1 **Intrinsic water use efficiency depends on stomatal aperture rather than stomatal**
2 **density in C₃ and C₄ grasses grown at glacial CO₂ and low light**

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4 *Running title: Stomatal aperture controls water use efficiency in grasses*

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22 **Highlights (30 words)**

23 Across C₃ and six C₄ grasses, intrinsic water use efficiency was strongly associated with
24 stomatal conductance, operational stomatal aperture, guard cell K⁺ influx and stomatal
25 opening speed on transition to high light.

26

27 ABSTRACT

28 We investigated how stomatal morphology and physiology control intrinsic leaf water use
 29 efficiency (*iWUE*) in grasses. Two C₃ and six C₄ grasses were grown at ambient (400 µl L⁻¹)
 30 or glacial CO₂ (180 µl L⁻¹) and high (1000 µmol m⁻² s⁻¹) or low light intensity (200 µmol m⁻²
 31 s⁻¹). C₄ grasses tended to have higher *iWUE* and CO₂ assimilation rates, and lower stomatal
 32 conductance (*g_s*), operational stomatal aperture (*a_{op}*) and guard cell K⁺ influx rate relative to C₃
 33 grasses, while stomatal size (SS) and stomatal density (SD) did not vary according to the
 34 photosynthetic type. Overall, *iWUE* and *g_s* depended most on *a_{op}* and density of open
 35 stomata. In turn, *a_{op}* correlated with K⁺ influx, stomatal opening speed on transition to high
 36 light and SS. Species with higher SD had smaller and faster-opening stomata. Although C₄
 37 grasses operated with lower *g_s* and *a_{op}* at ambient CO₂, they showed a greater potential to
 38 open stomata relative to maximal stomatal conductance (*g_{max}*), indicating heightened stomatal
 39 sensitivity and control. We uncover novel links between *a_{op}*, *g_s*, *iWUE* and K⁺ influx amongst
 40 grasses and differential K⁺ influx responses of C₄ guard cells to low light, revealing
 41 molecular targets for breeding crops with high *iWUE*.

42

43 **Keywords:** C₃ and C₄ grasses, guard cell K⁺ flux, intrinsic water use efficiency, stomatal
 44 conductance, stomatal aperture

45 INTRODUCTION

46 A key desirable feature of future ‘climate-ready’ crops is improved water use efficiency
 47 (WUE) to cope with decreased water availability due to a rising global population, shrinking
 48 arable lands, increasing temperature and declining rainfall (Flexas, 2016). Crop WUE is
 49 governed by multiple factors including leaf-level intrinsic WUE (*iWUE*) (Condon *et al.*,
 50 2004; Passioura, 1977). Leaf *iWUE* is defined as the ratio of CO₂ assimilation rate (A_{net}) over
 51 stomatal conductance (g_s) to water vapour (Farquhar and Sharkey, 1982). Photosynthesis and
 52 g_s are closely correlated over the long term (Wong *et al.*, 1979; Pinto *et al.*, 2016) such that
 53 selecting for reduced g_s , as a means of improving *iWUE*, often leads to reduced A_{net} and
 54 productivity (Blum, 2009; Ghannoum, 2016). However, g_s and A_{net} can be uncoupled in the
 55 short term and under various conditions (McAusland *et al.*, 2016; von Caemmerer *et al.*,
 56 2004) raising the prospect of improving stomatal traits without affecting CO₂ fixation, and
 57 hence yield (Ghannoum, 2016; Webster *et al.*, 2016). Therefore, developing the next
 58 generation of smart crops requires a greater understanding of how stomata regulate *iWUE*.
 59 Plant breeders often utilise variations within a narrow set of crop-related species to identify
 60 improved traits. The downfall of this strategy is the limited pool of genetic diversity available
 61 in a single species. Mining natural variation among diverse grasses will increase the potential
 62 of identifying beneficial stomatal traits (Anderson *et al.*, 2016; Cano *et al.*, 2019; Reeves *et al.*,
 63 2018; Watson-Lazowski *et al.*, 2019).

64 By controlling CO₂ entry into the leaf, stomata provide the initial limitation of CO₂ uptake.
 65 Grasses possess unique stomata composed of two dumbbell-shaped guard cells flanking
 66 subsidiary cells (Nunes *et al.*, 2020; Rudall *et al.*, 2017). Stomatal opening and closing
 67 govern gas exchange and are regulated by the movement of solutes and water in and out of
 68 the guard cells. These sophisticated structural and functional features enable fast and tight
 69 stomatal control in response to environmental fluctuations (Cai *et al.*, 2017; Chen and Blatt,
 70 2010; Chen *et al.*, 2010; Franks and Farquhar, 2007). They also contribute to the success,
 71 productivity and survival of grasses in semi-arid and arid habitats (Chen *et al.*, 2017; Taylor
 72 *et al.*, 2018; Taylor *et al.*, 2012). Therefore, it is not surprising that grasses including some of
 73 the most important cereal crops have been domesticated by our ancestors. Many grasses,
 74 including major crops (e.g., maize, sugarcane, sorghum and switchgrass) assimilate CO₂
 75 using the C₄ photosynthetic pathway (Brown, 1999; Sage, 2004). C₄ crops are becoming
 76 increasingly important for food and bioenergy security, with the global production of C₄

77 maize currently surpassing that of key C₃ cereals such as wheat and rice (Varshney *et al.*,
78 2012).

79 C₄ photosynthesis operates a carbon concentrating mechanism (CCM) which elevates CO₂
80 concentration ([CO₂]) around the carbon-fixing enzyme Rubisco (ribulose 1,5-
81 carboxylase/oxygenase) in the bundle sheath, thereby minimizing photorespiration and
82 allowing higher photosynthetic rates at relatively low stomatal conductance (Hatch, 1987;
83 Long, 1999). Consequently, *iWUE* is generally higher in C₄ relative to C₃ grasses (Taylor *et al.*,
84 2012). C₄ CCMs are broadly classified according to the major C₄ decarboxylase utilised,
85 either solely or with a secondary decarboxylase (Kanai and Edwards, 1999; Watson-
86 Lazowski *et al.*, 2018; Wingler *et al.*, 1999); nicotinamide adenine dinucleotide-malic
87 enzyme (NAD-ME), nicotinamide adenine dinucleotide phosphate-malic enzyme (NADP-
88 ME), and phosphoenol carboxykinase (PCK). Apart from differences in leaf anatomy and
89 biochemistry, the three C₄ subtypes also show variations in geographic distribution according
90 to rainfall and resource (e.g. light, water, nitrogen) use efficiency (Ghannoum *et al.*, 2011;
91 Ghannoum *et al.*, 2005; Hattersley, 1992). In particular, NAD-ME grasses exhibit higher
92 *iWUE* at glacial [CO₂] (gCO₂: 180 µl l⁻¹) (Pinto *et al.*, 2014) and higher plant WUE under
93 water stress (Ghannoum *et al.*, 2002) relative to NADP-ME and PCK counterparts. Recently,
94 we identified two subtype-specific differentially expressed transcripts linked to stomatal
95 function. βCA1, conserved only in NAD-ME species, is a gene known to sense CO₂ in plants;
96 and SASP, conserved only in PCK species, is involved in controlling stomatal aperture
97 (Watson-Lazowski *et al.*, 2020). Hence, the first objective of this study was to elucidate how
98 stomatal control of *iWUE* differs among grasses depending on the photosynthetic pathway
99 (C₃ versus C₄) or the C₄ biochemical subtypes.

