Stomatal acclimation over a subambient to elevated CO₂ gradient in a C₃/C₄ grassland


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ABSTRACT
An investigation to determine whether stomatal acclimation to [CO₂] occurred in C₃/C₄ grassland plants grown across a range of [CO₂] (200–550 umol mol⁻¹) in the field was carried out. Acclimation was assessed by measuring the response of stomatal conductance (gₛ) to a range of intercellular CO₂ (a gₛ-Cᵢ curve) at each growth [CO₂] in the third and fourth growing seasons of the treatment. The gₛ-Cᵢ response curves for Solanum dimidiatum (C₃ perennial forb) differed significantly across [CO₂] treatments, suggesting that stomatal acclimation had occurred. Evidence of non-linear stomatal acclimation to [CO₂] in this species was also found as maximum gₛ (gₛₘₐₓ; gₛ measured at the lowest Cᵢ) increased with decreasing growth [CO₂] only below 400 umol mol⁻¹. The substantial increase in gₛ at subambient [CO₂] for S. dimidiatum was weakly correlated with the maximum velocity of carboxylation (Vₖₐₘᵦ; r² = 0.27) and was not associated with CO₂ saturated photosynthesis (Aₛₘₐₓ). The response of gₛ to Cᵢ did not vary with growth [CO₂] in Bromus japonicus (C₄ annual grass) or Bothriochloa ischaemum (C₄ perennial grass), suggesting that stomatal acclimation had not occurred in these species. Stomatal density, which increased with rising [CO₂] in both C₃ species, was not correlated with gₛ. Larger stomatal size at subambient [CO₂], however, may be associated with stomatal acclimation in S. dimidiatum. Incorporating stomatal acclimation into modelling studies could improve the ability to predict changes in ecosystem water fluxes and water availability with rising CO₂ and to understand their magnitudes relative to the past.

Key-words: grassland ecosystems; stomata; stomatal limitation of photosynthesis; subambient CO₂; water use efficiency.

INTRODUCTION

Stomatal conductance (gₛ) is generally expected to decline in herbaceous plants with an increase in atmospheric CO₂ above the current concentration (Field, Jackson & Mooney 1995; Knapp et al. 1996; Drake, Gonzalez-Meler & Long 1997; Wand et al. 1999). There are many important physiological and ecological implications of such a decline. For example, lower gₛ may alter ecosystem hydrology by reducing transpiration and could increase the surface temperature through reduced evaporative cooling (Sellers et al. 1996; Jackson et al. 1998; Bounoua et al. 1999). Reduced gₛ, coupled with increased photosynthesis may also improve the water use efficiency (WUE) of many plants, which is of particular importance for productivity in arid and semi-arid regions (Polley, Johnson & Mayeux 1992; Polley et al. 1993; Sage 1995; Field et al. 1995; Owensby et al. 1999; Smith et al. 2000).

A major factor that could alter stomatal responses to CO₂ is the degree to which stomata acclimate to growth CO₂ concentration. Stomatal acclimation to CO₂ would be ecologically important if it either tempered or enhanced the reduction in gₛ with rising CO₂ (Santrůček & Sage 1996; Morison 1998). Acclimation is defined here as a change in stomatal function that occurs when plants are grown in contrasting CO₂ concentrations (Santrůček & Sage 1996; Morison 1998). For example, physiological acclimation would be demonstrated if the stomatal behaviour of plants grown at contrasting CO₂ concentrations differed when measured at the same CO₂ concentration. Stomatal acclimation to CO₂ may occur in several ways, including changes in maximum gₛ and stomatal sensitivity to CO₂. Other responses to CO₂, such as changes in stomatal morphology (density and size) may also influence gₛ, independently of physiological acclimation. Despite its potential importance for regulating plant water loss at high CO₂, stomatal acclimation has only been examined in a few species (Drake et al. 1997; Morison 1998).

The often dramatic response of terrestrial vegetation to elevated CO₂ has led to greater interest in plant responses to past CO₂ increases (Sage & Coleman 2001). Atmospheric CO₂ was as low as 180 umol mol⁻¹ during the last glacial maximum and has risen by 37% since the 1700s (Barnola et al. 1987; Jouzel et al. 1993). Past CO₂ increases have been implicated in shifting global distributions of C₃ and C₄ plants and increases in ecosystem productivity (Polley et al. 1993; Ehleringer, Cerling & Helliker 1997). Although studies suggest that carbon assimilation may be affected by subambient CO₂ (Sage & Reid 1992; Tissue et al. 1995; Anderson et al. 2001; Sage & Coleman 2001), com-
paratively little is known about stomatal responses to past atmospheric CO2 concentrations and their underlying mechanisms.

