1 **Mesophyll conductance in two cultivars of wheat (*Triticum aestivum*) grown in glacial to**
2 **super-elevated [CO₂]**

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15 Date of submission: 30th April 2020

16 Number of tables: 1

17 Number of figures: 11

18 Abstract word count: 176

19 Total word count (excluding references): 4923

20

21 Highlight: Mesophyll conductance increased with increasing [CO₂] from glacial to ambient
22 CO₂ levels, then declined at super-elevated CO₂ for both well-watered and water-limited
23 treatments. These responses of mesophyll conductance with varying [CO₂] have a
24 physiological basis.

25 Running title: Mesophyll conductance with [CO₂]

26

27

Abstract

Mesophyll conductance (g_m) is an important factor limiting photosynthesis. However, g_m response to long-term growth in variable $[CO_2]$ is not well understood, particularly in crop plants. Here, we grew two cultivars of wheat (Halberd and Cranbrook), known to differ in g_m under current environmental conditions, in four $[CO_2]$ treatments: glacial ($180 \mu\text{mol mol}^{-1}$), pre-industrial ($280 \mu\text{mol mol}^{-1}$), current ambient ($450 \mu\text{mol mol}^{-1}$) and super-elevated ($1000 \mu\text{mol mol}^{-1}$) in well-watered and moderate water limitation conditions, to develop an evolutionary and future climate perspective on g_m control of photosynthesis and water use efficiency (WUE). In the two wheat genotypes, g_m increased with rising $[CO_2]$ from glacial to ambient $[CO_2]$, but declined at super-elevated $[CO_2]$; however, the specific mechanism of g_m response to $[CO_2]$ remains unclear. Although g_m and g_m/g_{sc} (mesophyll conductance/stomatal conductance) were strongly associated with the variability of A and WUE, we found that plants with higher g_m may increase A without increasing g_{sc} , which increased WUE. These results may be useful to inform plant breeding programs and cultivar selection for Australian wheat under future environmental conditions.

Keywords: elevated CO_2 , genotype, leaf internal conductance, photosynthesis, *Triticum aestivum*, water stress

48 **Introduction**

49 Climate change is a major environmental challenge with significant threats to water
50 resources, crop development and food security. CO₂ concentration has increased by 47%
51 (278 ppm to 408 ppm) from 1750 to 2018 (IPCC, 2019) and is predicted to reach 550 μmol
52 mol^{-1} in the middle of this century and 700 $\mu\text{mol mol}^{-1}$ by the end of this century (Prentice *et*
53 *al.*, 2001). This rising atmospheric CO₂ concentration is altering global temperature and
54 precipitation patterns, which challenges agricultural productivity (Ainsworth *et al.*, 2008).
55 From the perspective of food security, a major uncertainty is the extent to which crops will
56 serve as a sink for extra atmospheric CO₂, and if this will affect food nutritive quality
57 (Prentice *et al.*, 2001). Therefore, a major challenge for plant physiologists, agronomists and
58 breeders is to select appropriate physiological traits that can maximize future crop production
59 and quality in a challenging climate.

60
61 Increasing CO₂ concentration generates partial closure of stomata, thereby decreasing leaf
62 conductance to CO₂ and H₂O vapour (Morison, 1985, 1987; Allen, 1990) and reducing leaf
63 transpiration (Kimball and Idso, 1983). This reduced stomatal conductance can increase leaf
64 temperature and potentially negate decreases in evapotranspiration (ET) (Kimball *et al.*,
65 1992, 1994, 1995). Elevated CO₂ can reduce plant water consumption, leading to reduced soil
66 water depletion (Hungate *et al.*, 1997; Leuzinger and Korner, 2007; Robredo *et al.*, 2007) but
67 it also often increases the leaf area index counteracting leaf-level reductions in transpiration
68 rate (Franks *et al.*, 2013). In addition, plant-scale water use may not decline in elevated CO₂.
69 Moreover, in the future, prolonged summer droughts and higher frequency of extreme
70 precipitation events will become more common (IPCC, 2007). Plant water status affects the
71 magnitude of plant carbon uptake in elevated CO₂ (Volk *et al.*, 2000; Knapp *et al.*, 1999;
72 Morgan *et al.*, 2001). So in the future, elevated CO₂ combined with water scarcity may
73 drastically affect crop production. Furthermore, the annual global wheat demand will increase
74 to about 900 Mt by 2050 with limited expansion of sown area due to increased population
75 and urbanisation (Alexandratos and Bruinsma, 2012).