100 Light regulates stomatal function via photosynthesis-dependent and independent processes
101 (Assmann and Jegla, 2016; Lawson, 2009). The photosynthesis-mediated (or red light)
102 response affects g_s by driving photosynthetic linear electron transport and is partially
103 mediated by decreasing intercellular [CO₂] (C_i) (Baroli *et al.*, 2008; Messinger *et al.*, 2006;
104 Mott, 2009). In addition, guard cells directly respond to red light, independent of mesophyll
105 photosynthesis, though the signal has not been conclusively identified (Busch, 2014; Lawson
106 and Matthews, 2020). Blue light directly affects the phototropins in guard cells through
107 photosynthesis-independent reactions leading to the activation of plasma membrane H⁺-
108 ATPase pumps (Christie, 2007; Hiyama *et al.*, 2017; Shimazaki *et al.*, 2007). The
109 hyperpolarised or depolarised plasma membrane activates inward- or outward-rectifying K⁺

channels, which are the key regulators of guard cell homeostasis, leading to stomatal opening or closure, respectively (Hosy *et al.*, 2003; Kim *et al.*, 2010; Schroeder and Fang, 1991). Photosynthesis and stomatal conductance respond with different speed to short-term changes in light intensity, causing transient disequilibrium in *iWUE* (Lawson and Vialet-Chabrand, 2019; McAusland *et al.*, 2016). Hence, the rates of stomatal closure or opening in response to light transients are valuable traits for optimised *iWUE* (Deans *et al.*, 2019; Knapp and Smith, 1987). Following long-term exposure to high light, stomatal density (SD) and stomatal pore lengths increase in certain species, such as tomato (Gay and Hurd, 1975), tobacco (Thomas *et al.*, 2003), *Arabidopsis ssd-1* mutants (Schluter *et al.*, 2003) and *Eucalytus globulus* (James and Bell, 2000). Conversely, decreased SD and stomatal index (SI), but not stomatal size (SS) were observed in tobacco leaves (Gerardin *et al.*, 2018; Thomas *et al.*, 2003) and *Coffea arabica* (Pompelli *et al.*, 2010) exposed to shade. However, it remains unclear if and how C₃ and C₄ stomata respond differently to light. Accordingly, our second objective was to determine how stomatal responses to short-term light and long-term light changes differ among C₃ and C₄ grasses, including the three C₄ subtypes.

A period of low (glacial) atmospheric [CO₂] (gCO₂) during the Oligocene is thought to be the primary driver for the evolution of C₄ photosynthesis (Christin *et al.*, 2008; Sage, 2004). Plants adapted to the geological decline in atmospheric [CO₂] by having higher SD compared to current atmospheric [CO₂] (Woodward, 1987; Woodward and Kelly, 1995). In response to short-term (instantaneous) changes in [CO₂], stomata respond by opening with decreasing [CO₂] and by closing with increasing [CO₂] both in darkness and light (Assmann, 1999; Azoulay-Shemer *et al.*, 2016; Lawson *et al.*, 2011). In the long term, stomata acclimate to changes in [CO₂] through biochemical and anatomical changes which may modify the instantaneous responses. Growth at gCO₂ increased g_s in C₃ and C₄ grasses (Maherali *et al.*, 2002; Pinto *et al.*, 2014; Sage and Coleman, 2001), and increased SD by 42% in *Arabidopsis* relative to ambient CO₂ (Li *et al.*, 2014). Conversely, growth at elevated [CO₂] reduced SD, g_s, and sensitivity to [CO₂] (Ainsworth and Rogers, 2007; Leakey, 2009; Maherali *et al.*, 2002; Woodward and Kelly, 1995; Xu *et al.*, 2016). To date, few studies investigated the mechanisms underpinning the acclimation of C₃ and C₄ stomata to [CO₂]. Hence, our third objective was to determine how stomatal responses to short-term light and long-term light changes to [CO₂] differ among C₃ and C₄ grasses, including the three C₄ subtypes.

To address these objectives, we grew closely related C₃ and C₄ grasses with different biochemical subtypes under gCO₂ (180 CO₂ µl L⁻¹) and/or low light (200 µmol m⁻² s⁻¹) as

well as control conditions ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ of light and $400 \mu\text{l CO}_2 \text{L}^{-1}$) with the aim of further exploring stomatal responses to these environmental stimuli. Light is expected to impart a greater stomatal and photosynthetic response than CO_2 because plants are continuously exposed to light fluctuations in their open environment, while atmospheric CO_2 gradually changed over time. Nevertheless, stomata have been shown to respond to both factors in the short and long term. Hence, we hypothesised that stomata will respond to gCO_2 by increased aperture and density (*Hypothesis 1*) while stomata respond to low light by reduced aperture and density (*Hypothesis 2*). We also hypothesised that both low light and gCO_2 will affect transient stomatal responses (*Hypothesis 3*). Elucidating these hypotheses will shed light on how stomatal traits (morphology, physiology, dynamics and/or ion flux) control $iWUE$ in C_3 and C_4 grasses acclimated to low light and gCO_2 . In turn, such new understanding will be useful for developing future cereal crops with improved $iWUE$.

MATERIALS AND METHODS

Plant materials and grow conditions

Eight C₃ and C₄ grasses (Poaceae) representing different photosynthetic and biochemical subtypes were grown in walk-in growth chambers (Biochambers, Winnipeg, Manitoba) controlled by LI-820 CO₂ gas analysers (LI-COR Inc., Lincoln, NE, USA) as described in (Watson-Lazowski *et al.*, 2020). They were two C₃: *Panicum bisulcatum* (Thunb.) and *Steinchisma laxa* (Zuloaga); two C₄ NAD-ME: *Panicum miliaceum* (L.) and *Leptochloa fusca* [(L.) Kunth.]; two C₄ NADP-ME: *Panicum antidotale* (Retz.) and *Setaria viridis* [(L.) Beauv.]; and two C₄ PCK: *Megathyrus maximus* (Jacq., synonym: *Panicum maximum*) and *Chloris gayana* (Kunth.). Two species, *L. fusca* (NAD-ME) and *C. gayana* (PCK) belong to subfamily Chloridoideae while the remaining six species belong to Subfamily Panicoideae (Grass Phylogeny Working, 2012). All species were individually planted in 3 L pots containing soil (Osmocote® Professional Seed Raising Mix, Scotts, Australia) and trace elements supplement (Osmocote® Plus Trace Elements, Scotts, Australia). Seeds were germinated in the control chamber (400 µl CO₂ L⁻¹ and 1000 µmol m⁻² s⁻¹). After germination, plants were transferred to respective growth chambers with different light intensity (high: 1000 µmol m⁻² s⁻¹ or low: 200 µmol m⁻² s⁻¹ at pot level) and CO₂ concentration (ambient: 400 µl l⁻¹ or glacial: 180 µl l⁻¹) treatments. High and low light treatments were abbreviated as HL and LL, respectively. Ambient and glacial [CO₂] were abbreviated as aCO₂ and gCO₂, respectively. The average day/night temperature was 28/22°C, relative humidity (RH) was 60%, and the photoperiod was 14 h. Plants were watered to full capacity every 1-2 days, fertilised as required and randomized twice a week to reduce the effects of within-chamber variation. There were 3-5 biological replicates per treatment.

Leaf gas exchange

Five weeks after germination, gas exchange was measured with LI-6400XT infrared gas analyser (LI-COR Inc., Lincoln, NE, USA) using the youngest fully expanded leaf from the main stem. Cuvette conditions were maintained at 60-65% RH, 28°C leaf temperature, and 350 mol s⁻¹ flow rate. Two types of measurements were taken between 10:00 and 15:00 inside the growth chambers for all eight grasses. Steady-state measurements of net CO₂ assimilation rate (A_{net}), stomatal conductance (g_s), intercellular CO₂ (C_i) and intrinsic water use efficiency (*iWUE* = A_{net}/g_s) were made at the four respective growth conditions.