Recent empirical and modelling studies suggest that plant and ecosystem responses to increasing CO2 may be non-linear (Ackerley & Bazzaz 1995; Luo, Sims & Griffin 1998; Luo & Reynolds 1999; Anderson et al. 2001). However, a majority of studies have examined plant responses to step increases in atmospheric CO2, generally comparing ambient to twice ambient manipulations (Drake et al. 1997; Wand et al. 1999). Because atmospheric CO2 is increasing gradually, results from step change experiments cannot be easily interpolated to intermediate CO2 concentrations, and threshold or non-linear responses to CO2 may go undetected (Luo & Reynolds 1999). Observations of plants grown across a range of CO2 (e.g. Polley et al. 1993) may therefore help refine predictions of ecosystem responses to future CO2 increases (Ackerley & Bazzaz 1995) and determine the magnitude of changes that have occurred since the start of the Industrial Revolution.

In this study, the influence of atmospheric CO2 on stomatal physiology was examined using experimental chambers that maintain a continuous gradient of CO2 from 200 to 550 µmol mol−1 (Johnson, Polley & Whitis 2000). A previous study in this C3/C4 grassland ecosystem reported 40–80% declines in g, along the gradient for several species (Anderson et al. 2001). Stomatal acclimation was examined in three dominant species of this grassland. First, we tested whether stomatal acclimation to CO2 occurred and examined whether there was a threshold or non-linear response to the CO2 gradient. Second, the widespread occurrence of photosynthetic acclimation to rising CO2 suggests that stomata may also acclimate to maintain the tight coupling between photosynthesis and conductance. We therefore determined whether stomatal acclimation was associated with changes in photosynthetic acclimation (e.g. Jarvis, Mansfield & Davies 1999). Finally, we examined the implications of variation in stomatal behaviour for carbon gain by assessing its effects on relative stomatal limitation of photosynthesis (Jones 1985) and intrinsic WUE.

METHODS

Study site and experimental system

The measurements were conducted in a grassland area near the USDA-ARS Grassland, Soil and Water Research Laboratory in Temple, TX (31°05′ N, 97°20′ W). The site has been managed as grassland for the last 50 years and was last grazed by cattle in 1992. Soils are in the Austin black soil series, classified as a fine-silty, carbonatic, thermic Udorthent (Haplustolls) with 35–55% clay in the top 40 cm (Johnson et al. 2000). To determine the potential for interspecific variation in responses to CO2, the study focused on three abundant species with different growth forms and photosynthetic pathways: Solanum dimidiatum Raf., a C3 perennial forb; Bromus japonicus L., a C3 annual grass; and Bothriochloa ischaemum (L.) Keng, a C4 perennial grass.

Other dominant species at the site include Solidago canadensis L. and Ratibida columnaris (Sims) D. Don. Mean annual precipitation (1913–99) is 877 mm and the mean minimum and maximum annual temperatures are 13.2 and 25.9 °C, respectively. C3 species are mostly active early in the growing season, and C4 species dominate by mid-summer.

The experimental system that was used consisted of two elongated chambers over parallel and adjacent plots of grassland, each 60 m in length, 1 m wide, and 1 m tall. Air was introduced into one end of each chamber and was progressively depleted of CO2 by photosynthesis as it was moved down the chamber by a blower. The desired CO2 concentrations were maintained by automatically varying the rate of air flow. During daytime, the CO2 concentration gradients in the subambient and superambient chambers ranged from 360 to 200 µmol mol−1 and from 550 to 360 µmol mol−1, respectively. At night, CO2 gradients were maintained at 150 µmol mol−1 above daytime levels by reversing air flow and using respiratory CO2 releases to create the gradient. Treatments began in May 1997 and operated each growing season (mid-February to mid-November) up to and including 2000.

The chambers were divided into 10 sections, each 5 m in length, with chilled-water cooling coils between sections to control temperature and humidity. Each section was enclosed in polyethylene film (which transmitted 85–95% of incident PPFD), and a rubber-coated barrier extended 1 m deep into the soil along the sides of each chamber. To maintain environmental conditions at ambient levels in chambers, air temperature and humidity were controlled by cooling and dehumidifying air before it entered each 5 m section of chamber. Irrigation was applied equally to each 5 m section to match ambient rainfall through July 1999. Thereafter, water was applied such that soil water content in sections matched that of adjacent grassland exposed to ambient CO2 as measured by neutron attenuation. During the drought years of 1999 and 2000, the total water applied to the chambers was 349 and 381 mm, respectively. There were no consistent effects of CO2 treatment on soil water content during these years (Polley et al. unpublished results). Therefore, stomatal responses to CO2 were not confounded by CO2-induced variation in soil water availability along the gradient. However, it should be noted that interspecific differences might have been affected by water availability because each species was measured at a different time of the year. Additional details of design, construction and operation of these chambers can be found in Johnson et al. (2000).

Steady-state gas exchange and stomatal acclimation

Steady-state leaf gas-exchange was measured at saturating irradiance with an open gas-exchange system (LI-6400; LI-Cor Inc., Lincoln, NE, USA) between 0900 and 1500 h central standard time. Incident irradiance during all measurements was provided by red-blue light-emitting diodes.