76
77 During photosynthesis, CO₂ diffuses from the atmosphere (C_a) to the sites of carboxylation
78 (C_c) inside the chloroplasts (Farquhar *et al.*, 1980). This CO₂ uptake is strongly influenced by
79 environmental factors that are predicted to change in the future, namely, atmospheric CO₂
80 concentration, temperature and water availability (Sage and Kubien, 2007; Lawlor and
81 Tezara, 2009; Alberth *et al.*, 2011). Mesophyll conductance is the conductance of CO₂ from

the leaf intercellular air space (C_i) to the sites of carboxylation (C_c), including gas and liquid phase transfer. Mesophyll conductance (g_m) can strongly influence the rate of photosynthesis (Warren *et al.*, 2003; Flexas *et al.*, 2008; Barbour *et al.*, 2010; Giuliani *et al.*, 2013; Jahan *et al.*, 2014; Xiong *et al.*, 2016; Ellsworth *et al.*, 2018; Tosens and Laanisto, 2018; Han *et al.*, 2018; Knauer *et al.*, 2019) with up to 25-30% reduction in photosynthetic rate (Epron *et al.*, 1995), and this limitation may be as large as that imposed by stomatal conductance (g_{sc}) (Warren and Adams, 2006; Flexas *et al.*, 2007a). Mesophyll conductance is a dynamic leaf trait that depends on genotype, environmental factors and anatomical differences; g_m may be affected by drought stress, CO_2 concentration and salinity during the growing period (Flexas *et al.*, 2008).

Leakey *et al.* (2009) reviewed the effect of long-term elevated CO_2 treatment on growth, and concluded that photosynthetic carbon gain is enhanced in C_3 plants by elevated CO_2 despite partial acclimation of photosynthetic capacity. Singsaas *et al.* (2003) reported that an increase in CO_2 concentration (200 $\mu mol\ CO_2\ mol^{-1}$ above atmospheric level) decreased g_m for some species [cucumber (*Cucumis sativus* L.), spinach (*Spinacia oleracea* L.) and linden bean (*Phaseolus vulgaris* L. var. Linden)] but not for all. Conversely, Bernacchi *et al.* (2005) showed that mesophyll conductance of soybean (*Glycine max*) was not altered by elevated-growth CO_2 levels. However, studies into the effects of elevated growth CO_2 on g_m are scarce (Singsaas *et al.*, 2003). To the best of our knowledge, there is no published report on the variation in mesophyll conductance of wheat with different growth CO_2 concentrations.

In C_3 leaves, water stress predominantly affects CO_2 diffusion in leaves through a decrease of stomatal and mesophyll conductance without decreasing the biochemical capacity to assimilate CO_2 (Flexas *et al.*, 2004). Tosens *et al.* (2012) worked on saplings of *Populus tremula* L. with drought and found that water stress reduced chloroplast surface area to leaf area ratio (S_c/S) and increased cell wall thickness and these are partly responsible for lower g_m . In contrast, g_m was unaffected by drought in bell pepper [*Capsicum annuum* L. var. *annuum* (Grossum Group) 'Quadrato d'Asti'] (Delfine *et al.*, 2001) and sugar beet (*Beta vulgaris* L.) plants (Monti *et al.*, 2006). Mesophyll conductance was strongly reduced for grapevines on the first day of water stress, but it was restored to the pre-stress values by the fourth day of water stress (Pou *et al.*, 2012). If we consider water stress and elevated CO_2 together, then existing studies suggest that plant responses to elevated [CO_2] and drought

stress are highly variable (Atwell *et al.*, 2007; Domec *et al.*, 2010; Wertin *et al.*, 2010, 2012; Ayub *et al.*, 2011; Duursma *et al.*, 2011; Warren *et al.*, 2011; Zeppel *et al.*, 2012; Franks *et al.*, 2013; Lewis *et al.*, 2013; Perry *et al.*, 2013; Duan *et al.*, 2014). In this study, the following hypotheses were tested: A) the historical rise in CO₂ from glacial to predicted future environment will affect g_m , and the responses of g_m with varying [CO₂] have a physiological basis; B) g_m is one of the dominant factors regulating photosynthesis; and C) g_m plays an important role in improving photosynthesis and water use efficiency (WUE) simultaneously in a future-stress climate scenario.