Measurements were also made at light-saturated ($2000 \mu\text{mol m}^{-2} \text{s}^{-1}$) and low CO_2 ($180 \mu\text{l L}^{-1}$ CO_2) conditions to induce maximal stomatal conductance (g_{sat}) and light-saturated photosynthesis (A_{sat}). Prior to each measurement, leaves were pre-adapted at the chamber conditions for 10-15 min until steady-state rates of CO_2 and H_2O exchange were reached. At least eight technical replicates across 3-5 biological replicates per species were measured.

Stomatal morphology using epidermal impressions

Stomatal impressions were characterised based on the methods described by (Sekiya and Yano, 2008; Weyers and Johansen, 1985) with some modifications. Epidermal imprints containing stomatal patterns were taken using clear nail varnish from the adaxial and abaxial surfaces of all eight grass species using the same leaf where the steady-state gas exchange was measured. Dried nail varnish impressions were transferred to glass slides (Knittel Glass, Germany), and images were captured using Nikon microscope equipped with CCD camera and DS-U3 controller (NIS-F1 Nikon, Tokyo, Japan). Images were captured under a field of view of 0.0768 mm^2 at $200\times$ and $400\times$ total magnification and processed using Nikon NIS Element imaging software (Nikon, Tokyo, Japan). To ensure uniformity, stomatal images were captured between two major veins of the leaf lamina and were analysed using Image J software (NIH, USA). Ten anatomical stomatal parameters (Caine *et al.*, 2019; Gerardin *et al.*, 2018) were measured (Figures S1 and S2): guard cell width (GCW), guard cell length (GCL), subsidiary cell width (SCW), aperture width (AW), aperture length (AL), operational stomatal aperture (a_{op}), stomatal size (SS), stomatal density (SD), stomatal index (SI), and density of open stomata (OD). Anatomical SS and a_{op} were calculated by assuming the area of an ellipse whereby:

$$a_{\text{op}} = \left(\left[\frac{AW}{2} \right] \times \left[\frac{AL}{2} \right] \times \pi \right) \quad (\text{eqt 1})$$

$$SS = \left([GCW + SCW] \times \left[\frac{GCL}{2} \right] \times \pi \right) - a_{\text{op}} \quad (\text{eqt 2})$$

such that $AW/2$ and $GCW+SCW$ were the short axes while $AL/2$ and $GCL/2$ were the long axes of a_{op} and SS , respectively. SD was calculated as the number of stomata per unit area while OD was calculated by counting the number of open stomata (i.e., showing a gap between the two guard cells) per unit area as observed from the nail varnish impressions (Figure S2). A stoma was counted if more than 50% was located within the field of view. SI was calculated as the ratio of stomata to total cell numbers in the epidermis (stomata plus non-stomatal cells) $\times 100$. Three random microscopic fields from the adaxial and abaxial

surfaces per biological replicate were measured totalling >300 stomata per species in each growth chamber and the mean of the adaxial and abaxial surfaces were used (*leaf side* $p = 0.75$, $df = 1,7$).

Maximal stomatal conductance using epidermal peels

Maximum stomatal aperture (a_{max}) was measured following (Liu *et al.*, 2014; Mak *et al.*, 2014) with some modifications. The mid-portion of the second fully expanded leaf was harvested and soaked immediately in stomata opening buffer (50 mM KCl, 5 mM Na-MES, pH 6.1) after which the leaf epidermis was promptly peeled. Peels were mounted in a 35-mm petri dish with 0.13 mm glass bottom (MatTek Corp, MA, USA) coated with silicone adhesive (B-521, Factor II, Lakeside, AZ 85929), and then 2 ml of opening buffer was added. Epidermal peels were incubated for 2 hr at 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance to induce maximal stomatal opening. a_{max} was calculated using the same equation (eqt 1) for calculating a_{op} . Maximum stomatal conductance (g_{max}) was calculated using a_{max} determined from epidermal peels. One-sided g_{max} was calculated using the equation presented by (Franks and Beerling, 2009a) based on the (Brown and Escombe, 1901) diffusion model of gases for plants:

$$g_{max} = \left(\frac{d}{v}\right) \left(\frac{SD \cdot a_{max}}{l + \frac{\pi}{2} \sqrt{\frac{a_{max}}{\pi}}}\right) \quad (\text{eqt 3})$$

where d = diffusivity of water vapor at 25°C (0.0000249 $\text{m}^2 \text{s}^{-1}$); v = molar volume of air (0.0224 $\text{m}^3 \text{mol}^{-1}$); SD = stomatal density (m^{-2}); a_{max} = maximum stomata aperture (m^2) and l is the stomata pore depth which is assumed to be equivalent to guard cell width (m). The total, two-sided g_{max} was calculated as g_{max} (abaxial) $\times 2$ since both leaf sides had similar SD.

Stomatal responses to light transitions

The rates of stomatal closure in response to transition to LL (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) followed by rate of stomatal opening in response to transition to HL (1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were measured under similar conditions as steady-state measurements for four Panicoidea species: *P. bisulcatum* (C₃), *P. miliaceum* (C₄-NAD-ME), *P. antidotale* (C₄-NADP-ME) and *M. maximus* (C₄-PCK). Initially, leaves were pre-adapted at 400 $\mu\text{l CO}_2 \text{l}^{-1}$ and 1000 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for 20-30 min until reaching steady-state CO_2 uptake. Subsequently, steady-state g_s was auto-logged every 10 s for 15 min after which light intensity was reduced to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The exponential decay of g_s was monitored every 10 s until a new steady-state was reached (~20 min). Light intensity was then increased to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to monitor

the rate of stomatal opening (Figure S3). The rate of stomatal closure was fitted using an exponential decay model as described by (Elliott-Kingston *et al.*, 2016):

$$g_s(t) = g_s(\infty_c) + (g_s[i] - g_s[\infty_c]) \cdot e^{-kct} \quad (\text{eqt 4})$$

where $g_s(t)$ is the stomatal conductance at time point (t), $g_s(\infty_c)$ is the stomatal conductance at the new steady-state after irradiance was switched to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, $g_s[i]$ is the initial steady-state conductance at $t = 0$, kc is the exponential decay constant for closing, and the closing half-time ($ct_{1/2}$) is the time required for g_s to decrease by 50% of the difference between its initial and final values. The rate of g_s opening was fitted using an exponential rise to maximum model:

$$g_s(t) = g_s(\infty_o) + (g_s[i] - g_s[\infty_o]) \cdot (1 - e^{-kot}) \quad (\text{eqt 5})$$

where $g_s(t)$ is the stomatal conductance at time point (t), $g_s(\infty_o)$ is the stomatal conductance at the new steady-state after irradiance was switched back to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, $g_s[i]$ is the initial steady-state conductance at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$, ko is the exponential rise constant for opening, and opening half-time ($ot_{1/2}$) is the time required for g_s to increase by 50% of the difference between its initial and final values. Lower $t_{1/2}$ indicates a faster response time. Closing and opening half times ($t_{1/2}$) were calculated using the respective exponential decay or rise constants (k) as:

$$ct_{1/2} = \log_e(2) - kc \quad (\text{eqt 6})$$

$$ot_{1/2} = \log_e(2) - ko \quad (\text{eqt 7})$$

Estimation of water and CO₂ losses during light transitions

Excess transpiration (ΔW , Figure S4a), the transpiration above final steady-state transpiration due to slow stomatal closure during transitions from HL ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) to LL ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$) was estimated according to (Deans *et al.*, 2019). It was defined as the time-integrated difference between the initial and final steady-state transpiration rates (E):

$$\text{Excess transpiration } (\Delta W) = \int_0^{t_\infty} (E[t] - E[\infty]) dt \quad (\text{eqt 8})$$

where $E[t]$ was the initial steady-state E at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $E[\infty]$ was the transpiration rate at time point 90% (t_{90}) wherein 90% of the residual E is no longer changing under the new steady-state ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$). Potential forgone potential photosynthesis (ΔC , Figure S4b) due to slow photosynthetic induction during transitions from LL to HL was defined as

the time-integrated difference between the final and initial A_{net} rates according to (Deans *et al.*, 2019):

$$\text{Forgone photosynthesis } (\Delta C) = \int_0^{\infty} (A_{\text{net}[\infty]} - A_{\text{net}[t]}) dt \quad (\text{eqt 9})$$

where $A_{\text{net}[\infty]}$ was the final steady-state assimilation rate at HL ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) and $A_{\text{net}[t]}$ was the residual steady-state assimilation rate at LL ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Microelectrode ion flux estimation