Stomatal acclimation to CO\textsubscript{2} in a C\textsubscript{3}/C\textsubscript{4} grassland

Measurements were taken when each species was at its peak abundance, which was June 2000 for \textit{S. dimidiatum}, April 2000 for \textit{Br. japonicus}, and August 1999 for \textit{Bo. ischaemum}. The youngest fully expanded leaf was sampled on three to four plants per section at a minimum of six treatment CO\textsubscript{2} concentrations (six chamber sections). Stomatal conductance (g\textsubscript{s}) was measured at saturating light levels of 1200, 1600, and 2000 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} for \textit{S. dimidiatum}, \textit{Br. japonicus} and \textit{Bo. ischaemum}, respectively. A Peltier cooling module maintained leaf temperatures at 20–23, 27–30 and 29–32 °C for \textit{Br. japonicus}, \textit{S. dimidiatum} and \textit{Bo. ischaemum}, respectively, matching the approximate ambient conditions at the time of the measurement. Leaf-to-air vapour pressure deficits (LAVPD) were maintained at levels that permitted the measurement of maximum g\textsubscript{s}, which were 0.9–1.1 kPa for \textit{S. dimidiatum} and \textit{Br. japonicus} and 1.4–1.6 kPa for \textit{Bo. ischaemum}. To calculate g\textsubscript{s} and intercellular CO\textsubscript{2} concentration (C\textsubscript{i}) a boundary layer conductance of 4.86 mmol m\textsuperscript{-2} s\textsuperscript{-1} was used for the grasses and 1.42 mmol m\textsuperscript{-2} s\textsuperscript{-1} was used for \textit{S. dimidiatum}. Boundary layer conductance was calculated on the basis of leaf area and fan speed using the energy balance algorithms of the LI-6400. Leaf area was measured with a portable leaf area meter (LI-3100; Li-Cor Inc.) or from leaf dimensions, depending on sample morphology. To examine long-term consequences of CO\textsubscript{2} for stomatal function, measurements were made during the third and fourth growing season of the experiment.

To examine stomatal acclimation to growth CO\textsubscript{2} concentrations, we measured the response of g\textsubscript{s} and the C\textsubscript{i}/C\textsubscript{s} ratio to a manipulation of C\textsubscript{i} (by changing the external CO\textsubscript{2} concentration, C\textsubscript{e}) within the leaf cuvette. Stomatal conductance versus C\textsubscript{i} (g\textsubscript{s}–C\textsubscript{i}) curves were measured on each species during the same period as steady-state gas exchange. Once clamped in the cuvette, leaves were exposed to the light, LAVPD, and temperature levels described above and the respective growth CO\textsubscript{2}. After steady-state conditions were achieved, the first measurement was taken, and C\textsubscript{i} was then reduced to the CO\textsubscript{2} compensation point and raised in steps (Santürçüek & Sage 1996). Stomatal conductance was recorded after steady-state conditions were re-established at each CO\textsubscript{2} level. Steady state was determined when the coefficient of variation of change in water vapour was < 0.05% (generally after 30–50 min).

To measure stomatal density (the number of stomates per mm\textsuperscript{2}) and the size of stomata, casts were made of leaves sampled in the field during April 2000. Stomatal size was defined as the length in micrometres between junctions of the guard cells at each end of the stomate (Malone et al. 1993), and was therefore related to the maximum potential opening of the stomatal pore, and not the amount of opening that occurs at a given C\textsubscript{i}. Measurements of stomatal density and stomatal size were made on leaf casts (e.g. Williams & Green 1988) for at least six individuals per species at each of six CO\textsubscript{2} concentrations (a total of at least 36 plants per species). For each impression, a leaf section located 2–3 cm from the petiole was pressed onto a micro-scope slide covered with polyvinylsiloxane dental impression material (‘Extrude’ Medium; Kerr Manufacturing Co., Orange, CA, USA). After the polymer hardened (approximately 5 min), the leaf was removed and the resulting leaf mould was later used as a cast for clear nail polish. Each impression was analysed at 400× (for \textit{S. dimidiatum} and \textit{Bo. ischaemum}) or 100× (for \textit{Br. japonicus}) using a light microscope interfaced with a solid-state TV camera (Model CCD-72-SX; DAGE-MTI Inc., Michigan City, IN, USA) using NIH Image 1.58 (U.S. National Institutes of Health; http://rsb.info.nih.gov/nih-image/). The stomatal density and stomatal size were sampled on three to six fields-of-view per slide, depending on the variation in the counts, and averaged for each slide.

The relative stomatal limitation of photosynthesis (l\textsubscript{g}) was calculated, based on the response of net photosynthesis (A) to variation in C\textsubscript{i} (A–C\textsubscript{i} curves). These curves were recorded concurrently with g\textsubscript{s}–C\textsubscript{i} curves on sampled plants and fitted with a non-linear regression model describing an exponential rise to a maximum:

\[
A = a(1 - \exp(-bc_i)) + c
\]

where c is the y intercept, 1/b is the rate constant, and a + c is CO\textsubscript{2} saturated A (A\textsubscript{max}). This model provided a good fit to our data (r\textsuperscript{2} > 0.97) and has been used previously for herbaceous plants (Jacob, Greitner & Drake 1995; Reid & Fiscus 1998). The relative stomatal limitation (l\textsubscript{g}) was calculated using the differential method of Jones (1985):

