

1 **Coordination of stomata and vein patterns with leaf width underpins water use**
2 **efficiency in a C₄ crop**

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25

26 **Abstract**

27

28 Despite its importance for crop productivity in drought-affected environments, the
29 underlying mechanisms of variation in intrinsic water use efficiency (*iWUE*) are not
30 well understood, especially in C₄ plants. Recently, Cano *et al.* (2019) discovered that
31 leaf width (*LW*) correlated negatively with *iWUE* and positively with stomatal
32 conductance for water vapour (g_{sw}) across several C₄ grasses. Here, we analysed these
33 relationships within 48 field-grown genotypes that cover a broad range of variation in
34 *LW* in *Sorghum bicolor*, a well-adapted C₄ crop to xeric and hot conditions, by
35 measuring and modelling leaf gas exchange and leaf energy balance three times a day,
36 using anatomical traits as potential drivers for *iWUE*. *LW* correlated negatively with
37 *iWUE* and stomatal density, but positively with g_{sw} , interveinal distance of
38 longitudinal veins (*IVDL*) and the percentage of stomatal aperture relative to
39 maximum. Energy balance modelling showed that wider leaves opened the stomata
40 more to generate a more negative leaf-to-air temperature difference especially at
41 midday, when air temperatures exceeded 40°C. These results highlight the important
42 role that *LW* plays in shaping *iWUE* through modification of vein and stomatal traits
43 and by regulating stomatal aperture. Therefore, *LW* could be used as a predictor for
44 higher *iWUE* among sorghum genotypes.

45

46 **Key words:** C₄ photosynthesis, *iWUE*, leaf boundary layer conductance, leaf
47 temperature, leaf size, natural genetic variation, stomatal density, vein density.

48 **Introduction**

49 Climate change is posing additional challenges on global agricultural production,
50 particularly in developing countries (Wheeler & von Braun 2013). The duration and
51 frequency of heat waves and the number of warmer days and nights have increased on
52 a global scale, with droughts becoming more common, especially in the tropics and
53 sub-tropics (Stocker *et al.* 2013). This, coupled with the prediction for human
54 population to exceed 9 billion by 2050, may threaten food security (Valin *et al.* 2014).
55 As the majority of the world's cereal crops are rain-fed and fresh water for
56 agricultural production is limited, increased investment in irrigation technology is
57 unlikely to alleviate the problems of water scarcity (Portmann, Siebert & Döll 2010;
58 Elliott *et al.* 2014). Instead, increased plant production per available water is needed
59 to sustain or improve crop productivity in rain-fed conditions (Godfray *et al.* 2010;
60 Beddington *et al.* 2012). C_4 crops play an important role for food security because the
61 CO_2 concentrating mechanism (CCM) in C_4 leaves leads to greater productivity and
62 leaf intrinsic water use efficiency (*iWUE*), which refers to the ratio of 'net
63 photosynthesis' (A_n) to 'stomatal conductance to water vapour' (g_{sw}), than in C_3 crops
64 (Leakey *et al.* 2019). Significant variation in *iWUE* exists among C_4 grass species
65 (Cano *et al.* 2019; Pathare, Koteyeva & Cousins 2020a) and within C_4 crops (Cruz de
66 Carvalho, Cunha & Marques da Silva 2011; Li *et al.* 2017; Feldman *et al.* 2018). The
67 mechanisms that underlie *iWUE* variation in C_4 crops are yet to be elucidated and
68 constitute the main objective of this study.

69

70 Intrinsic water use efficiency (*iWUE*) is the component of leaf water use efficiency
71 (*WUE*, the ratio of ' A_n ' to leaf transpiration 'E') that is regulated by the plant
72 (Osmond, Bjorkman & Anderson 1980). *iWUE* usually scales to plant *WUE*, defined

73 as the ratio of biomass produced by the amount of water transpired by the crop
74 (Condon *et al.* 2004). Generally, *iWUE* is stable across different climates, making it a
75 target trait for genetic selection (Osmond *et al.* 1980; Geetika *et al.* 2019). Measuring
76 *iWUE* requires sophisticated gas exchange equipment and is time-consuming, limiting
77 its usefulness as a selection tool in breeding programs (Farquhar *et al.* 1989).
78 Currently, there is no simple and fast alternative to gas exchange for measuring *iWUE*
79 in C₄ crops. The association between leaf carbon isotope composition ($\delta^{13}\text{C}$) and
80 *iWUE*, which has successfully been used to screen for WUE in C₃ species (Farquhar
81 & Richards 1984; Condon, Richards & Farquhar 1987), is not straightforward in C₄
82 photosynthesis (Henderson, Caemmerer & Farquhar 1992; Ellsworth & Cousins
83 2016). Improving *iWUE* in C₄ crops has received less attention than in C₃ crops,
84 possibly due to earlier studies reporting low genetic variability for plant *WUE*
85 (Donatelli, Hammer & Vanderlip 1992; Hammer, Farquhar & Broad 1997). Our
86 recently discovered positive correlation between *LW* and g_{sw} across C₄ grasses, the
87 stronger dependence of *iWUE* on g_{sw} than A_n , and the negative correlation between
88 *LW* and *iWUE*, uncovered promising avenues to improve *iWUE* in C₄ crops (Cano *et al.*
89 *et al.* 2019). These insights may also help identify physiological and anatomical
90 processes which increase *iWUE* without impairing photosynthesis, a main target for
91 crop breeding (Ghannoum 2009; Ghannoum 2016).

92

93 The relationship between *LW*, anatomical traits and *iWUE* in C₄ plants remains largely
94 unexplored and, to the best of our knowledge, has not been examined within a single
95 C₄ grass species. A few recent studies started exploring these relationships, including
96 correlations found between *LW*, g_{sw} and *iWUE* among C₄ grasses (Cano *et al.* 2019),
97 negative correlations between *iWUE* and vein density per leaf area (V_D) within an

98 annual C_4 herb (Reeves *et al.* 2018) and across C_4 grasses (Pathare *et al.* 2020b), and
 99 the positive correlation between quantum yield of photosynthesis and V_D in C_4 grasses
 100 (Ogle 2003). Wider leaves of C_4 grasses have lower V_D (Baird *et al.* 2021) as found
 101 also in eudicotyledons plants, where density of 1°, 2° and 3° veins declined with leaf
 102 size following a quasi-geometrical model (Sack *et al.* 2012). This geometrical model
 103 of leaf development was originally observed in the model C_3 species *Arabidopsis* in
 104 which major veins are formed during early leaf development, where the initial leaf
 105 size is determined by an early high rate of cell division, and the minor veins would
 106 develop afterwards during the high cell expansion phase (Donnelly *et al.* 1999). The
 107 parallel (striate) venation of the C_4 leaves arises in an hierarchical sequence similar to
 108 that observed in *Arabidopsis* (Nelson & Dengler 1997), as well as in monocotyledon
 109 C_3 grasses, where LW is negatively correlated with V_D (Smillie, Pyke & Murchie
 110 2012; Nawarathna *et al.* 2017; Baird *et al.* 2021). In some C_4 eudicot species,
 111 substantial leaf expansion continued after vein formation, and minor vein density
 112 declined as leaves continued to expand (McKown & Dengler 2009). V_D usually
 113 correlates positively with stomatal density (S_D) during leaf development to balance the
 114 water supply through veins with transpirational demand (Boyce *et al.* 2009; Brodribb
 115 & Jordan 2011; Carins Murphy, Jordan & Brodribb 2014; Fiorin, Brodribb &
 116 Anfodillo 2016), and hence wider leaves of C_4 grasses may also have lower S_D than
 117 narrow ones. Generally, a combination of small stomata (S_S) and greater S_D usually
 118 lead to higher g_{sw} across a wide range of environmental conditions and plant species
 119 (Salisbury 1927; Brodribb & Holbrook 2003; Franks & Beerling 2009). However, V_D ,
 120 S_S and S_D are not the only determinants of g_{sw} and wider leaves of C_4 grasses may have
 121 higher g_{sw} than narrower ones (Cano *et al.* 2019), even when the anatomical
 122 determinants of g_{sw} might suggest the opposite (e.g. lower S_D). Plants can alter

123 stomatal pore aperture by actively adjusting guard cell turgor pressure and thereby
 124 modulate g_{sw} (Hetherington & Woodward 2003; Kollist, Nuhkat & Roelfsema 2014).
 125 Accordingly, rice with reduced S_D increased g_{sw} to almost 40 % of the anatomically
 126 derived maximum g_{sw} (g_{sw_max}) through the upregulation of stomatal aperture under
 127 well-watered conditions, high light intensity and high temperature (40°C), achieving
 128 the same gas exchange rates and $iWUE$ as control rice which had much higher S_D and
 129 about 10 % of g_{sw_max} (Caine *et al.* 2019). Under conditions of high irradiance and high
 130 temperature which prevail where C_4 crops are grown, stomata should remain more
 131 open (to sustain transpirational cooling) if enough water is available in the soil (Drake
 132 *et al.* 2018; Caine *et al.* 2019). Therefore, the need to regulate leaf temperature may
 133 alter the physiology of stomatal anatomy.