Fluxes of K^+ , a major ion responsible for stomatal control, were measured for four Panicoidea species: *P. bisulcatum* (C_3), *P. miliaceum* ($\text{C}_4\text{-NAD-ME}$), *P. antidotale* ($\text{C}_4\text{-NADP-ME}$) and *M. maximus* ($\text{C}_4\text{-PCK}$). Measurements were made using microelectrode ion flux estimation on guard cells of leaf epidermal peels (Zhao *et al.*, 2019) with some modifications. Prior to measurements, ion-selective microelectrodes were prepared as described by (Chen *et al.*, 2005) and subsequently backfilled with a solution of 200 mM KCl. The tip was broken to achieve a 2-3 μm diameter and was front-filled with potassium ionophore I (Sigma, Switzerland), a liquid ion exchanger to achieve high resistance (4–6 G Ω). The microelectrodes were then mounted into the micromanipulator holder with AgCl wire together with a reference microelectrode backfilled with 1 M KCl in 2% agar. The microelectrodes were calibrated using 2, 5, and 10 mM KCl achieving a calibration slope of -55 mV and a correlation coefficient ≥ 0.999 .

Leaf epidermal peels were soaked in OB and promptly peeled to ensure stomatal integrity. The peels were mounted on a glass cover slide using a silicon prosthetic adhesive (Dow-Corning, USA) and immersed in a chamber filled with the stomatal measuring solution (10 mM KCl, 0.5 mM CaCl_2 , 5 mM MES-KOH, pH 6.1) in a 45-degree position. The peels equilibrated in the measuring solution were focused on the same plane together with the microelectrodes that were focused on top of the guard cells and moving in two positions, near the cells (10 μm) and away from the cell surface (40 μm) in 5-sec cycle, 80 μm amplitude for 10 min. The steady-state ion flux was calculated using the basic planar diffusion geometry described by (Newman, 2001) via the MIFEFLUX software and expressed per epidermal peel surface area. The same leaf and species used for g_s kinetics measurements were assayed.

Statistical analyses

ANOVA was performed using linear mixed-effects models (*lme*) in R (V.3.4.2; R Core Team, 2017). The full factorial experimental design included light (2 treatments) $\times$ CO₂ (2 treatments) and photosynthetic type or subtype, where species were nested inside the photosynthetic type (2 groups, $n = 2$ C₃ and $n = 6$ C₄ species) or biochemical subtypes (3 groups, $n = 2$ C₄ species per subtype). There were 3-5 plants (biological replicates) per species, and at least 3 technical replicates per plant (unbalanced ANOVA). Homoscedasticities and normalities were checked by examining the quantile plots and the re-fitted when necessary. Since the comparisons consisted of hierarchal (nested) terms, the *F*-statistic was re-calculated such that the signal (numerator mean squares) to noise (denominator mean squares) ratio reflected the true inference on the effects of [CO₂], light intensity, type/subtype and their respective interactions. The effect of glacial [CO₂] treatment was the average effects of chambers with gCO₂ regardless of light conditions (HL+gCO₂ and LL+gCO₂) while the effect of ambient [CO₂] was the average effects of aCO₂ chambers (HL+aCO₂ and LL+aCO₂) regardless of light treatment. Low light effect was the average effect of the chambers with LL conditions (LL+aCO₂ and LL+gCO₂) while high light effect was the mean variation within chambers with HL conditions (HL+aCO₂ and HL+gCO₂). The equivalent *p value* was calculated for a given degree of freedom. When significant, means were ranked using Tukey's *post-hoc* at a stringency of $\alpha=0.05$. Linear correlations between two variables were expressed in r^2 followed by significance values. Figures were plotted using the *ggplot2* package (Wickham, 2016). Principle components analysis (PCA) was generated in R using the *pca.comp* function, scaling each variable using the *scale=TRUE* function, then using *ggbiplot* to look for relationships between samples and traits.

RESULTS

Glacial CO₂ and low light differentially affect leaf gas exchange of C₃ and C₄ grasses

When measured under growth conditions, photosynthetic rate (A_{net}) and stomatal conductance (g_s) showed significant [CO₂], light and photosynthetic type, but not subtype, effects (Table 1). In the six C₄ grasses, A_{net} was higher under HL and reduced to a greater extent by LL (-63%) relative to the two C₃ (-44%) species (Figure 1a, Tables 1 and S1). In all species, g_s was greater under gCO₂ relative to aCO₂, while g_s was lower under LL relative to HL only in C₄ species, which maintained lower g_s relative to C₃ counterparts under gCO₂ and/or LL (Figure 1b, Tables 1 and S1). Intrinsic water use efficiency ($iWUE$) was higher in the C₄ than C₃ grasses, and lower under gCO₂ relative to aCO₂, but was not affected by LL (Figure 1c, Tables 1 and S1).

When measured under saturating light (2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and low [CO₂] (180 $\mu\text{l L}^{-1}$) to encourage stomatal opening, light-saturated photosynthesis (A_{sat}) and stomatal conductance (g_{sat}) were higher in C₄ relative to C₃ species, and tended to be lower under LL relative to HL in most C₄ species. Both A_{sat} and g_{sat} showed no significant CO₂ effect (Figure 1d, Tables 1 and S1). Under control conditions, the g_s/g_{sat} ratio averaged 0.7 and 0.4 in C₃ and C₄ species, respectively (Figure 1g, Tables 1 and S1). The g_s/g_{sat} ratio was higher under gCO₂ in all species (reaching close to 1 in C₃ species), and lower under LL in C₄ species, relative to the control (Tables 1 and S1). These results indicate that C₄ grasses operate with a much lower g_s , relative to g_{sat} , than C₃ grasses (Figure 1g, Tables 1 and S1).

Leaf surface morphological responses to glacial CO₂ and low light were driven by stomatal aperture

The stomatal complexes of the grass species were amphistomatous and paracytic, composed of two lateral, non-oblique (parallel to guard cells) subsidiary cells (SC) (Figure S2). The operational stomatal aperture (a_{op}) was the most responsive anatomically-measured traits in the grasses (Figure 2, Tables 1 and S2). Overall, a_{op} and density of open stomata (OD) were slightly lower in C₄ than C₃ species, mostly due to lowest a_{op} in the C₄-PCK relative to the other species (Figure 2a,d, Tables 1 and S2). Within C₄ species, SS was smaller in PCK and SI was higher in NADP-ME species (Figure 2, Tables 1 and S2). In partial support of *Hypothesis 1*, growth at gCO₂ led to higher a_{op} but had no effect on stomatal density (SD). Growth at gCO₂ also led to higher maximal stomatal conductance (g_{max}) and slightly lower

stomatal size (SS) relative to the control treatment (Figures 1e and 2, Tables 1 and S2). In full support of our *Hypothesis 2*, growth at LL led to lower a_{op} , SD, OD and stomatal index (SI) of most species (Figures 1e and 2, Tables 1 and S2).

