Mesophyll conductance increased with increasing [CO₂] from glacial to ambient CO₂ levels, then declined at super-elevated CO₂ for both well-watered and water-limited treatments. These responses of mesophyll conductance with varying [CO₂] have a physiological basis.
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 $\mu$mol mol$^{-1}$), pre-industrial (280 $\mu$mol mol$^{-1}$), current ambient (450 $\mu$mol mol$^{-1}$) and super-elevated (1000 $\mu$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
Introduction

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

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

During photosynthesis, CO₂ diffuses from the atmosphere (Cₐ) to the sites of carboxylation (Cₐ) inside the chloroplasts (Farquhar et al., 1980). This CO₂ uptake is strongly influenced by environmental factors that are predicted to change in the future, namely, atmospheric CO₂ concentration, temperature and water availability (Sage and Kubien, 2007; Lawlor and 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 ($g_{st}$) (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).

Leaky 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 µmol 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 µmol 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 µmol mol\(^{-1}\), using a Picarro \(^{13}\)CO₂ 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 (\( \delta^{13}CCO₂ \)) 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 µmol m\(^{-2}\)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 cm$^2$ (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 CO$_2$ mole fractions in the leaf chamber (CO$_2$
sample, ambient measurement CO$_2$) 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 CO$_2$
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 (\(\Delta_i\)) and measured discrimination (\(\Delta_{obs}\)), 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 14\(^{th}\) 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 344 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 \( (r^2 = 0.66, \text{ and } 0.62 \text{ respectively}) \) than between \( g_{sc} \) and \( A \) \( (r^2 = 0.37, \text{ and } 0.47 \text{ respectively}) \). 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 \( C_i/C_a \) and \( C_c/C_a \) 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 gₘ

Mesophyll conductance increased with increasing growth [CO₂] from 206 to 489 μmol mol⁻¹ and then decreased at 1085 μmol mol⁻¹ regardless of genotype and water treatment.

Similarly, Singsaas et al. (2003) observed that gₘ increased from 360 μmol mol⁻¹ (ambient) to 560 μmol 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 gₘ decreased from 500 to 750 μmol mol⁻¹ [CO₂] for other species [sweetgum (Liquidambar styraciflua L.) and aspen (Populus tremuloides L.)]. Mesophyll conductance also decreased from 390 to 780 µmol mol⁻¹ for Arabidopsis thaliana (Mizokami et al., 2019). In this study, the genotype ‘Halberd’ exhibited a nearly two-fold higher gₘ 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 gₘ with Cᵢ ranging from 50 to 1500 µbar among six C₃ species (depending on the species, gₘ varied five- to nine-fold) (Flexas et al., 2007b). So, the response of gₘ with different growth [CO₂] also depends on species- and/or different environmental condition (Singsaas et al., 2003).

The response of gₘ has a physiological basis

During gₘ calculation, we assumed values of two fractionation factors (b and f) and the non-photorespiratory respiration rate (Rₐ), and these choices may possibly have altered gₘ 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 △ᵢ - △ₗₐₜ₁ becomes smaller and gₘ larger, and if a higher value of b is chosen (31‰), then gₘ becomes smaller. However, regardless of the value of b that is used, gₘ is always higher for 489 μmol mol⁻¹ and lower for 206 μmol mol⁻¹.
$R_d$ values of 1.11 mol m$^{-2}$ s$^{-1}$ and 0.3 mol m$^{-2}$ 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 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$ and from 0.40 to 0.39 mol m$^{-2}$ s$^{-1}$ bar$^{-1}$ at 206 and 344 µmol mol$^{-1}$, respectively). Similarly, if the $R_d$ value is decreased by 30% at a high CO$_2$ mole fraction (1000 µmol mol$^{-1}$), then $g_m$ will increase slightly (from 0.24 to 0.25 mol m$^{-2}$ s$^{-1}$ 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 [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 [CO$_2$] increased the rate of photorespiration. If the highest $f$ value (16.2‰) for 206 and 344 µmol mol$^{-1}$ and the lowest value (11‰) for 1085 µmol mol$^{-1}$ were used, the nonlinear relationship was also obtained with increasing $g_m$ values from 206 to 1085 µmol 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 $\text{[CO}_2\text{]}$ 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 $\text{[CO}_2\text{]}$ 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 $\text{[CO}_2\text{]}$, and then decreased at super-elevated $\text{[CO}_2\text{]}$; however, LMA was not correlated with $g_m$ which may be due to the greater cell wall thickness which would constrain CO2 diffusion (Hassiotou et al., 2009; Tomas et al., 2013). There is another possibility that at super-elevated $\text{[CO}_2\text{]}$, 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 $\text{[CO}_2\text{]}$ has a physiological basis.

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

The diffusion conductance of CO2 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 $\text{[CO}_2\text{]}$. 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 $\text{[CO}_2\text{]}$ but also for future elevated $\text{[CO}_2\text{]}$, 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 $\text{[CO}_2\text{]}$. 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 $\text{[CO}_2\text{]}$, $g_m$ increased with increasing $g_{sc}$ (Fig. 11), and the correlation between $g_m$ and $g_{sc}$ also depended on $\text{[CO}_2\text{]}$. There was an interesting pattern between $g_m$ and $g_{sc}$ at four different $\text{[CO}_2\text{]}$; for a constant value of $g_{sc}$, $g_m$ increased with increasing CO2 mole fraction from 206 to 489 µmol mol$^{-1}$ and then decreased at 1085...
µmol 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 [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 [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 µmol mol\(^{-1}\) above ambient) than the control environment (ambient 370 µmol mol\(^{-1}\) 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 \(\Delta^{13}C\), but a significant correlation between \(C_{i}/C_{a}\) and \(\Delta^{13}C\) supports this theory. \(\Delta^{13}C\) 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 ($\Delta$) 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 µmol 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 [CO$_2$] from simulated glacial era to ambient condition and then declined at super-elevated [CO$_2$]. Although the specific mechanism of $g_m$ response to [CO$_2$] remains unclear, the response of $g_m$ to [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 [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 climactically 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**

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Table 1. Principal component analysis and its component loadings

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<th>Variable</th>
<th>PC1</th>
<th>PC2</th>
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<td>Variation explained (%)</td>
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<td>23.4</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Variable</td>
<td></td>
<td></td>
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<tr>
<td>$A$ (µmol m$^{-2}$ s$^{-1}$)</td>
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<td>-0.61</td>
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<tr>
<td>$g_{sc}$ (mol m$^{-2}$ s$^{-1}$)</td>
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<td>$g_m$ (mol m$^{-2}$ s$^{-1}$ bar$^{-1}$)</td>
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<tr>
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Figure legends

Fig. 1. Growth [CO₂] effect on photosynthetic rate (A), stomatal conductance to CO₂ (g_{sw}), mesophyll conductance (g_m) and leaf-intrinsic water use efficiency (A/g_{sw}) for leaves of two wheat cultivars. Values are mean ± 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 µmol mol⁻¹).

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

Fig. 4. The relationship between Δ¹³C 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 ± standard error, n = 5.

Fig. 5. The relationships between mesophyll conductance and Δ¹³C 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 = 0.031Δ¹³C - 0.37, r² = 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 ± standard error, n = 5, r² = 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 ± 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 ± 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 µmol mol⁻¹).
Figures

![Figure 1](https://example.com/figure1.png)

Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
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Fig. 9
Fig. 11