Materials and Methods

Plant material and growth conditions

Two cultivars (Cranbrook, Halberd) of wheat (*Triticum aestivum* L.) were grown in four controlled-environment growth rooms at the University of Sydney, Centre for Carbon Water and Food (CCWF), Camden (NSW, Australia). The wheat cultivars ‘Halberd’ and ‘Cranbrook’ are parental lines that were studied in depth in a quantitative trait loci (QTL) mapping population studied for $\Delta^{13}C$ of leaf tissue by Rebetzke *et al.* (2008). These two cultivars also have a wide range of g_m values under non-stress environmental conditions (Jahan *et al.*, 2014). These four growth rooms differed only in their (ambient growth) CO₂ mole fractions. Growth CO₂ levels were set to 180, 280, 450 and 1000 $\mu\text{mol mol}^{-1}$ to represent four [CO₂] from glacial, pre-industrial, ambient and super-elevated periods. Measured [CO₂] inside growth rooms were 206, 344, 489 and 1085 $\mu\text{mol mol}^{-1}$, using a Picarro ¹³CO₂ laser analyser (G1101-I, Picarro, CA, USA) to draw air from each room in sequence to the laser for 10 minutes each. Average [CO₂] and $\delta^{13}C$ of room air ($\delta^{13}C_{CO_2}$) were calculated over the last 5 minutes of sampling for the whole growing period and stopped 7 days prior to measurement. The running average over the 7 days prior to gas exchange measurement was used in g_m calculations described below, and for $\Delta^{13}C$ calculation of leaf tissue samples. The laser was calibrated as described by Thurgood *et al.* (2014). Apart from [CO₂], other environmental characteristics inside all the cabinets were the same: temperature was 25 °C during the 14-h light period (PPFD was 400 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at the top of the plants), and 17 °C during the 10-h dark period, while relative humidity was 75% at all times.

Seeds were planted in 2-L pots filled with commercial potting mix with slow-release fertilizer (Osmocote Exact, Scotts, Sydney, NSW, Australia). Seedlings were thinned to one per pot

after emergence. Sixteen days after emergence water treatments were started by withholding water to half of the pots as a drought treatment. At the temporary wilting point (7 days after the start of the drought treatment), weights were recorded for all drought pots, and this was taken as a target water content. A visual assessment of leaf wilting was used as an indicator of water status for water-stressed plants where temporary wilting point was defined as the first day on which leaves of droughted plants wilted. The target water content was maintained gravimetrically thereafter to introduce a mild water stress to half of total plants. The irrigated pots were well watered throughout the experiment.

Measurement of g_m

Two or three fully expanded youngest leaves per plant were selected to measure. Leaves were placed side-by-side in a 12 cm² (2×6 cm) leaf chamber (Li6400-11) attached to a LI-6400XT portable photosynthesis system (LiCor, Lincoln, NE, USA) fitted with a red-green-blue light (Li6400-18 RGB light source). At the time of measurement, all the plants were at tillering phase. During measurement, four different CO₂ mole fractions in the leaf chamber (CO₂ sample, ambient measurement CO₂) were used (180, 280, 450 and 1000 μmol mol⁻¹) with leaf temperature 25 °C, flow rate 350 μmol s⁻¹ and irradiance at 1300 μmol m⁻² s⁻¹ using the red and blue LEDs only. Mesophyll conductance was measured using a stable carbon isotope tunable-diode laser absorption spectrometer (TDLAS, model TGA100A, Campbell Scientific, Inc., Logan, UT, USA) coupled to the gas exchange system as described by Barbour *et al.* (2007, 2010). The highest possible accuracy of gas exchange is required particularly for A and C_i from gas exchange to obtain reliable estimates of g_m (Pons *et al.*, 2009). Similar CO₂ concentrations around and inside the leaf chamber, and realistic values of A and C_i supported the conclusion that there were no leaks in this study that needed to be considered. Furthermore, Pons *et al.* (2009) observed that it is not necessary to check for leaks when net photosynthetic rates (A) are high (such as in wheat).

Mesophyll conductance was calculated from the difference between predicted discrimination assuming infinite mesophyll conductance (Δ_i) and measured discrimination (Δ_{obs}), as described by Jahan *et al.* (2014), using equations developed by Evans *et al.* (1986) and Barbour *et al.* (2010), and including a ternary effect as described by Farquhar and Cernusak (2012). Here, average daytime $\delta^{13}C_{growth}$ $\delta^{13}C_{CO2}$ values were used for different ambient

growth CO₂ cabinets (-20.7, -18.4, -16.9 and -17.7 for 206, 344, 489 and 1085 µmol mol⁻¹, respectively) in calculation of g_m .

Leaf $\Delta^{13}C$

Leaf samples of known area were collected directly after gas exchange measurement for $\Delta^{13}C$ and percentage nitrogen concentration (%N). Leaves were oven dried at 65 °C for 72 hours and then dry mass was determined for LMA (leaf mass per area). Samples were then ground to a powder and approximately 1 mg of sample was weighed into tin cups. Isotope ratio mass spectrometry (IRMS) was used to determine the ratio of $\delta^{13}C$ in samples. Samples were analysed on a DeltaZQ V Advantage coupled to a Conflo IV and a FlashHT in dual-reactor setup (Thermo Fisher Scientific, Bremen, Germany). The precision for the standard materials was between 0.04 ‰ and 0.09 ‰.

Statistical analysis

The effects of CO₂ concentration (mole fraction), drought treatment, and their interactions were assessed separately for both cultivars, and for each measure, namely A , g_{sc} , g_m and A/g_{sw} (leaf-intrinsic water-use efficiency) using two-factor analysis of variance, as implemented in GenStat 14th edition (VSN International Ltd, Hemel Hempstead, UK); means were compared using Fisher's unprotected least significant difference tests. Differences were considered statistically significant when $P < 0.05$. Linear associations between the measures were evaluated using a general linear regression procedure in GenStat, making allowance for different regression slopes and intercepts between the two cultivars.

