

1 **Heat stress prevented the biomass and yield stimulation caused by elevated**
2 **CO₂ in two well-watered wheat cultivars**

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13 **Keywords:** Wheat, elevated CO₂, photosynthetic acclimation, temperature
14 response, heat stress, grain yield

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16 **Key message (<30 words)**

17 High temperatures increased photosynthetic rates only at eCO₂ and photosynthesis
18 was upregulated after recovery from heat stress at eCO₂ in Scout suggesting that
19 eCO₂ increased optimum temperature of photosynthesis.

20 **Abstract (250 words)**

21 To investigate the interactive effects of elevated CO₂ and heat stress (HS), we grew
22 two contrasting wheat cultivars, early-maturing Scout and high-tillering Yitpi, under
23 non-limiting water and nutrients at ambient (aCO₂, 450 ppm) or elevated (eCO₂, 650
24 ppm) CO₂ and 22°C in the glasshouse. Plants were exposed to two 3-day HS cycles
25 at the vegetative (38.1°C) and/or flowering (33.5°C) stage.

26 At aCO₂, both wheat cultivars showed similar responses of photosynthesis and
27 mesophyll conductance to temperature and produced similar grain yield. Relative to
28 aCO₂, eCO₂ enhanced photosynthesis rate and reduced stomatal conductance and
29 maximal carboxylation rate (V_{cmax}). During HS, high temperature stimulated
30 photosynthesis at eCO₂ in both cultivars, while eCO₂ stimulated photosynthesis in
31 Scout. Electron transport rate (J_{max}) was unaffected by any treatment. eCO₂ equally
32 enhanced biomass and grain yield of both cultivars in control, but not HS, plants. HS
33 reduced biomass and yield of Scout at eCO₂. Yitpi, the cultivar with higher grain
34 nitrogen, underwent a trade-off between grain yield and nitrogen. In conclusion,
35 eCO₂ improved photosynthesis of control and HS wheat, and improved biomass and
36 grain yield of control plants only. Under well-watered conditions, HS was not
37 detrimental to photosynthesis or growth but precluded a yield response to eCO₂.

38 Key words: Wheat, photosynthesis, grain yield, elevated CO₂, heat stress, climate
39 change

40 Introduction

41 Ongoing climate change is threatening the production of agricultural crops including
42 wheat (*Triticum aestivum*) (Asseng *et al.*, 2015; Mishra *et al.*, 2021). By the end of
43 this century, atmospheric carbon dioxide concentration ([CO₂]) is expected to reach
44 700 ppm, increasing surface temperatures by 1.1°C to 2.6°C (IPCC, 2014). For
45 every degree of the temperature increase, global wheat production is predicted to
46 decrease by 6–10% (Asseng *et al.*, 2015; García *et al.*, 2015). Crop models are
47 important tools for assessing the impact of climate change (Asseng *et al.*, 2013).
48 However, they largely lack the ability to consider genotype-specific responses to
49 elevated [CO₂] (eCO₂) and their interaction with other environmental conditions.
50 Hence, it is important to better understand how plants respond to eCO₂ interactions
51 with the environment. Photosynthesis, a fundamental process driving crop growth
52 and yield, can partially explain the interactive effects of eCO₂ with environmental
53 stresses and provide a mechanistic basis for crop models (Yin and Struik, 2009).

54 During photosynthesis, ribulose-1, 5-bisphosphate carboxylase (Rubisco) catalyzes
55 the carboxylation and oxygenation of ribulose-1, 5-bisphosphate (RuBP). eCO₂
56 increases photosynthetic rates (A_{sat}) and reduces photorespiration and stomatal
57 conductance (g_s). Generally, higher photosynthetic rates enhance the growth and
58 productivity of plants leading to increased leaf area, plant size and crop yield
59 (Krenzer and Moss, 1975; Sionit *et al.*, 1981; Hocking and Meyer, 1991; Mitchell *et al.*,
60 1993; Kimball *et al.*, 1995; Mulholland *et al.*, 1998; Cardoso-Vilhena and Barnes, 2001;
61 Högy *et al.*, 2009; Kimball, 2016; Fitzgerald *et al.*, 2016; Kimball, 1983). Following long
62 term CO₂ enrichment, photosynthetic capacity may diminish due to lower amount of
63 Rubisco (Nie *et al.*, 1995; Rogers and Humphries, 2000; Ainsworth *et al.*, 2003) or
64 reduced activation of Rubisco (Delgado *et al.*, 1994).

65 Optimum temperature range for wheat growth is 17-23°C, with a minimum of 0°C
66 and maximum of 37°C (Porter and Gawith, 1999). Global warming involves a gradual
67 increase in mean temperature as well as increased frequency and intensity of heat
68 waves. Heat can adversely affect crop growth and disrupt reproduction depending on
69 the timing, intensity and duration (Sadras and Dreccer, 2015). Higher daytime
70 temperatures (below damaging level) increase photosynthesis up to an optimum
71 temperature, above which photosynthesis decreases mainly due to higher

72 photorespiration (Berry and Bjorkman, 1980; Long, 1991). High night time
73 temperatures increase respiration and reduce overall photosynthetic carbon gain
74 (Prasad *et al.*, 2008). At the whole plant level, high temperatures accelerate growth
75 (Fischer, 1980) and shorten crop duration (Hatfield and Prueger, 2015), hence
76 reducing grain yield due to insufficient time to capture resources. Losses due to short
77 crop duration are usually higher than benefits of growth stimulation at high
78 temperature (Wardlaw and Moncur, 1995).

79 The severity of the damage caused by abrupt temperature increases above the
80 optimum range (termed heat stress, HS) depends on the magnitude and duration of
81 HS as well as the developmental stage of the plant (Wahid *et al.*, 2007). HS may
82 reduce photosynthesis due to reduced chlorophyll content, impaired photosystem II
83 and lower Rubisco activation (Berry and Bjorkman, 1980; Eckardt and Portis, 1997).
84 HS can directly damage cells and increase grain abortion resulting in reduced
85 growth, biomass and grain yield (Stone and Nicolas, 1996, 1998; Wardlaw *et al.*,
86 2002; Farooq *et al.*, 2011). Around anthesis, HS (>30°C) reduces seed setting due to
87 lower pollen viability, leading to poor fertilization, abnormal ovary development and
88 slower pollen growth (Balla *et al.*, 2019).

89 The interactive effects of eCO₂ and HS can be positive, negative or neutral (Wang *et al.*,
90 2008, 2011). Elevated CO₂ increases the temperature optimum of
91 photosynthesis (Long, 1991; Alonso *et al.*, 2009) by reducing photorespiration and
92 improving tolerance to photoinhibition (Hogan *et al.*, 1991). The impact of HS on
93 photosynthesis will depend on whether Rubisco, electron transport or end-product
94 synthesis is limiting at eCO₂ (Sage and Kubien, 2007). Enhanced growth and leaf
95 level water use efficiency (WUE) by eCO₂ may help compensate for the negative
96 impact of HS; conversely, heat-induced shortening of the grain-filling stage and grain
97 abortion could limit the benefits of eCO₂ (Lobell and Gourdjji, 2012). In addition,
98 decreased g_s under eCO₂ may limit transpirational cooling and therefore exacerbate
99 HS. Thus, HS counteracts the positive effect of eCO₂ on yield components and may
100 aggravate the negative effect of eCO₂ on grain quality due to the high sensitivity of
101 wheat to temperature stress especially during anthesis and grain-filling stage (Wang
102 & Liu, 2021).

103 Many studies have investigated the response of wheat to eCO₂ in enclosures and in
104 the field (Wang & Liu, 2021). However, only a few studies have considered eCO₂
105 interaction with temperature increases in wheat (Rawson, 1992; Delgado *et al.*,
106 1994; Morison and Lawlor, 1999; Jauregui *et al.*, 2015; Cai *et al.*, 2016) and rarely
107 with HS (Coleman *et al.*, 1991; Wang *et al.*, 2008). Studies considering HS have
108 addressed mainly the biomass or yield aspects and not the physiological processes
109 such as photosynthesis (Stone and Nicolas, 1994, 1996, 1998). Interactive effects of
110 eCO₂ and HS on photosynthesis have been reported in a limited number of studies
111 (Wang *et al.*, 2008, 2011; Macabuhay, 2016; Macabuhay *et al.*, 2018; Chavan *et al.*,
112 2019). Macabuhay *et al.*, (2018) studied interactive effects of eCO₂ and
113 (experimentally imposed) heatwaves on wheat (cv Scout and Yitpi) grown in a
114 dryland cropping system and concluded that eCO₂ may moderate some effects of
115 HS on grain yield but such effects strongly depend on seasonal conditions and
116 timing of HS. In another glasshouse experiment on the interactive effects of severe
117 HS and eCO₂ in wheat (cv Scout), we found that eCO₂ mitigated the negative
118 impacts of HS at anthesis on photosynthesis and biomass, but grain yield was
119 reduced by HS in both CO₂ treatments (Chavan *et al.*, 2019). However, HS can
120 occur throughout plant growth, including during vegetative, flowering or grain filling
121 stages. In addition, different crop genotypes may respond variably to the interaction
122 of eCO₂ with HS.

123 Here, we build on our previous work by comparing the interactive effects of eCO₂
124 and HS in two commercial wheat cultivars. Scout and Yitpi have similar genetic
125 background but distinct agronomic features. Scout is a mid-season maturity cultivar
126 with very good early vigor that can produce leaf area early in the season. Scout has
127 a putative water-use efficiency (WUE) gene, which has been identified using carbon
128 isotope discrimination (Condon *et al.*, 2004). Yitpi is a good early vigor, freely
129 tillering, late flowering and long maturity cultivar (Bahrami *et al.*, 2017; Pacificseeds,
130 2009; Seednet, 2005).

131 Although Scout is known to be a high yielding variety with very good grain quality
132 and high reproductive sink (Pacific seeds, 2009), we hypothesized that Yitpi will
133 produce higher grain yield due to its ability to initiate more tillers and its longer time
134 to flower and mature (Hypothesis 1). Fast growing plants with high sink capacity
135 show a greater eCO₂-induced growth stimulation (Poorter, 1993) and less

136 photosynthetic acclimation (Delgado *et al.*, 1994) compared to slow growing
137 counterparts with low sink capacity. Consequently, we hypothesized that Yitpi will
138 show greater photosynthetic, growth and yield response to eCO₂ due to its greater
139 vegetative sink capacity (tillering) relative to Scout with restricted tillering (Hypothesis
140 2). The greater growth stimulation at eCO₂ may buffer Yitpi against HS damage
141 compared to Scout. Thus, HS may decrease yield in Scout more than Yitpi and aCO₂
142 more than eCO₂ (Hypothesis 3). HS is more damaging at the reproductive relative to
143 the vegetative developmental stage (Farooq *et al.*, 2011). Hence, we expect less
144 damage in plants exposed to HS at the vegetative stage relative to the flowering
145 stage (Hypothesis 4).

