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2 **A dynamic hydro-mechanical and biochemical model of stomatal**
3 **conductance for C₄ photosynthesis**

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10 **Abstract**

11 C₄ plants are major grain (maize, sorghum), sugar (sugarcane) and biofuel (*Miscanthus*) producers,
12 and contribute ~20% to global productivity. Plants lose water through stomatal pores in order to
13 acquire CO₂ (assimilation, *A*), and control their carbon-for-water balance by regulating stomatal
14 conductance (*g_s*). The ability to mechanistically predict *g_s* and *A* in response to atmospheric CO₂,
15 water availability and time is critical for simulating stomatal control of plant-atmospheric carbon
16 and water exchange under current, past or future environmental conditions. Yet, dynamic
17 mechanistic models for *g_s* are lacking, especially for C₄ photosynthesis. We developed and coupled
18 a hydro-mechanical model of stomatal behaviour with a biochemical model of C₄ photosynthesis,
19 calibrated using gas exchange measurements in maize, and extended the coupled model with time-
20 explicit functions to predict dynamic responses. We demonstrated the wider applicability of the
21 model with three additional C₄ grass species in which interspecific differences in stomatal
22 behaviour could be accounted for by fitting a single parameter. The model accurately predicted
23 steady-state responses of *g_s* to light, atmospheric CO₂ and O₂, soil drying and evaporative demand,
24 as well as dynamic responses to light intensity. Further analyses suggest the effect of variable leaf
25 hydraulic conductance is negligible. Based on the model, we derived a set of equations suitable for
26 incorporation in land surface models. Our model illuminates the processes underpinning stomatal
27 control in C₄ plants and suggests the hydraulic benefits associated with fast stomatal responses of
28 C₄ grasses may have supported the evolution of C₄ photosynthesis.

29 **Keywords**

30 Mechanistic model, water use efficiency, transpiration, assimilation, time, maize.

31 **Running title**

32 C₄ stomatal model

33 Introduction

34 C₄ photosynthesis is a variant of the conventional C₃ photosynthetic pathway and evolved in hot,
35 open, semi-arid environments to reduce photorespiratory energy losses. Despite being the
36 photosynthetic type of ~3 % of species, C₄ plants contribute over 20 % to Earth's net primary
37 productivity (NPP, abbreviations listed in Table 1) (Ehleringer et al., 1997). Moreover, maize (*Zea*
38 *mays*, L.), a C₄ plant of the NADP-ME subtype, is the leading grain production cereal (FAO, 2012).
39 C₄ photosynthesis is shared between mesophyll (M) and bundle sheath (BS) cells, which are
40 coupled to allow the operation of a biochemical CO₂-concentrating mechanism (CCM) working
41 through an ATP-dependent carboxylation–decarboxylation cycle (Bellasio, 2017). The CCM
42 minimizes photorespiration by increasing the CO₂ concentration in the BS (C_{BS}), where Rubisco is
43 exclusively expressed, allowing high assimilation (A) at low rates of transpiration (E).
44 Consequently, C₄ plants, have higher photosynthetic water-use efficiency ($WUE=A/E$) compared
45 with C₃ plants (Ward et al., 1999; Anderson et al., 2001; Taylor et al., 2011; Cunniff et al., 2016).

46 Estimating fluxes of carbon and water in and out of plants is important for predicting NPP, and
47 studying plant responses to past and future environmental change (Ostle et al., 2009; de Boer et al.,
48 2011; Bonan et al., 2014; Paschalis et al., 2017). The pathway of CO₂ into the plant through
49 stomatal pores is the same as that for water vapour out, and plants regulate their carbon-for-water
50 budget by adjusting stomatal conductance (g_s). Stomata respond, not exclusively, to light,
51 temperature, atmospheric humidity and CO₂ concentration ($[CO_2]$) and the amount of water
52 supplied to and within leaves from the soil (Jarvis, 1976). Compared with C₃ species, C₄
53 photosynthesis is more sensitive to changes in g_s , leading to increased sensitivity to soil drying if
54 plant hydraulic conductance (K_{PLANT}) is not maintained (Ghannoum, 2009; Taylor et al., 2010;
55 Volder et al., 2010; Taylor et al., 2011; Osborne and Sack, 2012). It was recently shown that leaf
56 hydraulic conductivity (K_{LEAF}) is a critical bottleneck in the whole-plant hydraulic pathway and that
57 K_{LEAF} is light-dependent (Sack and Holbrook, 2006; Osborne and Sack, 2012). Because stomatal
58 movements are sensitive to leaf water status, g_s may in turn be influenced by the dynamics of K_{LEAF}
59 mediated by water availability at leaf level, but these effects have not been quantified. Moreover,
60 the stomatal and non-stomatal mechanisms governing the sensitivity of C₄ photosynthesis to soil
61 drying are not well understood and the dynamic feedbacks between soil moisture, g_s and A are
62 largely unknown (Wand et al., 1999; Ghannoum et al., 2000; Wand et al., 2001; Ghannoum, 2009).

63 Leaves continuously experience light- and shade-flecks with large transient variations in incident
64 light intensity ($PPFD$) due to changes in cloud cover, or shading by other leaves. Stomata and
65 photosynthesis continually respond to changes in $PPFD$, but the timing of stomatal and assimilation
66 responses is generally not synchronised, because stomatal movements can be an order of magnitude
67 slower than photosynthetic responses (McAusland et al., 2016). This lack of coordination between

68 carbon gain and water loss often results in suboptimal *WUE*, and photosynthetic losses (Lawson and
69 Blatt, 2014).

70 The importance of g_s at canopy, ecosystem and global scales is recognised, and models
71 describing stomatal behaviour coupled to leaf-level biochemical photosynthesis models form a
72 critical component of vegetation models (Ostle et al., 2009; Berry et al., 2010; Bonan et al., 2014;
73 Beerling, 2015; Sato et al., 2015). Within vegetation models, A is often predicted for C_3 and C_4
74 plants using sub-models dating from the 1980s (Berry and Farquhar, 1978), which have since been
75 updated (Yin and Struik, 2009; Yin and Struik, 2015). Photosynthesis models are generally coupled
76 to stomatal sub-models in order to estimate g_s from environmental or internal variables.
77 Historically, these stomatal models have been almost exclusively empirical or phenomenological,
78 and are often calibrated under non-limiting conditions (Ball et al., 1987; Collatz et al., 1992;
79 Leuning, 1995; Damour et al., 2010; Way et al., 2011). Empirical models may lose accuracy the
80 further the simulated conditions deviate from those under which the models were calibrated (Way et
81 al., 2011), and cannot provide insight into underlying physiological mechanisms (Buckley, 2017).

82 Alternatively, g_s may be simulated by defining the optimal trade-off between carbon gain and
83 water use (Givnish and Vermeij, 1976; Cowan and Farquhar, 1977; Damour et al., 2010; Manzoni
84 et al., 2013; Buckley et al., 2016; Paschalis et al., 2017). So-called optimality models have potential
85 application beyond plant-level, but lack biophysical underpinning and assume unlimited phenotypic
86 plasticity in response to environmental drivers, which limits their applicability in modelling plant
87 responses to atmospheric $[CO_2]$ and climate change (de Boer et al., 2011; Manzoni et al., 2013;
88 Buckley and Schymanski, 2014). In contrast, mechanistic models are underpinned by the
89 physiological mechanisms of stomatal functioning, but there are currently no such models coupled
90 with a biochemical model of C_4 photosynthesis.

91 Our objectives were to address three outstanding challenges. First, to develop and extend an
92 existing process-based framework for modelling stomatal conductance (Buckley et al., 2003;
93 Rodriguez-Dominguez et al., 2016) to C_4 species; second, to enable the resulting model to respond
94 to dynamic changes in *PPFD*; and third, to interrogate the model to broaden understanding of
95 stomatal behaviour in C_4 plants. First, we coupled a hydro-mechanical model of stomatal behaviour
96 with biochemical sub-models for enzyme-limited and light-limited C_4 photosynthesis that were re-
97 derived for this work. Then we developed and included sub-models accounting for CO_2 diffusion
98 through a finite mesophyll conductance, non-stomatal limitations, uneven transitions between
99 limitations to photosynthesis, acclimation of turgor pressure, and the effects of a light-dependent
100 induction of K_{LEAF} on g_s . Finally, we introduced time-explicit functions to simulate non steady-state
101 responses to dynamic environmental stimuli. We calibrated the model with a comprehensive gas
102 exchange experiment in C_4 maize and on three C_4 grasses and compared it against published
103 datasets to explore responses to soil water potential, $[CO_2]$, evaporative demand and *PPFD*.