In order to explore all nine gas-exchange parameters simultaneously, a principal components analysis (PCA) was undertaken on these data (variables scaled to have a unit standard deviation), using the prcomp function in R (<https://www.r-project.org/>). Loadings of the first two principal components were interpreted, and this was also supported by calculation of Pearson correlation coefficients between all nine parameters.

Results

Growth CO₂ effect

Plants were grown and measured at approximately the same CO₂ mole fraction period (grown at 206, 344, 489 and 1085 µmol CO₂ mol⁻¹, and measured at 180, 280, 450 and 1000 µmol CO₂ mol⁻¹, respectively). Photosynthetic rate (A) was positively related ($P < 0.001$) to growth

CO₂ mole fraction when measured under saturating light and with the CO₂ mole fraction surrounding the leaf controlled to match growth CO₂ mole fraction, varying between 9 μmol m⁻² s⁻¹ (206 μmol CO₂ mol⁻¹) and 27 μmol m⁻² s⁻¹ (1085 μmol CO₂ mol⁻¹) (Fig. 1A & 1E). The two genotypes responded somewhat differently to growth CO₂ mole fraction (i.e. there was a nearly-significant interaction effect between genotype and CO₂; $P = 0.06$), with ‘Halberd’ grown at 489 μmol CO₂ mol⁻¹ having significantly higher A (23 μmol m⁻² s⁻¹) than ‘Cranbrook’ (18 μmol m⁻² s⁻¹) grown at the same CO₂, despite the two genotypes having similar A at 1085 μmol CO₂ mol⁻¹. Irrigated plants had higher ($P = 0.03$) A compared to droughted plants.

Stomatal conductance (g_{sc}) was negatively related to growth CO₂ mole fraction ($P < 0.001$) when measured under saturating light and with the CO₂ mole fraction surrounding the leaf controlled to match growth CO₂ mole fraction, varying between 0.09 mol m⁻² s⁻¹ (1085 μmol CO₂ mol⁻¹) and 0.21 mol m⁻² s⁻¹ (206 μmol CO₂ mol⁻¹) (Fig. 1B & 1F). At any growth CO₂ mole fraction, irrigated plants had significantly higher g_{sc} than droughted plants.

Mesophyll conductance (g_m) was affected by growth CO₂ mole fraction ($P < 0.001$), and increased with increasing CO₂ concentration from 206 (0.26 mol m⁻² s⁻¹ bar⁻¹) to 489 μmol CO₂ mol⁻¹ (0.50 mol m⁻² s⁻¹ bar⁻¹), whereas at 1085 μmol CO₂ mol⁻¹, g_m was lower than but higher than 206 μmol mol⁻¹ (Fig. 1C & 1G). Irrigated plants had significantly higher g_m than droughted plants for ‘Halberd’ at 489 and 1085 growth CO₂ mole fraction. Genotype ‘Halberd’ also had higher g_m than ‘Cranbrook’ at 489 μmol mol⁻¹ growth CO₂ mole fraction.

Leaf-intrinsic water-use efficiency (the ratio of photosynthetic rate to stomatal conductance of water vapour; A/g_{sw}) increased significantly ($P < 0.001$) with increasing growth CO₂ mole fraction from 206 to 1085 μmol mol⁻¹ (Fig. 1C & 1G). At 1085 growth CO₂ mole fraction, ‘Cranbrook’ had higher ($P = 0.05$) A/g_{sw} than ‘Halberd’. There was a significant interaction effect ($P < 0.001$) between CO₂ and water availability on A/g_{sw} , with increasingly higher A/g_{sw} for droughted plants compared to irrigated plants as CO₂ mole fraction increased.

Relationship of g_{sc} and g_m with A , and A/g_{sw} with g_m/g_s

Significant positive relationships ($P < 0.001$) were found between g_{sc} and A , and g_m and A at four different growth CO₂ mole fractions (Fig. 2A & 2B). Between g_m and A (Fig. 2B), all the slopes were effectively equal, so that an increase in g_m is associated with the same increase in

A at any of the four CO₂ mole fraction. In pre-industrial and super-elevated [CO₂] periods, the relationship between g_m and A was stronger ($r^2 = 0.66$, and 0.62 respectively) than between g_{sc} and A ($r^2 = 0.37$, and 0.47 respectively). There was no significant water treatment effect on the association between g_{sc} and A, and between g_m and A. Leaf-intrinsic water-use efficiency (A/g_{sw}) had a significant positive ($P < 0.001$) relationship with g_m/g_{sc} for all four growth CO₂ mole fractions periods (Fig. 3).