\[
l_g = \frac{r_g}{r_g + r^*}
\]

where r\textsubscript{g} is the gas-phase resistance to CO\textsubscript{2} uptake (the supply function) and r\textsuperscript{*} is the slope of the A–C\textsubscript{i} curve (demand function). We calculated r\textsuperscript{*} as the first derivative of Eqn 1 at the operating C\textsubscript{i} and calculated r\textsubscript{g} as (C\textsubscript{i} – C\textsubscript{c})/A at the operating C\textsubscript{i} (Jones 1985). This model was also used to calculate l\textsubscript{g} for the C\textsubscript{4} species because A was not saturated at high CO\textsubscript{2} (550 \textmu mol mol\textsuperscript{-1}; Anderson et al. 2001), permitting the calculation of a positive slope for the A–C\textsubscript{i} curve at the operating C\textsubscript{i}.

To examine whether variation in stomatal conductance was associated with the maximum velocity of carboxylation (V\textsubscript{max}) and photosynthetic capacity (Jarvis et al. 1999), we compared maximum g\textsubscript{s} (g\textsubscript{max}), measured at the lowest CO\textsubscript{2} concentration to V\textsubscript{max} and A\textsubscript{max} (Eqn 1). V\textsubscript{max} was estimated from the biochemical model of von Caemmerer & Farquhar (1981):

\[
V_{\text{max}} = CE[\Gamma^* + K_C[1 + [O_2]/K_O]]
\]

where the constants were K\textsubscript{C} = 43.5 Pa, K\textsubscript{O} = 23.3 kPa, and \Gamma\textsuperscript{*} (CO\textsubscript{2} compensation point in the absence of mitochondrial respiration in the light) = 4.44 Pa at a temperature of 26 °C (Harley, Webber & Gates 1985; Reid & Fiscus 1998) and an [O\textsubscript{2}] of 21 kPa. Carboxylation efficiency (CE), which is the first derivative of Eqn 1 at the CO\textsubscript{2} compensation point, was calculated as

\[
CE = ab \times \exp(-bc_i)
\]
where \( C_i = (1/b) \ln[a/(a - A + c)] \) at \( A = 0 \) (Reid & Fiscus 1998).

We note that because the \( A-C_i \) curves were measured during the construction of the \( g_v-C_i \) curves, our calculations of \( V_{\text{max}} \) may be influenced by changes in the activation state of Rubisco over the course of the measurement. However, our observations of \( V_{\text{max}} \) using the ‘slow’ approach were similar to values derived from standard \( A-C_i \) curves measured on these species previously (Anderson et al. 2001).

### Statistical analyses

To examine whether CO₂ treatments influenced \( g_v-C_i \) and \( (C_i/C_a)-C_i \) curves, we used the analysis of repeated measures (ANOVAR) for physiological response curves (e.g. Potvin, Lechowicz & Tardif 1990) in SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). \( C_i \) was the within subjects factor whereas growth CO₂ was the between subjects factor. The statistical significance of relationships between measured variables and growth CO₂ was determined using linear, hyperbolic and power functions in SPSS 10.0. As multiple measurements (three to five) were taken in each treatment section, there was more than one \( Y \) for each \( X \) (where \( X = \) growth CO₂). Although means (± 1 SE) are presented in figures, all analyses were carried out using individual plants.

### RESULTS

Steady-state \( g_v \) declined strongly with increasing growth CO₂ for all species (Fig. 1). The decline was non-linear for \( S. \) dimidiatum \( [y = a + (b/x^2)] \) where \( a = 191.99 \) and \( b = 9.27 \times 10^4; \, r^2 = 0.94; \, P < 0.001 \), decreasing by 81% from 200 to 550 \( \mu \text{mol} \cdot \text{mol}^{-1} \) CO₂. Three-quarters of this decline occurred from subambient to ambient growth CO₂. The decline in \( g_v \) with growth CO₂ was linear for \( B. \) japonicus \( (r^2 = 0.74; \, P < 0.0001; \, a 49\% \) decline from 215 to 550 \( \mu \text{mol} \cdot \text{mol}^{-1} \) and weakly non-linear for \( B. \) ischaemum \( (y = ax^2, \, where \, a = -0.64 \, and \, b = 8.30 \times 10^4; \, r^2 = 0.76; \, P < 0.001; \, a 46\% \) decline from 215 to 540 \( \mu \text{mol} \cdot \text{mol}^{-1} \). \( S. \) dimidiatum had the highest overall \( g_v \) among the three species, followed by \( B. \) japonicus and \( B. \) ischaemum (Fig. 1).