134

135 Transpiration rate (E) is one effective way to dissipate the absorbed energy and cool
 136 down the leaf, although E is not only determined by the amount of available energy,
 137 but also by the product of 1) the difference in water vapour pressure between the sites
 138 of water evaporation in the leaf mesophyll and the free air beyond the adhering
 139 boundary layer next to the leaf surface (VPD) and 2) the combined total conductance
 140 to water vapour (g_{tw}) along the pathway (i.e. stomatal and boundary layer (BL)
 141 conductances acting in series) (Gates 1980; Nobel 2009). If the leaf temperature (T_{leaf})
 142 rises relative to air temperature, E would rise for the same g_{tw} due to the increase in
 143 VPD (Gates 1968; Jarvis & McNaughton 1986). Conversely, a reduction in g_{tw} would
 144 reduce E for the same VPD . BL is an aerodynamic characteristic of the leaf blade that
 145 is directly determined by leaf dimension in the direction of the wind and inversely
 146 related to the wind speed. Therefore, large leaves have a thicker BL which increases
 147 the resistance to the transfer of gases, e.g. out coming water vapour and incoming

148 CO₂, compared with smaller leaves (Gates 1980). Furthermore, *BL* exerts resistance to
149 the heat flux between the leaf and the surrounding air, such that overheated leaves
150 with a thicker *BL* would cool down slower than ones with a thinner *BL* (Gates 1980).
151 During hot days, the control of T_{leaf} is important not only for the water status of the
152 plant, but also to avoid reaching leaf temperatures that compromise the integrity of
153 membranes and leaf photochemistry (Leigh *et al.* 2012). Parkhurst and Loucks (1972)
154 quantitatively evaluated the importance of leaf size and *BL* on *WUE* for any given
155 combination of light, water and temperature by applying an energy budget of the leaf
156 and a function to describe the rate of photosynthesis. They concluded that in hot,
157 windy and dry environments with high radiation, small leaves confer a selective
158 advantage; while in hot environments with low radiation. Larger leaves may be more
159 beneficial for *WUE*. Crop canopies and leaves developed under full sunlight may
160 constitute examples of the first environment, while the interiors of tropical forests and
161 open environments in the tropics during the wet season may exemplify the latter.

162

163 Sorghum [*Sorghum bicolor* (L.) Moench.] offers great opportunities to study
164 anatomical and morphological adaptations to hot and xeric climates, and improving
165 *iWUE* in C₄ crops. Sorghum evolved, and then was domesticated, predominantly in
166 the hot tropics of Africa and South Asia, including populations adapted to open
167 habitats with hot and arid climate, and others developed in more mesic climates
168 influenced by cloud cover during the wet season in the tropics (Knox *et al.* 2012;
169 Morris *et al.* 2013). This array of climates might have produced a large variability of
170 *LW* in sorghum genotypes, likely linked to the different mechanisms to optimize
171 *iWUE* and plant production (Parkhurst & Loucks 1972). Notably, *LW* and S_D are
172 highly heritable traits in sorghum (Liang *et al.* 1973; Liang *et al.* 1975). The use of

173 the traditional varieties in current breeding programs for higher yield hybrids may
174 have increased the morphological and genetic diversity within sorghum as well (Casa
175 *et al.* 2008; Mace *et al.* 2013; Morris *et al.* 2013). In addition to being a source of
176 allele diversity, sorghum is known for its high productivity, especially in hot and arid
177 environments (Sanchez-Diaz & Kramer 1971; Muchow 1989; Staggenborg,
178 Dhuyvetter & Gordon 2008; Borrell *et al.* 2021), constitutes the staple food for more
179 than 500 million people in Africa and Asia (Assefa, Staggenborg & Prasad 2010;
180 Dahlberg *et al.* 2011) and is an important model for improvement of C₄ bioenergy
181 grasses (Carpita & McCann 2008; Wannasek *et al.* 2017).

182

183 In this study, we screened a large number of sorghum genotypes displaying high
184 variability in LW and grown in a rain-fed field to test whether LW correlates with
185 $iWUE$ within a single C₄ crop (sorghum) and to develop a mechanistic basis for this
186 relationship. Under these conditions, we expect that LW would affect both anatomical
187 traits and gas exchange variables, but also that the higher resistance to water and heat
188 dissipation imposed by a thicker BL in wide leaves could be counteracted by a lower
189 stomatal resistance, and the opposite in narrow leaves. Overall, we tested the
190 following hypotheses: **1)** LW correlates positively with g_{sw} and negatively with $iWUE$
191 within one C₄ species (sorghum); **2)** Wider leaves have lower vascular and stomatal
192 densities (V_D and S_D , respectively) but higher stomatal aperture, which in turn
193 determines the higher g_{sw} , and lower $iWUE$, of wider leaves relative to narrow ones;
194 and **3)** Wider leaves have higher g_{sw} to counteract BL thickness and keep leaf
195 temperature under safe values, especially at midday, in a process ruled by the energy
196 balance of field-grown sorghum.

2. Materials and methods

2.1 Genetic material

A sub-set of 48 sorghum genotypes [*Sorghum bicolor* (L.) Moench.] were selected to cover a broad range of genetic backgrounds and leaf width (Fig. S1) from a larger trial comprising 693 genotypes from the Sorghum Conversion Program (SCP). The SCP is a backcross breeding scheme in which genomic regions conferring early maturity and dwarfing from an elite donor were introgressed into approximately 800 exotic sorghum accessions representing the breadth of genetic diversity in sorghum (Stephens, Miller & Rosenow 1967). Forty-eight genotypes were the maximum number of genotypes that we were able to measure in the field experiment and they were restricted to one corner of the field to reduce spatial variability.

2.2 Field site and agronomy

The trial was planted on a near-level site on alluvial soil at the Hermitage Research Facility (28°12'S, 152°06' E; 480 m above sea level) in north-eastern Queensland, Australia. The soil had a high montmorillonite clay content with a full sub-soil moisture profile at sowing (McKeown 1978). 280 kg ha⁻¹ urea containing 46 % N was applied prior to sowing and the trial was kept weed and pest free with a pre-emergent herbicide (Atrazine 900 WG, Genfarm, Australia) and in-trial applications of insecticide (Alpha-Scud, Adama Agriculture Products, Australia) as needed.

The trial was planted with a vacuum precision planter at a target population of 50,000 plants/ha on 6 December 2016. Each plot planted with one genotype was 5 m long and 3 m wide and consisted of four rows. Row widths were 0.6 m between the middle

221 two rows and 0.75 m between the middle and outside rows. The trial was not
222 irrigated, but planted on near-full soil moisture profiles and received 127 mm of
223 rainfall from 1 December to 3 February, the last day of gas exchange measurements.
224 The estimated evapotranspiration for this period was 254 mm. Further climate data for
225 the growing season is presented in Fig. S2.

226

227 **2.2 Leaf gas exchange and leaf temperature measurements**

228 Leaf gas exchange was measured on 4 to 6 plants for each genotype selected from the
229 middle two rows of a plot. Fully sun-lit and relatively young, but fully expanded
230 leaves (depending on genotype this was usually leaf 12 to 18 counting from the base
231 of the plant) without appreciable damage were selected for the measurements. Gas
232 exchange was measured on the leaf blade avoiding the midrib using a LI-6400XT
233 (LICOR Biosciences, Lincoln, NE, USA) with the 2-cm² chamber (6400-40). CO₂
234 concentration was set at 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}\text{air}$, air flow rate through the cuvette was
235 300 $\mu\text{mol air s}^{-1}$ and light intensity of 1800 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (10% blue light)
236 with no control of leaf temperature nor incoming water vapour to better reproduce
237 field conditions. Gas exchange was measured on the flat portion of the leaf that was
238 orthogonal to the sun beam to ensure measuring in a photosynthetically activated
239 portion of the leaf during the day. As we measured only on sunny days and the
240 conditions inside the leaf chamber were close to ambient conditions, quasi steady state
241 readings were achieved after 2-3 minutes. This time was sufficient for full
242 equilibration of the gas exchange inside the chamber. Full acclimation to chamber
243 conditions usually takes longer because the incoming air, the temperature of chamber
244 walls, the set irradiance and the action of the fan that reduces *BL* thickness trigger
245 changes in leaf temperature (T_{leaf}) inside the chamber, moving it away from its

original temperature in the field. Hence by limiting the time that leaves stayed under chamber conditions, it was possible to capture the leaf-to-air temperature difference ($\Delta T_{\text{leaf-air}}$) closer to field conditions. T_{leaf} was measured with the inbuilt LICOR thermocouple that was in contact with the lower leaf surface. Gas exchange measurements were repeated on the same marked leaves three times a day: in the morning, midday and afternoon (10-11:30, 13-14:30 and 16-17:30 local time, respectively). The gas exchange was measured from 25 January to 3 February. At this time of the year, sunrise is at 5:30 and sunset at 18:30.

