

Stomatal sensitivity to vapour pressure difference over a subambient to elevated CO₂ gradient in a C₃/C₄ grassland

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ABSTRACT

In the present study the response of stomatal conductance (g_s) to increasing leaf-to-air vapour pressure difference (D) in early season C₃ (*Bromus japonicus*) and late season C₄ (*Bothriochloa ischaemum*) grasses grown in the field across a range of CO₂ (200–550 $\mu\text{mol mol}^{-1}$) was examined. Stomatal sensitivity to D was calculated as the slope of the response of g_s to the natural log of externally manipulated D ($dg_s/d\ln D$). Increasing D and CO₂ significantly reduced g_s in both species. Increasing CO₂ caused a significant decrease in stomatal sensitivity to D in *Br. japonicus*, but not in *Bo. ischaemum*. The decrease in stomatal sensitivity to D at high CO₂ for *Br. japonicus* fit theoretical expectations of a hydraulic model of stomatal regulation, in which g_s varies to maintain constant transpiration and leaf water potential. The weaker stomatal sensitivity to D in *Bo. ischaemum* suggested that stomatal regulation of leaf water potential was poor in this species, or that non-hydraulic signals influenced guard cell behaviour. Photosynthesis (A) declined with increasing D in both species, but analyses of the ratio of intercellular to atmospheric CO₂ (C_i/C_a) suggested that stomatal limitation of A occurred only in *Br. japonicus*. Rising CO₂ had the greatest effect on g_s and A in *Br. japonicus* at low D . In contrast, the strength of stomatal and photosynthetic responses to CO₂ were not affected by D in *Bo. ischaemum*. Carbon and water dynamics in this grassland are dominated by a seasonal transition from C₃ to C₄ photosynthesis. Interspecific variation in the response of g_s to D therefore has implications for predicting seasonal ecosystem responses to CO₂.

Key-words: elevated CO₂; grassland ecosystems; stomatal conductance; stomatal sensitivity; vapour pressure deficit.

Abbreviations: g_s , stomatal conductance for water vapour; D , leaf-to-air vapour pressure difference; g_{sref} , g_s at $D = 1$ kPa; A , net photosynthetic rate; C_i/C_a , the ratio of intercellular to external CO₂ concentration; $-dg_s/d\ln D$, stomatal

sensitivity to D , based on the slope of the response of g_s to the natural log of D .

INTRODUCTION

As primary physiological controls on the terrestrial flux of water to the atmosphere, stomata have long been the subject of studies evaluating plant responses to global climate change. The expectation that stomatal conductance (g_s) will decline with an increase in atmospheric CO₂ concentration (Field, Jackson & Mooney 1995; Drake, Gonzalez-Meler & Long 1997) has several implications for plant and ecosystem function. Barring an increase in leaf area, the most immediate consequence of decreasing g_s is a reduction in terrestrial evapotranspiration (Field *et al.* 1995; Jackson *et al.* 1998). By limiting transpiration, stomatal closure can also improve plant water use efficiency and therefore indirectly influence productivity in terrestrial ecosystems (Polley *et al.* 1993). Several environmental factors influence g_s in addition to CO₂ concentration (Cowan 1977). Therefore, predicting g_s in future environments requires an understanding of how CO₂ concentration and other environmental factors interact to influence stomatal behaviour (Medlyn *et al.* 2001; Wullschleger, Tschaplinski & Norby 2002).

Perhaps the most significant environmental variable controlling g_s is the difference in water vapour pressure between the inside and outside of leaves (D). Stomata typically close with increasing D , a response that is implicated in preventing excessive dehydration and hydraulic failure (Mott & Parkhurst 1991; Oren *et al.* 1999). A mechanistic understanding of the interactive effects of CO₂ and D on plant gas exchange is important because D varies temporally with climate and may increase during the next century with a greenhouse gas-induced global temperature increase of 1.5–4.5 °C (Kattenberg *et al.* 1996; Gregory, Mitchell & Brady 1997; MacCracken *et al.* 2001). Recent work indicates that growth at elevated CO₂ may reduce stomatal sensitivity to D (Bunce 1993, 1998; Heath 1998; Wullschleger *et al.* 2002). However, a mechanism by which this response occurs has not been identified. Based on a simple hydraulic model, Oren *et al.* (1999) predicted that in order to maintain constant leaf water potential and transpiration, stomatal sensitivity to D should be directly proportional to the magnitude of g_s . Therefore, a decrease in

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stomatal sensitivity to D that is correlated with CO_2 -induced reductions in the magnitude of g_s could be consistent with hydraulic regulation of stomatal function.

Changes in D may also influence the response of stomata to atmospheric CO_2 concentration (Morison & Gifford 1983). A reduction in g_s at high D may make stomata less responsive to increased CO_2 (Bunce 1998, 2000). Decreased stomatal sensitivity to CO_2 could affect the stimulation of photosynthesis by high CO_2 , potentially influencing productivity in arid and semi-arid regions in the future (Polley *et al.* 1993; Jackson *et al.* 1994; Owensby *et al.* 1999; Smith *et al.* 2000). In addition, predictions of reduced evapotranspiration and its influence on cloud cover and temperature at twice ambient CO_2 concentrations are based on the expectation of a 50% reduction in g_s (Henderson-Sellers, McGuffie & Cross 1995; Sellers *et al.* 1996). Thus, reduced stomatal sensitivity to CO_2 at high D may alter the expected response of climate to CO_2 on a global scale.

For some species, the response of stomatal conductance to CO_2 is particularly pronounced below the current ambient concentration (Maherali *et al.* 2002). Recent experiments indicate that the carbon and nitrogen dynamics of grasslands also respond more dramatically to increases in CO_2 from the Pleistocene (approximately $180 \mu\text{mol mol}^{-1}$; Petit *et al.* 1999) to the present concentration than from the present to future levels (Gill *et al.* 2002). Past CO_2 increases have been implicated in shifting the global distribution of C_3 and C_4 plants and increases in ecosystem productivity (Polley *et al.* 1993; Ehleringer, Cerling & Helliker 1997). Although carbon assimilation is affected by subambient CO_2 in both C_3 and C_4 plants (Anderson *et al.* 2001; Sage & Coleman 2001), comparatively little is known about stomatal responses to past atmospheric CO_2 concentrations and their interactions with environmental stresses such as increased D (Sage & Cowling 1999; Ward *et al.* 1999).