There was strong evidence for stomatal acclimation in \( S. \) dimidiatum. The \( g_v-C_i \) response curves for this species differed significantly across CO₂ treatments. Maximum \( g_v \) \( (g_{\text{max}}; \, g_v \text{ measured at the lowest } C_i) \), increased, and the \( g_v-C_i \) curves were progressively steeper for growth CO₂ concentrations below ambient levels \( (P < 0.05, \text{ ANOVAR, Fig. 2A}) \). We also found evidence of a non-linear response of stomatal acclimation to CO₂ as the \( g_v-C_i \) curves did not differ among plants exposed to growth CO₂ > 400 \( \mu \text{mol} \cdot \text{mol}^{-1} \). These differences in \( g_v-C_i \) curves also indicated that stomatal sensitivity to \( C_i \) was greater in plants grown at less than 400 \( \mu \text{mol} \cdot \text{mol}^{-1} \) CO₂. Although the \( (C_i/C_a)-C_i \) curves did not differ significantly \( (P > 0.10) \) among CO₂ treatments (Fig. 2B), average \( C_i/C_a \) was generally higher in subambient CO₂-grown plants measured at \( C_i < 400 \mu \text{mol} \cdot \text{mol}^{-1} \). This trend is consistent with stomatal acclimation observed in Fig. 2A. Modification of stomatal behaviour in \( S. \) dimidiatum was not strongly associated with the maximum velocity of carboxylation \( (V_{\text{max}}) \). The \( g_{\text{max}} \) was weakly, although significantly, correlated with \( V_{\text{max}} \) \( (r^2 = 0.27; \, P = 0.05, \text{ Fig. 3A}) \). Variation in CO₂ saturated photosynthesis \( (A_{\text{max}}) \), however, was not correlated with \( g_{\text{max}} \) \( (r^2 = 0.05; \, P = 0.32; \text{ Fig. 3B}) \). In contrast to \( S. \) dimidiatum, there was no evidence of stomatal acclimation in \( B. \) japonicus or \( B. \) ischaemum, as the \( g_v-C_i \) curves (Figs 4A & B) and \( (C_i/C_a)-C_i \) curves (data not shown) were statistically indistinguishable across growth CO₂ concentrations.

Interestingly, the stomatal density (Fig. 5A) increased linearly with growth CO₂ in \( S. \) dimidiatum \( (r^2 = 0.30; \, P < 0.001) \) and \( B. \) japonicus \( (r^2 = 0.41; \, P < 0.0001) \), but decreased linearly with growth CO₂ in \( B. \) ischaemum \( (r^2 = 0.15; \, P < 0.05) \). Stomatal size (Fig. 5B), measured as the length between junctions of the guard cells at each end of the stomate, decreased linearly with growth CO₂ in \( S. \) dimidiatum \( (r^2 = 0.49; \, P < 0.0001) \). In contrast, stomatal size increased weakly with growth CO₂ in \( B. \) japonicus \( (r^2 = 0.14; \, P < 0.05) \) and was not associated with growth CO₂ in \( B. \) ischaemum \( (r^2 = 0.06; \, P = 0.13) \).
Despite higher $g_s$ plants grown at lower CO$_2$ concentrations had greater relative stomatal limitation of photosynthesis ($I_p$) than plants grown at higher CO$_2$ concentrations (Fig. 6). The strongest effect was for *Br. japonicus*, in which the relationship was non-linear (second-order polynomial, $r^2 = 0.82; P < 0.0001$). Stomatal limitation of photosynthesis decreased linearly with increasing growth CO$_2$ for *S. dimidiatum* ($r^2 = 0.51; P < 0.001$) and *Bo. ischaemum* ($r^2 = 0.26; P = 0.03$). Although *S. dimidiatum* and *Br. japonicus* had similar $I_p$ at elevated growth CO$_2$, they diverged at subambient CO$_2$. Among species, stomatal limitation of photosynthesis was inversely proportional to $g_s$, being greatest in *Bo. ischaemum*, followed by *Br. japonicus* and *S. dimidiatum*.

Because plants were measured at the same LA VPD across treatments, the intrinsic water use efficiency ($A/g_s$) is representative of the water cost of photosynthesis in contrasting growth CO$_2$ environments. To illustrate the influence of stomatal acclimation to CO$_2$ on stomatal optimization of water loss relative to carbon gain, we plotted $A/g_s$, of all plants measured at a common $C_i$ of 200 µmol mol$^{-1}$ (where the strongest evidence for acclimation was observed, Fig. 2A) as a function of their growth CO$_2$ concentration (Fig. 7A–C). In *S. dimidiatum*, $A/g_s$ increased significantly with growth CO$_2$ ($r^2 = 0.59; P < 0.0001$). Thus, when measured at 200 µmol mol$^{-1}$ CO$_2$, $A/g_s$ for plants grown at 550 µmol mol$^{-1}$ was twice that of plants grown at 250 µmol mol$^{-1}$ CO$_2$. In contrast, growth CO$_2$ concentration had no effect on $A/g_s$ measured at a common $C_s$ for either grass species (Figs 7B & C).

**DISCUSSION**

We found clear evidence for stomatal acclimation to growth CO$_2$ in *Solanum dimidiatum*, a C$_3$ perennial forb, based on comparisons of the $g_s$–$C_i$ curves among plants grown across a range of CO$_2$ concentrations. The strongest

![Figure 3](image_url)

Figure 3. The relationship between maximum stomatal conductance ($g_{smax}$, measured at the lowest $C_i$) and $V_{cmax}$ (A) and $A_{max}$ (B) as determined from $g_s$–$C_i$ response curves. The $g_{smax}$ was significantly associated with $V_{cmax}$ ($r^2 = 0.27; P = 0.05$), but was not correlated with $A_{max}$ ($P > 0.05$).