105 Results

106 *Model calibration and output overview*

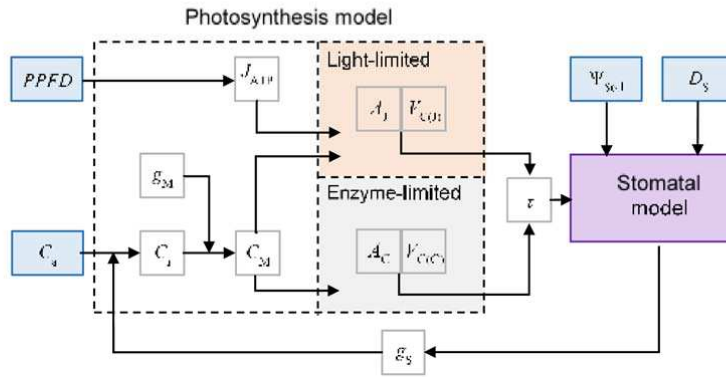
107 The coupled modelling scheme is depicted in Figure 1 to highlight which quantities are used as
108 model inputs and which are stated variables used to calculate photosynthetic and stomatal
109 responses. We derived photosynthetic parameters using data obtained from nine sets of
110 measurements on maize (*Zea mays* L., Table 1, see also Table S2, the full dataset is reported in
111 Supporting Information File S2), and three to eight sets of measurements on three C₄ grasses
112 (*Eragrostis curvula*, *Heteropogon contortus* and *Themeda triandra*, Table S3). Stomatal
113 movements are driven by both biochemical (ATP) and hydro-mechanical forcing, the latter of
114 which includes guard cell responses to the water status and turgor of the leaf, which are closely
115 related to K_{PLANT} and K_{LEAF} . In the stomatal component of the model, the biochemical driver of
116 stomatal responses is τ , a quantity related to the concentration of ATP in BS and M chloroplasts.
117 The influence of biochemical factors relative to hydro-mechanical forcing is determined by the
118 parameter β , while stomatal morphology is described by χ .

119 Below, we describe the modelled response of A , g_s , and key variables in their calculation, to
120 environmental drivers such as light, [CO₂] and soil water potential. We show the modelled dynamic
121 responses of A and g_s to transient changes in light intensity that represent leaf exposure to light-
122 flecks. Finally, we discuss a theoretical scenario in which K_{LEAF} was allowed to vary in response to
123 $PPFD$ in order to mimic the activation of aquaporins occurring upon transition from dark to light.

124 *Responses to irradiance*

125 Simulated stomatal conductance (g_s) and CO₂ assimilation (A) both increased non-linearly with
126 $PPFD$ (Figure 2A and C). To simulate $PPFD$ responses, external [CO₂], C_a was set at 400 μmol
127 mol^{-1} and the simulations were driven by varying $PPFD$ input at 150 discrete increments at which
128 C_M was iteratively fitted each time (Figure 1). The simulations compare well with maize data taken
129 from Bellasio et al. (2016a), (Figure 2, circles), measured under the same C_a and at eight $PPFD$
130 levels between 30 and 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Although here, A was overestimated at high $PPFD$, due to
131 a lower J_{ATPMAX} in the data of Bellasio et al. (2016a). This dataset is ideal for comparison with our
132 model because the measurements were taken using long acclimation time (>12 min) between $PPFD$
133 steps (and C_a steps, see below), meaning stomatal responses were likely captured at steady-state.
134 Stomatal responses were also simulated by coupling the photosynthetic sub-model with the stomatal
135 sub-model of Collatz et al. (1992), parameterised after Collatz et al. (1992), and shown for
136 comparison in Figure 2C. The enzyme- and light-limited potential rates of carboxylation [$V_{\text{C(C)}}$ and
137 $V_{\text{C(J)}}$, respectively] and the concentration of ATP in the BS chloroplasts, τ , are used to calculate g_s
138 and A . $V_{\text{C(C)}}$ shows an initial decline with increasing $PPFD$ below $\sim 200 \mu\text{mol m}^{-2} \text{s}^{-1}$, due to the re-
139 fixation of respired CO₂, and remains almost constant thereafter (Figure 2E). In contrast, $V_{\text{C(J)}}$

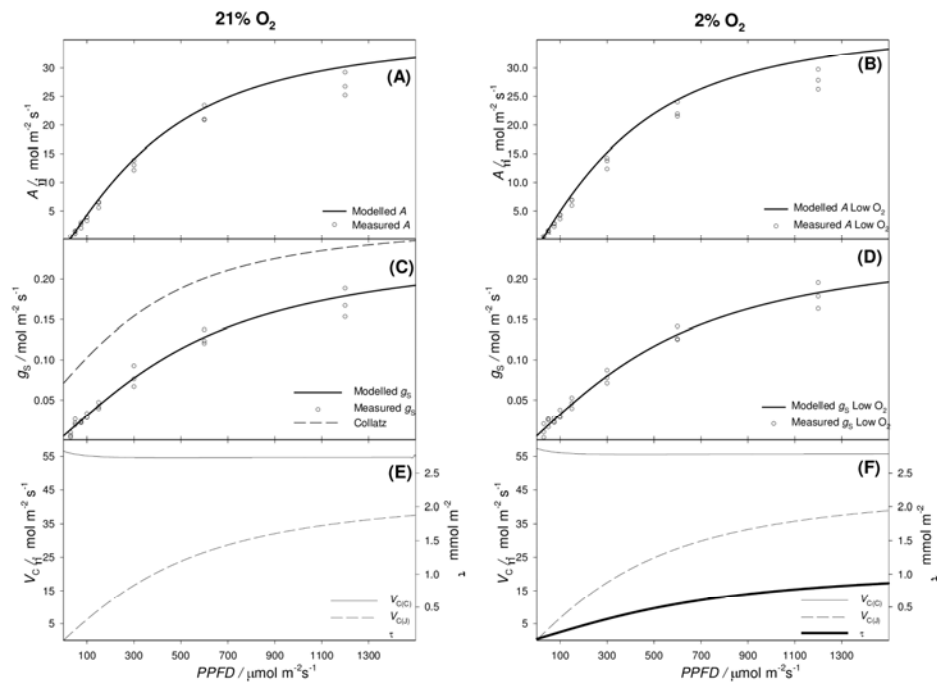
Figure 1. Modelling approach and framework. Blue boxes show input parameters/variables. Dashed boxes encapsulate the light-limited and enzyme-limited models for C_4 photosynthesis and the purple box represents the hydro-mechanical stomatal model. Photosynthetic photon flux density ($PPFD$) and CO_2 concentration external to the leaf [C_a] are inputs to the photosynthesis models. Electron transport and ATP production rates (J_{ATP}) and $[CO_2]$ in mesophyll (C_M) are fed into the light- and enzyme-limited models. The outputs from the photosynthesis models are used to calculate chloroplast ATP concentration, τ . τ is used in the stomatal model along with inputs for soil water potential, Ψ_{soil} and evaporative demand, D_s . The stomatal model also uses other set and fitted variables (e.g. π_e , $\chi\beta$, and K_{PLANT} [see Table 1]), along with τ_0 , which relates to the chloroplast ATP concentration in the dark and is calculated from g_s in the dark (g_{s0}) and D_s in the dark (D_{s0}). In the model simulations, C_M is calculated iteratively.



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140 increases non-linearly with $PPFD$ and has a characteristic saturating response dependent on the
 141 response of J_{ATP} to $PPFD$ (Eqn 8). Because τ , in turn, depends on the ratio of $V_{C(C)}/V_{C(J)}$ (Eqn 11), it
 142 responds to increasing $PPFD$ with the same saturating trend as $V_{C(J)}$. The predicted response
 143 patterns and magnitudes of A , g_s , $V_{C(C)}$, $V_{C(J)}$ and τ to increasing $PPFD$ were highly conserved

Figure 2. Responses to incident irradiance (*PPFD*) of assimilation rate, *A* (A – B), stomatal conductance, *g_s* (C – D), and quantities underpinning the calculation of *A* and *g_s* (E – F) at 21% O₂ (left) and at 2% O₂ (right). The model is plotted here against data taken from [Bellasio et al. \(2016a\)](#) (circles, *n*=3, all data shown). The model of Collatz *et al.* (1992) is shown for comparison in Panel C. In Panels E and F, the bold solid line shows the ATP concentration, τ , the thin solid line the potential enzyme-limited carboxylation rate, $V_{C(C)}$, and the dashed line represents the potential light-limited carboxylation rate, $V_{C(L)}$.



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144 between ambient (21 %) and low (2 %) [O₂], in close agreement with data taken from Bellasio et al.
 145 (2016a) (Figure 2B, D and F). The intercellular CO₂ concentration (*C_i*, not shown) was initially
 146 high due to respiration, but declined to a minimum of 217 $\mu\text{mol mol}^{-1}$ at a *PPFD* of 370 $\mu\text{mol m}^{-2}\text{s}^{-1}$
 147 ¹, and thereafter followed a gradual increase with *PPFD* in line with data taken from Bellasio et al.

(2016a) and previous reports (Sharkey and Raschke, 1981). To demonstrate applicability beyond maize, the model was parameterised with photosynthetic characteristics of three additional C₄ grasses (Table S3), while interspecific differences in stomatal behaviour were described by adjusting the combined parameter $\chi\beta$ after (Rodriguez-Dominguez et al., 2016). The model accurately predicted A and g_s at all *PPFD*s (Figure S1).