2.3 Leaf anatomical traits

Approximately 1 cm² leaf section was taken from the middle of the same leaf measured for gas exchange, about 1 cm away from the midrib, and fixed with FAA (glacial acetic acid, formaldehyde, 70% ethanol in 5:5:90 v/v.) for 48h and then preserved in 70% ethanol and in darkness until analysed as described in Rodríguez-Calcerrada *et al.* (2008). The same leaf section was used for measuring stomata and vein traits (Fig. S3) by first taking a leaf impression to measure stomatal traits for both leaf surfaces and then clearing the leaf section to measure the veins. Leaf stomata and vein traits were measured for three leaf sections from three individual plants for each genotype (a total of 144 samples from all genotypes).

To measure the stomatal traits, epidermal impressions were taken with acetate of cellulose from both leaf surfaces and then mounted on a slide to capture images by light microscopy using an Axio Scope.A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), as described by Zhao *et al.* (2017). Images from the microscope were

270 analysed using ImageJ (Schneider, Rasband & Eliceiri 2012). Four images of each
 271 leaf section (two from each leaf side) were taken at 20x magnification (field of view =
 272 0.15 mm²) using an optical microscope equipped with a digital camera (AxioCam
 273 MRc (Carl Zeiss Microscopy GmbH, Jena, Germany). Stomatal densities for each leaf
 274 surface (stomata that were counted only if more than 50% of the stoma was in the
 275 image) were measured separately because sorghum tends to have higher stomatal
 276 density on the lower (abaxial) compared with the upper (adaxial) surface (Liang *et al.*
 277 (1975). Total leaf stomatal density, S_D , was the sum of both adaxial and abaxial
 278 stomatal densities (i.e. $S_D = S_{Dada} + S_{Daba}$) and expressed per mm² of leaf area. Stomatal
 279 width (μm, SW), i.e. closed stomatal width (including two guard cells and two
 280 subsidiary cells), guard cell length (μm, GL) and pore length (μm, PL) were averaged
 281 from at least 60 randomly chosen stomata per leaf surface and genotype. Stomatal size
 282 (μm², S_S) was calculated by multiplying stomatal width and guard cell length ($S_S = SW$
 283 * GL). Theoretical maximum leaf stomatal conductance to H₂O ($g_{sw_max} = g_{sw_max_adaxial} +$
 284 $g_{sw_max_abaxial}$) may be estimated according to stomatal dimensions for each leaf side and
 285 environmental conditions following Franks and Farquhar (2001), which includes one
 286 end-correction.

$$287 \quad g_{sw_max} = \frac{D_{air-H_2O} S_D a_{max}}{v \left(l + \frac{\pi}{2} \sqrt{\frac{a_{max}}{\pi}} \right)} (1)$$

288 where D_{air-H_2O} is the diffusion coefficient of water vapour in air, which should be
 289 corrected for temperature using the equation by Marrero and Mason (1972) described
 290 in the Supplementary Methods A.

291 The other variables of equation 1 are v = molar volume of air (e.g. 27.3 10⁻³ m³ mol⁻¹
 292 at 42°C and 96 kPa), l = depth of stomatal pore (μm) which for grasses can be

approximated to the width of both guard cells (GW) for closed stomata (Franks & Farquhar 2007) and π is 3.1416. We have estimated the mean stomatal pore area when fully open (a_{max} , μm^2) as the product of closed guard cells width (GW) and pore length (PL) because, according to pictures provided in Franks and Farquhar (2007) and Franks *et al.* (2014), it provides a better approximation to the geometry of fully open stoma in grasses than other approaches described in the cited works:

$$a_{max} = GW * PL \quad (2)$$

Equation 1 can be used to estimate the operational stomatal pore area (a_{ope}) that matched with the measured stomatal conductance to water vapour (g_{sw}), which results in the next equation:

$$a_{ope} = \frac{\left(g_{sw}^2 v^2 \frac{\pi}{4} + 2 D_{air-H_2O} S_D g_{sw} v l \right) + \sqrt{g_{sw}^4 v^4 \frac{\pi^2}{16} + g_{sw}^3 v^3 \pi D_{air-H_2O} S_D l}}{2 D_{air-H_2O}^2 S_D^2} \quad (3)$$

The ratio of the operational stomatal pore area (a_{ope}) to a_{max} provides a measure of stomatal aperture, expressed as % stomatal aperture.

For vein analysis, all leaf sections were cleared and measured following the protocol described by Scoffoni and Sack (2013). Nine images (fields of view under 10x magnification) of each genotype were taken using the same light microscope described above. The length of all veins from each image were measured using Image J software (Schneider *et al.* 2012). Leaf vein density (V_D), sometimes called total vein length per leaf area (VLA), was calculated as the total longitudinal and transversal veins length per leaf area. The distances between longitudinal veins were measured between the centres of adjacent veins and averaged to get the interveinal distance of

longitudinal veins (*IVDL*). The total number of longitudinal veins across the leaf width was estimated as $1000 * LW(mm) / IVDL(\mu m)$. The interveinal distance of transversal veins (*IVDT*) were measured between the centres of adjacent transverse veins running between a pair of longitudinal veins according to Ueno *et al.* (2006).

319

2.4 Estimation of leaf boundary layer conductance (g_{blw_effect})

The effective boundary layer conductance for water vapour including the two sides of the leaf (g_{blw_effect} or BLCond according to LICOR nomenclature) was calculated as:

$$g_{blw_effect} = \frac{g_{blw}}{\frac{Stoma_{ratio}^2 + 1}{(Stoma_{ratio} + 1)^2}} (4)$$

Where the one-sided leaf boundary layer conductance for water vapour (g_{blw}) is calculated in $mol\ H_2O\ m^{-2}\ s^{-1}$ as described in the Supplementary Methods B and the stomatal ratio ($Stoma_{ratio}$) may be approximated to the ratio of S_{Dada} to S_{Daba} .

327

2.5 Estimation of leaf energy balance

The energy balance of a leaf in steady state has three major components: the net radiation 'R' ($W\ m^{-2}$), which includes short and long wave radiation, the sensible heat flux 'Q' ($W\ m^{-2}$) which represents the heat exchange between the leaf and the air, and the latent heat flux 'L' ($W\ m^{-2}$) or heat loss by leaf transpiration:

$$0 = R + Q + L (5)$$

Following the Manual of LICOR 6400 v.6, equation 5 was further detailed in the Supplementary Methods B and used to estimate $\Delta T_{leaf-air}$ and/or g_{sw} in leaves during

336 measurements within the cuvette and under field conditions. To estimate $\Delta T_{\text{leaf-air}}$ from
337 the energy balance model we used either the linearization method or the numerical
338 solver option of Excel to minimize the sum of square errors of equation 5. See Excel
339 spreadsheet for calculation on the Supplementary Material.

340

341 **2.6 Statistical analysis**

342 Statistical significance analysis of the means was performed by using repeated
343 measures ANOVA followed by Duncan's test. Relationships between the unweighted
344 variables were presented with Pearson correlation coefficients using IBM SPSS
345 statistics version 25.0 (SPSS Inc., Chicago, IL, USA). The significance of correlations
346 was tested by bivariate linear regression using IBM SPSS statistics taking the mean
347 values for each genotype as input data, with p values of <0.05 considered statistically
348 significant. All plots were performed with NCSS 2021 version (NCSS, LLC, Utah,
349 USA).