146 To test these hypotheses, Scout and Yitpi were grown at ambient or elevated CO₂
147 conditions and subjected to one or two heat stresses at the vegetative (HS1) and/or
148 flowering (HS2) stage. Growth, biomass and photosynthetic parameters were
149 measured at different time points across the life cycle of the plants. At the plant level,
150 we report that eCO₂ equally stimulated grain yield of Scout and Yitpi. While
151 moderate HS under well-watered conditions was not detrimental to photosynthesis
152 and growth in the long term due to the transpirational cooling, HS prevented the
153 wheat plant from reaping the benefits of eCO₂ on biomass and yield.

154 **Materials and methods**

155 *Plant culture and treatments*

156 The experiment was conducted in the glasshouse facility located at the Hawkesbury
157 campus of Western Sydney University (WSU). Seeds of commercial winter wheat
158 cultivars Scout and Yitpi were procured from Agriculture Victoria (Horsham).
159 Cultivars were selected based on their use in the Australian Grains Free Air CO₂
160 Enrichment (AGFACE) project investigating climate change impacts on wheat growth
161 and yield (Houshmandfar *et al.*, 2017). For germination, 300 seeds of each cultivar
162 were sterilized using 1.5 % NaOCl₂ for 1 min followed by incubation in the dark at
163 28°C for 48 hours in petri plates. Sprouted seeds were planted in germination trays
164 using seed raising and cutting mix (Scotts, Osmocote®) at ambient CO₂ (aCO₂, 400
165 µl L⁻¹), temperature (22/14 °C day/night), relative humidity (RH, 50 to 70%) and
166 natural light (midday average 500 µmol m⁻² s⁻¹) (Figure S1). The growth stages are
167 denoted by decimal code (DC) according to (Zadoks *et al.*, 1974) along with the time
168 points here after. Two-week-old seedlings (DC12) were transplanted to individual
169 cylindrical pots (15 cm diameter and 35 cm height) using sieved soil collected from
170 local site. At transplanting stage (T0) pots were distributed into two aCO₂ (400 µl L⁻¹)
171 and two eCO₂ (650 µl L⁻¹) chambers (Figure S1B). Some plants were exposed to
172 one or two HS cycles at the vegetative (HS1, 10 weeks after planting, WAP, DC 32)
173 and/or the flowering (HS2, 15 WAP, DC 63) stages for 3 days with temperature ramp
174 up from 14°C night temperature (8 pm to 6 am) to 38°C during mid-day (10 pm to 4
175 pm) at 60% daytime RH (Figures S1 and S2). The two HS cycles created four sets of
176 heat treatments at each CO₂ concentration as follows: (1) Control – plants were not
177 exposed to HS at any stage, (2) HS1 – plants were exposed to HS at vegetative
178 (DC32) stage only, (3) HS2 – plants were exposed to HS at reproductive (DC63)
179 stage only and (4) HS1+2 – plants were exposed to both the heat stresses HS1 and
180 HS2 (Figure S2).

181 Thrive all-purpose fertilizer (Yates) was applied monthly throughout the experiment
182 to maintain similar nutrient supply in all treatment combinations. Pots were regularly
183 swapped between left and right benches as well as between front and back for
184 randomization within chamber. Pots and treatments were also swapped between the
185 two ambient and two elevated CO₂ chambers for randomization among chambers.

186 *Growth and biomass measurements*

187 The full factorial experimental design included four chambers (two chambers for
188 each CO₂ treatment) and five destructive harvests at time points T0 (2 WAP, DC12),
189 T1 (6 WAP, DC28), T2 (10 WAP, DC35), T3 (17, DC65) and T4 (25 WAP, DC90)
190 (Figure S2). Ten plants per treatment per cultivar were measured and harvested at
191 each time point. Morphological parameters were measured followed by
192 determinations of root, shoot and leaf dry mass. Samples were dried for 48 hours in
193 the oven at 60°C immediately after harvesting. Leaf area was measured at time point
194 T1, T2 and T3 using a leaf area meter (LI-3100A, LI-COR, Lincoln, NE, USA). Plant
195 height, leaf number, tiller number and ear (grain inflorescence) number along with
196 developmental stage information (booting (DC45), half-emerged (DC55) or fully
197 emerged (DC60)) were recorded at time points T2 and T3).

198 *Leaf gas exchange measurements*

199 The youngest fully developed leaf (which was the flag leaf at T3) was used to
200 measure gas exchange parameters. Instantaneous steady state leaf gas exchange
201 measurements were performed at time points T1, T2 and T3 using a portable open
202 gas exchange system (LI-6400XT, LI-COR, Lincoln, USA) to measure light-saturated
203 (photosynthetic photon flux density (PPFD) =1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) photosynthetic rate
204 (A_{sat}), stomatal conductance (g_s), ratio of intercellular to ambient CO₂ (C_i/C_a), leaf
205 transpiration rate (E), dark respiration (R_d) and dark- and light-adapted chlorophyll
206 fluorescence (F_v/F_m and F_v'/F_m' , respectively). Dark adapted leaf measurements
207 were conducted by switching off light for 15 minutes. Steady state leaf gas exchange
208 measurements were also performed during and after heat shock along with recovery
209 stage. Plants were moved to a neighboring chamber with ambient CO₂ levels for
210 short time (20-30 min for each plant) where air temperature was separately
211 manipulated to achieve the desired leaf temperature. The LI-COR 6400-40 leaf
212 chamber fluorometer (LCF) was used to measure gas exchange at a PPFD of 1500
213 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at two CO₂ concentrations (400 and 650 $\mu\text{l L}^{-1}$) and two leaf
214 temperatures (25 and 35 °C). Photosynthetic down regulation or acclimation was
215 examined by comparing the measurements at common CO₂ (ambient and elevated
216 CO₂ grown plants measured at 400 $\mu\text{l L}^{-1}$ CO₂ partial pressure) and growth CO₂

217 (aCO₂ grown plants measured at 400 µl L⁻¹ CO₂ partial pressure and eCO₂ grown
218 plants measured at 650 µl L⁻¹ CO₂ partial pressure).

219 Dark respiration (R_d) was measured after a dark adaptation period of 15 minutes.
220 Photosynthetic water use efficiency (PWUE) was calculated as A_{sat} (µmol m⁻² s⁻¹)/ g_s
221 (mol m⁻² s⁻¹). The response of A_{sat} to variations in sub-stomatal CO₂ mole fraction
222 (C_i) (A-Ci response curve) was measured at T3 in 8 steps of CO₂ concentrations (50,
223 100, 230, 330, 420, 650, 1200 and 1800 µl L⁻¹) at leaf temperature of 25°C.
224 Measurements were taken around mid-day (from 10 am to 3 pm) on attached last
225 fully expanded or flag leaves of the main stems. Before each measurement, the leaf
226 was allowed to stabilize for 10-20 minutes until it reached a steady state of CO₂
227 uptake and stomatal conductance. Ten replicate plants per treatment were
228 measured.

229 *Mesophyll conductance and temperature response*

230 Mesophyll conductance (g_m) was determined by concurrent gas exchange and stable
231 carbon isotope measurements using portable gas exchange system (LI-6400-XT, LI-
232 COR, Lincoln, NE, USA) connected to a tunable diode laser (TDL) (TGA100,
233 Campbell Scientific, Utah, USA) for the two wheat cultivars grown at ambient
234 atmospheric CO₂ partial pressures. A_{sat} and ¹³CO₂/¹²CO₂ carbon isotope
235 discrimination were measured after T1 at five leaf temperatures (15, 20, 25, 30 and
236 35°C) and saturating light (1500 µmol quanta m⁻² s⁻¹). Leaf temperature sequence
237 started at 25°C decreasing to 15°C and then increased up to 35°C. Response of A_{sat}
238 to variations in C_i was measured at each leaf temperature. Dark respiration was
239 measured by switching light off for 20 minutes at the end of each temperature curve.
240 Measurements were made inside a growth cabinet (Sanyo) to achieve desired leaf
241 temperature. The photosynthetic carbon isotope discrimination (Δ) to determine g_m
242 was measured as follows (Evans *et al.*, 1986):

$$243 \quad \Delta = \frac{1000 \varepsilon (\delta^{13}C_{sam} - \delta^{13}C_{ref})}{1000 + \delta^{13}C_{sam} - \varepsilon (\delta^{13}C_{sam} - \delta^{13}C_{ref})} \quad (1)$$

244 Where,
245 (2)

$$\varepsilon = \frac{C_{ref}}{C_{ref} - C_{sam}}$$

246 C_{ref} and C_{sam} are the CO_2 concentrations of dry air entering and exiting the leaf
 247 chamber, respectively, measured by the TDI. g_m was calculated using correction for
 248 ternary and second-order effects (Farquhar and Cernusak, 2012; Evans and Von
 249 Caemmerer, 2013) following the next expression:

$$250 \quad g_m = \frac{\frac{1+t}{1-t} \left(b - a_i - \frac{eR_d}{A+R_d} \right) \frac{A}{C_a}}{(\Delta_i - \Delta_o - \Delta_e - \Delta_f)} \quad (3)$$

251 Where, Δ_i is the fractionation that would occur if the g_m were infinite in the absence
 252 of any respiratory fractionation ($e = 0$), Δ_o is observed fractionation, Δ_e and Δ_f are
 253 fractionation of ^{13}C due to respiration and photorespiration respectively (Evans and
 254 Von Caemmerer, 2013).

$$255 \quad \Delta_i = \frac{1}{1-t} a' \frac{1}{(1-t)} ((1+t)b - a') \frac{C_i}{C_a} \quad (4)$$

$$256 \quad \Delta_e = \frac{1+t}{1-t} \left(\frac{eR_d}{(A+R_d)C_a} (C_i - \Gamma^*) \right) \quad (5)$$

$$257 \quad \Delta_f = \frac{1+t}{1-t} \left(f \frac{\Gamma^*}{C_a} \right) \quad (6)$$

$$258 \quad t = \frac{(1+a')E}{2g_{ac}^t} \quad (7)$$

258 Where,

259 The constants used in the model were as follows: E denotes transpiration rate; g_{ac}^t is
 260 total conductance to diffusion in the boundary layer ($ab = 2.9\text{‰}$) and in air ($a =$
 261 4.4‰); a' is the combined fractionation of CO_2 across boundary layer and stomata;
 262 net fractionation caused by RuBP and PEP carboxylation ($b = 27.3\text{‰}$) (Evans *et al.*,
 263 1986); fractionation with respect to the average CO_2 composition associated with
 264 photorespiration ($f = 11.6\text{‰}$) (Lanigan *et al.*, 2008) and we assumed null fractionation
 265 associated with mitochondrial respiration in light ($e = 0$).