Leaf $\Delta^{13}C$

The relationship between $\Delta^{13}C$ and C_i/C_a was not significant ($P = 0.6$, $r^2 = 0.024$) (Fig. 4A), whereas the relationship between $\Delta^{13}C$ and C_c/C_a was significant ($P = 0.04$, $r^2 = 0.25$) (Fig. 4B), when C_c/C_a was calculated from gas exchange and online discrimination measurements made with the measurement CO₂ mole fraction close to that of growth CO₂ mole fraction. Moreover, there was a significant relationship between $\Delta^{13}C$ and g_m ($P = 0.002$, $r^2 = 0.5$) (Fig. 5). Mesophyll conductance with %N had a positive relationship but not significant ($P = 0.09$, $r^2 = 0.18$) (Fig. 6). Growth CO₂ had a significant effect on LMA ($P < 0.001$) where LMA decreased from 204 to 489 and then increased at super elevated CO₂ level (Fig. 7) however LMA did not show any significant relationships with g_m (Fig. 8).

Correlation between parameters and principal components analysis

The associations between all the parameters, including those mentioned above, can be summarised by correlations (Fig. 9). For example, the most positive correlation was between C_i/C_a and C_c/C_a ($r = 0.84$), while the most negative correlation was between %N and A/g_{sw} ($r = -0.80$). Exploring associations between all nine parameters further, based on the PCA, 68% of the variation in the nine-dimensional space was accounted for by the first two principal components (PC1 and PC2) (Table 1). In PC1, stomatal conductance (g_{sc}), %N and the ratios C_c/C_a and C_i/C_a have high positive loadings, whereas A/g_{sw} has a high negative loading, so can be interpreted as a contrast between these first four parameters and A/g_{sw} . On the other hand, in PC2, photosynthetic rate, mesophyll conductance and $\Delta^{13}C$ have high negative loadings, and A/g_{sw} and C_c/C_a have moderate negative loadings. From the plot of PC1 vs PC2, there is evidence of clustering of results on different growth [CO₂] (Fig. 10).

Discussion

Growth [CO₂] effects on g_m

Mesophyll conductance increased with increasing growth [CO₂] from 206 to 489 $\mu\text{mol mol}^{-1}$ and then decreased at 1085 $\mu\text{mol mol}^{-1}$ regardless of genotype and water treatment. Similarly, Singsaas *et al.* (2003) observed that g_m increased from 360 $\mu\text{mol mol}^{-1}$ (ambient) to 560 $\mu\text{mol mol}^{-1}$ CO₂ concentration for some species [cucumber (*Cucumis sativus* L.), spinach (*Spinacia oleracea* L.) and linden bean (*Phaseolus vulgaris* L. var. *Linden*)] and then g_m decreased from 500 to 750 $\mu\text{mol mol}^{-1}$ [CO₂] for other species [sweetgum (*Liquidambar styraciflua* L.) and aspen (*Populus tremuloides* L.)]. Mesophyll conductance also decreased from 390 to 780 $\mu\text{mol mol}^{-1}$ for *Arabidopsis thaliana* (Mizokami *et al.*, 2019). In this study, the genotype ‘Halberd’ exhibited a nearly two-fold higher g_m compared with ‘Cranbrook’ at ambient CO₂ growth environment, which is similar to the results reported by Jahan *et al.* (2014). Moreover, ‘Halberd’ shows a higher response at super-elevated [CO₂] compared to ‘Cranbrook’ (from Fig. 1C & 1G). Similarly, there was a species-dependent pattern on g_m with C_i ranging from 50 to 1500 μbar among six C₃ species (depending on the species, g_m varied five- to nine-fold) (Flexas *et al.*, 2007b). So, the response of g_m with different growth [CO₂] also depends on species- and/or different environmental condition (Singsaas *et al.*, 2003).

The response of g_m has a physiological basis

During g_m calculation, we assumed values of two fractionation factors (*b* and *f*) and the non-photorespiratory respiration rate (*R_d*), and these choices may possibly have altered g_m values with different [CO₂]. For example, various estimates of fractionation associated with carboxylation (*b*) may be found in the literature between 26‰ and 31‰ (Brugnoli and Farquhar, 2000; McNevin *et al.*, 2007; Lanigan *et al.*, 2008) and the discrimination equation is particularly sensitive to *b* (Seibt *et al.*, 2007). For this study, 29‰ for *b* was used for calculation. If a lower value of *b* is chosen (26‰; Lanigan *et al.*, 2008), then $\Delta_i - \Delta_{\text{obs}}$ becomes smaller and g_m larger, and if a higher value of *b* is chosen (31‰), then g_m becomes smaller. However, regardless of the value of *b* that is used, g_m is always higher for 489 $\mu\text{mol mol}^{-1}$ and lower for 206 $\mu\text{mol mol}^{-1}$.