3. Results

3.1 Variability of leaf gas exchange and relationships with leaf width (*LW*)

Measurements of g_{sw} , $iWUE$ and A_n varied widely across 48 *Sorghum bicolor* genotypes (Table S1) and throughout the day with higher gas exchange rates in the morning, lower A_n but not g_{sw} at midday, and lowest gas exchange rates in the afternoon (Table 1, Fig.1a). Among genotypes, g_{sw} varied 3-fold from 0.14 to 0.42 mol H₂O m⁻² s⁻¹ when measured at midday. Similarly, significant 2.4-fold variation was observed in A_n , with a minimum of 24.3 to a maximum of 58.8 μmol CO₂ m⁻² s⁻¹. The $iWUE$ showed less (1.6-fold) variation among genotypes, nonetheless ranging from 128 to 211 μmol CO₂ mol H₂O⁻¹ at midday and also exhibited lower variation across the day (Table 1), because A_n and g_{sw} were highly correlated at any time of the day (Fig. 1a, Table 1). $iWUE$ was significantly and negatively correlated with g_{sw} regardless of time of day ($r > -0.52$; $p < 0.001$) (Fig. 1b, Table 1). Although a negative correlation was also found between $iWUE$ and A_n (Fig. 1c), this was weaker ($r < -0.14$; $p < 0.05$), suggesting that $iWUE$ was mainly associated with changes in g_{sw} rather than A_n .

The sorghum genotypes varied substantially (2.6-fold) in leaf width (*LW*), ranging between 49 and 128 mm, with mean and median values of 83 and 84 mm, respectively (Table S2). Confirming our initial hypothesis, genotypes with wider leaves had substantially higher g_{sw} across the day ($r > 0.41$; $p < 0.001$) (Fig. 2b). Nevertheless, the strong linear correlation of *LW* and g_{sw} measured in the morning and at midday ($r > 0.57$; $p < 0.001$) disappeared in the afternoon (Table 1, Table S3). A positive correlation between *LW* and A_n was also found in the morning and midday, although these correlations were weaker than for g_{sw} and the slopes of the linear regressions were rather flat (Fig. 2a). *LW* and $iWUE$ were negatively correlated at morning and

midday (Fig. 2c, Table 1). Interestingly, sorghum genotypes with narrow leaves (e.g. $LW < 75$ mm) maintained rather constant g_{sw} or $iWUE$ during the course of the day (only 3 out of 16 genotypes decreased g_{sw} in the afternoon), while this number increased to 8 out of 25 for LW between 75 and 100 mm, and 6 out of 7 genotypes with $LW > 100$ mm showed a significant decrease of g_{sw} in the afternoon (Table S1).

380

3.2 Relationships between LW and leaf anatomical features

To investigate whether changes in LW were related to leaf anatomical traits important for gas exchange, we investigated vascular and stomatal features (Tables S2 and S4). A strong negative linear correlation was found between LW and V_D ($r = -0.59$, $p < 0.001$). Likewise, LW showed a significantly positive linear correlation with $IVDL$ ($r = 0.65$, $p < 0.001$) (Fig. 2d). The total number of longitudinal veins across the leaf width was positively correlated with LW (Fig. S4), suggesting that the formation of minor longitudinal veins was aligned with leaf width. LW and S_D were negatively correlated (Fig. 2f) as well as LW and $IVDL$ (Table 2), given that stomata develop in parallel lines to the longitudinal veins in grasses. Indeed, the ratio of S_D to V_D averaged 30 stomata per mm of vein length across the entire LW range (Fig. S5), and stomatal size was also not dependent on LW (Table 2). While there was only a small trend for maximum stomatal pore size (a_{max}) to be greater in wider leaves ($r = 0.33$; $p < 0.001$), operational stomatal aperture (a_{ope}) correlated more strongly with LW (e.g. $r = 0.59$; $p < 0.001$ at midday); and hence the % stomatal aperture showed a positive correlation with LW across all genotypes (Fig. 2e) (at midday $r = 0.48$; $p < 0.001$). This indicates that genotypes with wider leaves tended to have their stomata more open than narrow-leaf genotypes.

3.3 Correlations between gas exchange, leaf mass per area (*LMA*) and leaf anatomical traits

Neither A_n nor g_{sw} were correlated with any of the anatomical stomatal traits (e.g. S_D , S_s or a_{max}), but both showed positive and strong correlations with % stomatal aperture while $iWUE$ displayed a negative correlation with % stomatal aperture (Table 3, Fig. 3). In fact, there were significant negative correlations between the theoretical maximum stomatal conductance (g_{sw_max}) and the % stomatal aperture (Table 3) and between the % stomatal aperture and S_D (Fig. 4a). Hence, the degree of stomatal openness was the main factor determining the variation in stomatal conductance, and hence $iWUE$.

At the same time, A_n and g_{sw} were strongly positively correlated with $IVDL$, particularly at midday (Table 3, Fig. 3de), and $iWUE$ was negatively related to $IVDL$ at different times during hot days (Fig. 3f). In line with these findings, there was also a statistically significant relationship between $IVDL$ and % stomatal aperture (Fig. 4c), implying that stomata were more open as the distance between longitudinal veins increased and as S_D decreased.

Despite a 1.76-fold variation for *LMA*, i.e. 61.6 - 108.5 g m⁻² (Table S2), *LMA* was unrelated to leaf gas exchange variables (i.e. A_n , g_{sw} or $iWUE$), LW , V_D , $IVDL$ or S_D , but positively related to the distance between transverse veins ($IVDT$) and stomatal size (S_s) (Tables 2&3).

420

3.4. Regulation of leaf temperature and importance of leaf width related to boundary layer for leaf gas exchange.

423 Leaf temperature (T_{leaf}) rose from an average of 36 °C in the morning to almost 41 °C
 424 at midday then falling by one degree in the afternoon (Table 1, Fig. 4b). Although
 425 transpirational cooling was effective in decreasing T_{leaf} , i.e. higher g_{sw} was correlated
 426 with lower T_{leaf} at midday (Table 1), A_n was negatively correlated with T_{leaf} at midday
 427 and afternoon (Table 1), especially if T_{leaf} was above 40 °C (the maximum temperature
 428 reached in the morning), when A_n dramatically declined with increasing T_{leaf} (Fig. 4d).
 429 Given the boundary layer (BL) acts as a resistor in series with the stomata for both
 430 transpiration and heat loss, we estimated one-sided leaf BL conductance for water
 431 vapour (g_{blw}) and the BL thickness (δ_{bl}) at midday under field conditions (e.g. air
 432 temperature at 42 °C and wind speed of 0.1 m s⁻¹) for each LW to assess the impact on
 433 gas exchange. Widest leaves had 60% thicker δ_{bl} than the narrowest ones (Fig. 5a),
 434 and the effective two-sided BL g_{blw_effect} was highly correlated with $iWUE$, and
 435 negatively correlated with g_{sw} (Fig. 5b).
 436 Leaf to air temperature difference ($\Delta T_{leaf-air}$) and g_{sw} measured by LICOR were
 437 negatively correlated at any time of the day (Fig. 6). $\Delta T_{leaf-air}$ and g_{sw} estimated through
 438 the energy balance of the leaves inside the LICOR cuvette (Fig. 6b) and under field
 439 conditions (Fig. 6c) were generally in close agreement with the actual measured
 440 variables. The solver solution of the leaf energy balance within the cuvette produced
 441 correlations between $\Delta T_{leaf-air}$ and g_{sw} with slopes closer to the actual measurements
 442 (Fig. 6b) than using the linearization method (Fig. 6a). The best approximations to the
 443 measured values were field simulations in the morning and midday, but not in the
 444 afternoon due to the lower incoming radiation at this time of the day than inside the
 445 chamber (Fig. 6).
 446 To directly test the effect of LW on g_{sw} and on the transpiration rate (E) under field
 447 conditions, we simulated the energy leaf balance assuming that $\Delta T_{leaf-air}$ remains