In this study, we examined the influence of atmospheric CO_2 concentration on stomatal responses to D using an experimental system that maintains a continuous gradient of CO_2 from 200 to $550 \mu\text{mol mol}^{-1}$ in the field (Johnson, Polley & Whitis 2000; Gill *et al.* 2002). A previous study in this C_3/C_4 grassland ecosystem reported 40–80% declines in g_s measured at optimal D ($< 1.5 \text{ kPa}$) along the gradient for several species (Anderson *et al.* 2001). Based on the hydraulic model of Oren *et al.* (1999), we predicted that g_s in plants grown at elevated CO_2 would be less sensitive to variation in D than those grown at subambient CO_2 . Because stomatal closure limits the uptake of CO_2 , we also examined the sensitivity of net photosynthesis (A) to variation in D . Our grassland experimental system is dominated by C_3 photosynthesis early in the growing season and transitions to C_4 photosynthesis by the summer. To account for this functional change, we made measurements of the response of g_s to D on representative C_3 and C_4 grasses of this system. Changes in stomatal sensitivity to D have implications for the strength of plant responses to CO_2 (Morison & Gifford 1983; Seneweera, Ghannoum & Conroy 1998; Gunderson *et al.* 2002). Therefore we also examined how

variation in D affected the response of g_s and net photosynthesis to the CO_2 gradient.

METHODS

Study site and experimental system

Our experiment was conducted in a grassland near the USDA-ARS Grassland, Soil and Water Research Laboratory in Temple, TX ($31^\circ 05' \text{ N}$, $97^\circ 20' \text{ W}$). 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 Udorthentic Haplustoll with 35–55% clay in the top 40 cm (Johnson *et al.* 2000). To determine the potential for interspecific variation in responses to CO_2 , we focused on two abundant grass species with different photosynthetic pathways: *Bromus japonicus* Thunb. ex Murr., a C_3 annual grass and *Bothriochloa ischaemum* (L.) Keng, a C_4 perennial grass. Other species at the site include the forbs *Solanum dimidiatum* Raf., *Solidago canadensis* L. and *Ratibida columnaris* (Nutt.) Woot. & Standl. Mean annual precipitation (1913–99) is 877 mm and the mean minimum and maximum annual temperatures are 13.2 and 25.9 °C, respectively. C_3 species are mostly active early in the growing season and C_4 species dominate by mid-summer.

Our experimental system consisted of two elongated chambers over parallel and adjacent plots of grassland. Each chamber was 60 m long, 1 m wide, and 1 m tall. Air introduced into one end of each chamber was progressively depleted of CO_2 by photosynthesis as it moved down the chamber by a blower. Desired CO_2 concentrations were maintained by automatically varying the rate of air flow. During daytime, the CO_2 gradient in one chamber (subambient chamber) ranged from 360 to $200 \mu\text{mol mol}^{-1}$, whereas the gradient in the second chamber (elevated chamber) ranged from 550 to $360 \mu\text{mol mol}^{-1}$. At night, CO_2 treatments were maintained at $150 \mu\text{mol mol}^{-1}$ above daytime levels by reversing air flow and using respiratory CO_2 releases to create the gradient. Treatments began in May 1997 and operated each growing season (mid-February to mid-November) up to 2000.

The chambers were divided into 10 5-m-long sections, with a rubber-coated barrier extending to about 1 m deep into the soil along the sides of each chamber. Each section was enclosed in polyethylene film (which transmitted 85–95% of incident PFD), with chilled-water cooling coils between sections. Air temperature and humidity were controlled at ambient levels by cooling and dehumidifying air before it entered each 5 m section of chamber. Each 5 m section was irrigated to match ambient rainfall through July 1999. Thereafter, water was applied to all sections in the amount required to equalize soil water content in sections at ambient CO_2 with that of adjacent open grassland as measured by neutron attenuation (Polley, Johnson & Derner 2002). During the drought years of 1999 and 2000, the total water applied to the chambers was 349 and 381 mm, respectively. Additional details of design, con-

struction and operation of these chambers can be found in Johnson *et al.* (2000).

Steady-state gas exchange and responses to leaf-to-air vapour pressure difference

The consequences of CO_2 for stomatal responses to leaf-to-air vapour pressure difference (D) were examined during the fourth growing season of the experiment. 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. Gas exchange was measured when each species was near its peak biomass, which was April 2000 for *Br. japonicus* and August 2000 for *Bo. ischaemum* (Anderson *et al.* 2001; Maherali *et al.* 2002). The youngest fully expanded leaf was sampled on three to four randomly selected plants per 5-m-long section at a minimum of six treatment CO_2 concentrations (six chamber sections). Stomatal conductance (g_s) was measured at saturating light levels of 1600 and 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for *Br. japonicus* and *Bo. ischaemum*, respectively. A Peltier cooling module maintained leaf temperatures at approximately ambient conditions (20–23 °C and 29–32 °C for *Br. japonicus* and *Bo. ischaemum*, respectively). We maintained leaf-to-air vapour pressure deficit (D) at levels that permitted the measurement of maximum g_s , which were 0.9–1.1 kPa for *Br. japonicus* and 1.4–1.6 kPa for *Bo. ischaemum*. To calculate g_s and intercellular CO_2 concentration (C_i) we used a boundary layer conductance of 4.86 $\text{mol m}^{-2} \text{s}^{-1}$ for both species. 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 calculated from leaf dimensions.

To examine stomatal responses to D , we measured the response of g_s to a manipulation of D within the leaf cuvette. Once clamped in the cuvette, leaves were exposed to the light, D , and temperature levels described above and the respective growth CO_2 concentration. After steady-state conditions were achieved, the first measurement was taken and D was then increased by diverting a greater percentage of air through the desiccant (drierite). Stomatal conductance was recorded after steady-state conditions were re-established at each D level. Steady-state was determined when the coefficient of variation of change in water vapour was <0.05% (generally after 20–30 min). The response of net photosynthesis (A) was recorded simultaneously with g_s .

