that the growth $[CO_2]$ markedly changed the shape of the g_s/c_i response of *C. album*. In that case a non-linear g_s/c_i response curve with low sensitivity of g_s at high c_i in the control plants, changed to a linear response with high sensitivity of g_s at high $[CO_2]$ in high-$[CO_2]$-grown plants. In the present work the sensitivity to c_i (Fig. 1) was analysed using linear regressions (Table 1). A non-linear response of g_s to c_i between approximately 300–450 μmol mol^{-1} c_a has been observed widely in many different studies (e.g. Farquhar *et al.* 1978; Morison & Gifford 1983; Morison 1987; Berryman *et al.* 1994; Sage 1994; Beerling *et al.* 1998; Thomas & Eamus 1999) and is suggested by the individual g_s/c_i response of low-$[CO_2]$-grown plants in the present study (Fig. 1) although we had few measurement points within this range. The high-$[CO_2]$-grown plants showed low g_s throughout the response curve.

Some studies that have observed stomatal acclimation to c_i have found a parallel negative acclimation of photosynthesis (Tuba, Szente & Koch 1994; Šantrůček & Sage 1996) and some not (Heath & Kerstiens 1997; Morison 1998). In this study there was substantial negative photosynthetic acclimation to high $[CO_2]$. especially in the 1998 data (Fig. 4), broadly agreeing with other data from the site (Li *et al*. 1999), and many other studies (see Drake *et al*. 1997). In addition, the larger degree of photosynthetic acclimation in 1998 compared to 1997 was accompanied by lower g_s sensitivity to c_i. This might suggest that the acclimation of photosynthesis and conductance are linked, but it should be noted that the region of greatest distinction between high- and low-$[CO_2]$ grown-plants on the g_s/c_i response curve was at low c_i, whereas it was at high c_i for the A/c_i curves (compare Figs 2 & 4). Furthermore, the lack of correlation between A and g_s in the short term as c_a was varied contrasts with the correlation between A and g_s of different leaves at either growth $[CO_2]$ (Fig. 5). This coupling of g_s to mesophyll photosynthetic activity in the medium- and long-term, as often found (e.g. Wong *et al*. 1979, Wong, Cowan & Farquhar 1985; Ball, Woodrow & Berry 1987), is reflected in the much smaller coefficient of variation for c_i/c_a compared to that for A or g_s (e.g. 4, 42 and 40%, respectively, in 1997). The difference between the short- and long-term correlation of A and g_s remains an enigma, and suggests that stomata are not responding to some simple photosynthetic signal from the mesophyll alone.

The possible limitation of photosynthesis at high c_a by stomata is often deduced from the ratio of intercellular to atmospheric $[CO_2]$ (Drake *et al*. 1997). When measured at their respective growth $[CO_2]$, the leaves from the different growth treatments had the same c_i/c_a ratio (Table 2). However, the c_i/c_a ratios increased for plants grown in high $[CO_2]$, upon exposure to 350 μmol mol^{-1} c_a as previously observed by Šantrůček & Sage (1996), because of the negative acclimation of photosynthesis, confirming that stomata do not act to maintain c_i/c_a constant (e.g. Morison & Gifford 1983).

Another method of quantifying stomatal importance in the c_i/c_a relationship is by feedback loop analysis (Farquhar *et al*. 1978; Šantrůček & Sage 1996), which determines the degree to which the photosynthetic and stomatal feedback loops reduce the effect of a perturbation of c_a on c_i (Table 3). The open-loop gains G_g and G_A were modified in the high-$[CO_2]$-grown plants, reflecting the negative acclimation of A (Fig. 4) and the decreased sensitivity of g_s to low c_i values (Fig. 2). Šantrůček & Sage (1996) found comparable stomatal physiological gain (dg_s/dc_i) values of – 1080 and – 250 mol m^{-2} s^{-1} at 350 and 700 μmol mol^{-1} $[CO_2]$, respectively, for *C. album*. In the present study the largest negative values of G_g were reached at 600 μmol mol^{-1} c_a in the high-$[CO_2]$-grown plants, but were still decreasing at 700 μmol mol^{-1} c_a in low-$[CO_2]$-grown plants, indicating a shift in the point of highest sensitivity to c_i (Šantrůček & Sage 1996). The closed-loop gain $[G = 1/(1 - G_g - G_A)]$ was similar in the high- and low-$[CO_2]$-grown plants in 1997, but in 1998 was lower (larger reduction of the effect of perturbations in c_a on c_i) by a factor of approximately 0·16 in the low-$[CO_2]$-grown plants. In the previously mentioned Šantrůček & Sage (1996) study, G was reduced by a factor of approximately 0·5–0·25 for a comparable change in c_a. The similarity of A and g_s gain loops in both high- and low-$[CO_2]$-grown plants, when measured at their respective growth concentrations is another reflection of the tight coupling between A and g_s evident in Fig. 5.

In contrast to the results for *C. album* (Šantrůček & Sage 1996), the impact of acclimation of g_s and A with c_a on the so-called 'instantaneous' WUE was small (Table 2). The oak plants grown and measured in high $[CO_2]$ had only a 9 and 17% lower WUE in 1997 and 1998, respectively, than the low-$[CO_2]$-grown plants measured at high c_a. This is a small effect compared to the 2·5 to 3-fold increase of WUE with growth in high $[CO_2]$ which must have profound implications for the productivity of this species growing in an environment with high evaporative demand.

One of the problems in assessing the effect of c_a on any physiological process when comparing plants grown for long periods in different c_a treatments, is that observed differences may be caused by indirect changes in the environment (Norby *et al*. 1999), leading to changes in plant water and nutrient status. The measurements of g_s reported here were done on excised shoots in controlled conditions, so that any short-term effects of water stress should have been avoided. However, we cannot ignore possible long-term effects that might have differed between $[CO_2]$ treatments. The converse effect of growth at high $[CO_2]$ on g_s in block 4 in both 1997 (Fig. 1) and 1998 (data not shown), may indicate plant responses to general differences in growth conditions between the two chambers which were widely spaced in that particular block (see also Fig. 3), or could be due to shoot–shoot variation of g_s. The drought in the early part of 1998 was severe and led to a reduction in growth by 50% for high- and 66% for low-$[CO_2]$-grown plants compared with that in 1997 (personal communication, P Dijkstra). This difference in effect between treatments may well have affected the gas exchange responses we measured.

In conclusion, *Q. myrtifolia* showed a large reduction (36–46%) in g_s when grown in high $[CO_2]$, compared with low-$[CO_2]$-grown plants, and a small (9–16%) negative acclimation of g_s when plants from both growth treatments were measured at 700 μmol mol^{-1} c_a. Stomatal acclimation in the high-$[CO_2]$-grown plants was more clearly shown as a reduction in g_s sensitivity to a range of lower c_i whereas negative A acclimation (21–36%) in high-$[CO_2]$-grown plants was most evident at high c_i. There was no change in stomatal density. Although the mechanism for these changes in stomatal sensitivity is unknown, they suggest acclimation of guard cell responses to c_i independent of mesophyll photosynthetic changes. These large changes in stomatal conductance will not only be affected by other biotic and abiotic factors (e.g. Ceulemans, Janssens & Jach 1999) that show responses to increased $[CO_2]$, but will also have a large impact on ecosystem function through their effects on water relations.

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