448 constant when LW increases or decreases (Fig. 7). At low wind speed (e.g. 0.1 m s^{-1}),
 449 g_{sw} always increased with LW to keep constant $\Delta T_{\text{leaf-air}}$, but E remained almost
 450 unchanged with LW at this low wind speed (Fig. 7), due to the negative effect of LW
 451 on $g_{\text{blw_effect}}$ (Fig. 5). On average, at $\Delta T_{\text{leaf-air}}$ of $1.6 \text{ }^{\circ}\text{C}$, E was 20% lower than at $\Delta T_{\text{leaf-air}}$
 452 of $-1.6 \text{ }^{\circ}\text{C}$. At high wind speed (e.g. 2 m s^{-1}), E followed the same trend as g_{sw} , because
 453 of the lower magnitude of the $g_{\text{blw_effect}}$, and LW was an important regulator of E . At
 454 positive $\Delta T_{\text{leaf-air}}$, both g_{sw} and E were positively correlated with LW , while at negative
 455 $\Delta T_{\text{leaf-air}}$, they were negatively correlated with LW (Fig. 7). At high wind speed and to
 456 get the same $\Delta T_{\text{leaf-air}}$ of $1.6 \text{ }^{\circ}\text{C}$, E increased 39 % from the narrowest LW simulated
 457 (40 mm) to the widest (160 mm), i.e. from 4.5 to $6.2 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively.
 458 While to cool down the leaf, e.g. $\Delta T_{\text{leaf-air}}$ of $-1.6 \text{ }^{\circ}\text{C}$, E was reduced by 13.9 % from
 459 the narrowest to widest LW , i.e. from 12.3 to $10.6 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively.
 460 Altogether, LW modulated $iWUE$ in sorghum mainly through its influence on g_{sw} and
 461 the latter on $\Delta T_{\text{leaf-air}}$ due to the higher degree of stomatal aperture in wider leaves (Fig.
 462 8). LW was positively related with $IVDL$ and stomatal aperture, and negatively related
 463 with S_D . The higher g_{sw} in wider leaves measured in the morning and at midday was
 464 likely associated with the regulation of $\Delta T_{\text{leaf-air}}$ and E . In the morning, when wind
 465 speed was high as well as $g_{\text{blw_effect}}$, narrow and slightly hotter leaves than the
 466 surrounding air were more likely to reduce water losses through E (and g_{sw}) than in
 467 wider leaves (Fig. 7). At midday, when $g_{\text{blw_effect}}$ was minimal, larger stomatal
 468 apertures were needed in wide than in narrow leaves to reduce the elevated T_{leaf} .

469

470

4. Discussion

4.1. Leaf width (LW) predicts g_{sw} and $iWUE$ in sorghum

Variations in $iWUE$ among sorghum plants were mainly associated with changes in g_{sw} rather than A_n (Fig.1), as recently found among diverse C_4 grasses (Cano *et al.* 2019) and sugarcane clones (Li *et al.* 2017). This corroborates our *Hypothesis 1*, LW largely explained differences in g_{sw} (and hence, $iWUE$) in the morning and midday, but not in the afternoon, as wider leaves had higher g_{sw} than narrow leaves except in the afternoon (Fig. 2, Table S3). The occurrence of water stress during the gas exchange measurements may to some extent explain this lack of correlation between LW and g_{sw} in the afternoon. For example, the top soil at our field site was dry and some genotypes exhibited leaf rolling in the afternoon (Fig. S6). Furthermore, maximum g_{sw} measured in the morning (Table 1) was lower than for well-watered plants growing either in the glasshouse (Cano *et al.* 2019) or in the field (Wall *et al.* 2001), suggesting the occurrence of mild water stress at our study site (Ghannoum 2009). The decrease in g_{sw} in the afternoon was not equal in all genotypes; wide-leaf genotypes decreased g_{sw} to levels similar to that of narrow-leaf genotypes which, in turn, showed more constant gas exchange rates during the course of the day (Fig. 2, Tables S1 and S3). If wider-leaf genotypes had higher daily plant transpiration (because of the higher g_{sw} in the mornings and likely higher total leaf area than narrow-leaf genotypes for a similar culm number), we could expect lower leaf water potential in wider leaf genotypes that promoted a higher decline in g_{sw} in the afternoon than the narrow ones (Beardsell & Cohen 1975; Zhang & Davies 1990; Blum 2015). Inhibition of photosynthesis mediated by sugar accumulation in the leaves, as a consequence of the imbalance between photosynthesis and growth under water stress, could also explain gas exchange limitation in the late afternoon (Paul & Foyer 2001;

McCormick, Cramer & Watt 2008; Körner 2015). At midday, when leaf temperature rose above optimal temperature for photosynthesis ($\approx 36^\circ\text{C}$), approaching the thermal limit that compromises the stability of photochemistry ($\approx 43^\circ\text{C}$) in sorghum (Peacock 1982; Prasad, Boote & Allen 2006; Djanaguiraman *et al.* 2014; Prasad *et al.* 2019), g_{sw} was still high in wide-leaf genotypes (Fig. 4), implicating transpiration cooling as discussed in Section 4.4.

4.2. Vein density is highly determined by LW and negatively correlated with A_n in sorghum

As proposed in *Hypothesis 2*, a striking discovery of this study was the coordination between LW and $IVDL$, i.e. higher vein density as the leaf becomes narrower in sorghum (Fig. 2). A similar association between LW and $IVDL$ was found in rice (Feldman *et al.* 2014; Feldman *et al.* 2017) and between LW and $IVDL$ of second order veins in C_3 and C_4 grasses (Baird *et al.* 2021). Wide leaves had more veins across the leaf width (Fig. S4), suggesting that the development of the vasculature is co-ordinated with the tissue between the veins (mesophyll and bundle sheath) as LW increases in sorghum, and that the total number of minor veins in grasses scales with LW during the leaf expansion (Nelson & Dengler 1997). Higher V_D is often observed in species adapted to open environments with high irradiance and VPD , which usually leads to smaller and narrower leaves with higher $iWUE$ (Carins Murphy, Jordan & Brodribb 2012; Nardini, Pedà & Rocca 2012; Sack & Scoffoni 2013). Interestingly, increased vein density is among the first steps associated with the evolution of the C_4 syndrome in grasses in response to a drier climate and declining atmospheric CO_2 concentration during the Miocene. The evolution of greater $iWUE$ is an important step in C_4 evolution (Sage 2004; Edwards *et al.* 2010; Osborne & Sack 2012).

521 Nevertheless, increasing the density of minor veins may negatively affect A_n , despite
 522 enhanced $iWUE$, in sorghum (Fig. 3).
 523 High V_D is often correlated with high A_n in C_3 leaves (Brodribb, Feild & Jordan 2007)
 524 due to the major role of the stomata in limiting CO_2 diffusion. In contrast, the opposite
 525 trend was observed among sorghum genotypes (Fig. 3, Table 3) and in other C_4
 526 grasses (Pathare *et al.* 2020a). In C_4 plants, photosynthetic carbon reduction is
 527 primarily restricted to bundle sheath cells (BS) where Rubisco is strictly localised
 528 (Hatch 1987). In C_4 grasses with Kranz anatomy, the BS forms a concentric layer of
 529 chlorenchyma cells around individual minor veins, and hence reducing $IVDL$ (i.e.,
 530 high V_D) increases the density of BS on a leaf surface basis (Dengler *et al.* 1994; Ueno
 531 *et al.* 2006). It was suggested that enhancing light capture by BS cells would enhance
 532 the quantum yield of photosynthesis (ϕ , i.e. the initial slope of the A_n vs absorbed
 533 PPFD) in NADP-ME C_4 species, such as sorghum (Ehleringer, Cerling & Helliker
 534 1997). However this statement was partially based on the erroneous assumption that
 535 most of the chlorophyll is allocated to the BS, because in NADP-ME species most of
 536 the chlorophyll is located in the concentric mesophyll layer around the BS
 537 (Ghannoum *et al.* 2005). Hence, the negative regression between ϕ and $IVDL$ among
 538 C_4 grasses found by Ogle (2003) could be better explained through the negative link
 539 between the CO_2 leakiness and quantum yield (Farquhar 1983), indicating that lower
 540 $IVDL$ could be associated with lower leakiness. Based on direct observation of ϕ and
 541 $IVDL$, Ogle (2003) also predicted that the optimal $IVDL$ associated with ϕ in NADP-
 542 ME species was 63 μm , which for sorghum would match 70 mm of LW . The decline
 543 in A_n as $IVDL$ decreases (Fig. 3) could be explained by one a combination of the
 544 following four hypotheses, yet to be experimentally tested: lower $IVDL$ genotypes
 545 would have 1) lower Rubisco or PEPC content per unit of leaf area, 2) higher

leakiness, 3) lower chlorophyll per unit of leaf area, or 4) higher limitation to the diffusion of CO₂ by reduced stomatal conductance.