266 *Leaf nitrogen and carbon estimation*

267 Leaf discs were cut from the flag leaves used for gas exchange measurements at
 268 time points T2 and T3 then oven dried. Leaf discs were processed for nitrogen (N)
 269 and carbon (C) content using elemental analyzer (Dumas method). N and C were
 270 also estimated from other plant components including leaf, stem, root and grain

271 harvested at T1, T3 and T4. Ground samples were processed for C & N with a CHN
272 analyzer (LECO TruMac CN-analyser, Leco corporation, USA) using an automated
273 dry combustion method (Dumas method). Leaf N per unit area (N_{area}) was calculated
274 as $N \text{ (mmol g}^{-1}) \times \text{LMA (g m}^{-2})$. Photosynthetic nitrogen use efficiency (PNUE) was
275 calculated as $A_{sat} \text{ (}\mu\text{mol m}^{-2} \text{ s}^{-1})/\text{leaf } N_{area} \text{ (mmol m}^{-2})$. Protein content was
276 determined using N and multiplication factor of 5.7 (Mosse, 1990; Bahrami *et al.*,
277 2017).

278 *Statistical and temperature analysis*

279 All data analyses and plotting were performed using R computer software (R Core
280 Team, 2020). The effect of treatments and their interactions was analyzed using
281 linear modeling with ‘anova’ in R. Significance tests were performed with anova and
282 post hoc Tukey test using the ‘glht’ function in the multcomp R package. Coefficient
283 means were ranked using post-hoc Tukey test. The Farquhar-von Caemmerer-Berry
284 (FvCB) photosynthesis model was fit to the A_{sat} response curves to C_i (A-Ci
285 response curve) or chloroplastic CO_2 mole fraction (C_c), which was estimated from
286 the g_m measurements (A- C_c response curve). We used the plantecophys R package
287 (Duursma, 2015) to perform the fits, using measured g_m and R_d values, resulting in
288 estimates of maximal carboxylation rate (V_{cmax}) and maximal electron transport rate
289 (J_{max}) for D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) using
290 measured R_d values. Temperature correction parameter (Tcorrect) was set to False
291 while fitting A- C_i curves. Temperature response of V_{cmax} and J_{max} were calculated by
292 Arrhenius and peaked functions, respectively (Medlyn *et al.*, 2002). Estimated V_{cmax}
293 and J_{max} values at five leaf temperatures were then fit using nonlinear least square
294 (nls) function in R to determine energy of activation for V_{cmax} (EaV) and J_{max} (EaJ)
295 and entropy (ΔS_J). Temperature responses of V_{cmax} and R_d were fit using Arrhenius
296 equation as follows,

$$297 \quad f(Tk) = k_{25} \cdot \exp \left[\frac{E_a \cdot (Tk - 298)}{R \cdot 298 \cdot Tk} \right] \quad (8)$$

298 Where, Ea is the activation energy (in J mol^{-1}) and k_{25} is the value of R_d or V_{cmax} at
299 25 °C. R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$) and Tk is the leaf
300 temperature in K. The activation energy term Ea describes the exponential rate of
301 rise of enzyme activity with the increase in temperature. The temperature coefficient

302 Q_{10} , a measure of the rate of change of a biological or chemical system as a
303 consequence of increasing the temperature by 10 °C was also determined for R_d
304 using the following equation:

$$305 \quad R_d = R_{d25} \cdot Q_{10}^{[(T-25)/10]} \quad (9)$$

306 A peaked function (Harley *et al.*, 1992) derived Arrhenius function was used to fit the
307 temperature dependence of J_{max} , and is given by the following equation:

$$308 \quad f(Tk) = k_{25} \cdot \exp\left[\frac{H_a \cdot (Tk - 298)}{R \cdot 298 \cdot Tk}\right] \left[\frac{1 + \exp\left(\frac{298 \cdot \Delta S - H_d}{298 \cdot R}\right)}{1 + \exp\left(\frac{Tk \cdot \Delta S - H_d}{Tk \cdot R}\right)} \right] \quad (10)$$

309 Where, E_a is the activation energy and k_{25} is the J_{max} value at 25 °C, H_d is the
310 deactivation energy and S is the entropy term. H_d and ΔS together describe the rate
311 of decrease in the function above the optimum. H_d was set to constant 200 kJ mol⁻¹
312 to avoid over parametrization. The temperature optimum of J_{max} was derived from
313 Eqn 10 (Medlyn *et al.*, 2002) and written as follows:

$$314 \quad T_{opt} = \frac{H_d}{\Delta S - R \cdot \ln\left[\frac{E_a}{(H_d - E_a)}\right]} \quad (11)$$

315 The temperature response of A_{sat} was fit using a simple parabola equation (Crous *et al.*
316 *et al.*, 2013) to determine temperature optimum of photosynthesis:

$$317 \quad A_{sat} = A_{opt} - b \cdot (T - T_{opt})^2 \quad (12)$$

318 Where, T is the leaf temperature of leaf gas exchange measurement for A_{sat} , T_{opt}
319 represents the temperature optimum and A_{opt} is the corresponding A_{sat} at that
320 temperature optimum. Steady state gas exchange parameters g_m , g_s , C_i and J_{max} to
321 V_{cmax} ratio were fit using nls function with polynomial equation:

$$322 \quad y = A + Bx + Cx^2 \quad (13)$$

323

324 Results

325 Two commercial wheat cultivars Scout and Yitpi were grown under aCO₂ or eCO₂
326 (daytime average of 450 or 650 µl L⁻¹, respectively; 65% RH and 22°C), natural
327 sunlight and well-watered conditions (Figure S1). Both aCO₂ and eCO₂ grown plants
328 were exposed to two 3-day HS cycles at the vegetative (HS1, 10 WAP, DC32,
329 daytime average of 38°C) and flowering stage (HS2, 15 WAP, DC63, daytime
330 average of 33.5°C), while daytime RH was maintained at 60%. HS2 was lower in
331 intensity relative to HS1 due to the cool winter conditions. Both HS cycles had similar
332 overall effects on growth and yield parameters, refuting our fourth hypothesis that HS
333 during the reproductive stage is more damaging. Hence, we mostly compare the
334 control plants to those exposed to both heat stresses. Grain filling started 17 WAP
335 (DC65) and final harvest occurred 25 WAP (DC90) (Figure S2).

336 ***Photosynthetic temperature responses of the two wheat cultivars at aCO₂***

337 A-C_i curves together with g_m were measured at five leaf temperatures to characterize
338 the thermal photosynthetic responses of the two wheat cultivars grown at aCO₂
339 (Figure 1; Table 1). Overall, both cultivars had similar photosynthetic temperature
340 responses. A_{sat} and g_s increased with leaf temperature up to an optimum (T_{opt})
341 around 23.4°C and decreased thereafter, while C_i slowly decreased with
342 temperature. Mesophyll conductance (g_m) increased up to 25°C then plateaued
343 (Figure 1B). The temperature response of g_m as well as the values recorded at 25°C
344 (0.25-0.31 mol m⁻² s⁻¹ bar⁻¹) for Scout and Yitpi (Figure 1B) were similar to what has
345 been reported for wheat (0.39) and other crop species (cotton = 0.73, soybean =
346 0.49 and rice = 0.67) (Von Caemmerer and Evans, 2015; Jahan *et al.*, 2021). Scout
347 had slightly higher A_{sat} , g_s and g_m than Yitpi at most leaf temperatures (Figures 1A-
348 D). R_d linearly increased with temperature, and both cultivars had similar Q_{10} (Figure
349 1H, Table 1). V_{cmax} and J_{max} exponentially increased with leaf temperature, but J_{max}
350 declined above T_{opt} (30°C) in both cultivars (Figures 1E-F). There was no significant
351 difference in V_{cmax} , J_{max} or their activation energies between the two wheat cultivars
352 (Figure 1E-G, Table 1). The ratio of J_{max}/V_{cmax} was similar for the two cultivars and
353 linearly decreased with leaf temperature (Figure 1G).

354 ***eCO₂ stimulated photosynthesis and reduced stomatal conductance in both***
355 ***wheat cultivars***

356 Overall, the two wheat cultivars had similar A_{sat} , g_s , PWUE (A_{sat}/g_s), R_d , Fv/Fm, V_{cmax}
357 and J_{max} measured under most conditions (Figures 1-4, Tables S1-S3). Under
358 control (non-HS) conditions, eCO₂ enhanced A_{sat} measured at growth CO₂ (A_{growth})
359 and 25°C and reduced g_s in both cultivars at T1, T2 and T3 (Figure 2, Tables S1-S3).
360 When measured at common CO₂ and 25°C, eCO₂-grown plants had lower A_{sat} (-12%
361 at T2, $p < 0.01$) and g_s (-10% at T2, $p < 0.001$) relative to aCO₂. This photosynthetic
362 downregulation was more persistent in Yitpi compared to Scout (Figure 2, Tables
363 S1-S3).

364 ***High temperature during HS enhanced photosynthesis under eCO₂***

365 The two HS cycles did not reduce A_{growth} measured at 25°C during or after HS (Figure
366 3A-D, Tables S1-S3). During both HS1 and HS2, eCO₂ stimulated A_{growth} measured
367 at 25°C in Scout but not Yitpi. Relative to 25°C, A_{growth} increased at 35°C in Scout
368 (10-14%) and Yitpi (12-18%) grown at eCO₂ but not at aCO₂. Immediately after the
369 recovery from HS, A_{growth} was upregulated in eCO₂-grown Scout (Figures 3A-D and
370 S3). During both HS cycles, dark-adapted Fv/Fm measured at 25°C tended to be
371 lower in Yitpi grown at eCO₂ relative to aCO₂. In both cultivars, Fv/Fm decreased at
372 35°C relative to 25°C, indicating transient damage to PSII due to HS at both CO₂
373 treatments (Figure 3E-H, Tables S1-S3).