R_d values of $1.11 \text{ mol m}^{-2} \text{ s}^{-1}$ and $0.3 \text{ mol m}^{-2} \text{ s}^{-1}$ were used for genotypes ‘Cranbrook’ and ‘Halberd’, respectively for calculation of g_m (from Jahan *et al.*, 2014). Jahan *et al.* (2014) performed a sensitivity analysis and showed that, at high light intensity, g_m estimates were not appreciably affected by the value used for R_d . However, R_d was not measured at different CO_2 mole fractions in this experiment, and sometimes R_d has been reported to be substantially decreased at high CO_2 concentration (Bruhn *et al.*, 2007). Gu and Sun (2014) mentioned that the use of an underestimate of the true R_d results in a strong nonlinear dependence of the estimated g_m on C_i . If the R_d value was increased by 30% at a low CO_2 mole fraction, then g_m will decrease slightly (from 0.34 to $0.32 \text{ mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ and from 0.40 to $0.39 \text{ mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ at 206 and $344 \mu\text{mol mol}^{-1}$, respectively. Similarly, if the R_d value is decreased by 30% at a high CO_2 mole fraction ($1000 \mu\text{mol mol}^{-1}$), then g_m will increase slightly (from 0.24 to $0.25 \text{ mol m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$). So, estimates of g_m were not greatly affected by the value used for R_d at different CO_2 mole fractions.

The value of 16.2‰ for fractionation during photorespiration (f) was used. Jahan *et al.* (2014) tested the sensitivity of calculated g_m to the values used for f by changing f from 16.2‰ to 11‰ for both 21% and 2% O_2 concentrations, but did not find appreciable changes. However, it is possible that variable f could have an effect on the estimated values of g_m at different $[\text{CO}_2]$. Cano *et al.* (2014) corrected the g_m estimation equation based on results reported by Tholen *et al.* (2012, 2014), which promoted the view that a low $[\text{CO}_2]$ increased the rate of photorespiration. If the highest f value (16.2‰) for 206 and $344 \mu\text{mol mol}^{-1}$ and the lowest value (11‰) for $1085 \mu\text{mol mol}^{-1}$ were used, the nonlinear relationship was also obtained with increasing g_m values from 206 to $1085 \mu\text{mol mol}^{-1}$. Flexas *et al.* (2007b) measured g_m at both 21% and 1% O_2 with different C_i and found a decline of g_m at high CO_2 for both 21% and 2% O_2 levels; this suggests that variation during photorespiration can be neglected for g_m estimation. Moreover, if mitochondria are located predominately close to but behind the chloroplast, most of the photorespired CO_2 may enter the chloroplast from the back, which makes the effect of photorespiration small or insignificant on the gradient between p_i and p_c (Cernusak *et al.*, 2013). In one of our studies (unpublished), similar anatomical structures for wheat leaves were also observed when viewed under a light microscope, namely, that chloroplasts are located appressed to the cell wall with mitochondria located on the vascular side of the chloroplasts.

The reasons for g_m responses with $[CO_2]$ are varied. For example, Flexas *et al.* (2007b) worked on transgenic tobacco plants that differed in the amounts of aquaporin NtAQP1 and showed different slopes of g_m response to C_i , and suggested that NtAQP1 may also be involved in these responses. Mizokami *et al.* (2019) suggested that abscisic acid (ABA) contents in leaves plays an important role in the regulation of g_m with $[CO_2]$ for *Arabidopsis thaliana*. Other studies have shown that g_m was limited by their mesophyll structure (Evans *et al.*, 2009; Tosens *et al.*, 2012b). In our study, LMA increased from glacial to ambient $[CO_2]$, and then decreased at super-elevated $[CO_2]$; however, LMA was not correlated with g_m which may be due to the greater cell wall thickness which would constrain CO_2 diffusion (Hassiotou *et al.*, 2009; Tomas *et al.*, 2013). There is another possibility that at super-elevated $[CO_2]$, some parts of the chloroplast are not adjacent to air spaces and this could lead to reduced g_m despite it having a high level of photosynthesis. Changes in chloroplast position and/or size could also affect g_m (Tholen *et al.*, 2007). So, taken together there is evidence that the response of g_m with $[CO_2]$ has a physiological basis.

Relationship of g_s and/or g_m with A , and improving WUE

The diffusion conductance of CO_2 mostly includes g_s and g_m , and it has been illustrated that g_s and g_m both were important determinants of A (Flexas *et al.*, 2012; Tosen *et al.*, 2012, 2016; Tomas *et al.*, 2013). Additionally, this study observed that g_s and g_m are important determinants of A across all $[CO_2]$. In the PCA, g_s and g_m also loaded highly on PC1 with concurring photosynthetic rates. So, both g_s and g_m play a critical role for achieving A not only for ambient $[CO_2]$ but also for future elevated $[CO_2]$, and neither g_s or g_m should be excluded in analyses of photosynthetic rate and carbon-cycle models. Despite highly variable values of g_m , researchers have shown g_m to be the most limiting factor for A (Tomas *et al.*, 2013; Tosen *et al.*, 2016; Veromann-Jürgenson *et al.*, 2017). Similarly, stronger associations of g_m with A compared to g_s with A were observed for simulated preindustrial and super-elevated $[CO_2]$. Importantly, if g_s and g_m vary together, we need to understand how these traits affect resource use efficiency in a future changed climate.