4.3. Coordination of vein and stomatal traits partly explain the link between *iWUE* and *LW*

As proposed in *Hypothesis 2*, we also observed a significant increase in stomatal density (S_D) in narrower leaves along with the decrease in *IVDL* (Table 2), which led to an average of 30 stomata per mm of minor vein across the range of measured *LW* (Fig. S5). In monocots, the regular parallel venation arises in an hierarchical sequence, with the large longitudinal veins extending acropetally within the primordium and, as the leaf expands, small minor longitudinal and transverse vein strands form, beginning near the tip and progressing in a basipetal direction (Nelson & Dengler 1997). Stomatal differentiation, which determines S_D , occurs during the same development time as the venation. and stomata develop in parallel lines between the minor veins (Pantin, Simonneau & Muller 2012; Brodribb, McAdam & Carins Murphy 2017). Coordination among V_D and S_D has been observed in many species (Brodribb *et al.* 2017), including C₃ and C₄ grasses (Kawamitsu *et al.* 2002; Ocheltree, Nippert & Prasad 2012; Fiorin *et al.* 2016), to balance the supply of water through the veins with the transpiration rate and to support high rates of A_n with minimal water loss, i.e. high *WUE* (de Boer *et al.* 2012). This allometric scaling is also important to minimize water potential gradients inside the leaf through shortening the distance for water to move from the xylem to the stomata (Ocheltree *et al.* 2012; Fiorin *et al.* 2016). Also, an increase in S_D is linked to an increase of mesophyll porosity and to the surface of mesophyll available for CO₂ diffusion, reducing mesophyll resistance to CO₂ diffusion in C₃ and C₄ grasses (Hughes *et al.* 2017; Lundgren *et al.* 2019; Pathare

571 *et al.* 2020a). Although the scatter of data precluded correlations between S_D with
572 either $iWUE$ or g_{sw} in this study (Table 3), increased S_D along with lower $IVDL$ are
573 needed to get higher $iWUE$ in sorghum under field conditions (Fig. 3).

574 Regardless of the coordination among vein and stomatal traits in sorghum (Table 2),
575 measured g_{sw} was independent of its anatomical determinants, e.g. S_D , S_s or g_{sw_max} , but
576 highly dependent on the % stomatal aperture (Table 3, Fig. 3), corroborating our
577 *Hypothesis 2*. The correlation between g_{sw} and S_D or g_{sw_max} is usually high among
578 different plant species (Franks *et al.* 2014; Murray *et al.* 2020), it is only marginal if
579 significant within a single species (Ohsumi *et al.* 2007; Ouyang *et al.* 2017). The
580 aforementioned mild water stress during gas exchange measurements might have
581 influenced the anatomical dependence of g_{sw} , which was highly determined by the %
582 of stomatal aperture (Fig. 3). Stomata may change their aperture due to a change in
583 guard cell turgor pressure to minimize water loss, thereby keeping the leaf hydrated,
584 while allowing diffusion of CO_2 into the leaf to perform photosynthesis (Franks &
585 Farquhar 2001; Lawson & Matthews 2020). However, stomata also regulate leaf
586 temperature through latent heat loss via transpiration. The role of stomatal
587 conductance in regulating leaf temperature (Fig. 4) is well known and is described by
588 the energy balance of the leaf (Gates 1968; Nobel 2009; Monteith & Unsworth 2013),
589 as discussed in Section 4.4. We also theorize that anatomical traits may influence
590 stomatal behaviour independently of LW , although this effect would be of lower
591 magnitude than the LW dependent effects. Mesophyll located at a greater distance
592 from the veins, and from the stomata, in wider leaves (Fig. 2) may evaporate more
593 water than from the epidermis to avoid reaching higher temperature locally, which
594 could drive higher stomatal aperture in those genotypes. Our data support this
595 hypothesis given the positive correlation of % stomatal aperture with increasing $IVDL$

(Fig. 4c), and negative correlation with S_D (Fig. 4a), at any time of the day. Latent heat loss is needed to cool down any part of the leaf that is absorbing high irradiance and the evaporation of liquid water is mostly driven by the partial pressure of water vapour at the sites of evaporation (Rockwell, Holbrook & Stroock 2014), but when absorbed PAR is high, e.g. $1800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, small differences in temperature within the leaf usually occur, driving anisothermal vapour transport (AVT) (Buckley *et al.* 2017). When the evaporative demand is really high, AVT might also slightly induce unsaturation in the intercellular airspaces near the stomata (Buckley *et al.* 2017; Cernusak *et al.* 2018). To avoid local overheating in the distant mesophyll tissue between stomata, i.e. genotypes with large $IVDL$, the stomata would need to be more open to enhance the gradient in water vapour partial pressure and facilitate evaporation in the most distant tissue (Fig. 4). Furthermore, if $g_{\text{blw_effect}}$ is low by either low wind speed or in wider leaves, leaf transpiration will be limited as the capacity to cool down the leaf (see Section 4.4). Also, given that leaf thickness drives the increase of transdermal temperatures, i.e. the difference between maximum and minimum temperatures across leaf thickness, which in turn drives AVT through the intercellular air spaces (Buckley *et al.* 2017), the positive correlation of LMA , as a surrogate of leaf thickness, and stomatal size found in our study (Table 2) would facilitate the control of temperature within the leaf and buffer the leaf from rapid swings in temperature in sorghum.

4.4 Boundary layer thickness partly explains the relationship between leaf width and g_{sw}

Direct measurements and simulations confirmed that g_{sw} was negatively correlated with the leaf-to-air temperature difference ($\Delta T_{\text{leaf-air}}$), and that increased LW enhanced

621 g_{sw} to sustain a determined $\Delta T_{leaf-air}$ (either positive or negative) especially when wind
 622 speed is low, and hence the boundary layer is thicker (*BL*) (*Hypothesis 3*). For the
 623 diffusion of gases, the *BL* acts as a resistance in series with stomatal resistance, and
 624 *BL* also regulates convective heat loss used to cool down the leaves (Gates 1968;
 625 Jarvis & McNaughton 1986). Hence, a thicker *BL* may slow the transfer of heat to the
 626 ambient air, requiring wide leaves to open their stomata more to increase the latent
 627 heat loss at the evaporation sites within the leaf to reduce T_{leaf} , thereby reducing *iWUE*
 628 (Fig. 5) (Parkhurst & Loucks 1972). In other words, to get the same $\Delta T_{leaf-air}$, wider
 629 leaves need to open their stomata more than narrower leaves (Fig. 7). To reduce ΔT_{leaf-}
 630 air to the same extent, wider leaves would need to open their stomata more especially if
 631 g_{sw} is low, for example, due to mild water stress (Monteith & Unsworth 2013). The
 632 interplay between $\Delta T_{leaf-air}$ and g_{sw} is mediated by g_{blw_effect} , which depends on *LW* and
 633 wind speed, and may constitute the mechanistic basis for the close correlation
 634 between *LW* and g_{sw} in sorghum in the morning and midday (Fig. 8). In the afternoon,
 635 there were both high air temperature and wind speed, and simulations indicated that to
 636 keep a negative $\Delta T_{leaf-air}$, and hence to reduce T_{leaf} that was already negatively
 637 impacting A_n (Table 1), g_{sw} should slightly decrease with *LW* (Fig. 7), producing the
 638 lack of correlation between g_{sw} and *LW* measured in the afternoon (Table 1). This is in
 639 accordance with the likely higher water stress in wide leaf genotypes discussed in
 640 Section 4.1. In any case, data in the afternoon should be interpreted with caution
 641 because PPFD was much lower outside the chamber than within (Fig. S2), and the
 642 energy balance of the leaves changed dramatically (Fig. 6c), thus we cannot discard
 643 the decoupling of the leaf within the cuvette due to the stomatal kinetics to changes in
 644 PPFD (McAusland *et al.* 2016). An alternative explanation for the correlation between
 645 *LW* and g_{sw} is that if g_{sw} were similar in wide and narrow leaves, the intrinsically

646 lower $g_{\text{blw_effect}}$ of wider leaves (Fig. 5) would result in lower A_n in wider leaf
647 genotypes under low wind speed in the field, given that stomatal and BL conductances
648 are in series in the pathway of both H_2O and CO_2 and that C_i was generally low
649 anytime of the day in our plants(Farquhar *et al.* 1989).