374 Following long-term recovery from HS1 and/or HS2, the eCO₂ stimulation of A_{growth}
375 was still marginally apparent in all T3 plants, being the strongest in eCO₂-grown Yitpi
376 (Figure 4A-B, Tables S1-S3). The reduction of g_s at eCO₂ was weak in all plants
377 (Figure 4C-D, Tables S1-S3). Hence, PWUE was stimulated by eCO₂ in all
378 treatments, while PNUE was unaffected (Figure 4E-H, Tables S1-S3). There was a
379 good correlation between A_{growth} and g_s ($r^2 = 0.51$, $p < 0.001$) across all treatments
380 (Figure 5A).

381 V_{cmax} and J_{max} were derived from A-C_i response curves measured at 25°C during the
382 recovery stage after HS2. For control and HS plants, growth at eCO₂ marginally
383 reduced V_{cmax} in Scout (-14%, $p = 0.09$) and Yitpi (-15%, $p = 0.06$) but had no effect
384 on J_{max} . HS had no effect on V_{cmax} or J_{max} in either cultivar (Figure 4I-L, Tables S1-

385 S3). V_{cmax} and J_{max} correlated well ($r^2 = 0.75$, $p < 0.001$) across treatments (Figure
386 5B).

387 ***Yitpi produced more tillers and grains than Scout***

388 When compared at aCO₂, the two wheat cultivars differed in phenology and growth
389 habit. Scout developed faster and flowered earlier than Yitpi. At T2, 43% of tillers
390 had ears in Scout compared to 11% in Yitpi (Figure S4). At T2, Scout was 74% ($p <$
391 0.001) taller than Yitpi but at T3 both cultivars had similar height (Figure 6I-J, Tables
392 S4 and S5). In contrast, Yitpi accumulated more biomass relative to Scout by
393 producing more tillers. At T3, Yitpi had 42% ($p < 0.005$) more total plant biomass,
394 130% ($p < 0.001$) more tillers, 254% ($p < 0.001$) larger leaf area, 128% ($p < 0.001$)
395 more leaves and 61% ($p < 0.001$) larger leaf size compared to Scout (Figure 6,
396 Tables S4 and S5).

397 At the final harvest (T4), Yitpi had more plant biomass (84%, $p < 0.001$), tillers (88%,
398 $p < 0.001$) and number of grains (54%, $p < 0.001$). Conversely, Scout had larger
399 grain size (+31%, $p < 0.001$), a higher proportion (100%) of its tillers developed ears
400 and more ears filled grains compared to higher tillering Yitpi (88%). Hence, both
401 cultivars had relatively similar grain yield (g/plant) (Figure 7A-F, Tables S4 and S6).
402 Higher (178%, $p < 0.001$) harvest index (HI) in Scout was due to early maturity and
403 consequent leaf senescence leading to loss of biomass at final harvest (Tables S5).
404 The final harvest was undertaken four weeks after all ears had matured on Scout to
405 give ample time for grain filling in Yitpi (Figure 7, Tables S4 and S6).

406 ***eCO₂ similarly stimulated wheat biomass and grain yield under non-HS*** 407 ***conditions***

408 The increase in plant biomass at eCO₂ depended on the growth stage (Figures 6-7,
409 Tables S4 and S5). However, the overall stimulation was not different between the
410 two cultivars as evident from the non-significant eCO₂ x cultivar interaction at all
411 harvests (Table S4). By T3 (anthesis), when both cultivars were still within the
412 exponential growth stage, eCO₂ stimulated plant biomass of Yitpi (+29%, $p < 0.001$)
413 and Scout (+9%, $p < 0.001$) under control conditions. The number of tillers, total leaf
414 area, mean leaf size or leaf mass area were not significantly affected by eCO₂ in
415 either cultivar (Figure 6, Tables S4 and S5). eCO₂ increased allocation to stem

416 relative to leaf biomass, particularly in Yitpi. Accordingly, there was a strong
417 correlation across treatments between stem and leaf biomass ($r^2 = 0.83$, $p < 0.001$)
418 and between total biomass and leaf area ($r^2 = 0.83$, $p < 0.001$) in Scout but not in
419 Yitpi. However, the two cultivars followed common relationship for root *versus* shoot
420 biomass ($r^2 = 0.41$, $p < 0.001$) and leaf area *versus* leaf number ($r^2 = 0.82$, $p < 0.001$)
421 across all treatments suggesting no effect of cultivar, eCO₂ or HS on these common
422 allometric relationships (Figure S5, Table S5).

423 At the final harvest T4 (seed maturity), eCO₂ enhanced biomass and equally
424 stimulated grain yield by increasing grain number in both cultivars (+64% in Scout
425 and +50% in Yitpi) under control conditions only (Figure 7A-D, Tables S4-S6).
426 Harvest index was not directly affected by any treatments but showed a significant
427 interaction ($p < 0.05$) between CO₂ and cultivar, such that HI was higher in Yitpi
428 under eCO₂ (Tables S5 and S6).

429 **eCO₂ did not stimulate the grain yield of HS plants**

430 At T3, moderate HS (34-38°C) applied under well-watered conditions and 60% RH
431 during the vegetative (HS1 applied after T1) and flowering (HS2 applied after T2)
432 stages had no significant impact on plant biomass of either wheat cultivar or CO₂
433 treatment. By T4, there were significant HS x CO₂ x cultivar interactions ($p < 0.01$)
434 for biomass and grain yield. HS1+2 reduced the biomass and grain yield of eCO₂-
435 grown Scout relative to aCO₂-grown counterparts. Unlike control plants, the biomass
436 and yield of HS plants were not enhanced by eCO₂ (Figure 7, Tables S4 and S5).

437 **eCO₂ reduced grain N in Yitpi but not in Scout**

438 Neither eCO₂ or HS had a significant effect on flag leaf N content in either cultivar at
439 T2 or T3, but eCO₂ reduced aggregate leaf N content (-18%) at T3 in Yitpi only
440 (Cultivar x CO₂ $p < 0.05$) (Table S7). Yitpi had higher grain N content (+26%) than
441 Scout in control plants grown at aCO₂ (Figure 7G-H, Table S6). In control plants,
442 eCO₂ significantly reduced grain protein content in Yitpi (-18%, $p < 0.05$) but not in
443 Scout due to significant cultivar X CO₂ interaction ($p < 0.01$), while HS had no effect
444 on protein content in either cultivar (Figure 7G-H, Table S6).

445 Discussion

446 ***Two wheat cultivars with contrasting morphology and phenology, but similar*** 447 ***photosynthesis and grain yield***

448 The effects of future climate conditions, including eCO₂, will depend on the
449 environmental conditions (e.g., water and heat stress) and the crop's agronomic
450 features. Here, we compared the interactive effects of eCO₂ and HS on two
451 commercial wheat cultivars, Scout and Yitpi, with contrasting phenology and growth
452 habit. Plants were grown under well-watered and fertilized conditions to remove any
453 confounding effects of water or nutrient limitations on the eCO₂ or HS responses. RH
454 was kept constant to minimize the negative impact of dry air during HS. Finally, we
455 compared the effects of applying HS at the vegetative and flowering stages.

456 Free tillering Yitpi produced substantially more tillers, leaf area and biomass relative
457 to the faster developing Scout. Accordingly, our first hypothesis predicted that Yitpi
458 will have higher grain yield. The results only partially supported this hypothesis,
459 because relative to Yitpi, Scout had higher harvest index (HI) due to its early
460 maturing and senescing habit. While Yitpi initiated more tillers, a lower proportion of
461 these tillers produced ears and filled grains. In contrast, Scout produced less tillers
462 but flowered earlier which allowed enough time for all its tillers to produce ears and
463 fill bigger grains by the final harvest. Hence, both cultivars had relatively similar
464 yields due to bigger grain size in Scout and higher grain number in Yitpi. It is worth
465 noting that some field trials have reported slightly higher grain yields in Scout than
466 Yitpi (National variety trial report, GRDC, 2014). Our results are consistent with a
467 previous study using different wheat cultivars with contrasting source-sink
468 relationships which reported that the freely tillering cultivar "Silverstar" translated into
469 more spikes while restricted tillering cultivar "H45" had more and heavier kernels per
470 spike than "Silverstar" (Tausz-Posch et al., 2015). Thus, early vigor and maturity
471 compared to high tillering capacity seem to be equally beneficial traits for high grain
472 yield in the Australian environment.

473 The two wheat cultivars showed similar photosynthetic traits and response to
474 temperature and eCO₂. In contrast to our expectations that Scout would have higher
475 WUE due to its selection based on a carbon isotope discrimination gene (Condon et

476 *al.*, 2004), both wheat cultivars showed similar PWUE under most measurement and
477 growth conditions in this study (Figure 4E-F, Table 2).

478 ***Elevated CO₂ stimulated photosynthesis but reduced photosynthetic capacity***
479 ***in both cultivars***

480 Long term exposure to eCO₂ may reduce photosynthetic capacity due to lower
481 amount of Rubisco in a process referred as 'acclimation' (Nie *et al.*, 1995; Rogers
482 and Humphries, 2000; Ainsworth *et al.*, 2003). Alternatively, eCO₂ may 'down-
483 regulate' photosynthetic capacity by reducing Rubisco activation or other regulatory
484 mechanisms without affecting Rubisco content (Delgado *et al.*, 1994). In the current
485 study, eCO₂ similarly increased A_{growth} (+21%) measured at growth CO₂ and reduced
486 both A_{sat} (-12%) measured at common CO₂ (Figure 2, T2) and V_{cmax} (Figure 4I-J) in
487 both cultivars. In contrast, leaf N (and possibly Rubisco) was not significantly
488 affected in either cultivar (Table S4). Hence, the wheat cultivars have likely
489 undergone a photosynthetic downregulation -rather than acclimation- in response to
490 eCO₂ (Delgado *et al.*, 1994; Leakey *et al.*, 2009). These results partially countered
491 our second hypothesis suggesting that Yitpi will show less photosynthetic
492 acclimation due to its higher sink capacity. The interaction of eCO₂ with plant traits
493 are complex. On the one hand, eCO₂ is expected to cause less feedback inhibition
494 on photosynthesis in plants with high sink capacity (Ainsworth *et al.*, 2004). On the
495 other, fast-growing plants show a proportionally larger response to eCO₂ (Poorter
496 and Navas, 2003). Hence, high tillering in Yitpi and fast development in Scout both
497 led to a relatively small observed photosynthetic downregulation in response to
498 growth at eCO₂. This allowed a sustained photosynthetic stimulation, which in turn
499 led to a significant biomass and yield enhancement by CO₂ enrichment in both wheat
500 cultivars (Figures 6-7). Photosynthetic responses of wheat in current study are in
501 agreement with earlier enclosure studies which generally have higher response to
502 eCO₂ than the FACE studies (Kimball *et al.*, 1995; Hunsaker *et al.*, 1996; Osborne *et*
503 *al.*, 1998; Kimball *et al.*, 1999; Long *et al.*, 2006; Cai *et al.*, 2016).