In this study, from glacial to super-elevated $[CO_2]$, g_m increased with increasing g_{sc} (Fig. 11), and the correlation between g_m and g_{sc} also depended on $[CO_2]$. There was an interesting pattern between g_m and g_{sc} at four different $[CO_2]$; for a constant value of g_{sc} , g_m increased with increasing CO_2 mole fraction from 206 to 489 $\mu mol\ mol^{-1}$ and then decreased at 1085

$\mu\text{mol mol}^{-1}$ (Fig. 11); g_m was altered by different CO_2 mole fractions to the same degree as g_{sc} . The higher value of g_{sc} was not associated with a higher value of g_m at low growth CO_2 mole fraction conditions. These relationships are supported by the PCA, where in PC1, g_{sc} had a high (positive) loading and g_m had a moderate (positive) loading.

To improve WUE, it will be important to manage g_{sc} , g_m and g_m/g_{sc} simultaneously. Researchers observed that improving water-use efficiency through increased g_m would require g_{sc} and g_m to be uncoupled (Barbour *et al.*, 2010; Gu *et al.*, 2012; Jahan *et al.*, 2014) because increasing g_m alone would result in increasing A which potentially increases leaf WUE. Moreover, researchers know that g_m can be affected by g_{sc} , and the response of g_m to CO_2 was faster than that of g_{sc} (Tazoe *et al.*, 2011; Mizokami *et al.*, 2019). In a water-stressed condition, g_m increased more than g_{sc} (Han *et al.*, 2016), and this plays an important role in the change of g_m/g_{sc} . Our results show that leaf-intrinsic WUE (A/g_{sw}) was strongly correlated with g_m/g_{sc} for all four $[\text{CO}_2]$ periods (Fig. 3). We also observed that some of the increase in g_m/g_{sc} was not associated with the same improvement rate of leaf-intrinsic WUE (A/g_{sw}) for all growth $[\text{CO}_2]$ treatments; plants in the super-elevated treatment had higher rates of WUE than in the simulated glacial, pre-industrial or ambient treatments. Moreover, in this study we also have an interaction between the effect of growth CO_2 and water treatment (here plants are only exposed to a mild water stress) on leaf-intrinsic WUE (A/g_{sw}). Similarly, wheat plant water use efficiency (WUE) was 12% and 7% greater for Free-Air CO_2 Enrichment (FACE) ($200 \mu\text{mol mol}^{-1}$ above ambient) than the control environment (ambient $370 \mu\text{mol mol}^{-1} \text{CO}_2$) for first year and second year measurements, respectively (Hunsaker *et al.*, 2000). Condon (2002) stated that field scale WUE (grain produced per unit water transpired) could be interpreted by leaf-intrinsic WUE. Barbour *et al.* (2010) stated that selecting for high g_m to improve A/g_{sc} will also improve crop WUE if allocation of carbon to the harvested plant organ is not reduced. Hence, for wheat genotypes, higher leaf-intrinsic WUE has the potential to improve crop WUE.

The carbon isotope composition of leaf tissue strongly reflects photosynthetic discrimination (Farquhar *et al.*, 1989), so the ratio of chloroplast to ambient CO_2 mole fractions, C_i/C_a , should be taken into account for evaluating A , g_{sc} and g_m . The observation here of no significant correlation between C_i/C_a and $\Delta^{13}\text{C}$, but a significant correlation between C_i/C_a and $\Delta^{13}\text{C}$ supports this theory. $\Delta^{13}\text{C}$ in leaf dry matter has been widely used as a long-term

estimation method for C_i/C_a and WUE between species or genotypes (Brugnoli and Farquhar, 2000), assuming either constant g_m , or that g_m scales with g_{sc} . Moreover, ^{13}C discrimination (Δ) and %N in leaf dry matter also shows a similar trend to that observed in g_m (increased with increasing growth CO_2 from 206 to 489, then decreased at $1085 \mu\text{mol mol}^{-1}$). Therefore, g_m plays an important role in regulating A , which could alter WUE for stressed crops in a future environment.