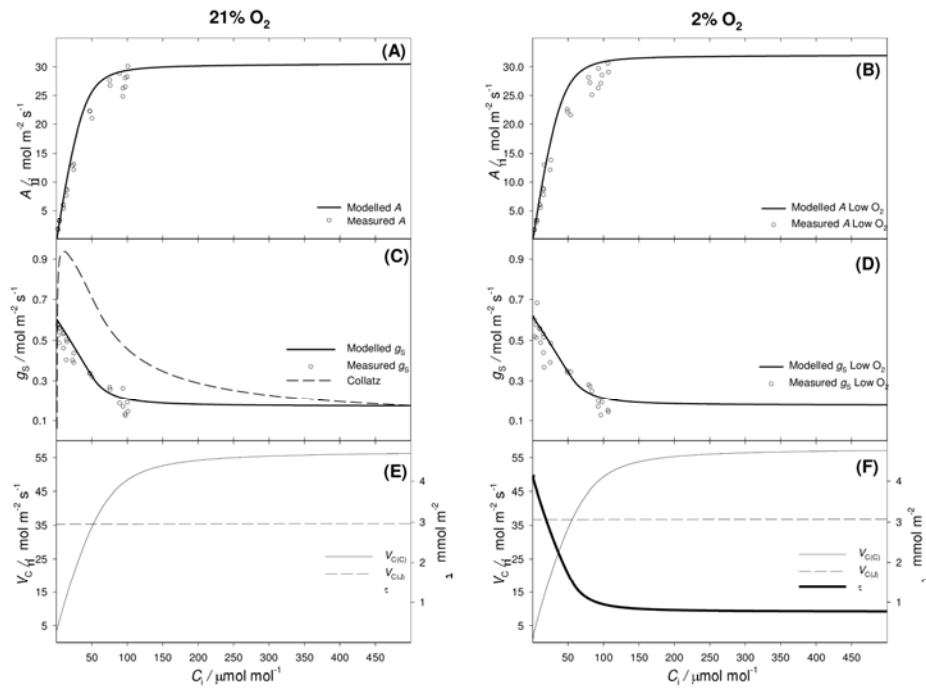
Responses to intercellular CO₂ concentration

Simulated responses of A , g_s , $V_{C(C)}$, $V_{C(J)}$ and τ to increasing C_i (Figure 3) in maize were obtained by varying the C_a input at 89 discrete increments, and iteratively fitting C_M at each value of C_a , under a set *PPFD* of 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Predicted A and g_s again compared well with data from Bellasio et al. (2016a), which were measured under the same *PPFD* as the simulations (Figure 3, circles), and by setting reference [CO₂] at 9 levels between 400 and 10 $\mu\text{mol mol}^{-1}$. Simulated A increased rapidly between a C_i of 0 and 100 $\mu\text{mol mol}^{-1}$, due to the C₄ CCM, then levelled out at the *PPFD*-saturated value, while g_s initially decreased quickly as C_i approached 150 $\mu\text{mol mol}^{-1}$ before continuing to decrease more gradually with increasing C_i (Figure 3A – D). Between a C_i of 0 and 50 $\mu\text{mol mol}^{-1}$, τ declined almost linearly, but the rate of decline decreased until it flattened to ~ 1 mmol m^{-2} at around a C_i of 150 $\mu\text{mol mol}^{-1}$ (Figure 3E). As $V_{C(C)}$ surpassed $V_{C(J)}$ at a C_i of 90 $\mu\text{mol mol}^{-1}$, photosynthesis switched from enzyme-limited to light-limited, and the response of τ to C_i then decreased more rapidly before levelling out. Stomatal responses simulated with the stomatal sub-model of Collatz et al. (1992) are shown for comparison (Figure 3C). Because of the low sensitivity of C₄ photosynthesis to [O₂], both the simulated and observed responses of A , g_s , $V_{C(C)}$, $V_{C(J)}$ and τ to increasing C_i at 2% O₂ (Figure 3B, D and F) were indistinguishable from those at 21% O₂.

Responses to soil water potential

When C₄ plants experience water limitation, a portion of the overall decrease in A is driven by biochemical limitations, unrelated to stomatal movements, and is generally referred to as non-stomatal limitation (Ghannoum et al., 2003; Ghannoum, 2009). This is not accounted for by the C₄ photosynthetic model, which overestimates A at low Ψ_{Soil} (Quirk et al., under review). Here, we account for non-stomatal limitations through an empirical correction (Eqn 15), which links the inputs $V_{C\text{MAX}}$, and $J_{\text{ATP}\text{MAX}}$ to Ψ_{Soil} (Figure S3), preserving the ratio between $J_{\text{ATP}\text{MAX}}$ and $V_{C\text{MAX}}$. Because the correction is applied to model inputs, outputs are mutually consistent and can be used for theoretical and physiological analyses. Simulated responses of A , g_s , $V_{C(C)}$, $V_{C(J)}$ and τ to increasingly negative soil water potential, Ψ_{Soil} (Figure 4) were obtained by varying Ψ_{Soil} at 257 discrete increments, and iteratively fitting C_M at each value of Ψ_{Soil} , under a set *PPFD* of 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and C_a of 400 $\mu\text{mol mol}^{-1}$. As Ψ_{Soil} started to decrease, simulated g_s declined almost linearly, driven solely by the decrease in leaf turgor. In contrast, A was initially light-limited and insensitive to g_s (Figure 4A). As Ψ_{Soil} continued to decrease, stomatal closure exerted more influence on $V_{C(C)}$,

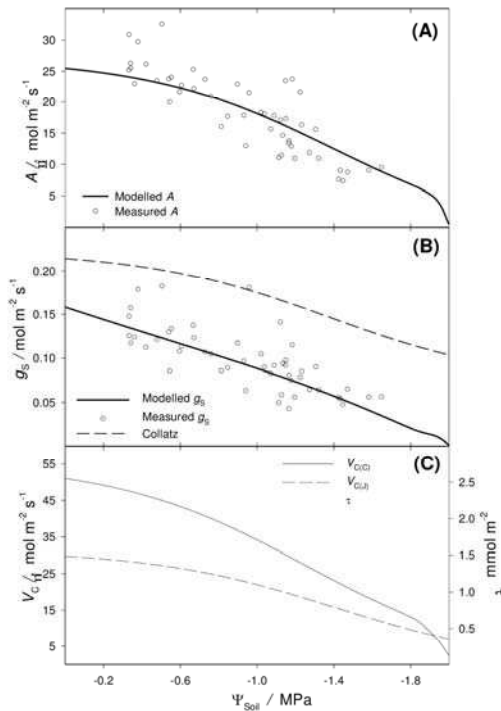
Figure 3. Responses to intercellular CO_2 concentration, C_i of assimilation rate, A (A – B), stomatal conductance, g_s (C – D), and quantities underpinning the calculation of A and g_s (E – F), at 21% O_2 (left) and at 2% O_2 (right). The model (lines) is plotted here against data taken from [Bellasio et al. \(2016a\)](#) (circles, $n=3$, all data shown). The output of Collatz *et al.* (1992) is shown for comparison in Panel C. In Panels E and F, the bold solid line shows the ATP concentration (τ) the thin solid line the potential enzyme-limited carboxylation rate ($V_{C(C)}$), and the dashed line represents the potential light-limited carboxylation rate ($V_{C(L)}$).



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183 causing τ to rise sharply and the g_s response to deviate from near-linearity. When $V_{C(C)}$ declined
 184 below $V_{C(L)}$ at a Ψ_{Soil} around -1.3 MPa, A became enzyme-limited and began to decrease more
 185 rapidly with Ψ_{Soil} . As soil continued to dry the loss of guard cell turgor eventually induced stomatal

Figure 4. Responses to soil water potential, Ψ_{Soil} of assimilation rate, A (A), stomatal conductance, g_s (B), and quantities underpinning the calculation of A and g_s (C). The model (lines) is plotted here against independent data obtained by instantaneous gas exchange measurements on maize plants in controlled-environment growth cabinets (circles, all data shown) under a $PPFD$ of $700 \mu\text{mol m}^{-2} \text{s}^{-1}$, and reference CO_2 of $400 \mu\text{mol mol}^{-1}$. The output of Collatz *et al.* (1992) is shown for comparison in Panel B. In Panel C the bold solid line shows the ATP concentration (τ), the thin solid line the potential enzyme-limited carboxylation rate ($V_{C(C)}$), and the dashed line represents the potential light-limited carboxylation rate ($V_{C(L)}$).



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186 closure and g_s reached the minimal value of g_{s0} (Figure 4B). The predicted response of g_s to Ψ_{Soil}
 187 compared well with independent instantaneous measurements (Figure 4A–B, circles). There was
 188 close agreement between measured and simulated A at all values of Ψ_{Soil} . Stomatal responses

189 simulated with the stomatal sub-model of Collatz et al. (1992) are shown for comparison (Figure
190 3C).

191 *Responses to evaporative demand (vapour pressure deficit)*

192 The response of g_s to water vapour pressure deficit (VPD), represented here by the input term D_s
193 (the water vapour mole fraction difference between the leaf and air, which is VPD divided by
194 atmospheric pressure), was simulated by varying D_s and iteratively fitting C_M at each value under a
195 set $PPFD$ of $750 \mu\text{mol m}^{-2} \text{s}^{-1}$ and four levels of C_a (Figure 5A). Stomatal conductance declined
196 hyperbolically with increasing D_s (as evaporative demand increased), with the response influenced
197 by C_a (Figure 5). At sub-ambient $[\text{CO}_2]$ of $200 \mu\text{mol mol}^{-1}$, g_s was more sensitive to D_s , due to
198 higher levels of τ which is itself induced by lower $V_{C(C)}/V_{C(D)}$ (not shown). The simulated trends
199 were in line with the data of Morison and Gifford (1983), measured under the same $PPFD$ of the
200 simulations. However, Morison and Gifford (1983) showed a higher sensitivity of g_s to changes in
201 VPD , which we partly attribute to growth differences, and partly to the fact that any feed-forward
202 action of humidity on stomatal movement is neglected in our model (Buckley, 2005).