504 ***Elevated CO₂ stimulated grain yield similarly in both wheat cultivars***

505 In disagreement with our second hypothesis, eCO₂ similarly stimulated plant
506 biomass and grain yield in early-maturing Scout and high tillering Yitpi (Figures 6-7,
507 Table S4-S5). In Scout, the biomass stimulation was associated with increased

508 tillering (one extra tiller per plant). In contrast, Yitpi produced many tillers at aCO₂
509 and the additional fixed carbon at eCO₂ was allocated to the existing tillers. At seed
510 maturity, eCO₂ stimulated grain yield similarly in both cultivars as a result of the
511 trade-off between grain yield components (Dias de Oliveira *et al.*, 2015). In
512 particular, eCO₂ stimulated grain number in both cultivars, while grain size increased
513 in Scout only (Figure 7, Table S6). Generally, eCO₂ stimulates grain yield by
514 increasing the number of tillers and consequently, ears per plant (Zhang *et al.*, 2010;
515 Bennett *et al.*, 2012), which has also been reported in FACE studies (Högy *et al.*,
516 2009; Tausz-Posch *et al.*, 2015; Fitzgerald *et al.*, 2016). However, in our study, the
517 increase in grain yield at eCO₂ was mainly due to the increase in the number of
518 grains per ear. In line with our results, Tausz-Posch *et al.*, (2015) reported
519 comparable grain yield stimulation by eCO₂ in two different wheat cultivars with
520 contrasting source-sink relationships. Moreover, grain yield of twenty wheat cultivars
521 that differed in tillering propensity, water soluble carbohydrate accumulation, early
522 vigor and transpiration efficiency responded similarly to eCO₂ in glasshouse settings
523 (Ziska *et al.*, 2004; Bourgault *et al.*, 2013).

524 ***Elevated CO₂ reduced grain N in Yitpi only***

525 Overall, there is a negative relationship between grain yield and quality (Taub *et al.*,
526 2008; Pleijel and Uddling, 2012). Hence, increased grain yield at eCO₂ results in
527 lower grain N and hence protein content (Seneweera and Conroy, 1997; Bahrami *et al.*
528 *et al.*, 2017). In our study, eCO₂ reduced grain N in Yitpi under control conditions.
529 Scout was characterized by having larger grains which accumulated less N than
530 Yitpi. Moreover, eCO₂ reduced total leaf N (-18%) at T3 and grain N (-17 %) at T4 in
531 Yitpi but not in Scout. This is consistent with the results from FACE study with same
532 cultivars which reported -14% reduction in N content by eCO₂ in above ground dry
533 mass in Yitpi but not in Scout under well-watered conditions (Bahrami *et al.*, 2017).
534 The higher biomass accumulation in free tillering Yitpi may have exhausted the
535 nutrient supply, such that further biomass stimulation by eCO₂ lead to a significant
536 dilution in N content (Taub and Wang, 2008).

537 Wheat cultivars with early vigour such as Scout have greater root biomass
538 accumulation as well as greater early N uptake which may have avoided a negative
539 effect of eCO₂ on leaf and grain N (Liao *et al.*, 2004; Bahrami *et al.*, 2017).

540 Accordingly, Scout maintained a higher N utilization efficiency (grain yield per total
541 plant N) relative to Yitpi under all treatments (Table S6). Increased grain yield is
542 strongly associated with higher grain number per unit area (Zhang *et al.*, 2010;
543 Bennett *et al.*, 2012) which dilutes the amount of N translocated per grain. Quality
544 deterioration due to lower protein via reduced N is of critical concern in future high
545 CO₂ climate considering that even additional supply of N does not prevent N dilution
546 in grain under eCO₂ (Tausz *et al.*, 2017). In addition, eCO₂ has strong detrimental
547 effect on other nutrient availability and remobilization from leaves to grains (Tcherkez
548 *et al.*, 2020).

549 ***HS had little effects on wheat photosynthesis or yield at aCO₂***

550 A key finding of this study was that the application of HS events (HS1, HS2 or
551 HS1+2) was not detrimental to aCO₂-grown wheat plants (Figures 5 and 7, Tables
552 S4-S6). Thus, our hypothesis that HS will reduce photosynthesis, biomass and yield
553 at aCO₂ was rejected. This finding is in contrast to previously reported studies where
554 HS reduced the grain yield and negatively affected the growth and development in
555 wheat (Stone and Nicolas, 1996, 1998; Farooq *et al.*, 2011; Coleman *et al.*, 1991).
556 During heat waves in the field, the vapor pressure deficit (VPD) increases and soil
557 moisture decreases leading to lower stomatal conductance and consequently lower
558 transpiration rate. Thus, plants are unable to cool down and leaf temperatures rise
559 beyond optimum levels causing damage. The negligible effect of HS in our study
560 could be explained by the ability of well-watered plants to maintain leaf temperature
561 below damaging levels due to transpirational cooling (Perera *et al.*, 2019; Deva *et al.*,
562 2020) even with air temperatures reaching up to 38°C. At moderate (~60%)
563 relative humidity, there is sufficient water vapour gradient to sustain high
564 transpiration rates when soil water is available, as was the case in our experiment. In
565 most cases, g_s was not significantly affected (Tables S1 and S2), and even slightly
566 higher at T3 in HS-plants relative to the control (Figure 4c,d) Well-watered crops can
567 maintain grain-filling rate, duration and size under HS (Dupont *et al.*, 2006), and high
568 temperatures can increase crop yields if not exceeding critical optimum growth
569 temperature (Welch *et al.*, 2010). Also, in the current study, the night temperatures
570 were not increased during HS which favors plant growth by reducing respiratory
571 losses (Prasad *et al.*, 2008).

572 In particular, HS did not elicit a direct negative impact on photosynthesis or
573 chlorophyll fluorescence in either cultivar or CO₂ treatment. During HS, high
574 temperature transiently reduced maximum efficiency of PSII (Fv/Fm) in both cultivars
575 and CO₂ treatments (Figure 3E-H). However, unchanged Fv/Fm measured at 25°C
576 confirmed that photosynthesis did not suffer long-term damage during or after HS.
577 Moreover, HS was not severe enough to negatively affect A_{growth} measured at 25°C.
578 These results are corroborated by the insensitivity of V_{cmax} and J_{max} to HS (Figure 4I-
579 L), but contrast with previously reported studies where HS reduced photosynthesis in
580 wheat at the vegetative (Wang et al., 2008) and the flowering (Chavan *et al.*, 2019;
581 Balla *et al.*, 2019) stages. HS lowers membrane thermostability by inducing reactive
582 oxygen species (ROS) and altering the membrane protein structures, which lead to
583 changes in the fluidity of the thylakoid membrane and separation of light harvesting
584 complex from the photosystems (Wahid *et al.*, 2007; Poudel, 2020). We were unable
585 to measure leaf temperatures in the current study, but we speculate that, in well-
586 watered wheat plants growing at moderate RH, leaf temperatures might not have
587 increased beyond damaging levels to the membranes during the HS events.
588 Repeated HS may result in priming which involves pre-exposure of plants to a
589 stimulating factor such as HS (Wang *et al.*, 2017) and enable plants to cope better
590 with later HS events (Balla *et al.*, 2021). However, there was no difference between
591 HS applied at the vegetative (HS1) and/or flowering stage (HS2) in rejection of our
592 fourth hypothesis, and this may additionally be due to the short term duration of the
593 two HS cycles (3 days each). Hence, our study demonstrated the benign effect that
594 HS has on crop yield when separated from water stress and plants are able to
595 transpire.

596 ***HS precluded an eCO₂ response in biomass and grain yield***

597 In our study, the impact of HS depended on the wheat cultivar and growth CO₂
598 (Tables S1 and S4). Elevated CO₂ and temperature interactions can be complex,
599 dynamic and difficult to generalize as they can go in any direction depending on
600 plant traits and other environmental conditions (Rawson, 1992). Plant development
601 is generally accelerated by increased temperature; eCO₂ can accelerate it further in
602 some instances or may have neutral or even retarding effects in other cases
603 (Rawson, 1992).

604 While eCO₂ stimulated wheat biomass and grain yield under control (non-HS)
605 conditions, HS precluded a yield response to eCO₂ in Yitpi and reduced biomass and
606 yield in eCO₂-grown Scout relative to aCO₂-grown counterparts (Figure 7). These
607 results are in contrast with previous studies that reported similar wheat yield
608 reduction at ambient or elevated CO₂ in response to severe (Chavan *et al.*, 2019) or
609 moderate HS (Zhang *et al.*, 2018). The results also partially refuted our third
610 hypothesis that HS may decrease yield more at aCO₂ than eCO₂, while partially
611 agreeing that HS will have a more negative impact on Scout relative to Yitpi, albeit
612 for different reasons than what we originally suggested. The negative effect of HS on
613 Scout biomass and grain yield at eCO₂ occurred despite the eCO₂ stimulation of
614 A_{growth} under HS (T3, Figure 4). However, over the long term, A_{growth} was stimulated
615 in eCO₂-grown Yitpi and not Scout (Figure 4).

616 Lack of a biomass stimulation despite high photosynthetic rates during HS under
617 eCO₂ could be due to the short duration of HS (3 days), which may not have been
618 long enough to stimulate biomass gain. In addition, nutrient limitation at eCO₂ may
619 have restricted the eCO₂ growth response. Typically, eCO₂ studies show reduced N
620 content in wheat and other crops (Taub and Wang, 2008; Leakey *et al.*, 2009;
621 Bahrami *et al.*, 2017). Hence, the wheat plants may have exhausted available
622 nutrients due to increased demand by growing sinks at eCO₂, which may limited
623 further stimulation by high temperature. HS may be more damaging at eCO₂ due to
624 reduce transpirational cooling as a result of reduced g_s at eCO₂, leading to higher
625 leaf temperatures. However, A_{growth} increased in response to high temperature
626 (35°C) under eCO₂ but not under aCO₂ during HS (Figure 3A-D), which refutes the
627 suggestion of HS-damage to photosynthesis.