Statistical analyses

To examine whether CO_2 treatments influenced the response of g_s to D , 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). D was the within subjects factor whereas growth CO_2 was the between subjects fac-

tor. Differences in the slope of the response of g_s , A or the C_i/C_a ratio to D among growth CO_2 concentrations were determined by testing for an interaction between D and CO_2 . Univariate (within subjects) tests of significance (at $P = 0.05$) are reported for each comparison. For descriptive purposes, all g_s , A and C_i/C_a versus D response curves were fitted with linear or quadratic least squares regression models. Quadratic models were only employed when they explained more (as determined by r^2 and P -values) of the variation in the data than linear models. Although means (± 1 SE) are presented in figures, all analyses were performed using individual plants.

To determine whether stomatal sensitivity to D varied with g_s (e.g. Morison & Gifford 1983; Oren *et al.* 1999), we fitted our data to the empirical function described in Oren *et al.* (1999):

$$g_s = -m \times \ln D + b \quad (1)$$

where m and b are parameters generated by least-squares regression. The parameter b is defined as $g_{s\text{ref}}$, or the reference g_s at $D = 1$ kPa and m is the stomatal sensitivity to D , or $-dg_s/d\ln D$. This equation was selected because it fitted our data well ($r^2 > 0.95$), and has been used to analyse the responses of g_s to D for a variety of species (Oren *et al.* 1999). The statistical significance of the relationship between $dg_s/d\ln D$ and $g_{s\text{ref}}$ was determined using least-squares linear regression.

Evaluation of empirical data with a hydraulic model

Although the signalling mechanism by which stomata respond to leaf-to-air vapour pressure difference is not known, empirical observations suggest that g_s responds to transpiration (E) (Mott & Parkhurst 1991), and may therefore be regulated by a feedback response to the water status of leaves as defined by leaf water potential. We evaluated whether observed differences in $-dg_s/d\ln D$ across growth CO_2 concentrations in each species could be explained by hydraulic regulation of stomatal function. If the response of g_s to D is coupled to the maintenance of constant leaf water potential and transpiration under steady-state conditions, stomatal behaviour can be predicted using an Ohm's law analogy (Oren *et al.* 1999):

$$g_1 = (K_L) \times (1/D) \times (\Psi_s - \Psi_L) \quad (2)$$

where g_1 is the leaf conductance to water vapour (the boundary layer conductance, g_{bl} , and g_s in series), K_L is the leaf specific hydraulic conductance of the soil-leaf pathway, and Ψ_s and Ψ_L are the soil and leaf water potential, respectively. Equation 2 was used to generate theoretical responses of g_s to D for each CO_2 concentration. K_L was manipulated to generate species-specific maximum g_1 values for each treatment CO_2 concentration. We used the appropriate range of D (e.g. 1–3.6 kPa for *Br. japonicus*; 1.5–4.0 kPa for *Bo. ischaemum*, Figs 1a & 2a) for each species. The equation was parameterized with g_{bl} used in gas exchange calculations and $(\Psi_s - \Psi_L)$ was assumed to be

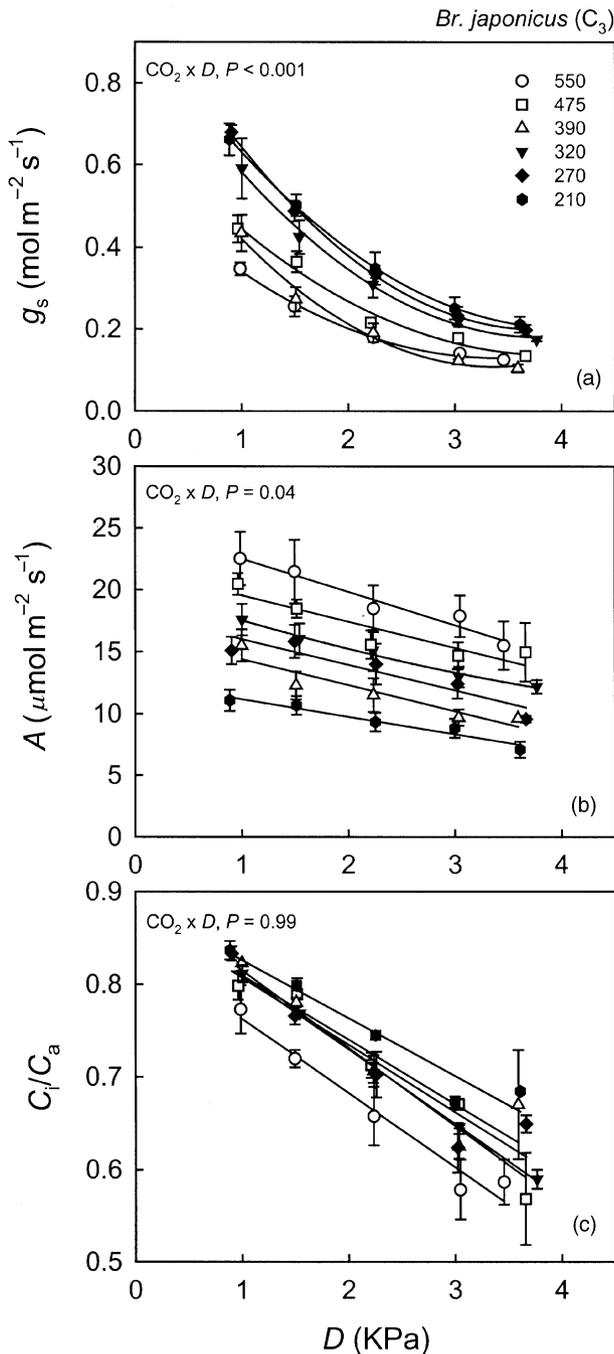


Figure 1. The response of mean (± 1 SE) g_s (a), A (b), and C_i/C_a (c) to D for *Br. japonicus* plants grown across a range of atmospheric CO_2 . Symbols correspond to different growth CO_2 concentrations ($\mu\text{mol mol}^{-1}$). Each data point represents the mean of three to five individuals.

equal to 1. Further details of these calculations are described in Oren *et al.* (1999). The data generated from Eqn 2 were fitted with Eqn 1 to calculate $-dg_s/d\ln D$ and $g_{s\text{ref}}$ (g_s at $D = 1$ kPa) as described previously. We evaluated the predictions of the hydraulic model by comparing the magnitude of the theoretical slope between $-dg_s/d\ln D$ and $g_{s\text{ref}}$ (Oren *et al.* 1999) with the slope generated from empirical

data. We tested whether the empirical slope differed statistically from the modelled slope using a two-tailed Student's *t*-test (Sokal & Rohlf 1995).