![Figure 2](image_url)

Figure 2. The response of mean (± 1 SE) $g_s$ to $C_i$ ($g_s$–$C_i$ response curve; (A) and $C_i/C_a$ to $C_i$ ([($C_i/C_a$)–$C_i$] response curve; (B) for *S. dimidiatum* plants grown across a range of atmospheric CO$_2$. For $g_s$ responses, a different letter next to each curve indicates that it is significantly different ($P < 0.05$, ANOVAR) from the other curves. Symbols correspond to different growth CO$_2$ concentrations (µmol mol$^{-1}$).
response of \( g_s \) to \( C_i \) occurred in plants grown at 250 and 294 \( \mu \text{mol mol}^{-1} \) \( \text{CO}_2 \), followed by plants grown at 354 \( \mu \text{mol mol}^{-1} \) \( \text{CO}_2 \) (Fig. 2A). In contrast, \( g_s-C_i \) curves did not differ for plants grown at greater than 400 \( \mu \text{mol mol}^{-1} \) \( \text{CO}_2 \). Although other studies have observed stomatal acclimation from ambient to elevated \( \text{CO}_2 \) (Morgan et al. 1994; Tuba, Szente & Koch 1994; Chen, Begonia & Hesketh 1995; Šantrůček & Sage 1996; Bunce 2001; Lodge et al. 2001), no evidence of such a pattern was found for any species in the present study (Figs 2A & 4). The results indicate that in \( S. \text{dimidiatum} \), stomatal acclimation to growth \( \text{CO}_2 \) occurred non-linearly, below an apparent threshold of 350–400 \( \mu \text{mol mol}^{-1} \) \( \text{CO}_2 \). To our knowledge, this is the first study to document stomatal acclimation to subambient \( \text{CO}_2 \) in the field.

Neither grass species, \( B. \text{japonicus} \) or \( B. \text{ischaemum} \), showed evidence of stomatal acclimation to growth \( \text{CO}_2 \); there were no differences in the \( g_s-C_i \) response curves for either species across growth \( \text{CO}_2 \) treatments (Fig. 4A & B). This result was surprising, given systematic differences in absolute photosynthetic rates (Anderson et al. 2001) and \( g_s \) (Fig. 1) between the \( C_3 \) annual \( B. \text{japonicus} \) and the \( C_4 \) perennial \( B. \text{ischaemum} \). Our observations for \( B. \text{japoni}-

![Figure 4](image-url)  

**Figure 4.** The response of mean (± 1 SE) \( g_s \) to manipulation of \( C_i \) (\( g_s-C_i \) response curve) for \( B. \text{japonicus} \) (A) and \( B. \text{ischaemum} \) (B) plants grown across a range of atmospheric \( \text{CO}_2 \). Symbols correspond to different growth \( \text{CO}_2 \) concentrations (\( \mu \text{mol mol}^{-1} \)).

cus and \( B. \text{ischaemum} \) therefore suggest that stomatal responses to \( \text{CO}_2 \) in \( C_3 \) and \( C_4 \) grass species, like growth responses, may be quite similar (Wand et al. 1999). In contrast, stark differences in stomatal acclimation to \( \text{CO}_2 \) between \( S. \text{dimidiatum} \) and \( B. \text{japonicus} \) indicate that species with similar photosynthetic pathways may have vastly different stomatal responses to \( \text{CO}_2 \).

Previously, we observed significant up-regulation of the maximum velocity of carboxylation (\( V_{\text{max}} \)) at subambient growth \( \text{CO}_2 \) in \( S. \text{dimidiatum} \) (Anderson et al. 2001). The observation that \( g_s \) is often coupled to photosynthetic capacity (e.g. Wong, Cowan & Farquhar 1979) raises the possibility that stomatal acclimation in \( S. \text{dimidiatum} \) represents a response to photosynthetic acclimation rather than a direct response to growth \( \text{CO}_2 \). A significant positive relationship was observed between \( g_{\text{max}} \) and \( V_{\text{max}} \) (Fig. 3A), but variation in \( V_{\text{max}} \) could only account for 27% of the variation in \( g_{\text{max}} \). The \( \text{CO}_2 \) saturated photosynthetic rate (\( A_{\text{max}} \)), in contrast, was not related to \( g_{\text{max}} \) (Fig. 3B). Although the mechanism for stomatal acclimation to atmospheric \( \text{CO}_2 \) concentration is not known, the present results suggest that stomatal acclimation to \( \text{CO}_2 \) may not be driven solely by adjustments in photosynthetic bio-

![Figure 5](image-url)  

**Figure 5.** The response of mean (± 1 SE) stomatal density (A) and stomatal size (B) to a range of atmospheric \( \text{CO}_2 \) for the three study species. Statistically significant differences (\( P < 0.05 \)) in the magnitude of each variable among species are indicated by different letters. A * next to each letter indicates that the slope of the relationship is significantly (\( P < 0.05 \)) different from zero.
chemistry (e.g. Bunce 2001; Lodge et al. 2001; but see Jarvis et al. 1999).