628 Higher g_s during HS at moderate RH in well-watered conditions may increase A_{growth}
629 by increasing C_i in both aCO₂ and eCO₂ grown plants. Furthermore, lower
630 photorespiration under eCO₂ allows additional increase in A_{growth} with temperature
631 when measured at 35°C relative to 25°C (Long, 1991). Under aCO₂, photorespiration
632 increases with temperature reducing A_{growth} measured at 35°C relative to 25°C. Our
633 results also point to a shift in T_{opt} of photosynthesis (~ 24°C at aCO₂) to higher
634 temperatures for plants grown at eCO₂ (Sage and Kubien, 2007). This would come
635 about as a result of lower photorespiration at eCO₂ as well as the slight upregulation
636 of photosynthetic rates observed in eCO₂-grown Scout at the recovery stage of HS

637 (Figure 3A, C). However, at T3, A_{growth} was similar between aCO₂ and eCO₂ grown
638 plants (Figure 4A) indicating the short-term nature of this photosynthetic
639 upregulation.

640 **Conclusions**

641 The two wheat cultivars, Scout and Yitpi differed in growth and development but
642 produced similar grain yield. Under control conditions, eCO₂ stimulated biomass and
643 yield similarly in both cultivars. HS was not damaging to photosynthesis, growth,
644 biomass or grain yield under well-watered and moderate RH conditions. However,
645 HS interacted with eCO₂, leading to similar or lower biomass and grain yield at eCO₂
646 relative to both aCO₂ in plants exposed to HS. This interactive effect precluded the
647 positive effects of eCO₂ in HS-plants. eCO₂ improved photosynthetic rates in control
648 and HS plants. Also, high temperature stimulated photosynthesis under eCO₂ but not
649 under aCO₂ during HS which suggests increased optimum temperature of
650 photosynthesis at eCO₂. We speculate that in the current study, HS plants were able
651 to cool down using high transpiration which helped to maintain lower leaf
652 temperatures despite high air temperatures during HS. The current study provides
653 important insights into the effect of short-term moderate temperature increases in
654 well-watered conditions under future elevated CO₂, potential role of transpirational
655 cooling during HS and interactions between HS and eCO₂ which will be useful in
656 breeding cultivars for future climate and improving crop model accuracy to predict
657 crop performance in future high CO₂ environment with frequent heat waves.

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668 **Conflicts of interest**

669 The authors declare that they have no conflict of interest.

670 **Availability of data**

671 All data supporting the findings of this study are available within the paper and within
672 its supplementary materials published online. Reuse of the data is permitted after
673 obtaining permission from the corresponding author.

674 **Code availability**

675 Software application and codes used are all publicly available.

676 **Authors contributions**

677 All authors conceived the project. SGC maintained the plants and collected the data.
678 SGC and RAD analysed the data. SGC and OG prepared the manuscript with input
679 from other co-authors.

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Table 1 Summary of modelled parameters for temperature response of photosynthesis. Summary of coefficients derived using nonlinear least square fitting of CO₂ assimilation rates and maximal rate of carboxylation (V_{cmax}) and maximal rate of RuBP regeneration (J_{max}) determined using A-C_i response curves and dark respiration measured at five leaf temperatures 15, 20, 25, 30 and 35°C. Values are means with standard errors. Derived parameters include temperature optima (T_{opt}) of photosynthesis (A_{opt}); activation energy for carboxylation (EaV); activation energy (EaJ), entropy term (ΔSJ) and T_{opt} and corresponding value for J_{max} with deactivation energy (Hd) assumed constant; and activation energy (EaR) and temperature coefficient (Q_{10}) for dark respiration. Letters indicate significance of variation in means.

Parameter	Constant	Scout	Yitpi
A_{sat} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	T_{opt} (°C)	23.4 ± 1 a	23.4 ± 0.7 a
	A_{opt}	24.6 ± 1 a	22 ± 0.6 b
g_m ($\text{mol m}^{-2} \text{s}^{-1} \text{bar}^{-1}$)	T_{opt} (°C)	27.9 ± 2.2 a	32.5 ± 6.9 b
	g_m at 25°C	0.31 ± 0.01 a	0.25 ± 0.01 b
	g_m at T_{opt}	0.30 ± 0.01	0.25 ± 0.01
V_{cmax} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	V_{cmax} at 25°C	192.7 ± 17.1 a	198.4 ± 17.7 a
	EaV (kJ mol ⁻¹)	43.3 ± 8.74 a	46.4 ± 8.7 a
J_{max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	J_{max} at 25°C	187.9 ± 13.1 a	186.1 ± 5.7 a
	T_{opt} (°C)	29.6 ± 0.3 a	30.5 ± 0.3 a
	J_{max} at T_{opt}	205.7 ± 10.2	215.4 ± 13.4
	EaJ (kJ mol ⁻¹)	37.7 ± 13.2 a	41.1 ± 5.8 a
	ΔSJ (J mol ⁻¹ K ⁻¹)	648.3 ± 5.3 a	647 ± 2.4 a
	Hd (kJ mol ⁻¹)	200	
Rd ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Rd at 25°C	1.25 ± 0.02 a	1.25 ± 0.02 a
	EaR (kJ mol ⁻¹)	30.9 ± 1.6 a	33.2 ± 1.7 a
	Q_{10}	1.51 ± 0.03 a	1.56 ± 0.04 a

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Figure Legends

Figure 1 Temperature response of photosynthetic parameters: CO₂ assimilation rate (a), mesophyll conductance (b), stomatal conductance (c) and intercellular CO₂ (d), V_{cmax} (e), J_{max} (f), J_{max} / V_{cmax} (g) and dark respiration (h) over leaf temperatures (15, 20, 25, 30 and 35 °C) in plants grown at aCO₂. Scout and Yitpi are depicted using circles with solid cultivars and triangles with broken cultivars respectively. Data in panels (a), (b), (c), (d), (e), (f) and (h) are fit using nonlinear least square (nls) function in R.

Figure 2 Response of leaf gas exchange parameters to eCO₂ under non-HS conditions. Measurements were made at 25°C before each harvest (T1, T2 and T3) for CO₂ assimilation rates (a, b) and stomatal conductance (c, d) in Scout (Circles) and Yitpi (Triangles). Plants were grown and measured at aCO₂ (blue solid cultivars), grown and measured at eCO₂ (red solid cultivars), and grown at eCO₂ and measured at 400 µL CO₂ L⁻¹ (red dashed cultivars). Statistical significance levels (t-test) for the growth condition within each cultivar are shown and they are: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure 3 Response of photosynthesis and chlorophyll fluorescence to HS in Scout and Yitpi grown at aCO₂ or eCO₂. CO₂ assimilation rates (a, b, c, d) and dark-adapted chlorophyll fluorescence, Fv/Fm (e, f, g, h) were measured at growth CO₂ and 25 °C in Scout (Circles) and Yitpi (Triangles). Open and closed symbols represent control and HS plants, respectively. In addition, plants were measured at 35°C (*) during both HS cycles.

Figure 4 Response of photosynthetic parameters to eCO₂ and HS at anthesis (T3) in Scout and Yitpi. CO₂ assimilation rate (a, b), stomatal conductance (c, d), photosynthetic water use efficiency (e, f) and photosynthetic nitrogen use efficiency (g, h) were measured at growth CO₂. V_{cmax} (i, j) and J_{max} (k, l) were derived from ACi curves measured at 25°C. Cultivars indicate means and shaded region is 95% confidence interval. Data shown for control (not exposed to any heat stress) and plants exposed to both heat stress cycles (HS1+2). Statistical significance levels (t-test) for the growth condition within each cultivar are shown and they are: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Figure 5 Relationships with leaf gas exchange and grain yield across treatments. CO₂ assimilation rate plotted as a function of stomatal conductance (a) (both aCO₂ and eCO₂ grown plants measured at 400 µl L⁻¹), J_{max} plotted as a function of V_{cmax} (b) and grain protein plotted as a function of yield (c) in Scout

(Circles) and Yitpi (Triangles). Ambient and elevated CO₂ are depicted in blue and red, respectively. Control and heat stressed plants depicted using open and closed symbols. Panel a depicts data for control, HS1, HS2 and both heat stresses (HS1+2), while panels b and c include only control and HS1+2.

Figure 6 Response of plant growth and morphological traits to elevated CO₂ and HS: Total dry mass (a, b), tillers or number of tillers (c, d), leaf area (e, f), leaf number (g, h) and height (i, j) were measured at different time points across the life cycle of wheat cultivars Scout (Circles) and Yitpi (Triangles). Ambient and elevated CO₂ are depicted in blue and red color, respectively. Open symbols connected with solid cultivars and closed symbols connected with dashed cultivars represent control and HS plants, respectively. HS1 and HS2 depict the timing of HS applied at 10 and 15 weeks after planting respectively.

Figure 7 Response of total plant dry mass and grain parameters to growth at eCO₂ and HS at maturity (T4): Total dry mass (a, b), grain dry mass (c, d), grain number (e, f) and grain nitrogen (g, h) were measured at the final harvest. Cultivars indicate means and shaded region is 95% confidence interval. Ambient and elevated CO₂ are depicted in blue and red color respectively. Heat stress levels include plants not exposed to any heat stress (control) and both heat stresses (HS1+2). Statistical significance levels (t-test) for the growth condition within each cultivar is shown and they are: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Supplementary Materials

Table S1. Summary of statistics for gas exchange parameters.

Table S2. Response of Scout gas exchange parameters to elevated CO₂ and heat stress.

Table S3. Response of Yitpi gas exchange parameters to growth at elevated CO₂ and heat stress.

Table S4. Summary of statistics for plant dry mass and morphological parameters.

Table S5. Response of plant dry mass and morphological parameters to elevated CO₂ and HS.

Table S6. Summary of plant nitrogen content parameters.

Figure S1. Glasshouse growth conditions.

Figure S2. Experimental design.

Figure S3. Photosynthetic response to growth at eCO₂ and heat stresses (HS1 and HS2).

Figure S4. Response of ear development in Scout and Yitpi to eCO₂ and HS at the booting stage (T2).

Figure S5. Relationship between dry mass and morphological parameters measured at anthesis (T3).