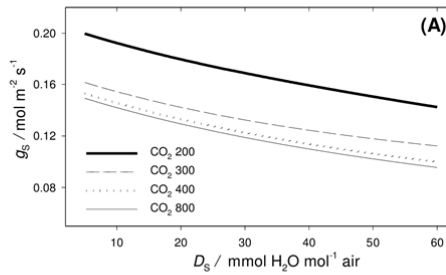
203 *Dynamic responses to an increase in PPFD*

204 Simulated dynamic responses of A and g_s to an increase in $PPFD$ from 50 to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$
205 compared well with data for maize taken from Chen et al. (2013) (Figure 6). For the experimental
206 measurements, maize leaves were acclimated at a $PPFD$ of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ for at least 10 min
207 before $PPFD$ was increased to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Chen et al., 2013). For the simulations, C_a was set
208 at $400 \mu\text{mol mol}^{-1}$ and D_s at 10mmol mol^{-1} , and then run with a one-second time interval,
209 simulating the kinetics of J_{ATP} and response of g_s dynamically (Equation 16 and 17). C_M was
210 iteratively fitted at each time point. Assimilation responded immediately to the increase in $PPFD$,
211 but took ~ 10 min to reach steady state (Figure 6A). In contrast, the response of stomata was delayed
212 relative to A , reaching steady state after ~ 15 min (Figure 6B–C).

213 *Leaf hydraulic conductance*

214 Leaf hydraulic conductance (K_{LEAF}) was found to be light-dependent in dicots (Sack and
215 Holbrook, 2006) and maize (Kim and Steudle, 2007). The light induction is probably mediated by
216 an increased transcription of aquaporins (Cochard et al., 2007). Here, we interrogated the model to
217 assess whether a light-inducible K_{LEAF} would have any effect on g_s , mediated by decreased leaf
218 water availability under low light. We described K_{LEAF} induction through a simple Michaelis-
219 Menten saturating response, as $K_{LEAF} = K_{LEAF \text{ MIN}} + \frac{PPFD(K_{LEAF \text{ MAX}} - K_{LEAF \text{ MIN}})}{PPFD + K_M(K_{LEAF})}$, where $K_{LEAF \text{ MIN}}$ is
220 the value of K_{LEAF} in the dark, $K_{LEAF \text{ MAX}}$ is the fully induced value of K_{LEAF} and $K_M(K_{LEAF})$ is the
221 $PPFD$ at half K_{LEAF} saturation. K_{PLANT} was calculated as $K_{PLANT} = \frac{1}{\frac{1}{K_{STEM}} + \frac{1}{K_{LEAF}}}$. K_{STEM} was assumed

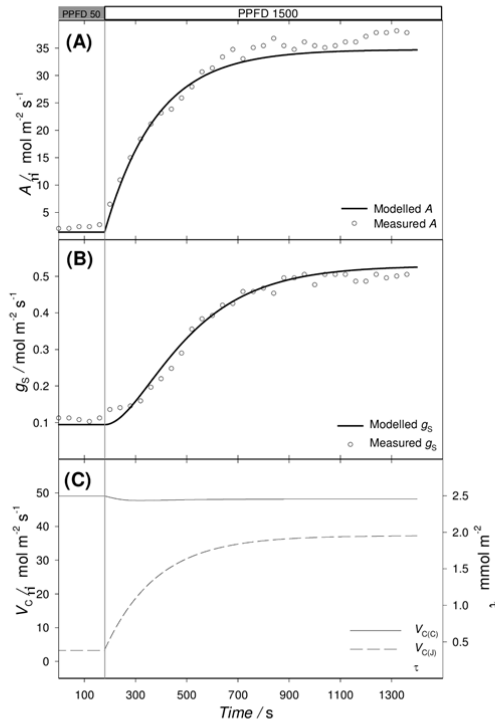
Figure 5. Modelled response of stomatal conductance, g_s to water pressure deficit, VPD – expressed here as mole gradient ($D_s=10VPD$) – obtained at four different CO_2 concentrations: $200 \mu\text{mol mol}^{-1}$ (thick solid line), $400 \mu\text{mol mol}^{-1}$ (dashed line), $600 \mu\text{mol mol}^{-1}$ (dotted line), $800 \mu\text{mol mol}^{-1}$ (thin solid line), under a $PPFD$ of $750 \mu\text{mol m}^{-2} \text{s}^{-1}$. Other inputs are listed in Table 1.



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222 not to vary with $PPFD$, and knowing that $\frac{1}{K_{LEAF}} \approx 0.3 \frac{1}{K_{PLANT}}$ (Sack and Holbrook, 2006), we set a
 223 $K_{LEAF \text{ MAX}}$ of $40 \text{ mmol H}_2\text{O m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ and a K_{STEM} of $20 \text{ mmol H}_2\text{O m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$, so that
 224 $K_{PLANT} \approx 12 \text{ mmol H}_2\text{O m}^{-2} \text{s}^{-1} \text{MPa}^{-1}$ under a $PPFD$ of $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$.

Figure 6. Dynamic responses of assimilation rate, A (**A**), stomatal conductance, g_s (**B**), and quantities underpinning the calculation of A and g_s (**C**) following an increase in incident light intensity, $PPFD$ from 50 to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (vertical grey line). Modelled responses (lines) were obtained by setting $g_{s0} = 0.06 \text{ mol m}^{-2} \text{s}^{-1}$, $\chi\beta = 0.39 \text{ mol air mmol}^{-1} \text{ATP s}^{-1} \text{MPa}^{-1}$, while other inputs are listed in Table 1. The data points (circles) are taken from Chen et al. (2013) in which maize leaves were acclimated at a $PPFD$ of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for >10 min, before $PPFD$ was increased to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In Panel **C** the bold solid line shows the ATP concentration (τ), the thin solid line the potential enzyme-limited carboxylation rate ($V_{C(C)}$) and the dashed line represents the potential light-limited carboxylation rate ($V_{C(L)}$).



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225 Firstly, we simulated well-watered conditions with a realistic $K_{\text{LEAF MIN}} = 0.5 K_{\text{LEAF MAX}}$ (Sack and
 226 Holbrook, 2006). We used three different $K_M(K_{\text{LEAF}})$ ($\mu\text{mol m}^{-2} \text{s}^{-1}$): a $K_M(K_{\text{LEAF}}) = 1$ to represent
 227 induction in the dark, $K_M(K_{\text{LEAF}}) = 200$, which is the most realistic case, and $K_M(K_{\text{LEAF}}) = 500$ to
 228 represent induction at high $PPFD$ (Figure S2). In each case, the effect on g_s was negligible (Figure

229 S3 A). For instance, at a $PPFD$ of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, g_S was 0.36 % and 0.6 % lower with a
230 $K_M(K_{LEAF})$ of 200 or $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ than with a $K_M(K_{LEAF})$ of $1 \mu\text{mol m}^{-2} \text{s}^{-1}$. In a more stringent
231 scenario we set $K_{LEAFMIN}=0.25 K_{LEAF MAX}$ (Cochard et al., 2007), again, we found that any effect on
232 g_S was negligible (Figure S3 B). For instance at a $PPFD$ of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ g_S was 0.65% and
233 1.2% lower with a $K_M(K_{LEAF})$ of 200 or $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ than with a $K_M(K_{LEAF})$ of $1 \mu\text{mol m}^{-2} \text{s}^{-1}$.
234 We finally simulated an extreme case of reduced water availability ($\Psi_{Soil}=-1$ MPa), high
235 evaporative demand ($D_S=50$ mmol $\text{H}_2\text{O mol air}^{-1}$) and $K_{LEAF MIN}=0.25 K_{LEAF MAX}$. Even in this case
236 the effect on g_S was negligible (Figure S3 C). For instance, at a $PPFD$ of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ g_S was
237 1.9% and 3.5% lower with a $K_M(K_{LEAF})$ of 200 or $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ than with a $K_M(K_{LEAF})$ of
238 $1 \mu\text{mol m}^{-2} \text{s}^{-1}$. Overall, the outputs indicate that the light induction of K_{LEAF} does not substantially
239 reduce g_S mediated by decreased water availability, although we cannot exclude other feedback
240 mechanisms.

242 Discussion

243 We successfully coupled a hydro-mechanical stomatal model to a newly derived enzyme- and
244 light-limited biochemical model of leaf-level C_4 photosynthesis calibrated for maize and
245 demonstrated the ability of the resulting model to simulate the behaviour of three additional C_4
246 grass species. We establish that in maize, during a transient increase in $PPFD$ (light-fleck), stomata
247 respond after assimilation, and that slower stomatal responses to light- and shade-flecks can
248 substantially limit the water use optimality of leaves (see dedicated paragraph below). We also
249 show that light induction of K_{PLANT} does not reduce g_s through effects of decreased leaf-level water
250 availability. Our model allowed accurate simulation of steady-state photosynthetic and stomatal
251 responses to changes in $PPFD$ in four C_4 species, atmospheric $[CO_2]$, soil moisture and evaporative
252 demand (Figures 2–5), as well as dynamic responses to light-flecks following the incorporation of
253 time-explicit constraints on J_{ATP} and τ (Figure 6).

254 The hydro-mechanical and biochemical rationale underpinning the stomatal model is described
255 fully in Buckley et al. (2003) and we followed the simplified implementation of Rodriguez-
256 Dominguez (2016) for wider applicability. In this formulation, any turgor difference or hydraulic
257 resistance between guard and epidermal cells is neglected and all quantities can be measured except
258 one combined parameter, $\chi\beta$ that can be fitted. The relative simplicity of the modelling approach
259 and the equations we have derived make them suitable for implementation in larger scale vegetation
260 modelling.