RESULTS

Stomatal conductance (g_s) declined non-linearly with increasing leaf-to-air vapour pressure difference (D) in the

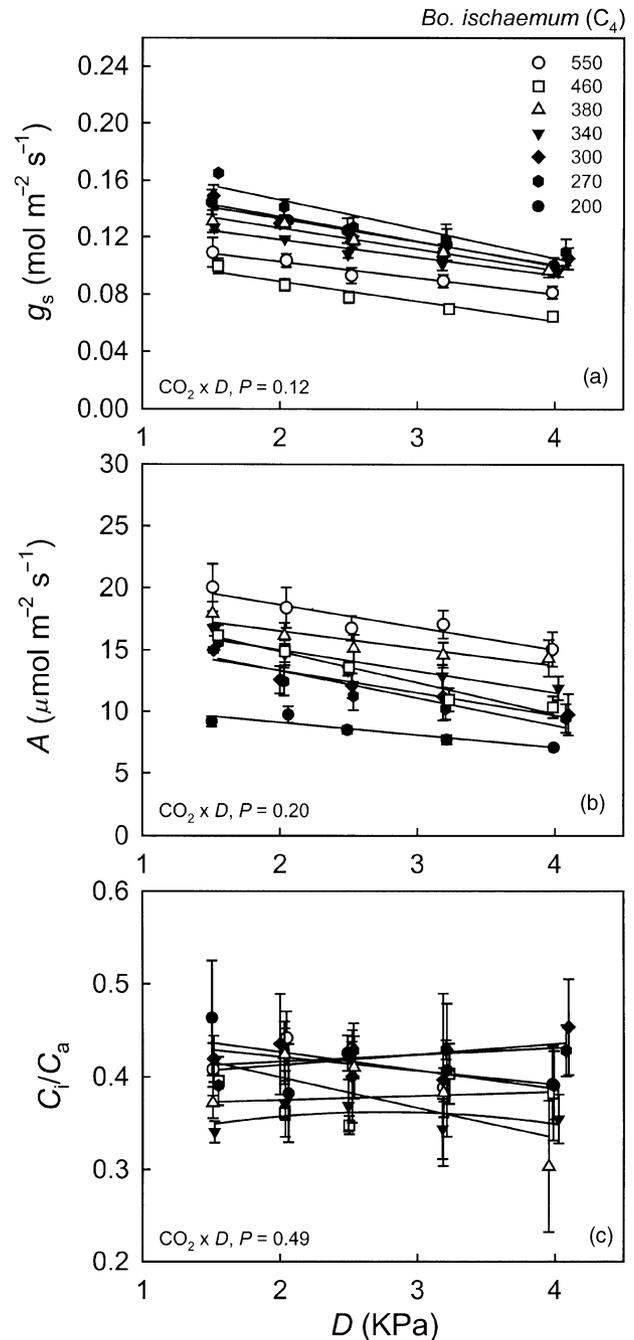


Figure 2. The response of mean (± 1 SE) g_s (a), A (b), and C_i/C_a (c) to D for *Bo. ischaemum* plants grown across a range of atmospheric CO_2 . Symbols correspond to different growth CO_2 concentrations ($\mu\text{mol mol}^{-1}$). Each data point represents the mean of three to five individuals.

C_3 *Br. japonicus* at all CO_2 concentrations (Fig. 1a; $P < 0.001$). A significant ($P < 0.001$) interaction between CO_2 and D in the ANOVA indicated that the response of g_s to D differed across CO_2 treatments. The strongest stomatal responses to D occurred in plants grown at $< 350 \mu\text{mol mol}^{-1}$ relative to those grown at $> 350 \mu\text{mol mol}^{-1}$. Net photosynthetic rate (A) also decreased with increased D ($P < 0.001$). The response of A to D differed across CO_2 concentrations (ANOVA, $CO_2 \times D$ interaction, $P = 0.04$, Fig. 1b), with photosynthesis responding most strongly to increased D in plants grown at $> 350 \mu\text{mol mol}^{-1} CO_2$. The ratio of intercellular to external CO_2 concentration (C_i/C_a) decreased with D at all CO_2 concentrations (Fig. 1c; $P < 0.001$). The response of C_i/C_a to D appeared to be the same regardless of growth CO_2 concentration, as determined by the lack of a significant $CO_2 \times D$ interaction (ANOVA, $P = 0.99$).

Stomatal conductance decreased linearly with rising leaf-to-air vapour pressure difference ($P < 0.001$) in the C_4 *Bo. ischaemum* at all CO_2 concentrations (Fig. 2a). There was no $CO_2 \times D$ interaction (ANOVA, $P = 0.12$), indicating that the responses of g_s to D were similar across growth CO_2 . Net photosynthesis also decreased with rising D ($P < 0.001$), but the response of A of D was statistically similar at all growth CO_2 concentrations (ANOVA, $CO_2 \times D$ interaction, $P = 0.20$, Fig. 2b). The C_i/C_a ratio was not affected by D ($P = 0.45$, Fig. 2c). This lack of response to D appeared to occur at all growth CO_2 concentrations (ANOVA, $CO_2 \times D$ interaction, $P = 0.49$).