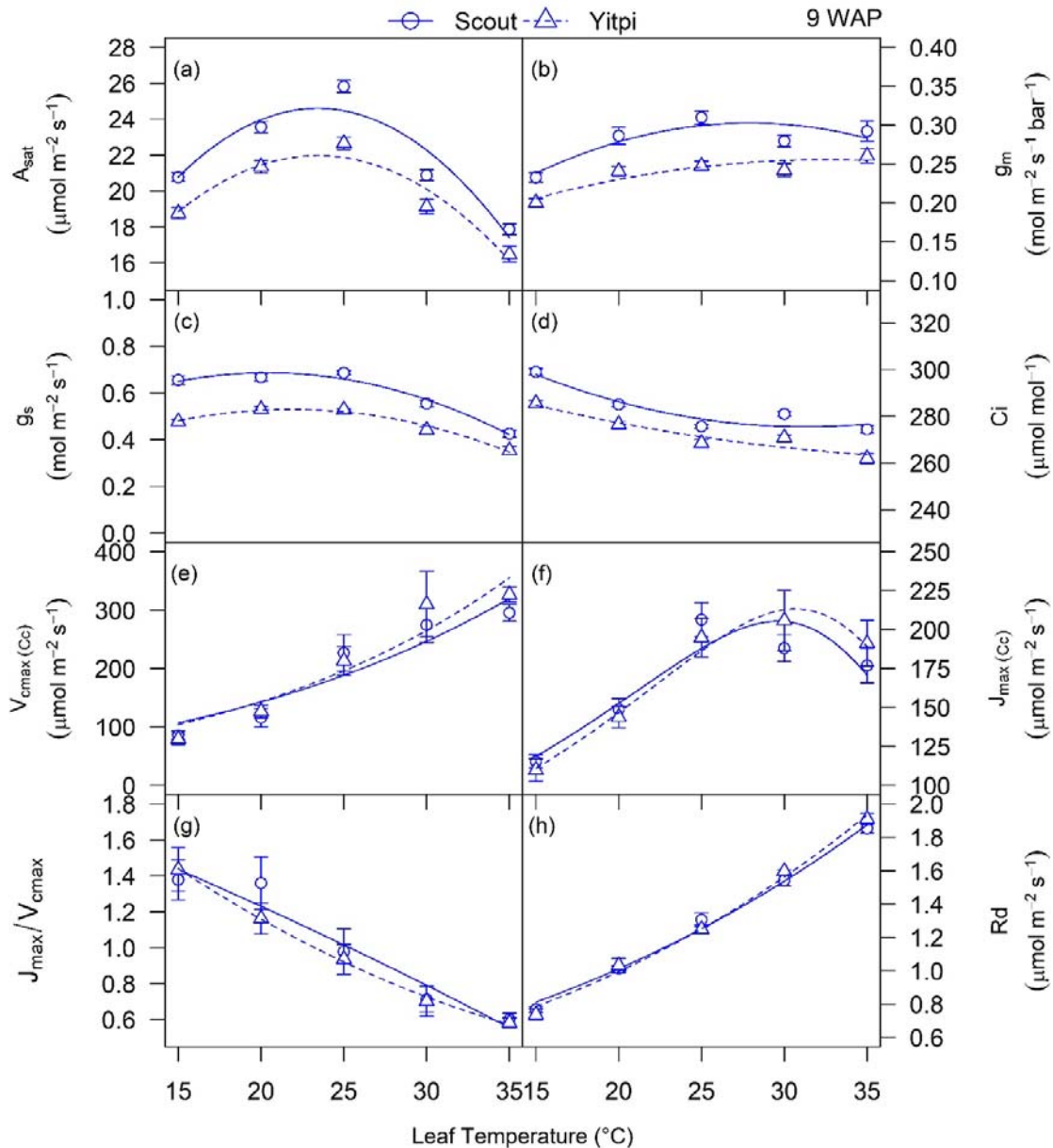


Figure 1 Temperature response of photosynthetic parameters: CO₂ assimilation rate (a), mesophyll conductance (b), stomatal conductance (c) and intercellular CO₂ (d), V_{cmax} (e), J_{max} (f), J_{max} / V_{cmax} (g) and dark respiration (h) over leaf temperatures (15, 20, 25, 30 and 35 °C) in plants grown at aCO₂. Scout and Yitpi are depicted using circles with solid cultivars and triangles with broken cultivars respectively. Data in panels (a), (b), (c), (d), (e), (f) and (h) are fit using nonlinear least square (nls) function in R.

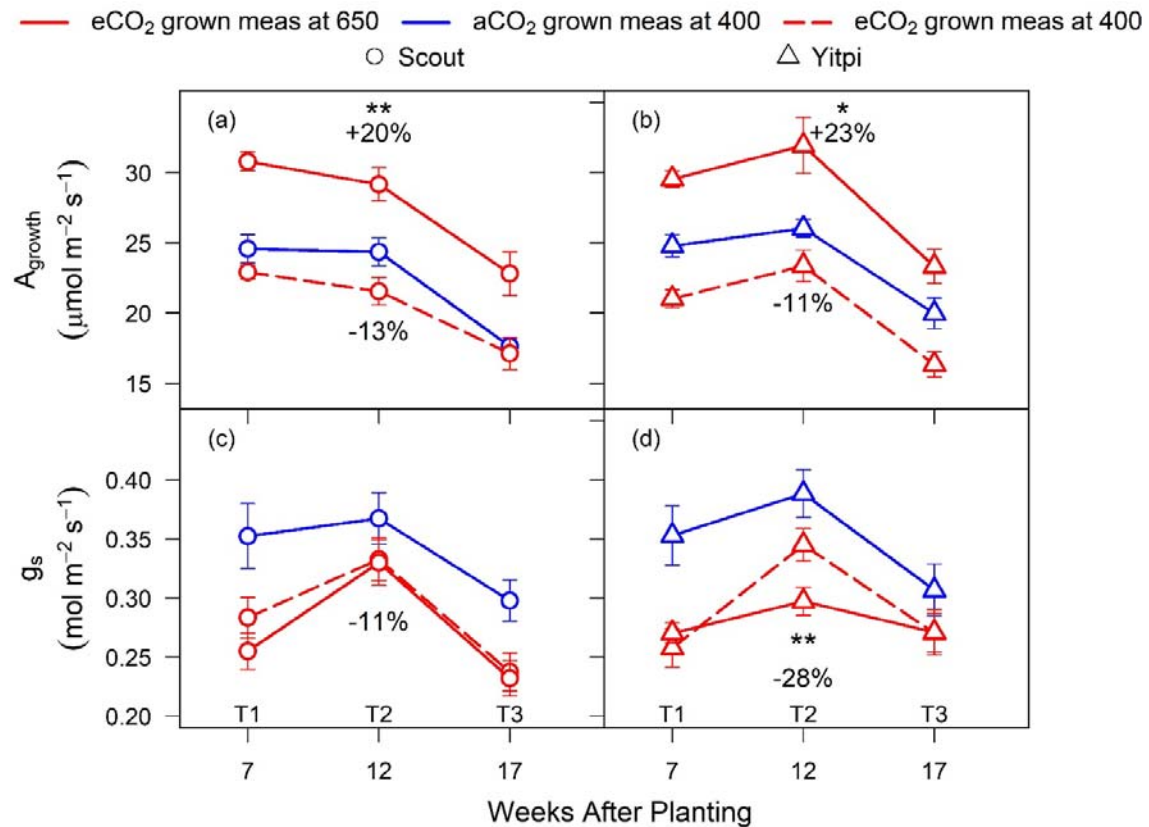


Figure 2 Response of leaf gas exchange parameters to eCO₂ under non-HS conditions. Measurements were made at 25°C before each harvest (T1, T2 and T3) for CO₂ assimilation rates (a, b) and stomatal conductance (c, d) in Scout (Circles) and Yitpi (Triangles). Plants were grown and measured at aCO₂ (blue solid cultivars), grown and measured at eCO₂ (red solid cultivars), and grown at eCO₂ and measured at 400 μL CO₂ L⁻¹ (red dashed cultivars). Statistical significance levels (t-test) for the growth condition within each cultivar are shown and they are: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