Physiologically induced maximal stomatal aperture (a_{max}) was 3-5-fold greater than a_{op} captured using *in situ* epidermal impressions (Table S2). Under control conditions, the g_s/g_{max} ratio was slightly higher in C₃ (0.24) than C₄ (0.19) species (Figure 1h, Tables 1 and S1). The g_s/g_{max} ratio was higher under gCO₂ in all species, and lower under LL in the C₄ species, relative to the control (Tables 1 and S1). The resultant g_{sat}/g_{max} was relatively constant and lower in the C₃ relative to the C₄ species (Figure 1f, Tables 1 and S1). Hence, C₄ grasses had a greater potential of stomatal opening (measured as g_{sat}) relative to g_{max} (Figure 1f).

Correlations among stomatal variables of C₃ and C₄ grasses

Overall, g_s correlated best with a_{op} ($r^2 = 0.472$; Figure 3a) and OD ($r^2 = 0.473$, Table 2), and weakly with g_{max} ($r^2 = 0.177$, Table 2). In turn, a_{op} correlated well with SS ($r^2 = 0.340$; Figure 3b), and SS showed a negative trade off with SD ($r^2 = 0.307$; Figure 3c). In addition, a_{max} correlated positively with SS ($r^2 = 0.647$) and a_{op} ($r^2 = 0.378$), and negatively with SD ($r^2 = 0.297$) (Table 2). Maximal anatomical (g_{max}) and diffusional (g_{sat}) stomatal conductances did not correlate (Table 2). Moreover, g_{max} correlated best with OD ($r^2 = 0.336$) due to a stronger correlation with SD ($r^2 = 0.288$) than g_s (0.177) (Table 2). A good illustration of these correlations is within PCK species, which had smaller a_{op} and SS, greater SD but similar g_{max} relative to other species (Figures 1 and 2, Tables 1 and S2).

Smaller and more closed stomata opened faster in response to high light transition

Stomatal kinetics in response to short-term light transitions and guard cell K⁺ influx were measured in only one species per type or subtype (one C₃ and three C₄ grasses). Stomatal closing ($ct_{1/2}$) and opening ($ot_{1/2}$) half-times varied between grass species (Table 1). *M. maximus* had a lower $ot_{1/2}$ relative to the other species (Figure 4a-b), consistent with its lower a_{op} and SS (Figure 2a-b). In disagreement with our *Hypothesis 3*, $ct_{1/2}$ showed no consistent response to growth conditions. In addition, $ot_{1/2}$ was not affected by growth [CO₂] and was marginally lower ($p = 0.0609$) under LL in only two C₄ species relative to the control treatment (Figure 4a-b, Tables 1 and S3). $ct_{1/2}$ and $ot_{1/2}$ were not correlated, but $ot_{1/2}$

positively correlated with a_{op} ($r^2 = 0.378$, Figure 4c) and to a lesser extent with SS ($r^2 = 0.284$), g_s ($r^2 = 0.283$) and g_{max} ($r^2 = 0.260$), such that smaller and relatively closed stomata opened faster in response to increased light intensity during measurements (Table 2).

Growth conditions had no significant effect on calculated water loss during transition from HL to LL (excessive transpiration, ΔW) or calculated CO_2 loss during transition from LL to HL (forgone photosynthesis, ΔC) (Tables 1 and S3). ΔW and ΔC correlated with $ct_{1/2}$ and $ot_{1/2}$, respectively (Table 2). ΔW correlated positively with a_{op} ($r^2 = 0.397$, Figure 4d) and negatively with A_{sat} , while ΔC correlated positively with g_{sat} and negatively with SD (Table 2). During light transitions, the C_3 grass tended to lose more water under all treatments and fix less CO_2 under high light relative to the C_4 grasses (Table S3).

Guard cell K^+ flux was affected similarly by gCO_2 but differently by LL in C_3 and C_4 grasses

P. bisulcatum (C_3) maintained a higher net K^+ influx rate into guard cells compared to the three C_4 grasses under HL conditions (Figure 5a, Tables 1 and S3). Relative to the control treatment, net K^+ influx rate was higher under gCO_2 for all four species and lower under LL only in *P. bisulcatum* (Figure 5a, Tables 1 and S3). Net K^+ influx positively correlated with g_s ($r^2 = 0.563$, Figure 5b), a_{op} ($r^2 = 0.830$, Figure 5b), g_{max} ($r^2 = 0.669$), ΔW ($r^2 = 0.588$), and OD ($r^2 = 0.365$) (Table 2).

Determinants of $iWUE$ across C_3 and C_4 grasses

Overall, $iWUE$ showed strong negative correlations with g_s ($r^2 = 0.398$, Figure 6b), a_{op} ($r^2 = 0.472$, Figure 6c) and guard cell K^+ influx ($r^2 = 0.587$, Figure 6d), and weakly correlated with OD (+ve, $r^2 = 0.213$) and A_{net} (-ve, $r^2 = 0.179$, Figure 6a) (Table 2). When the key stomatal traits were analysed by PCA, $iWUE$ and g_s were diametrically opposite (Figure 6e). The parameters a_{op} , g_s , g_{max} and K^+ influx clustered together, while a_{max} , g_{sat} and SI clustered together. The first (a_{op}) cluster was flanked by SS/ $ot_{1/2}$ and SD/ $ct_{1/2}$. Thus, gCO_2 had an opposite and stronger influence on stomatal traits than LL in the tested grass species (Figure 6e).

DISCUSSION

Determinants of grass stomatal conductance and responses

This study used eight closely-related C₃ and C₄ grasses to explore stomatal characteristics and acclimation to low [CO₂] and low light, and how these affect stomatal conductance (g_s) and intrinsic leaf water use efficiency ($iWUE$). C₄ grasses tended to have lower g_s , operational stomatal aperture (a_{op}) and guard cell K⁺ influx rate, and higher CO₂ assimilation rates (A_{net}) and $iWUE$ relative to C₃ grasses. In addition, g_s was equally sensitive to gCO_2 among the grasses, but was more sensitive to LL in C₄ than C₃ species. Stomatal size (SS) and stomatal density (SD) showed no trend between the two photosynthetic types. These results confirm what was previously reported in a small subset of species possessing dumbbell-shaped stomata (Maherali *et al.*, 2002; Malone *et al.*, 1993).

More significant than the differences in these species were the overall correlations and how they varied with the growth environment. For all species, g_s depended most on a_{op} , density of open stomata (OD) and stomatal opening half-time ($ot_{1/2}$). In turn, a_{op} was largely predicted by the guard cell K⁺ influx rate ($r^2 = 0.830$, Figure 5b), and correlated with $ot_{1/2}$ and SS. Although C₄ grasses generally operated with lower g_s and a_{op} at current aCO_2 , they showed a greater potential to open stomata (g_{sat}) relative to maximal stomatal conductance (g_{max}), indicating heightened stomatal sensitivity and control as a result of the C₄-CCM. Finally, species with more stomata (higher SD) had smaller stomata (lower SS) and opened their stomata faster on transition to HL (lower $ot_{1/2}$).

Stomatal aperture and guard cell K⁺ influx

During stomatal opening and closing, K⁺ fluxes across the guard cell membranes are key determinants of the complex signalling pathways in response to variations of CO₂, light and other stimuli (Kim *et al.*, 2010). Hence, the strong correlations of a_{op} and g_s measured under growth conditions with guard cell K⁺ influx (Table 2, Figure 5) are based on well-understood guard cell function, whereby K⁺, anion and solute influx are accompanied by H₂O uptake leading to stomatal opening (Chen *et al.*, 2017; Chen *et al.*, 2012). What is particularly significant in our study is that g_s measured *in planta* using leaf gas exchange correlated well with K⁺ influx measured on epidermal peels (Figure 5). This indicates that K⁺ homeostasis of guard cells of isolated epidermal peels generally reflects *in vivo* leaf gas exchange. This was

true for the C₃ grass under all conditions, and for C₄ grasses under control and gCO₂, but not LL, conditions.