261 In the stomatal model, g_s is proportional to stomatal aperture, which is in turn governed by
262 changes in guard cell and epidermal turgor (Franks et al., 1995). The model is based on the
263 hydroactive feedback hypothesis that passive shifts in guard cell turgor caused by changes in leaf
264 water balance are amplified by active adjustment of guard cell osmotic pressure in proportion to
265 leaf turgor. The ultimate result is that g_s is proportional to leaf turgor, all else being equal. This
266 assumption is well supported by experimental evidence and apparently involves leaf-endogenous
267 ABA synthesis [see Buckley (2017)]. Leaf turgor varies from a maximum (π_c), to zero, mediated by
268 the equilibrium between water demand (D_s and g_s) and water supply, dependent on Ψ_{Soil} and
269 K_{PLANT} . The latter was measured under growth conditions, under a VPD of ~ 1.7 KPa, and calculated
270 using operational transpiration measurements made under the same conditions. Around the level of
271 K_{PLANT} found for maize (Table 1), the model was relatively insensitive to variable K_{PLANT} (Figure
272 S5). When K_{PLANT} was increased by 30 %, maximum g_s decreased by only ~ 3 %, and *vice versa*,
273 however, the specific response to $[CO_2]$, $PPFD$, VPD or Ψ_{Soil} was not affected. Furthermore, in
274 simulations using variable K_{PLANT} , decreasing water availability under low light did not lower g_s
275 (Figure S5). Higher sensitivities were found at lower values of K_{PLANT} (Figure S5), indicating a shift
276 from biochemical to hydraulic control over g_s , which may occur, for instance, under water stress,
277 where xylem cavitation causes severe dynamic declines in K_{PLANT} .

278 In the model, $\chi\beta$ scales the strength of the hydroactive loop to the turgor-mediated hydropassive
279 feedback. Interspecific differences in stomatal responses between maize and three other grass
280 species were successfully described simply by varying $\chi\beta$. In most angiosperms the hydropassive
281 feedback has been identified as the main determinant in the regulation of stomatal conductance,
282 mediated by hydraulic conductance (Buckley, 2005; Brodrribb and McAdam, 2017). C_4 plants may
283 have shifted their control of stomatal aperture towards a closer link with photosynthesis driven by
284 improved water economy. This may allow tighter stomatal control, but requires increased water
285 supply at leaf level (Quirk *et al.*, under review). The resulting improvement in water relations may
286 have constituted a key evolutionary driver of C_4 photosynthesis and even of grasses in general [see
287 below and (Osborne and Sack, 2012; Griffiths *et al.*, 2013)]. In fact, prior to the evolution of C_4 it is
288 well recognised that improved plant hydraulics conferred benefits through the decrease of
289 interveinal distance and the acquisition of a larger BS (Osborne and Sack, 2012; Griffiths *et al.*,
290 2013; Bellasio and Lundgren, 2016).

291 Steady-state guard cell osmotic pressure is also proportional in the model to τ , a quantity related
292 to the ATP concentration in the BS chloroplast. The quantity τ is a measure of the balance between
293 the light and dark reactions of photosynthesis, capturing stomatal responses to factors that are
294 thought to be mediated partly by photosynthetic processes (e.g., light and CO_2). In our model, τ is
295 simulated empirically using a model for mesophyll chloroplastic ATP concentration originally
296 derived for C_3 plants by Farquhar and Wong (1984). Tau is predicted through constraints on PGA
297 reduction, RuBP regeneration and the RPP cycle (collectively, C_3 activity), which are valid in C_4
298 plants. Here, the C_3 activity is shared between the M and the BS chloroplasts (Bellasio and
299 Griffiths, 2014c; Bellasio, 2017), and τ , therefore, collectively describes the energy status of the M
300 and the BS.

301 Tau behaves in a manner broadly consistent with evidence suggesting that stomata respond to
302 some measure of the poise between the supply and demand for energy carriers in photosynthesis
303 (Wong, 1979; Messinger *et al.*, 2006; Busch, 2014; Mott *et al.*, 2014) – i.e., increasing with $PPFD$
304 and decreasing with C_i . The use of τ as a predictor of stomatal behaviour is empirically based,
305 which is justified by its capacity to predict parallel events occurring in M or BS chloroplasts, as
306 well as in guard cells, but no direct connection is implied [for a detailed discussion see (Farquhar
307 and Wong, 1984)]. Ultimately, the mechanism of the stomatal response is not fully understood
308 (Buckley, 2017), and it is, therefore, not clear whether the τ model faithfully replicates the
309 mechanistic underpinnings. For example, the τ formulation assumes that the potential capacities for
310 ATP generation and consumption, sensed by the quantities $V_{C(I)}$ and $V_{C(C)}$, respectively, are
311 independent of one another. Realistically, however, the actual rate of J_{ATP} will promptly respond to
312 a decrease in C_i , mediated by an increase in non-photochemical energy dissipation, while the
313 activity of light reactions will promptly respond to photophosphorylation levels (Kramer *et al.*,

2004; Foyer et al., 2012). Additionally, the τ model does not simulate responses to blue light, which are independent of photosynthesis (Shimazaki et al., 1986), nor the role of starch degradation in stomatal function (Horrer et al., 2016). Nevertheless, our results suggest that τ is a reliable predictor of stomatal behaviour in C_4 plants, as it has proved to be in many C_3 plants (Buckley et al., 2003; Diaz-Espejo et al., 2012; Rodriguez-Dominguez et al., 2016).

Response to light and shade flecks

The steady-state formulation of the model inherently precludes direct prediction of dynamic features such as the approach of stomatal conductance to a new steady-state following a change in *PPFD*. To overcome this limitation, we extended the model to simulate dynamic responses with a newly derived framework, which fused the simplicity of the approach of Violet-Chabrand et al. (2016) with the rigour of Gross et al. (1991). The principle of this dynamic model is that J_{ATP} responds instantaneously when *PPFD* decreases, but with a delay when *PPFD* increases. Similarly, stomata will respond to any perturbation with a delay due to the kinetics of adjustment of guard cell osmotic pressure, but the time constant for that delay can differ between opening and closing movements (Lawson and Blatt, 2014). The model captures the dynamics of stomatal responses to light and CO_2 on the timescale of minutes. Although our formulation does not incorporate the transient ‘wrong-way responses’ (WWR) of g_s following changes in leaf water status, we note that WWR duration varies widely across species (Buckley et al., 2011), and our model may prove adequate for species with short WWRs. We did not attempt to assess this for maize. In future studies of photosynthetic efficiency at timescales shorter than one minute [e.g. (Pearcy et al., 1997)], the explicit calculation of metabolite pools, which have a central role in C_4 photosynthesis (Stitt and Zhu, 2014), will be key. Further, in our simplified day, other daytime factors, which may influence g_s dynamics, like leaf temperature and *VPD*, as well as circadian rhythms, were not accounted for.

The dynamic stomatal response following an increase in *PPFD* typically has three phases, all of which our model reliably simulated (Figure 6): an induction or lag (up to 10 min), a period of increasing g_s , and a plateau (Lawson and Blatt, 2014). Our model suggests the lag phase may result mechanistically from the lag in ATP production, in line with the conclusions of Barradas and Jones (1996). The duration of these phases and the speed of stomatal movements have important implications for NPP and *WUE*. Crops will experience light- and shade-flecks across the canopy as a result of changes in cloud cover and solar angle as well as self-shading or shading by neighbouring plants (Lawson and Blatt, 2014). Lag times reported for C_3 plants suggest that longer shade intervals may induce slower stomatal opening upon re-illumination than shorter shade intervals (Lawson and Blatt, 2014). Our model can account for the duration of shade intervals by varying the time constants for responses of both stomata and J_{ATP} . During a long shade interval, g_s will more closely approach steady-state closure, but it will also take longer to reach steady-state

350 upon re-illumination. Conversely, during a short-lived shade-fleck, g_s will remain further from
351 steady-state closure, enabling stomata to re-open more quickly upon re-illumination. It is also
352 important to note that, because the modelled stomatal response depends on τ , which is a function of
353 J_{ATP} , the model predicts that g_s will always lag behind photosynthetic acclimation. If this prediction
354 is correct, then perfect synchronicity between photosynthetic and g_s induction dynamics – which
355 was identified as a desirable target for stomatal manipulation (Lawson and Blatt, 2014) – is
356 impossible.