To determine if stomatal sensitivity to leaf-to-air vapour pressure difference was influenced by the magnitude of g_s , we employed a model described by Oren *et al.* (1999) in which sensitivity is defined as the slope of response of g_s to D . Stomatal sensitivity to D ($dg_s/d\ln D$, Eqn 1) was strongly associated with g_{sref} in *Br. japonicus* ($R^2 = 0.91$, $m = 0.56$, $P < 0.001$, Fig. 3a). Much of the variation in g_{sref} , in turn was driven by growth at contrasting CO_2 concentrations, in which plants grown at higher CO_2 had lower g_{sref} . Although the relationship between $dg_s/d\ln D$ and g_{sref} in *Bo. ischaemum* featured more variation, it was still statistically significant ($R^2 = 0.48$, $m = 0.37$, $P < 0.001$, Fig. 3b).

To determine whether stomatal responses to D were consistent with the regulation of constant leaf water potential and transpiration, we compared the magnitude of the slope of the relationship between stomatal sensitivity to D and g_{sref} from our gas exchange data with that generated from a hydraulic model (Oren *et al.* 1999). In *Br. japonicus*, there was no statistical difference ($t = 1.33$, d.f. = 16, $P > 0.05$) between the calculated theoretical $-dg_s/d\ln D$ versus g_{sref} slope of 0.62, and the empirical slope of 0.56 (a difference of 11%) (Fig. 3a). In contrast, the theoretical $-dg_s/d\ln D$ versus g_{sref} slope of 0.53 for *Bo. ischaemum* was significantly greater by 43% ($t = 2.10$, d.f. = 26, $P < 0.05$) than the empirical slope of 0.37 (Fig. 3b).

Based on a comparison of relative responses averaged across CO_2 treatments, the C_3 annual grass *Br. japonicus* was more responsive to leaf-to-air vapour pressure difference than the C_4 perennial *Bo. ischaemum*. Relative g_s

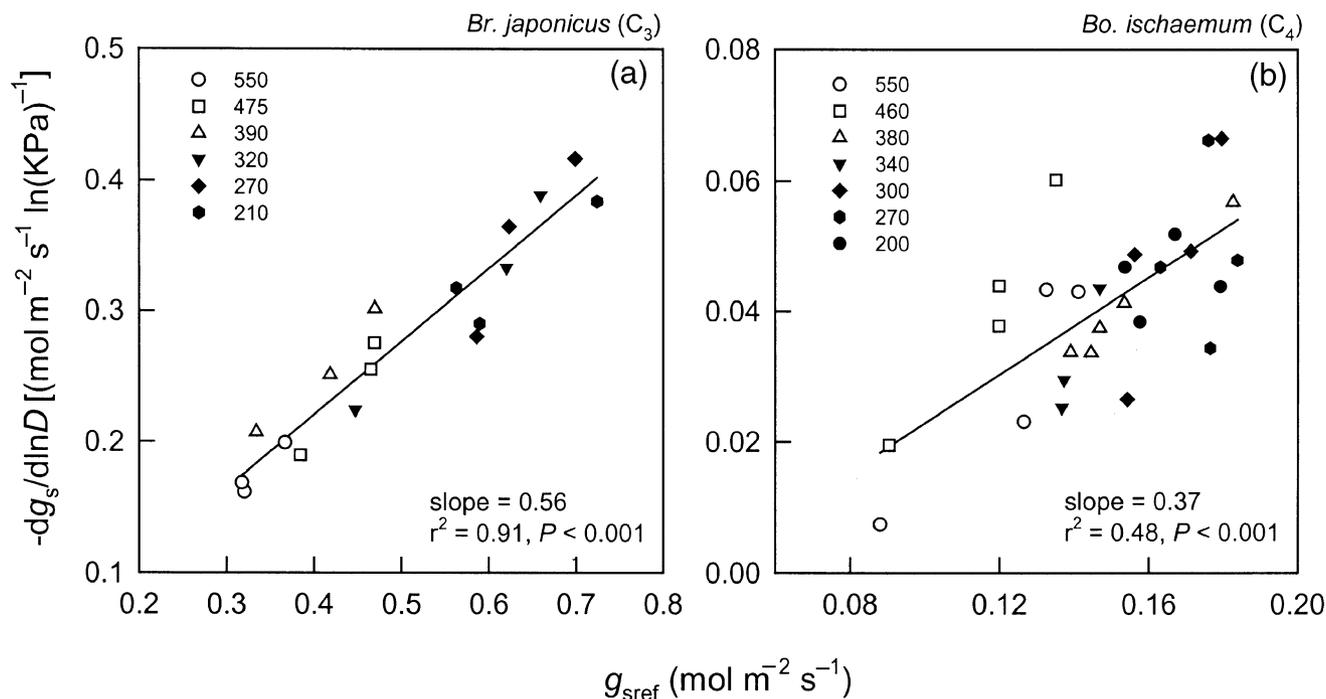


Figure 3. The relationship between stomatal sensitivity to D (the slope of the response of g_s to D ; $-dg_s/d\ln D$) and the magnitude of g_s at 1 kPa (g_{sref}) for *Br. japonicus* (a) and *Bo. ischaemum* (b). Note the differences in scales between plots. Each data point represents a single individual.