As in previous studies in grasslands, increasing growth CO₂ reduced steady-state gₛ in all species (Fig. 1; Johnson, Polley & Mayeux 1993; Jackson et al. 1994; Knapp et al. 1996; Niklaus, Spinnler & Körner 1998; Lee et al. 2001). Our results suggest that stomatal acclimation to CO₂ in S. dimidiatum was responsible for driving both the large (81%) and non-linear decrease in steady-state gₛ along the CO₂ gradient (Fig. 1A). For example, steady-state gₛ at a CO₂ concentration of 200 µmol mol⁻¹ was 75% higher than it would have been if acclimation had not occurred (based on a comparison of the gₛ–Cᵢ curves of plants grown at 250 µmol mol⁻¹ CO₂ versus those grown at greater than 400 µmol mol⁻¹ CO₂; Fig. 2A). In contrast, gₛ declined linearly and more modestly (46–49%) with CO₂ in the two grass species in which stomatal acclimation was not observed.

Some studies suggest that decreased stomatal density is a mechanism for reducing gₛ in response to rising CO₂ (Woodward 1987; Beerling & Woodward 1993; Kurschner et al. 1997). Our results from a C₃/C₄ grassland provide little support for this pattern, as the species showing the strongest decline in gₛ with rising CO₂ (S. dimidiatum, Fig. 1A), actually increased stomatal density with increasing growth CO₂ (Fig. 5A). Similarly, the relationship between stomatal density and growth CO₂ was very different between Br. japonicus and Bo. ischaemum, species that had nearly identical declines in gₛ in response to increased CO₂ along the gradient. Of the three species measured only Bo. ischaemum decreased stomatal density in response to rising CO₂ (Fig. 5A), but the strength of this relationship was relatively weak (r² = 0.15). These results suggest that gₛ responses to CO₂ need not correlate in magnitude or direction with stomatal density responses to CO₂. However, it should be noted that measurements of gₛ and stomatal density were not made at the same time; therefore we cannot rule out the possibility that phenological variation in stomatal morphology also contributed to the lack of correlation between sto-

**Figure 6.** The response of mean (± 1 SE) relative stomatal limitation of photosynthesis (lₛ) to increasing growth CO₂ for C₃ (A) and C₄ (B) species. Note the difference in scales on the Y-axis between (A) and (B).

**Figure 7.** Mean (± 1 SE) intrinsic leaf water use efficiency (A/gₛ) measured at 200 µmol mol⁻¹ C₄ for each species in each growth CO₂ treatment. A/gₛ increased significantly with growth CO₂ only in S. dimidiatum. Note the difference in scales on the Y-axis between (A), (B) and (C).
matal density and \( g_s \). Nevertheless, our results suggest that the response of stomatal density to \( CO_2 \) is not necessarily generalizable across species or growth forms (e.g. Knapp et al. 1994).

Stomata were significantly larger in \( S. \) dimidiatum plants grown at subambient \( CO_2 \) (Fig. 5B). As a potential developmental response to \( CO_2 \) starvation, the increased stomatal size facilitates \( CO_2 \) diffusion into the leaf (Parkhurst 1994) because conductance is proportional to the square of the effective radius of the stomatal pore (Nobel 1991). Therefore, larger stomatal size at subambient \( CO_2 \) may contribute to the substantial increases in \( g_s \) that were observed in \( S. \) dimidiatum (Fig. 1A). Similarly, small stomatal size at elevated \( CO_2 \) could constrain \( g_s \) upon exposure to subambient \( CO_2 \), as was observed in our study (Fig. 2A). Large stomatal size in \( S. \) dimidiatum, however, does not appear to prevent guard cell control of water loss, as \( g_s \) measured at high \( C_\text{\textalpha} \) was the same for all plants regardless of growth \( CO_2 \) (Fig. 2A). A reduction in \( g_s \) for elevated \( CO_2 \)-grown \( S. \) dimidiatum, despite an increase in stomatal density, also suggests that increased stomatal size may be the primary morphological adjustment implicated in stomatal acclimation to \( CO_2 \). The observation that stomatal size varied weakly with \( CO_2 \) treatment in \( Br. \) japonicus and \( Bo. \) ischaemum (e.g. Malone et al. 1993), where stomatal acclimation did not occur (Fig. 4), is consistent with this view.

To determine whether stomatal acclimation could influence \( CO_2 \) diffusion and carbon assimilation, relative stomatal limitation of photosynthesis (\( l_p^\prime \); Jones 1985) was calculated from \( A–C_i \) curves measured concurrently with \( g_s–C_i \) curves (Fig. 6). In general, the magnitude of \( l_p^\prime \) was inversely proportional to \( g_s \) among species (\( S. \) dimidiatum < \( Br. \) japonicus < \( Bo. \) ischaemum). Stomatal closure at higher growth \( CO_2 \), however, did not lead to an increase in \( l_p^\prime \), suggesting that the stimulation of photosynthesis by \( CO_2 \) more than compensated for any diffusional limits imposed by stomata (Tissue et al. 1995; Drake et al. 1997). The value of \( l_p^\prime \) was approximately similar for both \( C_\text{\textalpha} \) species at elevated \( CO_2 \) (approximately 10–15%) but \( l_p^\prime \) in \( Br. \) japonicus increased disproportionately relative to \( S. \) dimidiatum at subambient growth \( CO_2 \) concentrations. These results suggest that stomatal acclimation may have a significant role in reducing \( l_p \) in \( S. \) dimidiatum at subambient \( CO_2 \) when compared with the non-acclimating \( Br. \) japonicus.