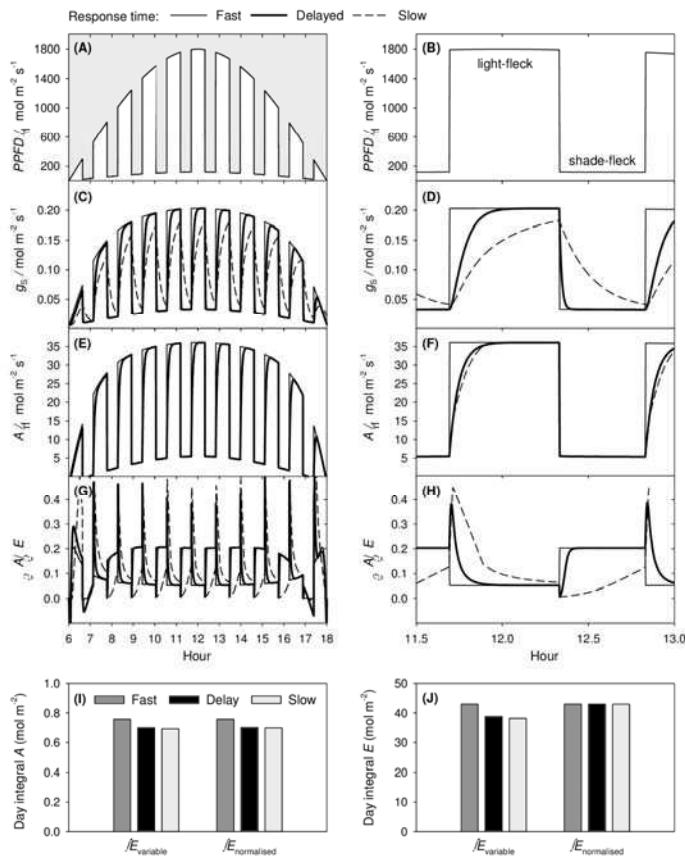
357 To illustrate the broader implications of stomatal response speed we simulated a daytime *PPFD*
358 cycle at 10 s resolution, with ten, equally spaced ‘cloud spells’ of 30 min duration and *PPFD* of
359 $1/15^{\text{th}}$ that of the ‘clear sky’ intensity at a given time (other inputs are given in Table 1). The time
360 constants for stomatal responses were set at three levels that bracketed the kinetics derived for
361 maize in this study with upper (instantaneous) and lower bounds [values derived for *Arabidopsis*
362 *thaliana* (Violet-Chabrand et al., 2016)], while the time constants for J_{ATP} responses were kept at
363 maize physiological levels. Our calculations show that if stomata responded instantaneously to
364 changes in *PPFD*, total carbon gain over the day would increase by 8 % (using maize g_s kinetics) or
365 9.2 % (using *Arabidopsis* g_s kinetics) (Figure 7 I, left), while total water loss over the day would
366 increase by 11 % (Figure 7 J, left), reducing whole-day *WUE* by ~ 3 %, in both cases.

367 The water savings in the slow-response simulations result from the asymmetry between stomatal
368 opening and closure. Because stomata take longer to open than close, an opening-closing cycle
369 results in lower g_s on average compared with leaves with stomata that respond instantaneously. An
370 alternative comparison is to investigate how stomatal response speed influences total daily carbon
371 gain, while treating total daily water loss as a constraint imposed by the environment, and holding it
372 equal between simulations. This requires adjusting the overall scale of g_s whilst simultaneously
373 adjusting the time constant for g_s responses in order to compensate for the tendency of faster-
374 responding stomata to result in greater overall water use. We achieved this by iteratively varying the
375 parameter $\chi\beta$ such that the integral of E over the day was equal across all simulations (Figure 7 J,
376 right). Although these conditions result in similar daily A to those calculated previously (~ 8 % gain
377 in A for instantaneous responses over both maize and *Arabidopsis* kinetic parametrisations, Figure
378 7 I compare left with right), the lower daily A obtained with delayed responses, compared with
379 instantaneous responses, is now explained entirely by suboptimal temporal patterns of g_s .

380 *Water use optimality*

381 Optimality of water use is measured by the marginal carbon gain obtainable for a given marginal
382 water loss, $\partial A/\partial E$ (the small increase in A that results if stomata are a little bit more open, all other
383 conditions being equal). A pattern of g_s regulation is optimal, in the sense that it maximises total
384 carbon gain for a given amount of water loss, if $\partial A/\partial E$ is constant over a given time interval
385 [generally a day, but the time span and target value of $\partial A/\partial E$ are complex and debated topics, see

Figure 7. Diurnal profiles of g_s , A and the marginal carbon revenue of water use $\partial A/\partial E$ for C_4 maize. Stomatal responses were instantaneous (*fast*), with a C_4 delay derived for maize here (*delayed*), or slow (using *Arabidopsis thaliana* kinetics). Panel (A) shows $PPFD$ over a hypothetical day, incorporating a *sin* rise to peak $PPFD$ of $1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ at midday and regular shading intervals corresponding to $1/15^{\text{th}}$ that of the clear sky $PPFD$. Right-hand line plots show the same curves on an expanded x -axis scale spanning the 90 min interval around midday. Panels show responses to $PPFD$ of g_s (C – D), A (E – F), and $\partial A/\partial E$ (G – H), respectively. Integrated total daily A (I) and transpiration, E (J) are shown for each stomatal response speed when the day integral of E depended on stomatal speed ($\int E_{\text{variable}}$), and when $\int E$ was constrained to equal that of fast stomatal responses ($\int E_{\text{normalised}}$). $\partial A/\partial E$ was calculated by re-running the model at each time point with a 1‰ increase in g_s and all other inputs maintained from the previous run.



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386 (Buckley et al., 2016)]. The same principle also applies to water allocation between parts of a plant
 387 or a canopy exposed to variable $PPFD$. Interestingly, $\partial A/\partial E$ was more variable in the delayed-
 388 response simulations than in the instantaneous-response simulations, both over the course of the day
 389 (Figure 7 G) and during a light-to-shade fleck (Figure 7 H), indicating that faster stomata were

390 closer to optimality. However, even where stomatal responses were instantaneous, $\partial A/\partial E$ varied
391 between light- and shade-flecks. This indicates that the stomatal behaviour predicted by our model
392 is suboptimal, which opens up the intriguing question of whether suboptimal water allocation is an
393 element contributing to the poor performance of C₄ photosynthesis under low light (Sage, 2014).
394 Further experimental work should aim to test optimality *in vivo* at the plant or field scale (Buckley
395 et al., 2014). Further, it may be interesting to see whether C₄ plants have mechanisms to re-
396 acclimate g_s following progressive shading by the overgrowing crop canopy, as was shown for BS
397 conductance (Bellasio and Griffiths, 2014a, b).

398 We note that the improved optimality discussed above for C₄ maize was associated with a 10
399 times smaller time constant for stomatal opening responses than for C₃ *Arabidopsis thaliana*
400 (Violet-Chabrand et al., 2016). This large improvement may result in part from the dumbbell-shaped
401 guard cells of grasses, which facilitate rapid stomatal movements (Franks and Farquhar, 2007;
402 McAusland et al., 2016), and in part from C₄ photosynthesis itself, which is generally associated
403 with faster stomatal responses (Knapp, 1993; Franks and Farquhar, 2007; McAusland et al., 2016).
404 We propose the association between improved hydraulics (discussed above) and faster stomatal
405 regulation as an important, yet overlooked, driver of the evolution of grasses and C₄ photosynthesis
406 [e.g. (Raven, 2002)]. In an evolutionary context, C₄ grasses evolved under high light and declining
407 CO₂ in open grasslands characterised by semi-aridity (Osborne and Sack, 2012). Plants exposed to
408 low CO₂ reduce the size of stomata, whilst increasing stomatal density (Franks et al., 2012). The
409 integrated or net lag time of many smaller stomata to reach maximal or minimal aperture was
410 shown to be shorter relative to fewer larger stomata (Lawson and Blatt, 2014). In this context our
411 findings further the proposal of Osborne and Sack (2012), that C₄ photosynthesis was partly
412 selected for and co-opted as a water-conserving mechanism, and indicate that quicker responses of
413 stomata for C₄ plants relative to C₃ plants would confer benefits in A and $\partial A/\partial E$.

414 **Conclusion**

415 We developed a coupled biochemical and hydro-mechanical model of stomatal conductance for
416 C₄ photosynthesis, and extended it with time-explicit functions allowing prediction of dynamic
417 responses to environmental stimuli. We calibrated the C₄ model using gas exchange measurements
418 for maize and three C₄ grass species (*Eragrostis curvula*, *Heteropogon contortus* and *Themeda*
419 *triandra*), and validated it against independent datasets. We showed that following a light-fleck,
420 stomata respond after the assimilation response, not because stomatal responses are inherently slow,
421 but because the stomatal response is itself functionally dependent on assimilation. We also
422 demonstrate that the slower stomata are to respond to fluctuations in light, the lower the water use
423 optimality. We propose that fast stomatal responses, reported for maize and other C₄ grasses, may
424 have contributed to the evolution of C₄ photosynthesis mediated by the increase of water use
425 optimality in open, semi-arid environments. Finally, we showed that light induction of leaf

426 hydraulic conductance does not cause any substantial reduction in stomatal conductance mediated
 427 by decreased water availability at leaf level. Overall, the coupled model has clear promise as a
 428 predictive and analytical tool for stomatal research in C_4 species, and support (but do not prove) the
 429 hypothesis that the process framework underpinning the hydro-mechanical stomatal model remains
 430 valid for C_4 plants. The equations derived for the model are suitable for incorporation in land
 431 surface models and for detailed ecophysiological studies. Combined with the ability to predict
 432 dynamic scenarios, the model has potential for superseding the long-dominant empirical approach
 433 for stomatal modelling.

434 **Materials and Methods**

435 A biochemical model of C_4 photosynthesis comprising light- and enzyme-limited formulations,
 436 as well as models of CO_2 diffusion through stomata and mesophyll, was developed and coupled
 437 with a hydro-mechanical model of stomatal behaviour to yield a steady-state modelling framework
 438 – a schematic overview of which is shown in Figure 1. This framework was augmented with
 439 submodels accounting for non-stomatal limitations, uneven transition between limitations through
 440 the leaf profile, acclimation of turgor pressure under reduced water availability, and time delay
 441 functions to simulate the dynamic behaviour of stomatal responses to environmental stimuli,
 442 particularly fluctuations in light intensity.