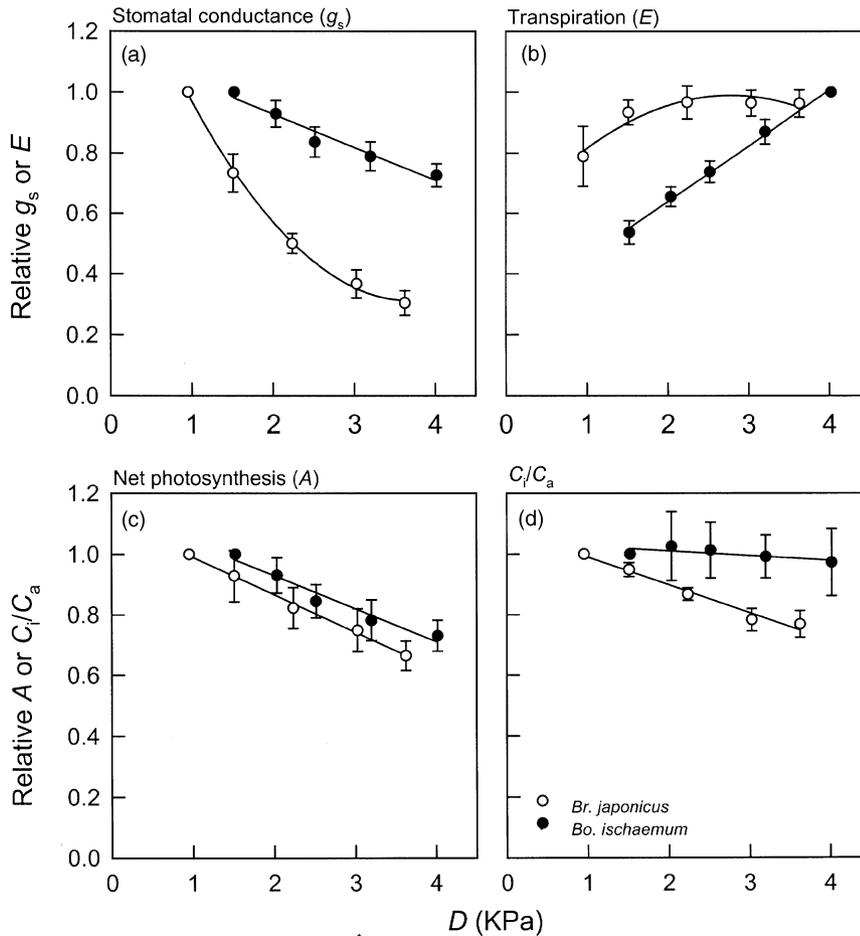


Figure 4. A comparison of the mean (± 1 SD) relative response of g_s (a), E (b), A (c) and C_i/C_a (d) to D in *Br. japonicus* and *Bo. ischaemum* pooled across CO_2 treatments.

declined non-linearly by approximately 65% with increasing D in the C_3 species, whereas relative g_s declined linearly by 25% in the C_4 species (Fig. 4a). The strong decrease in g_s for *Br. japonicus* caused relative leaf transpiration (E) to be approximately constant at a $D > 1.5$ kPa (Fig. 4b). In contrast, E increased linearly with increasing D in *Bo. ischaemum*. Despite these differences in stomatal sensitivity to D , the accompanying decline in photosynthesis was quite similar between the species (Fig. 4c). Relative A declined linearly by approximately 34% in *Br. japonicus* and by approximately 27% in *Bo. ischaemum* (Fig. 4c). In *Br. japonicus*, the C_i/C_a ratio declined by approximately 25% with increasing D whereas C_i/C_a was unaffected by D in *Bo. ischaemum* (Fig. 4d).

In all cases, stomatal conductance and photosynthesis responded linearly to increases in CO_2 from subambient to elevated levels (Fig. 5). In *Br. japonicus*, g_s declined significantly with increasing CO_2 at all levels of D ($P < 0.0001$). The response of g_s to CO_2 was strongest (i.e. the steepest slope) at the lowest D (1 kPa, Fig. 5a), as indicated by a significant $CO_2 \times D$ interaction ($P < 0.001$). In consequence, the slope of the g_s response to CO_2 was 3.6 times higher at 1 kPa than at 3.6 kPa. Stomatal conductance also declined with rising CO_2 ($P < 0.001$) in *Bo. ischaemum*, but

the trajectory of the response was similar across levels of D (Fig. 5c).

Photosynthesis was stimulated by rising CO_2 at all levels of D ($P < 0.0001$) in *Br. japonicus*. The strongest response occurred at low D (ANOVAR, $CO_2 \times D$ interaction, $P = 0.04$, Fig. 5b), causing the slope of the response of A to CO_2 to be 1.3 times higher at 1 kPa than at 3.6 kPa. Although A increased with increasing CO_2 ($P < 0.001$) in *Bo. ischaemum*, the strength of the response was not altered by D (Fig. 5d).

DISCUSSION

Based on previous experiments with a variety of species (Morison & Gifford 1983; Bunce 1993, 1998; Heath 1998; Wullschlegel *et al.* 2002), we predicted that rising CO_2 concentration would make stomata less sensitive to increasing leaf-to-air vapour pressure difference (D). We found support for this prediction in the C_3 annual *Br. japonicus* (Fig. 1a). The slope of the relationship between stomatal sensitivity to D and g_{sref} (Fig. 3a) was also indistinguishable from the predicted slope generated by the Oren *et al.* (1999) hydraulic model. Stomatal closure in *Br. japonicus* also appeared to allow the maintenance of relatively con-