650 In terms of water loss by leaf transpiration, the large effect of LW on g_{sw} was almost
651 compensated at low wind speed (e.g. midday) by the lower $g_{\text{blw_effect}}$, resulting in
652 similar transpiration rates (E) along the leaf width range (Fig. 7a&c). During high
653 wind speed, E decreases in narrow leaves to keep a constant positive $\Delta T_{\text{leaf-air}}$ (Fig. 7a),
654 otherwise E would increase in narrow leaves in an attempt to keep a negative constant
655 $\Delta T_{\text{leaf-air}}$ (Fig. 7c). Under field conditions, we could expect narrow leaves to sustain a
656 nearly positive $\Delta T_{\text{leaf-air}}$, and negative $\Delta T_{\text{leaf-air}}$ in wider ones (Table 1) to optimize
657 WUE , especially when wind speed is high. As a corollary, we could expect narrow
658 leaf genotypes to be more heat resistant than wide ones (Cano *et al.*, unpublished
659 results). In the absence of water stress, the openness of stomata in wider leaves would
660 be even higher, hence decreasing $iWUE$ more than in the narrow leaf genotypes, as
661 previously observed in soybean (Baldocchi *et al.* 1985). In water stress prone sites,
662 like shallow soils, plants with likely smaller canopies produced by narrow-leaf
663 genotypes may be advantageous because water stress would be delayed in two ways,
664 by a) lowering total plant transpiration over time (Tardieu, Simonneau & Muller
665 2018), and b) increasing daily leaf $iWUE$, and hence likely increasing WUE , as
666 discovered here. One additional advantage of narrow leaves would be that they might
667 allow higher light penetration within the canopy which, in combination with an erect
668 leaf angle, may further increase WUE and productivity at the canopy level (Duncan *et*
669 *al.* 1967; Drewry, Kumar & Long 2014). This should enable plant density to be
670 increased to sustain crop production in places where water is not limiting. Enhancing

671 plant density would reduce the number of tillers, given that narrow leaf sorghum
672 genotypes have higher susceptibility to grow extra tillers than wide leaf one (Lafarge,
673 Broad & Hammer 2002; Alam *et al.* 2014).

674

675 In summary, we have demonstrated that screening for genotypes with narrow leaf
676 blades should be an effective and easy method to achieve higher *iWUE* in sorghum,
677 since both *LW* and *S_D* are highly heritable traits in sorghum (Liang *et al.* 1973; Liang
678 *et al.* 1975). *LW* regulated vein and stomatal densities, and higher stomatal aperture
679 was needed in wide leaves to enhance *g_{sw}*, counteract the lower *g_{blw_effect}*, and regulate
680 the leaf to air temperature difference through transpiration. Despite narrow-leaf
681 genotypes exhibiting reduced *A_n*, the much higher *iWUE* of the narrow-leaf genotypes
682 during at least morning and midday may be advantageous to increase plant
683 productivity in drought-prone sites. However, we need to assess how this affects plant
684 and canopy water use and net photosynthetic rate under different environments.

685 Altogether, we have highlighted the important role of *LW* and $\Delta T_{\text{leaf-air}}$ in regulating
686 *g_{sw}*, *iWUE* and *WUE* at the leaf level, and discussed implications across the sorghum
687 canopy with important consequences for plant growth, water use and crop evaporation
688 (Borrell *et al.* 2014; George-Jaeggli, Mortlock & Borrell 2017). Parkhurst and Loucks
689 (1972) concluded: “[plant breeders] should be able to improve water-use efficiencies
690 of crop species by producing plants with a given total area divided into smaller units”.
691 Our results corroborate this statement.

692

693

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705

706 **Author Contribution**

707 FJC, BG-J, AB, DJ and OG designed the experiment based on an original idea of FJC.
708 FJC and BG-J undertook the gas exchange measurements. LP analysed gas exchange
709 data and measured anatomical traits with the help of YA-S, FK and FJC. FJC
710 modelled the leaf energy balance and ran the simulations. LP and FJC drafted the first
711 version of the manuscript and all of the authors contributed to the final version of the
712 manuscript. OG coordinated the project execution.

713

714 **Conflict of Interest**

715 The authors have no conflict of interest to declare.

716

717 **Data availability**

718 The data generated and analysed during this study are available from the
719 corresponding author.

720

721 **Supporting Information**

722 Additional supporting information may be found in the online version of this article.

723 **Supplementary Methods A.** Correction of the diffusion coefficient of water vapour
724 in air (D_{air-H_2O}) for temperature and air pressure.

725 **Supplementary Methods B.** Estimation of leaf boundary layer conductance ($g_{blw_{effect}}$).

726 **Supplementary Methods C.** Leaf energy balance and simulations.

727 **Table S1.** Means and standard error (SE) of leaf gas exchange and leaf-to-air
728 temperature differences across 48 sorghum genotypes.

729 **Table S2.** Means and standard error (SE) of various leaf vein traits and *LMA* across
730 48 sorghum genotypes.

731 **Table S3.** Generalized linear models (GLM) to test the interactions of gas exchange
732 variables with leaf width measured three times a day.

733 **Table S4.** Means of stomatal traits on adaxial and abaxial leaf surfaces from 48
734 sorghum genotypes

735 **Supplementary Fig. S1.** Variation in leaf width (*LW*) among the 48 *Sorghum bicolor*
736 genotypes.

737 **Supplementary Fig. S2.** Climate data at the field site during the measurements.

738 **Supplementary Fig. S3.** Example of images analysed for vein and stomatal traits.

739 **Supplementary Fig. S4.** Total number of longitudinal veins across the leaf width.

740 **Supplementary Fig. S5.** The ratio of S_D to V_D averaged at 30 stomata per mm of vein
741 length across the entire *LW* range.

742 **Supplementary Fig. S6.** Pictures that evidenced the occurrence of water deficit at the
743 field site.

744 **Supplementary Material.** Excel spreadsheet to calculate the components of the leaf
745 energy balance and run the simulations.
746

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Table 1. Unweighted means (\pm standard error) of leaf gas exchange parameters, leaf temperature (T_{leaf}) and leaf-to-air temperature difference ($\Delta T_{\text{leaf-air}}$) in 48 sorghum genotypes measured three times a day in the field are represented by bold numbers. Pearson correlation coefficient matrix between leaf width and diurnal leaf gas exchange and temperature are shown below the means for each time of the day.

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	g_{sw} (mol H ₂ O m ⁻² s ⁻¹)	$iWUE$ ($\mu\text{mol CO}_2$ mol H ₂ O ⁻¹)	A_n ($\mu\text{mol CO}_2$ m ⁻² s ⁻¹)	T_{leaf} (°C)	$\Delta T_{\text{leaf-air}}$ (°C)
Morning	0.25\pm0.00^B	181.9 \pm 1.2^{AB}	44.75\pm0.56^C	36.12 \pm 0.12^A	-0.11\pm0.06^A
Leaf width (mm)	0.574**	-0.539**	0.369*	ns	ns
g_{sw}		-0.622**	0.853**	ns	-0.539**
$iWUE$			ns	ns	0.426**
A_n				ns	-0.361*
T_{leaf}					ns
Midday	0.24\pm0.00^B	175.8 \pm 1.4^A	41.86 \pm 0.65^B	40.59\pm0.11^C	-1.23\pm0.08^C
Leaf width (mm)	0.651**	-0.689**	0.363*	ns	-0.419*
g_{sw}		-0.496**	0.848**	-0.475**	-0.676**
$iWUE$			ns	ns	0.535**
A_n				-0.524**	-0.636**
T_{leaf}					0.396*
Afternoon	0.20\pm0.00^A	183.3 \pm 1.1^B	36.41\pm0.68^A	39.49\pm0.12^B	-0.42\pm0.08^B
Leaf width (mm)	ns	ns	ns	ns	ns
g_{sw}		-0.494**	0.955**	ns	-0.479**
$iWUE$			ns	ns	0.310*
A_n				-0.293*	-0.412*
T_{leaf}					ns

1107
1108 Different superscripts (A, B, C) indicate statistically significant differences between means at
1109 the 5% level using Duncan test and repeated measures ANOVA. *significant at $p < 0.05$; **significant at
1110 $p < 0.001$; ns not significant.