443 *The biochemical model of C_4 photosynthesis – enzyme-limited*

444 To solve inconsistencies in the published equations, an enzyme-limited C_4 photosynthesis model
 445 was newly derived starting from Eqn 4.10, 4.12 and 4.16 in von Caemmerer (2000). Briefly, this
 446 formulation calculates assimilation based on CO_2 concentration at the M carboxylating sites,
 447 assuming fully activated Rubisco and PEPC, and saturating concentrations of RuBP and PEP, as:

$$448 \quad A_C = \frac{-q + \sqrt{q^2 - 4pr}}{2p}, \quad 1$$

448 where:

$$449 \quad p = \frac{\alpha K_C}{0.047 g_{BS} K_O} - \frac{1}{g_{BS}};$$

$$450 \quad q = C_M + \frac{V_P}{g_{BS}} - \frac{R_M}{g_{BS}} + K_C + \frac{O_M K_C}{K_O} + \frac{R_{LIGHT} \alpha K_C}{0.047 g_{BS} K_O} - \frac{R_{LIGHT}}{g_{BS}} + \frac{V_{C_{MAX}}}{g_{BS}} + \frac{\alpha \gamma^* V_{C_{MAX}}}{0.047 g_{BS}};$$

$$451 \quad r = R_{LIGHT} \left(C_M + \frac{V_P}{g_{BS}} - \frac{R_M}{g_{BS}} + K_C + \frac{O_M K_C}{K_O} \right) - C_M V_{C_{MAX}} - \frac{V_P V_{C_{MAX}}}{g_{BS}} + \frac{R_M V_{C_{MAX}}}{g_{BS}} + \gamma^* O_M V_{C_{MAX}}.$$

452 In Eqn 1, 0.047 is a coefficient scaling O_2 and CO_2 diffusivity (von Caemmerer, 2000); α is the
 453 fraction of O_2 evolution in the BS; K_C and K_O are the Rubisco Michaelis-Menten constants for CO_2
 454 and O_2 , respectively; R_M is the fraction of R_{LIGHT} in the mesophyll ($0.5 R_{LIGHT}$); g_{BS} is the fitted BS
 455 conductance to CO_2 diffusion; γ^* is half the reciprocal of Rubisco CO_2/O_2 specificity; C_M is
 456 calculated using an M supply function, Eqn 16; O_M is the O_2 concentration in mesophyll (see Table

1 for details); $V_{C_{MAX}}$ is the CO₂-saturated rate of Rubisco carboxylation; and, finally V_P , the rate of PEPC carboxylation, was calculated using a Michaelis-Menten equation as:

$$V_{P(C)} = \frac{C_M V_{P_{MAX}}}{C_M + K_P}, \quad 2$$

where $V_{P_{MAX}}$ is the maximal rate of PEP carboxylation, and K_P is the PEPC Michaelis-Menten constant for HCO₃⁻ (Table 1). The concentration of CO₂ in the BS was estimated by mass balance of the M:

$$C_{BS(C)} = \frac{V_P - A - R_M}{g_{BS}}, \quad 3$$

The O₂ concentration in the BS is:

$$O_{BS} = \frac{\alpha A}{0.047 g_{BS}} + O_M. \quad 4$$

The rate of enzyme-limited Rubisco carboxylation is:

$$V_{C(C)} = \frac{C_{BS} V_{C_{MAX}}}{C_{BS} + K_C \left(1 + \frac{O_{BS}}{K_O}\right)}, \quad 5$$

and is used to calculate the Rubisco oxygenation rate:

$$V_{O(C)} = V_{C(C)} 2 \gamma * \frac{O_{BS}}{C_{BS}}. \quad 6$$

Biochemical modelling of C₄ photosynthesis – light-limited

Light-limited C₄ photosynthesis was modelled after von Caemmerer (2000). Briefly this formulation assumes that light limits assimilation, mediated by the ATP made available through photophosphorylation [for more on assumptions see (Bellasio and Griffiths, 2014a, b)]. The total ATP production rate is assumed to be entirely used by C₄ activity and C₃ activity and split between those by a parameter, called x , which is assumed constant at 0.4. Assimilation is:

$$A_J = \frac{-b - \sqrt{b^2 - 4ac}}{2a}, \quad 7$$

where:

$$a = 1 - \frac{7\alpha\gamma^*}{3 \cdot 0.047};$$

$$b = -\left\{ \frac{x J_{ATP}}{2} - R_M + g_{BS} C_M + \frac{(1-x) J_{ATP}}{3} - R_{LIGHT} + \frac{7 g_{BS} \gamma^* O_M}{3} + \frac{\alpha\gamma^*}{0.047} \left(\frac{(1-x) J_{ATP}}{3} + \frac{7 R_{LIGHT}}{3} \right) \right\};$$

$$c = \left(\frac{x J_{ATP}}{2} - R_M + g_{BS} C_M \right) \left(\frac{(1-x) J_{ATP}}{3} - R_{LIGHT} \right) - g_{BS} \gamma^* O_M \left(\frac{(1-x) J_{ATP}}{3} + \frac{7 R_{LIGHT}}{3} \right);$$

in which J_{ATP} was calculated from an empirical non-rectangular hyperbola as:

$$J_{ATP} = \frac{Y(J_{ATP})_{LL} PPFD + J_{ATP_{MAX}} - \sqrt{(Y(J_{ATP})_{LL} PPFD + J_{ATP_{MAX}})^2 - 4\theta J_{ATP_{MAX}} Y(J_{ATP})_{LL} PPFD}}{2\theta} \quad 8$$

where, $J_{ATP_{MAX}}$ is the light-saturated electron transport rate, $Y(J_{ATP})_{LL}$ is the conversion efficiency of PPFD into J_{ATP} , and θ is an empirical factor, defining the curvature (Table 1).

478 Taken from Eqn 16 and 17 in Bellasio et al. (2016a), the light-limited rate of Rubisco oxygenation
 479 was solved as:

$$V_{O(J)} = \frac{(1-x)J_{ATP} - 3(A+R_{LIGHT})}{5} \quad 9$$

480 $C_{BS(J)}$ and $O_{BS(J)}$ were calculated through Eqn 3 and 4 respectively, where $V_{P(J)}=0.5xJ_{ATP}$, and,
 481 finally, $V_{C(J)}$ was calculated by inverting Eqn 6, with $V_{O(J)}$ calculated through Eqn 9.

482 *The hydro-mechanical model of stomatal behaviour*

483 The hydro-mechanical model used here is a simplified formulation of the model of Buckley *et al.*
 484 (2003) following the derivation of Rodriguez-Dominguez et al. (2016). The model assumptions are
 485 described in the *Discussion*. The model calculates g_S as:

$$g_S = \max \left(g_{S0}, \frac{\chi \beta \tau (\Psi_{Soil} + \pi_e)}{1 + \chi \beta \tau R D_S} \right), \quad 10$$

486 where $\chi\beta$ is a lumped parameter scaling turgor-to-conductance and the hydro-mechanical-to-
 487 biochemical response; Ψ_{Soil} is soil water potential; π_e is epidermal osmotic pressure; τ is related to
 488 the ATP concentration in BS chloroplasts; R is the effective hydraulic resistance to the epidermis,
 489 calculated as $1/K_{PLANT}$; and D_S is the leaf-to-boundary layer H_2O mole fraction gradient, a measure
 490 of vapour pressure deficit, VPD . The parameter τ encompasses the biochemical components of the
 491 model and is calculated from one of two values depending on the limit to photosynthesis, after
 492 Farquhar and Wong (1984), using output from the enzyme- and light-limited photosynthesis
 493 models:

$$\tau = \tau_0 + \begin{cases} \tau_C & \text{if } V_{C(C)} < V_{C(J)} \\ \tau_J & \text{else} \end{cases} \quad 11$$

494 When assimilation is enzyme-limited, $V_{C(C)} < V_{C(J)}$, τ_C is calculated as:

$$\tau_C = \alpha_t - p \frac{V_{C(C)}}{V_{C(J)}}, \quad 12$$

495 where α_t is the total concentration of adenylates; and p is the concentration of photophosphorylation
 496 sites (Table 1).

497 When assimilation is light-limited, $V_{C(C)} > V_{C(J)}$, τ_J is:

$$\tau_J = \frac{(\alpha_t - p) \left(\frac{V_R}{E_T} - 1 \right)}{\frac{V_{C(C)}}{V_{C(J)}} \frac{V_R}{E_T} - 1}, \quad 13$$

498 where V_R is the potential RuBP pool size and E_T is the total concentration of Rubisco carboxylating
 499 sites (Table 1).

500 The basal level of ATP activity due to other metabolic processes including mitochondrial
 501 respiration is defined as τ_0 . Here, we calculated τ_0 , based on the gas exchange data, by inverting
 502 Eqn 10:

$$\tau_0 = \frac{1}{\chi\beta\left(RD_{S0} - \frac{\Psi_{Soil0}}{g_{S0}} - \frac{\pi_e}{g_{S0}}\right)}, \quad 14$$

503 where g_{S0} ($\text{mol m}^{-2} \text{s}^{-1}$) is stomatal conductance measured in the dark; and D_{S0} is the evaporative
504 demand measured in the dark, and Ψ_{Soil0} is the corresponding Ψ_{Soil} (here, 0 MPa).