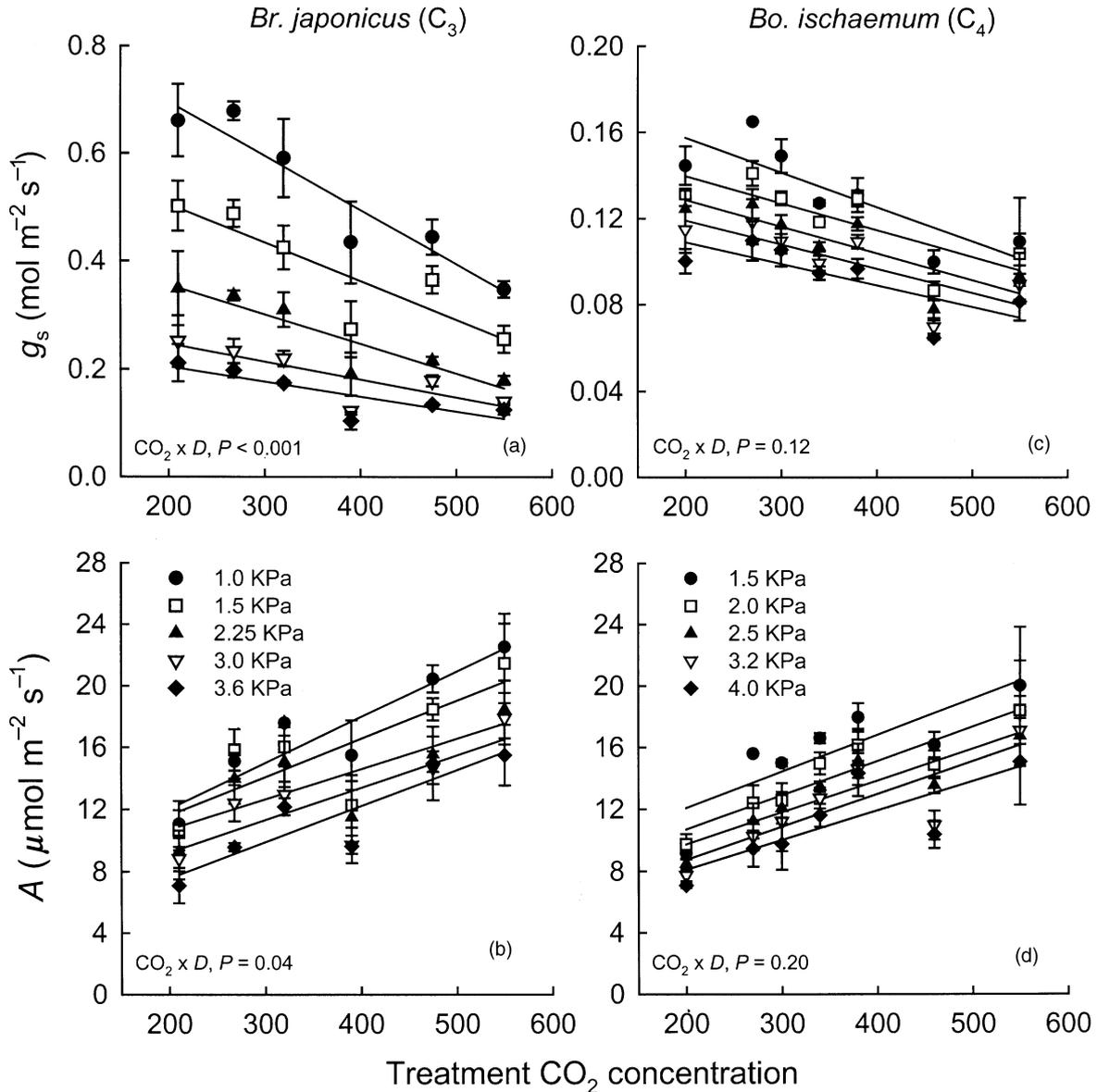


Figure 5. The response of mean (± 1 SE) g_s (a, c), A (b, d) to growth CO_2 concentration ($\mu mol mol^{-1}$) at contrasting D in *Br. japonicus* and *Bo. ischaemum*. Each data point represents the mean of three to five individuals. Responses to growth CO_2 concentration were fitted with linear least squares regression models.

stant leaf transpiration (E) with increasing D (Fig. 4b). Thus, decreased stomatal sensitivity to D at high CO_2 was consistent with stomatal regulation of constant water potential and transpiration in this C_3 annual grass.

We found no evidence that stomatal sensitivity to D was affected by growth CO_2 concentration in the C_4 perennial *Bo. ischaemum* (Fig. 2a). The slope of the $-dg_s/d \ln D$ versus g_{sref} relationship for this species (Fig. 3b) was also significantly smaller than predicted from the hydraulic model. Stomatal responses to D in *Bo. ischaemum* may be governed by factors other than strict hydraulic maintenance of constant water potential, perhaps by non-hydraulic signaling via changes in abscisic acid (ABA) delivery to stomatal guard cells (Bunce 1998; Heath 1998). It is also possible that

stomatal control of water loss in this species is poor; for example, leaf transpiration increased linearly with increasing D despite progressive stomatal closure (Fig. 4a & b). *Bothriochloa ischaemum* is dominant during the most drought-prone part of the growing season in this grassland (Polley *et al.* 2002). By maintaining open stomata under prolonged water limitation and therefore permitting continued CO_2 uptake, low stomatal sensitivity to D may represent an adaptation to drought (Ogle & Reynolds 2002). The lower than predicted empirical slope of 0.37 for *Bo. ischaemum* is also similar to the 0.40 slope reported for other drought-tolerant species, such as the desert shrubs *Ephedra nevadensis* and *Larrea tridentata* (Oren *et al.* 1999; Pataki *et al.* 2000; Ogle & Reynolds 2002).

The relative insensitivity of stomata to D in *Bo. ischaemum* could be a general feature of plants with low maximum g_s (e.g. Wullschleger *et al.* 2002), perhaps because of a comparatively greater effect of cuticular conductance on gas exchange. Although it is a small proportion of leaf water vapour flux, cuticular conductance is an unmeasured component of g_s (Kerstiens 1996; Meyer & Genty 1998). As stomata close in response to rising D , cuticular conductance may remain constant and constitute an increasingly greater proportion of measured g_s . For species with low stomatal conductance, the insensitivity of cuticular conductance to D could dampen the overall response of g_s to D . In support of this hypothesis, Kerstiens (1997) has observed that an artificial increase in cuticular conductance was correlated with decreased stomatal sensitivity to D in three hypostomatous species. Nonetheless, it is not known whether cuticular permeability has any influence on the reduction in stomatal sensitivity to D at high CO_2 .

In several grassland ecosystems, increased CO_2 reduces canopy transpiration and increases soil moisture content (Fredeen *et al.* 1997; Owensby *et al.* 1999; Grunzweig & Korner 2001; Morgan *et al.* 2001; Polley *et al.* 2002). These patterns raise the possibility that CO_2 could have indirectly affected stomatal functioning in our study species via a feedback on soil moisture availability. Although there were some CO_2 effects on soil water content in this experiment (see Polley *et al.* 2002), they were generally small and only appeared during the late summer drought. Therefore, stomatal responses to CO_2 and D were probably not confounded by CO_2 -induced variation in soil water availability in the early season *Br. japonicus*. In contrast, increased soil moisture at high CO_2 during the late season drought could have affected stomatal sensitivity to D in *Bo. ischaemum*. The absence of differences in stomatal sensitivity across the gradient, however, suggests that neither CO_2 nor soil moisture affected stomatal sensitivity in the C_4 species. It is also possible, though, that increased soil moisture and CO_2 had opposite effects on stomatal sensitivity to D . These contrasting effects could have cancelled each other out and prevented the detection of a significant CO_2 effect for *Bo. ischaemum* in our experiment.