1111 **Table 2.** Pearson correlations coefficient matrix between leaf width and leaf anatomical traits averaged of each genotype.
1112

	Leaf width (mm)	%Stomatal aperture	IVDL (μm)	IVDT (μm)	V_D (mm mm ⁻²)	S_{Dada} (n° mm ⁻²)	S_{Daba} (n° mm ⁻²)	S_D (n° mm ⁻²)	S_s (μm^2)	a_{max} (μm^2)	LMA (g m ⁻²)	g_{sw_max} (mol H ₂ O m ⁻² s ⁻¹)
Leaf width		0.477**	0.646**	ns	-0.590**	ns	-0.315*	-0.307*	ns	0.333*	ns	ns
%Stomatal aperture			0.492**	ns	-0.425**	-0.595**	-0.539**	-0.601**	ns	ns	ns	-0.656**
IVDL				0.325*	-0.930**	-0.296*	-0.290*	-0.312*	ns	-0.411*	ns	ns
IVDT					-0.381*	ns	ns	ns	0.350*	0.304*	0.444*	ns
V_D						0.311*	0.304*	0.327*	ns	-0.363*	ns	ns
S_D adax							0.761**	0.927**	0.173*	ns	ns	0.656**
S_D abax								0.949**	0.179*	ns	ns	0.686**
S_D									0.188*	ns	ns	0.716**
S_s										0.351*	0.381*	0.450**
a_{max}											0.333*	0.540**
LMA												ns

1113
1114 Percentage of stomatal aperture at midday based on theoretical maximum a_{max} at midday (%Stomatal aperture); $IVDL$, interveinal distance of longitudinal veins; $IVDT$,
1115 interveinal distance of transverse vein; V_D , Vein density; S_{Dada} , stomatal density on adaxial leaf side; S_{Daba} , stomatal density on abaxial leaf side; S_D , sum of stomatal density
1116 on both leaf sides; S_s , the average of Stomatal size on both leaf sides; LMA is the leaf mass per area; g_{sw_max} , the theoretical maximum leaf stomatal conductance for water
1117 vapour
1118 *significant at $p<0.05$; ** significant at $p<0.001$; ns not significant.
1119
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1121 **Table 3.** Pearson correlation coefficients between averaged leaf gas exchange variables, leaf-to-air temperature difference ($\Delta T_{\text{leaf-air}}$) and leaf anatomical traits per genotype.
 1122 Leaf gas exchange values are the average of each genotype at midday.
 1123

	V_D (mm mm ⁻²)	$IVDL$ (μm)	S_D (n° mm ⁻²)	S_s (μm^2)	%Stomatal aperture	g_{sw_max} (mol H ₂ O m ⁻² s ⁻¹)	LMA (g m ⁻²)
$iWUE$	0.295*	-0.331*	ns	ns	-0.461**	ns	ns
g_{sw}	-0.503**	0.576**	ns	ns	0.753**	ns	ns
A_n	-0.422**	0.475**	ns	ns	0.592**	ns	ns
$\Delta T_{\text{leaf-air}}$	ns	-0.280*	ns	ns	-0.385**	ns	ns

1124
 1125 V_D , vein density; $IVDL$, interveinal distance of longitudinal veins; S_D , stomatal density including both leaf sides; S_s , Stomatal size of both leaf sides; percentage of stomatal
 1126 aperture based on theoretical maximum (%Stomatal aperture); g_{sw_max} , the theoretical maximum leaf stomatal conductance for water vapour.
 1127 *significant at $p<0.05$; ** significant at $p<0.001$; ns not significant.

Figure legends

Fig. 1 Relationships among net photosynthesis rates (A_n), stomatal conductance to water vapour (g_{sw}) and intrinsic water use efficiency ($iWUE$) in the morning, midday and afternoon. Means are represented for each genotype ($n=4-6$ plants/genotype) of the 48 genotypes. The average values of gas exchange variables of each genotype from morning, midday and afternoon were used for plotting side bars coloured with black, red and green, respectively. All linear regressions are significant (*significant at $p<0.05$; ** significant at $p<0.001$).

Fig. 2 Correlations between leaf width and photosynthesis rates (A_n) (a), stomatal conductance to water vapour (g_{sw}) (b), intrinsic water use efficiency ($iWUE$) (c), interveinal distance of longitudinal veins ($IVDL$) (d), % stomatal aperture (e) and stomatal density including the two leaf surfaces (S_D) (f). Each point represents the mean for each genotype ($n=4-6$ plants). A side bar was plotted with black, red and green colour for displaying the significant changes of in values of % stomatal aperture at morning, midday and afternoon of the day. All linear regressions are significant (*significant at $p<0.05$; ** significant at $p<0.001$).

Fig. 3 Correlations between % stomatal aperture and photosynthesis rate (A_n) (a), % stomatal aperture and stomatal conductance to water vapour (g_{sw}) (b), % stomatal aperture and intrinsic water use efficiency ($iWUE$) (c), interveinal distance of longitudinal veins ($IVDL$) and photosynthesis rates (A_n) (d), interveinal distance of longitudinal veins ($IVDL$) and stomatal conductance to water vapour (g_{sw}) (e), and interveinal distance of longitudinal veins ($IVDL$) and intrinsic water use efficiency ($iWUE$) (f). Each point represents the mean for each genotype ($n=4-6$ plants). All linear regressions are significant (*significant at $p<0.05$; ** significant at $p<0.001$).

Fig. 4 Correlations between the stomatal density including two leaf surfaces (S_D) and the % stomatal aperture (a), leaf width and leaf temperature (T_{leaf}) (b), the interveinal distance of longitudinal veins ($IVDL$) and % stomatal aperture (c), and leaf temperature (T_{leaf}) and net photosynthesis (A_n) (e). Each point represents the mean for each genotype ($n=4-6$ plants). A side bar displays the significant changes in leaf temperature (T_{leaf}) at three time points of the day. All linear regressions are significant, except for T_{leaf} and leaf width (*significant at $p<0.05$; ** significant at $p<0.001$).

Fig. 5 The effect of leaf width on one-sided leaf boundary layer conductance to water vapour (g_{blw}) and boundary layer thickness (δ_{bl}) (a) and the correlations among the effective two-sided leaf boundary layer conductance to water vapour (g_{blw_effect}) with stomatal conductance to water vapour (g_{sw}) and intrinsic water use efficiency ($iWUE$) measured at midday (b). Each point represents the mean for each genotype ($n=4-6$ plants). Note that to calculate the g_{blw_effect} we have taken into account the measured ratio of S_D in the adaxial to abaxial surfaces. Conditions to calculate g_{blw} and g_{blw_effect} are for midday with wind speed 0.1 m s^{-1} and air temperature of 42°C .

Fig. 6. The negative correlations between measured stomatal conductance to water vapour (g_{sw}) and leaf-to-air temperature difference ($\Delta T_{leaf-air}$). Each point represents the averaged value per genotype measured in the morning, at midday or in the afternoon (black circle, red square and green triangles symbols and dashed lines, respectively) and simulated variables (five-star symbols with grey, gold and blue colours,

respectively and solid lines) under three scenarios: a) $\Delta T_{\text{leaf-air}}$ calculated using the linearized form of the energy balance equation of the leaf within the cuvette (equation C11 in Supplementary Material C) vs. the measured g_{sw} . b) Solving the energy balance equation of the leaf within the cuvette for T_{leaf} using the solver function of Excel and recalculate g_{sw} from the measured transpiration rate (E) at the new T_{leaf} . $\Delta T_{\text{leaf-air}}$ was calculated using the new T_{leaf} and the measured T_{air} . c) Solving E from the leaf energy balance for each leaf under the field conditions (e.g. including changes in incoming PPFD, wind speed and actual leaf width described in Supplementary Material C) using the solver function of Excel by assuming that leaves had the same T_{leaf} and T_{air} as measured by LICOR, and hence the same $\Delta T_{\text{leaf-air}}$, then g_{sw} was recalculated to match the simulated E . R^2_{mes} and R^2_{sim} represent the coefficients of determination calculated with measured variables and simulated variables, respectively.

Fig. 7 The effect of changing leaf width when maintaining a positive (left panels) or negative (right panels) leaf-to-air temperature difference ($\Delta T_{\text{leaf-air}}$) on the relative change of the transpiration rate (E) (a,c) and the stomatal conductance to water vapour (g_{sw}) (b,d) for two scenarios of wind speed: low wind speed (0.1 m s^{-1} , open symbols, dashed lines) and high wind speed (2.0 m s^{-1} , closed symbols, continuous lines). The simulations were performed using the solver function in Excel (see Supplementary Material) to minimize square error of the energy balance equation by changing g_{sw} to keep constant $\Delta T_{\text{leaf-air}}$ for a leaf in the field with incipient PPFD of $2100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, $T_{\text{air}} = 43.85 \text{ }^{\circ}\text{C}$ and $T_{\text{ground}} = 41.26 \text{ }^{\circ}\text{C}$. Values used as reference were those corresponding with leaf width of 160 mm , wind speed 0.1 m s^{-1} and $\Delta T_{\text{leaf-air}} = -1.6 \text{ }^{\circ}\text{C}$, when E was $9.30 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ and g_{sw} was $0.318 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$.

Fig. 8 Correlation network among the main morphological, anatomical and physiological variables that explained the variability of g_{sw} and $iWUE$ at midday among sorghum genotypes. All variables were measured, except $g_{\text{blw_effect}}$ which was modelled as described in Fig. 5. Each line represents a significant correlation ($p < 0.05$), either positive (continuous line) or negative (dashed line), among the studied variables. Thick lines represent Pearson correlation coefficients equal or higher than 0.5 , while thin ones show lower correlation, although still significant.