Stomata have distinct responses and signalling pathways for CO₂ and light (Assmann and Jegla, 2016; Engineer *et al.*, 2016). In our study, higher guard cell K⁺ influx at gCO₂ relative to aCO₂ was proportional to g_s, indicating a common gCO₂ effect towards increased guard cell K⁺ uptake and signalling response in grasses. However, there was different stomatal acclimation and possibly signalling responses to light between C₃ and C₄ grasses because K⁺ influx was not affected by growth at LL in C₄ grasses while it decreased in proportion with g_s in the C₃ grass (Figure 5). These results may have useful implications for future investigation on the way stomata respond and acclimate to CO₂ and light in C₃ and C₄ grasses.

In addition to different signalling mechanisms, possible explanations for the differential stomatal responses to [CO₂] relative to light among the grass species may relate to guard cell wall properties. In vascular plants, stomatal cell walls undergo reversible deformation during opening and closing, requiring a strong and flexible structure. The composition (e.g., cellulose, pectin, lignin, phenolics) of guard cell wall differs between phylogenetic groups impacting stomatal function (Rui *et al.*, 2018). Noteworthy, whilst C₃ wheat and C₄ sorghum possess similar type III dumbbell shaped stomata, wheat guard cells exhibited different relative retardance (cellulose crystallinity) than sorghum (Shtein *et al.*, 2017). The relevance of these differences to other C₃ and C₄ grass species is yet to be established. Moreover, polar stiffening is emerging as a greater player in guard cell movement than radial stiffening (Carter *et al.*, 2017). Understanding how signalling pathways and cell wall composition of guard cells differ between C₃ and C₄ grasses and acclimate to the environment may potentially be exploited for crop improvement and will be the subject of future research.

Stomatal opening speed

Stomata constantly respond to changing light intensity on a scale of seconds through to seasons. Stomatal opening and closing usually occur at a slower rate than photosynthetic activation (Henry *et al.*, 2020), creating asynchrony between CO₂ uptake and H₂O loss which compromises *iWUE*. Hence, dynamic stomatal responses are critical for the optimisation of *iWUE*, in addition to advanced stomatal morphology and patterning (Cai *et al.*, 2017; Lawson

and Vialet-Chabrand, 2019; Slattery *et al.*, 2018; Vialet-Chabrand *et al.*, 2017). In this study, leaves with more open stomata (higher a_{op}) tended to have lower $iWUE$, slower opening (i.e., greater $ot_{1/2}$), larger excessive transpiration and forgone photosynthesis on transition to HL (Tables 2, S2 and S3). Under gCO_2 and LL, stomatal opening was generally faster (i.e., smaller $ot_{1/2}$) in CO_2 -saturated C_4 grasses relative to their C_3 counterparts (Figure 4b). In contrast, CO_2 -limited C_3 leaves showed slower stomatal opening at gCO_2 , possibly due to hydraulic constraints as highlighted below.

C_4 photosynthesis is more complex than C_3 photosynthesis, involving both C_3 and C_4 metabolic cycles operating across two photosynthetic tissues (Hatch, 1987). Hence, efficient C_4 photosynthesis requires the establishment of substantial metabolite gradients (Arrivault *et al.*, 2016; Leegood and von Caemmerer, 1988). Accordingly, it has been shown that C_4 plants generally have less photosynthetic efficiency and productivity than C_3 plants under dynamic irradiance, such as sun flecks (Kubásek *et al.*, 2013). However, a thorough examination, revealed that C_4 photosynthesis is buffered against light transients due to the flexible use of the C_4 acid decarboxylases (Bellasio and Griffiths, 2013; Wingler *et al.*, 1999) and reducing equivalents stored in the large metabolite gradients (Slattery *et al.*, 2018). Our results concur with the latter consideration, and further suggest that C_4 grasses can efficiently utilise high light transients due to their highly responsive stomata. Amongst the three subtypes of C_4 grasses, the PCK species showed faster stomatal opening in response to HL. The PCK subtype is characterised by high photosynthetic efficiency at LL (Sagun *et al.*, 2019; Sonawane *et al.*, 2018) and more efficient Rubisco (Sharwood *et al.*, 2016). Moreover, the two PCK species had smaller stomatal size, which is in line with the general view that the rapidity of g_s responses in dumbbell-shaped guard cells in grasses could be attributed to size (McAusland *et al.*, 2016).

Stomatal closing speed

Stomatal $ct_{1/2}$ showed no consistent relationship with measured parameters regardless of photosynthetic type or growth conditions. Rather, C_3 and NAD-ME grasses had more excessive transpiration (ΔW) due to their slower stomatal closure rate on transition to LL. This may highlight the CO_2 limitation of the C_3 *versus* C_4 pathway and of NAD-ME *versus* NADP-ME and PCK subtypes (Pinto *et al.*, 2016; Sonawane *et al.*, 2018). Slower stomatal closing rate may also be related to hydraulic conductivity (Taylor *et al.*, 2018). Although

stomatal $ct_{1/2}$ did not directly correlate with any parameter (other than $ot_{1/2}$), there were some interesting observations. Firstly, ΔW derived from $ct_{1/2}$ and transpiration rates at initial and final steady states, correlated strongly with guard cell K^+ influx rate ($r^2 = 0.588$). Secondly, grass leaves with more frequent stomata (high SD) showed less forgone photosynthesis (ΔC) on opening to HL ($r^2 = 0.292$). Among C_3 kidney-shaped stomata, ΔW and stomatal closing were not correlated (Deans *et al.*, 2019). These aspects warrant further investigation using a larger set of C_3 and C_4 species.

Trade-off between SS and SD

So far, we argued that g_s responses were dominated by a_{op} and underpinned by guard cell K^+ fluxes. Moreover, a_{op} correlated with SS (Figure 3b). Smaller SS is a well-characterized strategy to speed up solute flux in and out of guard cells by increasing the surface area to volume ratio (Chen *et al.*, 2012), and shown to occur in several species (Lawson and Blatt, 2014; Papanatsiou *et al.*, 2016; Tanaka *et al.*, 2013). However, SS exhibited no significant correlations with g_s , g_{sat} or g_{max} in our study (Table 2). Hence, the advantage of grass stomata structure may enable small changes in guard cell width to translate into larger changes in a_{op} (Hetherington & Woodward, 2003; Franks & Farquhar, 2007). This strategy enables grass stomata to rapidly respond under gCO_2 .

In line with other studies (Franks and Beerling, 2009a; Franks *et al.*, 2009; Franks *et al.*, 2012), SS negatively correlated with SD in our study (Figure 3c). Notably, the two PCK species tended to have smaller SS and higher SD relative to the other C_4 grasses (Figure 2). The particular interest in SD stems from its apparent environmental plasticity and the prospect that genetic manipulation of SD may impact crop *iWUE* (Buckley *et al.*, 2020). Indeed, genetically manipulated C_3 plants such as rice and wheat with moderate SD reductions were shown to conserve water without suffering a yield penalty (Caine *et al.*, 2019; Dunn *et al.*, 2019). In the current study, SD was generally not affected by gCO_2 , and could not predict g_s . Only when we accounted for the frequency of open stomata, OD, a good relationship was obtained between OD and g_s (Table 2). These results agree with the conclusions made by (Dow *et al.*, 2014a) that g_s response to $[CO_2]$ is a pore-specific property that is scaled by SD. In our study, OD was a more physiologically relevant attribute than SD.