Stomatal acclimation, by increasing water loss from the leaf surface, could alter intrinsic WUE (defined here as \( A/g_s \)). Although rising \( CO_2 \) increases WUE in this grassland (Anderson et al. 2001), stomatal acclimation appears to modify the trajectory of this response in \( S. \) dimidiatum. The influence of stomatal acclimation on WUE was apparent in the strong effect that growth \( CO_2 \) concentration had on \( A/g_s \), even when plants were measured at the same \( C_\text{\textalpha} \). For example, \( A/g_s \) increased significantly with growth \( CO_2 \) in \( S. \) dimidiatum measured at 200 \( \mu \)mol mol\(^{-1} \) (Fig. 7A). In contrast, \( A/g_s \) measured at a common 200 \( \mu \)mol mol\(^{-1} \) \( C_\text{\textalpha} \) did not vary with growth \( CO_2 \) in either of the non-acclimating grass species (Fig. 7B & C).

The observation that \( A/g_s \) was lower in \( S. \) dimidiatum than it would have been without acclimation suggests that, in the past, this phenotype could have had a negative impact on plant productivity in water-limited grasslands (Polley et al. 1993; Sage 1995; Hsiao & Jackson 1999). Therefore, what is the ecological significance of stomatal acclimation to subambient \( CO_2 \)? One possibility is that maximizing leaf \( A/g_s \) is not necessarily an adaptive response to variation in \( CO_2 \). For example, maintaining a high \( A/g_s \) may be a disadvantage if conserved water that is stored in soil is lost to competitors or through soil evaporation (DeLucia & Schlesinger 1991; Jones 1993). It is also likely that the strength of stomatal regulation of water loss in a given species is dependent on other correlated physiological and morphological traits (Givnish 1986). For example, weak stomatal control of water loss may be associated with species that have access to stable sources of water via deep roots or that possess xylem that is resistant to drought-induced cavitation (Jones 1993; Jackson, Sperry & Dawson 2000). Given our limited understanding of the whole-plant context of stomatal responses to \( CO_2 \) and the mechanisms by which these responses occur, the adaptive significance of stomatal acclimation to subambient \( CO_2 \) remains uncertain.

It is not known to what degree comparisons of extant plants grown in subambient and elevated \( CO_2 \) concentrations are representative of actual changes in plant function from the past to the present and future. As we have no information on the genotypes of past populations, it is not known if our study species have evolved in response to rising \( CO_2 \). We observed strong evidence of stomatal acclimuation in \( S. \) dimidiatum at growth \( CO_2 \) concentrations as high 294 \( \mu \)mol \( mol^{-1} \), a level which occurred relatively recently (around 1900 AD). Although a step change in atmospheric \( CO_2 \) can act as a selective agent on plant populations (Ward et al. 2000), the relatively short time span involved suggests that populations of a perennial species such as \( S. \) dimidiatum may not have evolved substantially in response to the gradual increase in \( CO_2 \). In consequence, our results suggest that the greatest stomatal response to \( CO_2 \) (approximately 76%) in \( S. \) dimidiatum has already taken place. Further reductions in \( g_s \) will likely occur as atmospheric \( CO_2 \) increases, but will be of much smaller magnitude. In contrast, the absence of stomatal acclimation in \( Br. \) japonicus and \( Bo. \) ischaemum suggests that future stomatal responses to rising \( CO_2 \) may mimic those of the past in these species.

Our results have implications for modelling efforts aimed at predicting how stomatal responses to \( CO_2 \) may feedback on ecosystem hydrology and climate (Henderson-Sellers, McGuffie & Gross 1995; Pollard & Thompson 1995; Sellers et al. 1996; Jackson et al. 1998). When stomatal acclimation is included in these models, it is usually assumed to occur linearly with \( CO_2 \) (e.g. Sellers et al. 1996). In contrast to these assumptions, we observed stomatal acclimation only at subambient \( CO_2 \), a phenomenon that caused reductions in \( g_s \), from subambient to ambient \( CO_2 \) to be greater than those observed from ambient to elevated \( CO_2 \).
occurrence of stomatal acclimation and substantial reductions in $g$, from pre-industrial to current ambient $CO_2$ concentration suggest that large changes in evapotranspiration and soil drainage in grassland ecosystems may have already occurred. Incorporating these physiological perspectives into modelling studies could improve our ability to predict changes in ecosystem water fluxes and water availability with rising $CO_2$ and to understand their magnitudes relative to the past.

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