Conclusion

In two wheat genotypes, g_m increased with increasing $[\text{CO}_2]$ from simulated glacial era to ambient condition and then declined at super-elevated $[\text{CO}_2]$. Although the specific mechanism of g_m response to $[\text{CO}_2]$ remains unclear, the response of g_m to $[\text{CO}_2]$ may be due to aquaporins (Flexas *et al.*, 2007b) and/or leaf anatomical changes (Tholen *et al.*, 2007) which have a physiological basis. However, g_m and g_m/g_{sc} are strongly associated with the variability of A and leaf-intrinsic WUE, especially for future super-elevated $[\text{CO}_2]$. Moreover, g_m may be more important than g_{sc} in the change of g_m/g_{sc} , so plants with higher g_m may improve A without increasing g_{sc} , which ultimately increases leaf-intrinsic WUE (A/g_{sw}) for a climatically changed future environment. These data will inform plant breeding programs for Australian wheat under future environmental conditions. Additional gains could also be made by searching for genomic regions associated with these WUE-related traits through quantitative trait loci (QTL) or genome-wide association study (GWAS). Such a breeding program may then be further enhanced by inclusion of this genomic information in their selection schemes.

Acknowledgements

Firstly, I would like to express my sincere thanks to my PhD supervisor, Professor Margaret Barbour, who gave support throughout my experiment and editorial assistance. I thank Svetlana Ryazanova for technical assistance with gas exchange and stable isotope measurements. This work was supported by the Grains Research and Development Corporation (US00056, and GRS 10660 to support my study).

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Table 1. Principal component analysis and its component loadings

	PC1	PC2
Variation explained (%)	44.2	23.4
Variable		
A ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	-0.12	-0.61
g_{sc} ($\text{mol m}^{-2} \text{s}^{-1}$)	0.43	0.05
g_{m} ($\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$)	0.23	-0.45
A/g_{sw} ($\mu\text{mol mol}^{-1}$)	-0.44	-0.27
$C_{\text{c}}/C_{\text{a}}$	0.38	-0.24
$C_{\text{i}}/C_{\text{a}}$	0.42	0.04
%N	0.38	0.18
$\Delta^{13}\text{C}$	0.17	-0.47
LMA	-0.17	0.11

Figure legends

Fig. 1. Growth [CO₂] effect on photosynthetic rate (A), stomatal conductance to CO₂ (g_{sc}), mesophyll conductance (g_m) and leaf-intrinsic water use efficiency (A/g_{sw}) for leaves of two wheat cultivars. Values are mean $\pm$ standard error, $n = 5$.

Fig. 2. The relationships between photosynthetic rate and stomatal conductance to CO₂ (A), and photosynthetic rate and mesophyll conductance (B) for both wheat genotypes at four different growth [CO₂] (206, 344, 489 and 1085 $\mu\text{mol mol}^{-1}$).

Fig. 3. Correlation between leaf-intrinsic WUE (A/g_{sw}) and the ratio of g_m and g_{sc} for both wheat genotypes at four different growth [CO₂] (206, 344, 489 and 1085 $\mu\text{mol mol}^{-1}$).

Fig. 4. The relationship between $\Delta^{13}\text{C}$ in leaf dry matter and the instantaneous ratios C_i/C_a (A) and C_c/C_a (B) in two wheat genotypes at four different growth CO₂ mole fractions with two water treatments. Gas exchange parameters were measured at similar CO₂ mole fraction as growth CO₂. Values are means $\pm$ standard error, $n = 5$.

Fig. 5. The relationships between mesophyll conductance and $\Delta^{13}\text{C}$ in leaf dry matter for two wheat genotypes at four different growths CO₂ mole fraction with two water treatments. The line is a least squares linear regression across all treatments: $g_m = 0.031\Delta^{13}\text{C} - 0.37$, $r^2 = 0.50$, $P = 0.002$.

Fig. 6. The relationship between % N in leaf dry matter and mesophyll conductance in two wheat genotypes at four different growth CO₂ mole fractions with two water treatments. Gas exchange parameters were measured at similar CO₂ mole fraction as growth CO₂. Values are means $\pm$ standard error, $n = 5$, $r^2 = 0.15$, $P = 0.09$.

Fig. 7. The response of leaf mass per area (LMA) at four growth CO₂ mole fractions under two water treatments for genotype Halberd (A), and Cranbrook (B). Values are means $\pm$ standard error, $n = 5$.

Fig. 8. The relationship between leaf mass per area (LMA) and mesophyll conductance for two wheat genotypes at four different growths CO₂ mole fraction with two water treatments. Values are means $\pm$ standard error, $n = 5$, $r^2 = 0.18$, $P = 0.13$.

Fig. 9. Visualization of the correlation matrix between all nine parameters.

Fig.10. Plots of the first two principal component scores (PC1, PC2) for wheat plants. The growth [CO₂] for each plant is indicated by the colour code.

Fig. 11. The relationships between stomatal conductance to CO₂ and mesophyll conductance for both wheat genotypes at four different growth [CO₂] (206, 344, 489 and 1085 $\mu\text{mol mol}^{-1}$).

Figures

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