505 *Non-stomatal limitations*

506 The biochemical model described above assumes that photosynthetic potential is maintained
507 regardless of plant water status. Although, it is well-known that C_4 plants respond to decreasing
508 Ψ_{Soil} with an overall downregulation of photosynthetic activity, through processes collectively
509 referred to as non-stomatal limitation. These include source-sink feedbacks, reduced supply of
510 substrates to carboxylases, limitations imposed by the diffusion of metabolites between M and BS,
511 and a downregulation of photosynthetic potential (V_{CMAX} , V_{PMAX} and J_{ATPMAX}). Here, for simplicity
512 we combine these limitations and describe them as a downregulation of V_{CMAX} and J_{ATPMAX} using
513 an attenuating function from Osborne and Sack (2012):

$$Parameter = \frac{Parameter_{MAX}}{1 + e^{-\frac{\Psi_{Soil} + b}{c}}}, \quad 15$$

514 where $Parameter_{MAX}$ may be V_{CMAX} or J_{ATPMAX} fitted under well-watered conditions, b is the water
515 potential when $Parameter = 0.5 Parameter_{MAX}$, which we set to equal osmotic pressure at full
516 watering (-1.2 MPa), while c defines the shape of the sigmoidal curve and was set at 0.5; outputs
517 are shown in Figure S4. In the simulations where Ψ_{Soil} is constant, $Parameter = Parameter_{MAX}$.

518 *Combining the submodels*

519 The original model of von Caemmerer (2000) assumes a discrete ‘jump’ at the point
520 corresponding to the transition between light-limited and enzyme-limited assimilation. Buckley et
521 al. (2016) noted that, realistically, this transition would be smooth and occur at different C_M
522 depending on the position of the chloroplasts through the leaf profile. Here we describe the
523 smoothed relationship (A_{MOD}) in terms of a non-rectangular hyperbola as:

$$A_{MOD} = \frac{A_C + A_J - \sqrt{(A_C + A_J)^2 - 4\theta_A A_C A_J}}{2\theta_A}, \quad 16$$

524 where θ_A was set at 0.93. A set of equations to simulate all the key photosynthetic quantities,
525 consistent with Eqn 16, is reported in Supplementary Information Note S1.

526 Mesophyll CO_2 concentration, C_M , is:

$$C_M = C_i - \frac{A_{MOD}}{g_M}, \quad 17$$

527 where $C_i = C_a - \frac{A_{MOD}}{g_s}$, and g_M is mesophyll conductance to CO_2 diffusion (Table1). Seed values of
 528 C_M were used to calculate Eqn 1, 2, and 7, and then iteratively fitted to Eqn 17 with g_s calculated
 529 through Eqn 10 (see Figure 1).

530 *Dynamic stomatal responses*

531 One important factor for stomatal dynamics has been identified as the delay to reach a steady-
 532 state stomatal aperture after a change in *PPFD* (Lawson and Blatt, 2014). Here, stomatal dynamics
 533 were accounted for by describing the time dependence of J_{ATP} and τ with a set of recursive
 534 equations analogous to Vialet-Chabrand et al. (2016):

$$\tau_t = \tau_{t-1} + \begin{cases} \frac{\tau - \tau_{t-1}}{K_i} & \text{if } \tau_{t-1} < \tau \\ \frac{\tau - \tau_{t-1}}{K_d} & \text{else} \end{cases}, \quad 18$$

535 where τ_t and τ_{t-1} are the values at the time step t or at the previous step $t-1$; τ is the steady state value
 536 (Eqn 11), K_i and K_d are the time constants for an increase and decrease in g_s , respectively.

537 Similarly, we write:

$$\begin{cases} J_{ATP t} = J_{ATP t-1} + \frac{J_{ATP} - J_{ATP t-1}}{K_J} & \text{if } J_{ATP t-1} < J_{ATP} \\ J_{ATP t} = J_{ATP} & \text{else} \end{cases}, \quad 19$$

538 where K_J is the time constant for an increase in J_{ATP} , $J_{ATP t}$ and $J_{ATP t-1}$ are the values at the time step t
 539 or at the previous step $t-1$; J_{ATP} is the steady state value (Eqn 8).

540 *Plant growth conditions, leaf measurements and model parameterisation*

541 *Zea mays* L. (F1 Hybrid PR31N27, Pioneer Hi-bred, Cremona, IT), *Eragrostis curvula*,
 542 *Heteropogon contortus* and *Themeda triandra* plants were grown in 2.5 dm³ pots filled with three-
 543 parts commercial loam-free top soil (Boughton Ltd. Kettering, UK) plus one-part John Innes No.3
 544 compost (John Innes Manufacturers Association, Reading, UK). Plants were grown in controlled-
 545 environment growth rooms (BDR16, Conviron Ltd, Winnipeg, Canada) with a 14-hr photoperiod of
 546 700 (maize) or 350 (grasses) $\mu\text{mol m}^{-2} \text{s}^{-1}$ *PPFD* Light was provided from a 3:1 mix of 39 W
 547 white-fluorescent tubes (Master TL5, Philips, Eindhoven, Netherlands) and 39 W red-blue
 548 fluorescent tubes (Grolux T5, Havells-Sylvania, Newhaven, UK), augmented with six 105 W
 549 halogen light bulbs (GLS, Havells-Sylvania). Air temperature was 27 °C / 18 °C (day / night) and
 550 relative humidity was 70 % / 50 % (day / night). Plants were manually watered every one-to-three
 551 days to provide variation in soil water availability over natural wetting and drying cycles.

552 To determine transpiration (E), assimilation (A) and g_s under operational growth conditions,
 553 instantaneous leaf gas exchange at midday was measured within the growth chambers on young,
 554 fully expanded leaves under a *PPFD* of 700 (maize) or 350 (grasses) $\mu\text{mol m}^{-2} \text{s}^{-1}$, and reference
 555 [CO_2] of 400 $\mu\text{mol mol}^{-1}$ with an infra-red gas analyser (IRGA, LI6400XT, LI-COR, USA), fitted

556 with a 6400-40 leaf chamber fluorometer for maize and a red-blue LED light source (6400-02B, LI-
 557 COR Biosciences) for the three C₄ grass species (Bellasio et al., 2016b). Leaf water potential at
 558 midday (Ψ_{MD}) and midnight (Ψ_{MN}) (a proxy for soil water potential, Ψ_{Soil}) were measured on the
 559 apical portion of fully light exposed leaves cut the day and night following instantaneous gas
 560 exchange measurements with varying time since last watering to yield measurements at a range of
 561 soil water availability. Leaf water potential was measured using a pressure chamber (PMS
 562 Instrument Company, Model 1000, Albany, USA). Plant hydraulic conductance (K_{PLANT}) was
 563 calculated as $E/(\Psi_{MN}-\Psi_{MD})$ (Fini et al., 2013).

564 Photosynthetic response curves (an $A-C_i$ and $A-PPFD$ curve under ambient (21 %) and low (2 %)
 565 O₂ (maize only) were measured at the bench in a randomised order on $n = 9, 8, 5$ and 3 (maize,
 566 *Eragrostis Heteropogon* and *Themeda*, respectively) plants. Primary gas exchange data were
 567 corrected for CO₂ diffusion (Boesgaard et al., 2013) as:

$$A = Photo + \frac{0.4(400-C_a)}{100 Area} \quad 19$$

568 where *Photo* is the uncorrected assimilation as calculated by the LI-COR software, 400 is the
 569 external CO₂ concentration, C_a is the CO₂ concentration in the cuvette, and *Area* is leaf area within
 570 the cuvette: 2 cm² (maize) or 6 cm² (grasses). C_i was recalculated using *A* calculated with Eqn 19.
 571 Diffusion-corrected data are reported in Supporting Information File S2. Data were analysed
 572 following the 13-step protocol of Bellasio et al. (2016a) to derive a suite of photosynthetic
 573 parameters (Table 1, and Table S2).

574 To parameterise the model, g_{BS} , $J_{ATP_{MAX}}$, R_{LIGHT} , $V_{P_{MAX}}$, θ , and $Y(ATP)_{LL}$ were derived through
 575 the analysis described above (Table 1 and S3); $V_{C_{MAX}}$ is not well constrained by gas exchange
 576 (Pinto et al., 2014), and for maize was taken from von Caemmerer (2000), or calculated as
 577 $J_{ATP_{MAX}}/4$ (grasses); K_C , K_O , and K_P were taken from Ubierna et al. (2016); g_M was taken from
 578 Barbour et al. (2016), O_M was assumed to equal ambient [O₂]; g_{S0} and D_{S0} were averaged from
 579 measurements taken in the dark; V_R was set at 150 mmol m⁻² after Farquhar and Wong (1984); p
 580 and E_t were set at the values reported in Farquhar and Wong (1984); a_t was initially set at the values
 581 reported in Farquhar and Wong (1984) and then empirically adjusted (-30%) such that the output
 582 from Eqn 10 fitted observations at low C_i (Fig 3C); π_e was linked to Ψ_{Soil} through a simple linear
 583 relationship, $\pi_e = 1.2 - 0.4 \Psi_{Soil}$, derived by liner regression of data from Sharp and Davies (1979).
 584 When responses were simulated under well-watered conditions, Ψ_{Soil} was set at 0 MPa. We derive χ
 585 and β as a single quantity, $\chi\beta$ after Rodriguez-Dominguez et al. (2016). $\chi\beta$ is dependent on stomatal
 586 morphology, which is in turn under environmental control (Franks and Farquhar, 2007), and we
 587 consequently expect $\chi\beta$ to differ between species and respond to environmental growing conditions.
 588 $\chi\beta$ was initially set at the values reported in Buckley et al. (2003) and then empirically adjusted
 589 such that the output from Eqn 10 fitted observations at high *PPFD* (for maize Figure 2 C, for
 590 grasses Figure S3 A, B and C). The time constants defining increases and decreases in g_S (K_i , K_d) or

591 increase in $J_{ATP}(K_J)$ were derived through curve-fitting with the dataset reported in Figure 6. For
592 clarity and simplicity the fitting described in this paragraph was done by manual adjustment,
593 avoiding automated routines. All model parameters and values are listed in Table 1 and S3
594 (grasses).

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