It has been predicted that g_{max} adapts to long term changes in atmospheric $[CO_2]$ by adjustments in SS and/or SD. In particular, growth at elevated $[CO_2]$ reduces photosynthetic

capacity in C_3 plants, and it is suggested that optimisation of operational g_s requires concomitant reduction in g_{max} to keep it within the sensitive range of guard cell osmotic pressure (Franks *et al.*, 2012). However, our data do not support the predictions about SS and SD adjustments as they both changed little under gCO_2 or LL, even in C_3 species (Figure 2). Our results agree with other studies using C_4 grasses grown at low $[CO_2]$ (Maherali *et al.*, 2002), as well as a large survey of published studies at elevated $[CO_2]$ where SD varied by an average of 5% whilst g_s declined by 22% (Ainsworth and Rogers, 2007). Therefore, the global inverse relationship between SD and atmospheric $[CO_2]$ documented in the fossil records may partly be due to the larger changes in $[CO_2]$ across the geological timescale (Franks and Beerling, 2009b; Woodward, 1987; Woodward and Kelly, 1995), or to variations among species (Aasamaa and Aphalo, 2016; Evans-Fitz Gerald *et al.*, 2016), leaf positions or sun/shade aspects (Poole *et al.*, 1996).

Maximal stomatal conductance

In this study, we estimated maximal stomatal conductance anatomically (g_{max}) by measuring SD and a_{max} , and physiologically (g_{sat}) by measuring leaf gas exchange at low CO_2 and high light (Figure 1). g_{sat} showed no correlation with any measured stomatal traits, while g_{max} weakly correlated with g_s , SD and $ot_{1/2}$ (Table 2). These results partially fit with work using Arabidopsis SD mutants demonstrating that operational stomatal conductance can be estimated as a function of g_{max} , independently of SD (Dow and Bergmann, 2014; Dow *et al.*, 2014a; Dow *et al.*, 2014b). For the two C_3 species, g_s was a constant fraction of g_{sat} and g_{max} when measured at aCO_2 , while g_s/g_{sat} was higher at gCO_2 (Figures 1f-h, Tables 1 and S1). For the C_4 species, the g_s/g_{sat} and g_s/g_{max} ratios were higher at gCO_2 and lower at LL. Moreover, the C_3 species had higher g_s/g_{sat} while the C_4 species had higher g_{sat}/g_{max} (Figures 1f-h, Tables 1 and S1). These results indicate that while C_3 grasses operate with higher g_s , the C_4 grasses have a greater potential (measured as g_{sat}) for stomatal opening relative to their g_{max} . In turn, these results indicate the greater sensitivity of C_4 stomata to open at gCO_2 which reflects both their low CO_2 -limitation due to the CCM and their reduced risk of hydraulic failure at low CO_2 . Both factors allow C_4 stomata to open more in response to gCO_2 (Pinto *et al.*, 2014). These conclusions are supported by previous work showing that g_s was more sensitive to changing intercellular CO_2 in C_4 relative to C_3 species within the dicot genus *Flaveria* (Huxman and Monson, 2003; Vogan and Sage, 2011).

583

584 ***Determinants of grass iWUE***

585 Across all species and treatments, *iWUE* was most affected by g_s , a_{op} and guard cell K^+
 586 influx, which all strongly correlated among each other. Overall, *iWUE* correlated more
 587 strongly with g_s than A_{net} . The correlation with A_{net} here was stronger than what has been
 588 previously reported in other studies utilising C_4 grasses (Cano *et al.*, 2019). C_4 photosynthesis
 589 is largely CO_2 -saturated at aCO_2 , hence changes in g_s have little effect on A_{net} , but large
 590 effects on *iWUE* (Ghannoum, 2016; Li *et al.*, 2017). In contrast, *iWUE* is strongly affected by
 591 both A_{net} and g_s in C_3 plants (Farquhar and Sharkey, 1982). The inclusion of two C_3 grasses
 592 and the gCO_2 treatment has strengthened the influence of A_{net} on *iWUE* in our set of mostly
 593 C_4 grasses. Consequently, *iWUE* increased as A_{net} increased and g_s , a_{op} , SS and K^+ flux
 594 decreased (Figure 6).

595 **Conclusions**

596 In conclusion, we reveal new insight on the variation in stomatal behaviour of C_3 and C_4
 597 grasses, highlighting both conserved and distinct responses determined by photosynthetic
 598 type (C_3 and C_4) and environmental condition (gCO_2 and LL). Across species and treatments,
 599 stomatal conductance (g_s) and *iWUE* correlated well with guard cell K^+ influx and a_{op} , but
 600 poorly with SD. Grasses with smaller stomata had smaller a_{op} and faster stomatal opening on
 601 transition to HL, which allowed them to maintain greater steady-state and dynamic *iWUE*. In
 602 addition, C_4 grasses exhibited different responses of g_s and guard cell K^+ influx to LL
 603 compared to C_3 stomata allowing them to maintain higher *iWUE* at LL. These mechanistic
 604 links between stomatal traits highlight future avenues for improving photosynthesis and water
 605 use efficiency via improved understanding of stomatal function in C_3 and C_4 cereal species.

606

607 **AUTHOR CONTRIBUTION**

608 All authors contributed to experimental design. WKI and AJWL carried out the experimental
 609 work and data analysis. WKI and OG wrote the manuscript with contribution from AJWL
 610 and ZHC.

611

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Table 1 Statistical analysis summary of the main effects and interactions of CO₂, light, species, and photosynthetic type or subtype. For leaf gas exchange and stomatal morphology parameters, two separate *glm* analyses were used with eight grass species nested in either Type (2 C₃ and 6 C₄) or Subtype (n=2 in each of the three subtypes). Species was used as a random effect while the functional type was utilised as the fixed effect. For stomatal kinetics and guard cell K⁺ influx parameters the analysis used species (n=4), [CO₂] light. Bold numbers indicate significant effect at $p \leq 0.05$.

Parameters	Treatment effects (<i>p</i>)			C ₃ vs C ₄ comparison (<i>p</i>)				C ₄ subtype comparison (<i>p</i>)			
	[CO ₂]	Light	[CO ₂] × Light	Type	[CO ₂] × Type	Light × Type	[CO ₂] × Light × Type	Subtype	[CO ₂] × Subtype	Light × Subtype	[CO ₂] × Light × Subtype
<i>Gas exchange</i>											
CO₂ assimilation rate, A _{net} (μmol CO ₂ m ⁻² s ⁻¹)	0.0053	<0.0001	0.0737	<0.0001	0.4121	<0.0001	0.8632	0.1136	0.9376	0.8394	0.8939
Stomatal conductance, g _s (mol H ₂ O m ⁻² s ⁻¹)	0.0010	<0.0001	0.0150	<0.0001	0.2407	0.0457	0.0975	0.3982	0.2417	0.2116	0.1492
Intrinsic WUE (A_{net}/g_s), iWUE (μmol [CO ₂] (mol H ₂ O) ⁻¹)	0.0003	0.7736	0.7377	<0.0001	0.0030	0.0796	0.1999	0.1466	0.3154	0.4223	0.3670
Intercellular [CO₂], C _i (μmol mol ⁻¹)	0.0004	0.0387	0.0910	<0.0001	0.1037	0.8701	0.7850	0.5611	0.9984	0.6518	0.7473
Light-saturated assimilation rate, A _{sat} (μmol CO ₂ m ⁻² s ⁻¹)	0.6187	0.0540	0.2764	<0.0001	0.6252	0.5549	0.8241	0.4117	0.9679	0.7549	0.8314
Light-saturated maximum conductance, g_{sat} (mol H ₂ O m ⁻² s ⁻¹)	0.3722	0.0229	0.8880	0.0370	0.1063	0.8643	0.4035	0.6205	0.5288	0.0791	0.1526
Ratio of g_s to g_{sat} (g_s/g_{sat})	<0.0001	<0.0001	0.0001	<0.0001	0.0553	0.0159	0.9640	0.6836	0.0021	0.0199	0.1069
Ratio of g_s to g_{smax} (g_s/g_{smax})	0.0281	0.0001	0.3615	0.0056	0.8843	0.5803	0.6493	0.6264	0.5774	0.4936	0.5068
Ratio of g_{sat} to g_{smax} (g_{sat}/g_{smax})	0.0684	0.3994	0.8289	0.0522	0.3436	0.4537	0.8816	0.4264	0.8566	0.3527	0.5509
<i>Stomatal morphology</i>											
Stomatal aperture, a_{op} (μm ²)	0.0004	0.0122	0.0512	0.0014	0.5394	0.8786	0.7544	0.0007	0.9098	0.0296	0.5506
Stomatal size, SS (μm)	0.0290	0.1646	0.4172	0.1003	0.5280	0.5713	0.5369	0.0105	0.7969	0.2765	0.7675
Stomatal index, SI (%)	0.2515	0.0368	0.0271	0.8142	0.8235	0.8395	0.7259	0.0002	0.8411	0.6705	0.8260
Stomatal density, SD (mm ⁻²)	0.2852	0.0316	0.5648	0.6816	0.6819	0.9819	0.8269	0.0635	0.9113	0.5053	0.9388
Density of open stomata, OD (mm ⁻²)	0.0516	0.0185	0.7977	0.0099	0.8417	0.6593	0.4327	0.1050	0.9179	0.4943	0.7076
Maximum stomatal aperture, a _{max} (μm ²)	0.2066	0.1403	0.2313	0.7906	0.5810	0.3568	0.6479	0.0631	0.9103	0.3347	0.9822
Maximum anatomical conductance, g _{smax} (mol H ₂ O m ⁻² s ⁻¹)	0.0326	0.1596	0.1662	0.6725	0.6725	0.4793	0.9363	0.7078	0.8140	0.4142	0.7898