Photosynthetic rate declined in response to rising D in both species (Figs 1b & 2b), a response that has been observed in other studies (Forseth & Ehleringer 1983; Bunce 1993; Seneweera *et al.* 1998). The observation that C_i/C_a declined with increasing D in the *Br. japonicus* (Figs 1c & 4d), suggests that stomatal limitation was the primary cause of decreased photosynthesis at high D in this C_3 grass (Sage 1994). We also observed that the response of A to D was strongest at high C_a in *Br. japonicus* (Fig. 1b), a result that can be predicted from the relatively uniform decline in C_i/C_a with increasing D across growth CO_2 concentrations (Fig. 1c). For example, we note that C_i declined from 402 to 321 $\mu\text{mol mol}^{-1} CO_2$ at the highest treatment C_a , but only declined from 167 to 143 $\mu\text{mol mol}^{-1} CO_2$ at the lowest treatment C_a (Fig. 1c). Assuming that the $A-C_i$ response of *Br. japonicus* does not change along the CO_2 gradient (as shown in Anderson *et al.* 2001), a greater

change in absolute C_i with increasing D at high versus low C_a will also cause A to be more strongly affected by D at high versus low C_a .

Decreased photosynthesis at high D in *Bo. ischaemum* did not appear to be caused by stomatal limitation because constant C_i/C_a was maintained with increasing D (Figs 2c & 4d). It is possible that photosynthesis declined because increased transpirational water losses at high D (Fig. 4b) reduced leaf relative water content (RWC), which in turn inhibited metabolic processes. Such a response has been observed in both C_3 and C_4 species and may be the result of the inhibition of ATP synthesis by an increase in ion concentration (primarily Mg^{2+}) with decreasing RWC in the chloroplast (Lawlor 2002). The lack of response of C_i to decreasing photosynthesis may also be an artefact of non-uniform (patchy) stomatal closure (Mott 1995). The overestimation of C_i under these circumstances is particularly acute when stomatal conductance is low, as was the case for *Bo. ischaemum* (Buckley, Farquhar & Mott 1997; Meyer & Genty 1998). Nevertheless, we lack the necessary data in the present study to determine whether metabolic inhibition of photosynthesis or non-uniform stomatal closure was responsible for the decline in A and constant C_i/C_a with increasing D in the C_4 species.

Several studies suggest that exposure to high leaf-to-air vapour pressure difference may temper the relative response of stomatal conductance to atmospheric CO_2 concentration (Bunce 1998, 2000; Heath 1998; Wullschleger *et al.* 2002). We found support for this hypothesis in *Br. japonicus*. For example, the slope of the response of g_s to increasing growth CO_2 concentration was 3.6 times higher at the lowest versus highest D (Fig. 5a) in this C_3 species. In contrast, exposure to increasing D did not alter the slope of the response of g_s to increasing CO_2 in *Bo. ischaemum* (Fig. 5d). Interspecific variation in the response of stomatal conductance to CO_2 with changing D has implications for predicting seasonal ecosystem responses to CO_2 in this grassland because of the transition from early season C_3 (March–June) to late season C_4 (July–November) photosynthesis (Anderson *et al.* 2001; Mielnick *et al.* 2001). For example, if D increases in the future (MacCracken *et al.* 2001), CO_2 enrichment will reduce absolute g_s less in the C_3 species than currently expected. After the seasonal transition to C_4 photosynthesis, however, the expected decrease in stomatal conductance at elevated CO_2 will probably be sustained at high D . Interspecific variation in the response of g_s to D may also be particularly important for the regulation of canopy gas exchange with rising CO_2 in this grassland since stomatal acclimation to CO_2 does not occur in either species (Maherali *et al.* 2002).

One of our goals in utilizing an experimental gradient approach was to determine whether there is a non-linear or threshold response of g_s to changes in CO_2 concentration. Quantifying the trajectory of the response of plant processes to CO_2 has implications not only for understanding the nature of the responsible mechanisms, but also for

predicting plant responses to the gradual rise in atmospheric CO_2 concentration (Anderson *et al.* 2001; Gill *et al.* 2002). For instance, Sage & Cowling (1999) suggest that plant stresses may magnify the negative effects of growth at low CO_2 . A prediction from this hypothesis is that stresses such as high D could produce a non-linear stomatal response to rising CO_2 . Previously, we found that g_s decreased linearly in response to rising CO_2 in *Br. japonicus* and *Bo. ischaemum* under optimal conditions (Anderson *et al.* 2001; Maherali *et al.* 2002). The results from the present study suggest that high D does not alter the linear nature of the stomatal response to CO_2 .

We demonstrated that stomata were less responsive to D when grown at increasing CO_2 concentration in the C_3 grass *Br. japonicus*. We also found support for hydraulic regulation as a mechanism responsible for decreasing stomatal sensitivity to D with increasing CO_2 concentration. Our findings for *Br. japonicus* corroborate stomatal responses to CO_2 and D observed in other species (Heath 1998; Bunce 2000; Wullschlegel 2002). In contrast, stomatal sensitivity to D in the C_4 grass *Bo. ischaemum* was unaffected by CO_2 . The relative insensitivity of stomata to D in this species may be associated with drought tolerance, or be a general feature of species with low maximum g_s (e.g. Wullschlegel *et al.* 2002). Our results highlight the importance of the leaf-to-air vapour pressure difference as a factor that alters the effect of CO_2 on gas exchange. For the species with the most sensitive stomata, *Br. japonicus*, the effect of CO_2 on stomatal conductance and photosynthesis was strongest at low D . More generally, the influence of D on the strength of the gas exchange response to CO_2 could help account for variation in the effect size of CO_2 treatments among species (Curtis & Wang 1998) as well as seasonal variation in the field (Bunce 2000; Gunderson *et al.* 2002). Therefore, understanding how the interaction between CO_2 and D affects gas exchange among species is necessary to scale individual plant responses to CO_2 to the level of the ecosystem.

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