<i>Stomatal kinetics and K⁺ flux</i>	Main effects (<i>p</i>)			Interactions (<i>p</i>)			
	[CO ₂]	Light	Species	[CO ₂] × Light	[CO ₂] × Species	Light × Species	[CO ₂] × Light × Species
Closing half-time, ct_{1/2} (min)	0.4239	0.6658	<0.0001	0.6811	<0.0001	<0.0001	0.0001
Opening half-time, ct_{1/2} (min)	0.8976	0.0609	<0.0001	0.7284	0.0023	0.0062	0.0018
Excessive transpiration, ΔW (mol m⁻²)	0.3888	0.1440	<0.0001	0.2505	0.0473	<0.0001	0.4580
Forgone photosynthesis, ΔC (mmol m⁻²)	0.2408	0.2077	<0.0001	0.6223	0.0274	<0.0001	0.6005
Net K⁺ influx, K⁺ flux (nmol m⁻² s⁻¹)	<0.0001	<0.0001	<0.0001	NA	<0.0001	0.0032	NA

Figure Legends

Fig. 1 Effects of glacial [CO₂] and/or low light on leaf gas exchange parameters of two C₃ and six C₄ grasses (two of each subtype): **(a)** Net CO₂ assimilation rate at growth conditions (A_{net}), **(b)** stomatal conductance at growth conditions (g_s), **(c)** maximum stomatal conductance calculated from anatomical traits (g_{max}), **(d)** light-saturated stomatal conductance at g_{CO_2} (g_{sat}), and **(e)** intrinsic water-use efficiency ($iWUE = A_{net}/g_s$) measured at growth conditions. Data represent means $\pm$ SE ($n = 2$ C₃ or 6 C₄) with species as replicate within each group. Means with the same letter are not significantly different at $p \leq 0.05$ using Tukey's HSD *post hoc*. Letters on top of the columns describe treatment effect for each group. Letters inside the columns indicate group differences at each of the growth condition. Plots **f-h** show the distribution of **(f)** g_{sat}/g_{max} , **(g)** g_s/g_{sat} and **(h)** g_s/g_{max} . The box and whiskers represent the 25-75 and 0-100 percentiles, respectively. The square and line inside the box represent the mean and median of the data, respectively.

Fig. 2 Acclimation of stomatal traits to glacial [CO₂] and/or low light in two C₃ and six C₄ grasses (two of each subtype): **(a)** operational stomatal aperture (a_{op}), **(b)** stomatal size (SS), **(c)** stomatal density (SD), **(d)** density of open stomata (OD), **(e)** stomatal index (SI). Data represent means $\pm$ SE ($n=2$ species in each group). Means with the same letter are not significantly different at $p \leq 0.05$ using Tukey's HSD *post hoc*. Letters on top of the columns describe treatment effect for each group. Letters inside the columns indicate group differences at each of the growth condition.

Fig. 3 Relationships between various stomatal traits among two C₃ and six C₄ grasses (two of each subtype) acclimated to glacial [CO₂] and/or low light. Points represent the species mean $\pm$ SE at each growth condition, which were indicated by different colours. Fitted linear models are as follows: **(a)** $g_s = 0.0067 \times a_{op} + 0.0742$; **(b)** $a_{op} = 0.0485 \times SS + 8.3318$; **(c)** $SD = 176.19 - 0.1041 \times SS$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Shaded regions represent the 95% confidence interval of the linear model using all the data.

Fig. 4 Stomatal **(a)** closing half-time and **(b)** opening half-time in response to light transitions for four grass species acclimated to glacial [CO₂] and/or low light. Means with the same letter are not significantly different at $p \leq 0.05$ using Tukey's HSD *post hoc*. Letters on top of the columns describe treatment effect for each species. Letters inside the columns indicate species differences at each of the growth condition. Species abbreviations: *P. bisulcatum* (PB, C₃), *P. miliaceum* (PM, C₄-NAD-ME), *P. antidotale* (PA, C₄-NADP-ME) and *M. maximus* (MM, C₄-PCK). Linear correlations **(c)** between opening half-time and stomatal aperture and **(d)** between excessive transpiration and stomatal aperture. Fitted linear models are as follows: **(c)** $O_{t1/2} = 0.021 \times a_{op} + 0.3165$; **(d)** $\Delta W = 1.3594 \times a_{op} + 12.634$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fig. 5 Net K^+ influx of guard cells (**a**) and its relationship with stomatal conductance (**b**) and stomatal aperture (**c**) in four grass species acclimated to glacial $[CO_2]$ or low light. Values are means $\pm$ SE of $n=7-9$ stomata and ranked using Tukey's HSD *post hoc* test at $p \leq 0.05$. Fitted linear models are as follows: (**b**) $g_s = 0.0003 \times K^+flux + 0.1319$, and (**c**) $a_{op} = 0.0494 \times K^+flux + 12.87$. Other details are described for Figure 4.

Fig. 6 Relationships of intrinsic water-use efficiency ($iWUE$) with (**a**) net photosynthetic rates, (**b**) stomatal conductance, (**c**) stomatal aperture, and (**d**) net K^+ guard cell influx in eight grasses grown at glacial $[CO_2]$ or low light. Points represent species means at each growth condition $\pm$ SE and shaded regions represent the 95% confidence interval of the linear models. Fitted linear model equations were as follows: (**a**) $iWUE = 1.6167 \times A_{net} + 43.216$; (**b**) $iWUE = 117.36 - 149.8 \times$; (**c**) $iWUE = 126.04 - 1.5842 \times a_{op}$; and (**d**) $iWUE = 125.27 - 0.0829 \times K^+flux$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (**e**) PCA biplot of stomatal traits underpinning $iWUE$ was generated using *pca.comp* and visualised using *ggbiplot* in R. The influence of key traits on the principle components separating each sample, across the two principle components accounting for the largest variation. Arrows show the direction and strength of the influence of each trait, while the black circles show where each sample falls across the two principle components.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6