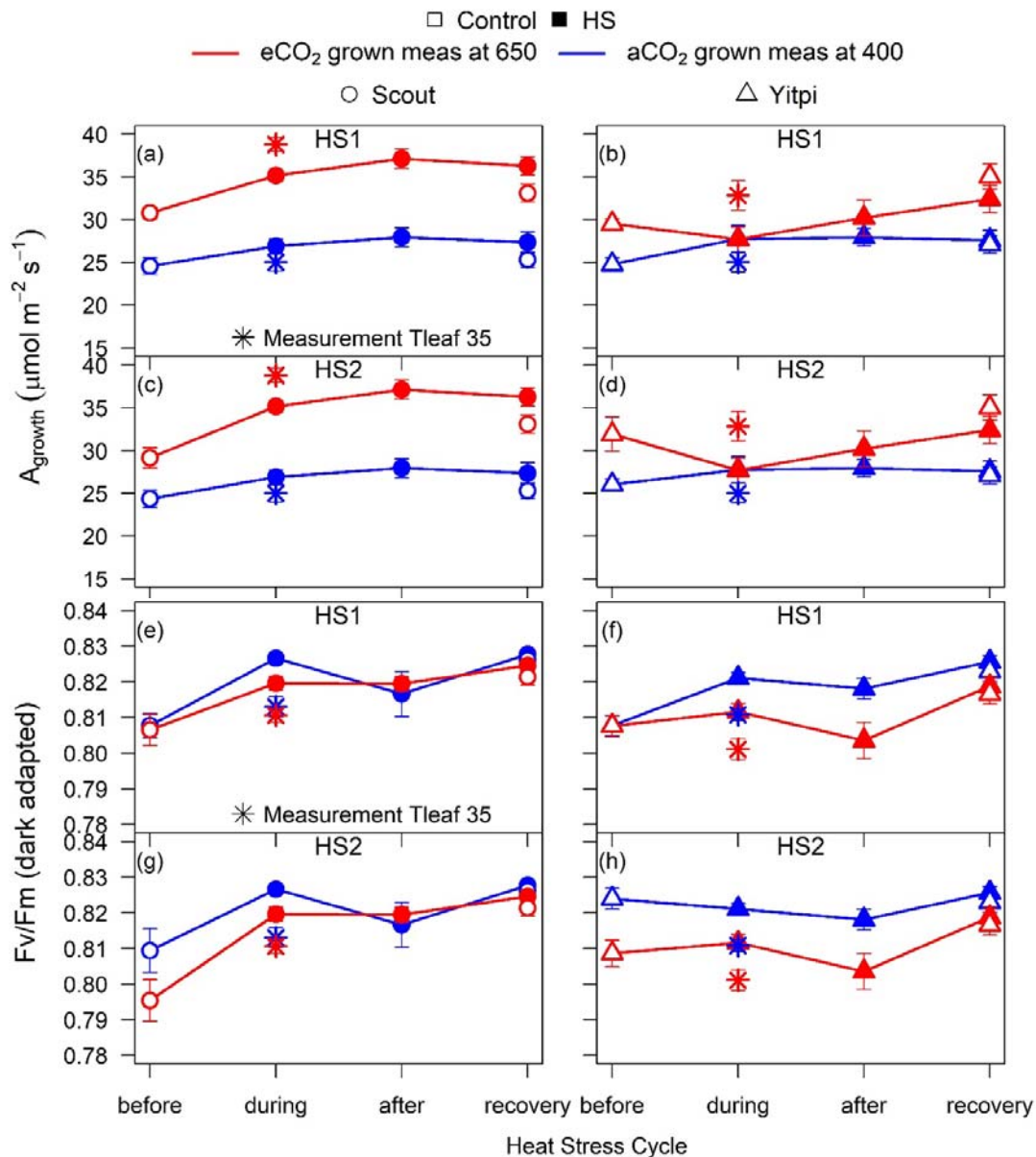


Figure 3 Response of photosynthesis and chlorophyll fluorescence to HS in Scout and Yitpi grown at aCO₂ or eCO₂. CO₂ assimilation rates (a, b, c, d) and dark-adapted chlorophyll fluorescence, F_v/F_m (e, f, g, h) were measured at growth CO₂ and 25 °C in Scout (Circles) and Yitpi (Triangles). Open and closed symbols represent control and HS plants, respectively. In addition, plants were measured at 35°C (*) during both HS cycles.

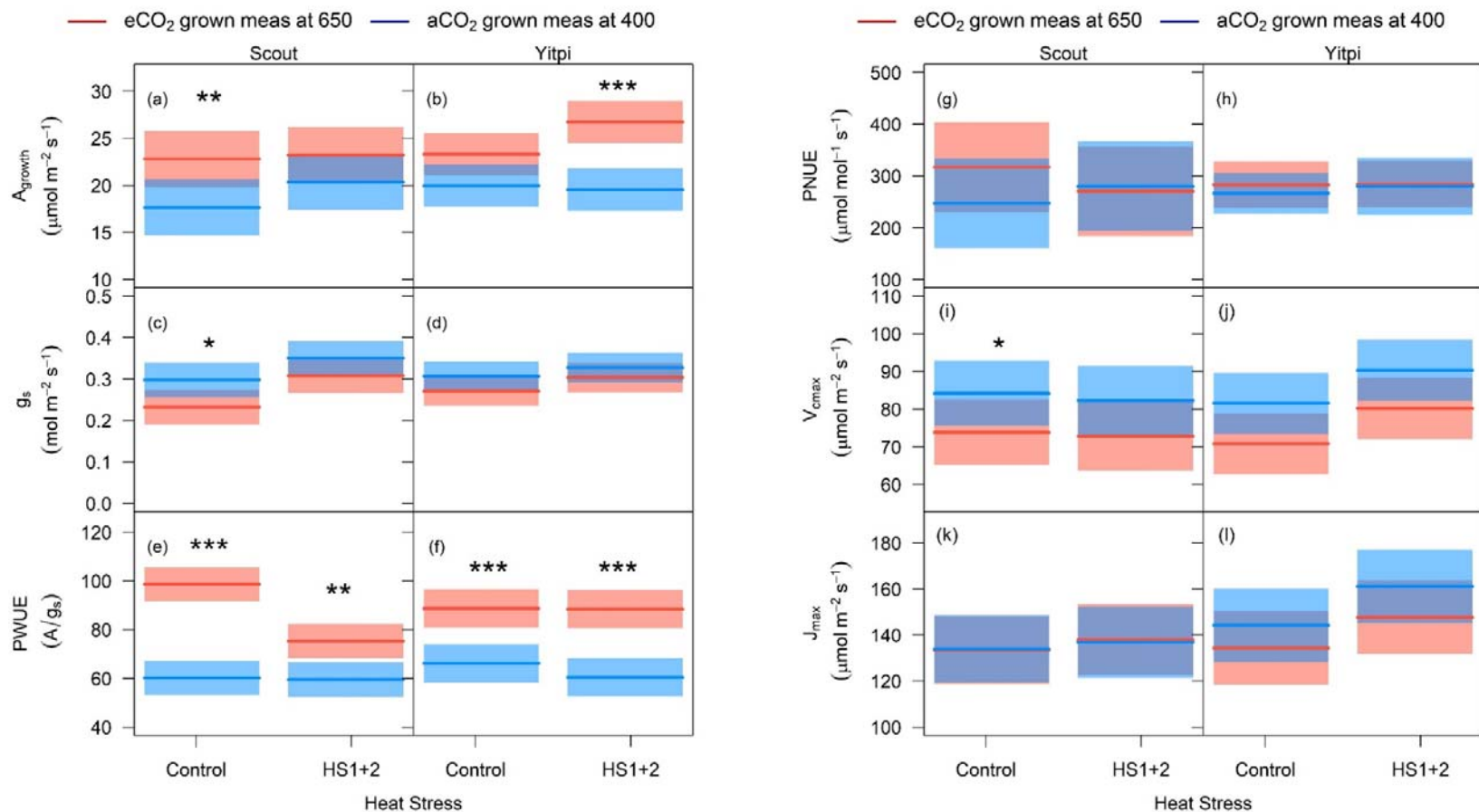


Figure 4 Response of photosynthetic parameters to eCO₂ and HS at anthesis (T3) in Scout and Yitpi. CO₂ assimilation rate (a, b), stomatal conductance (c, d), photosynthetic water use efficiency (e, f) and photosynthetic nitrogen use efficiency (g, h) were measured at growth CO₂. V_{cmax} (i, j) and J_{max} (k, l) were derived from ACi curves measured at 25°C. Cultivars indicate means and shaded region is 95% confidence interval. Data shown for control (not exposed to any heat stress) and plants exposed to both heat stress cycles (HS1+2). Statistical significance levels (t-test) for the growth condition within each cultivar are shown and they are: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

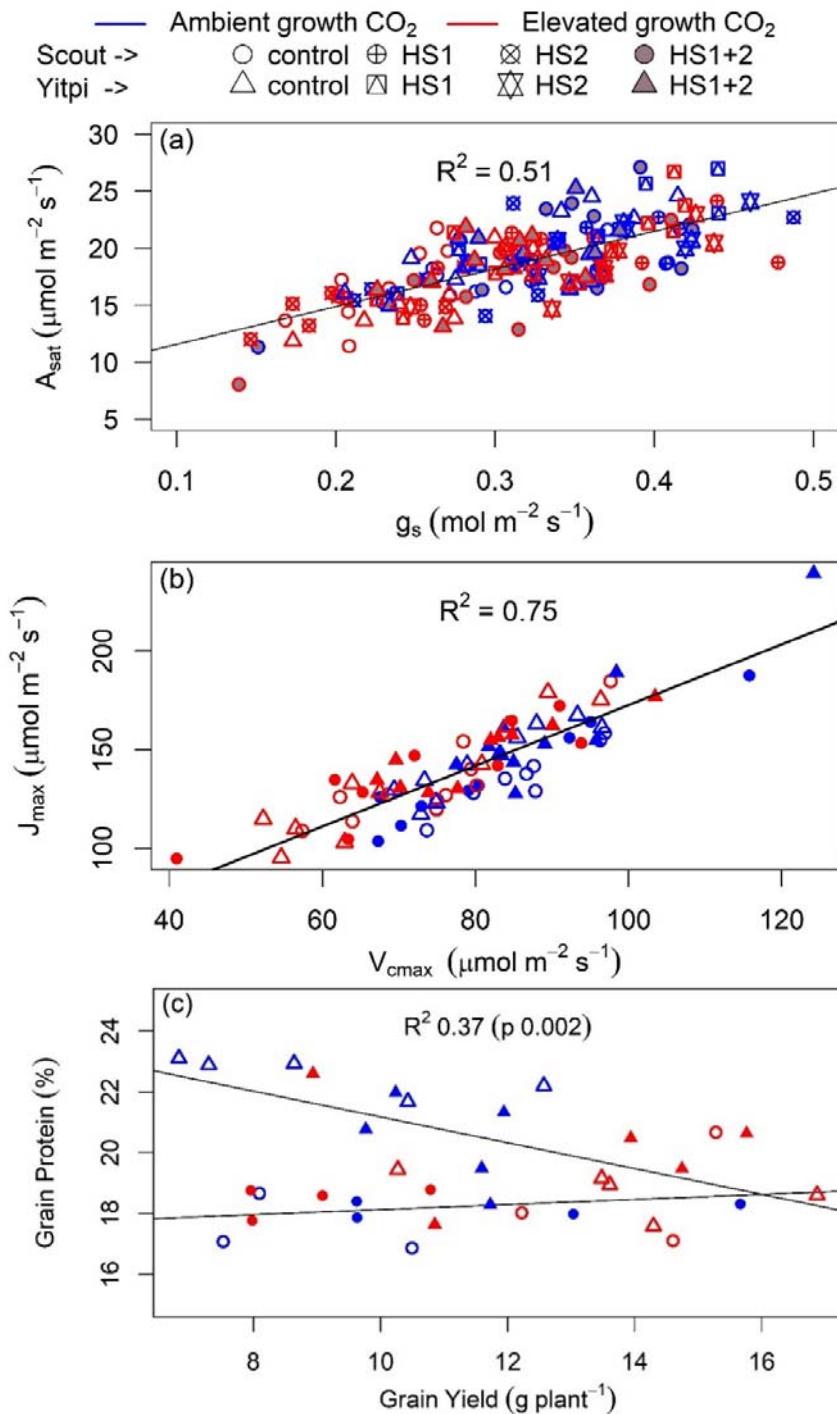


Figure 5 Relationships with leaf gas exchange and grain yield across treatments. CO₂ assimilation rate plotted as a function of stomatal conductance (a) (both aCO₂ and eCO₂ grown plants measured at 400 μl L⁻¹), J_{max} plotted as a function of V_{cmax} (b) and grain protein plotted as a function of yield (c) in Scout (Circles) and Yitpi (Triangles). Ambient and elevated CO₂ are depicted in blue and red, respectively. Control and heat stressed plants depicted using open and closed symbols. Panel a depicts data for control, HS1, HS2 and both heat stresses (HS1+2), while panels b and c include only control and HS1+2.

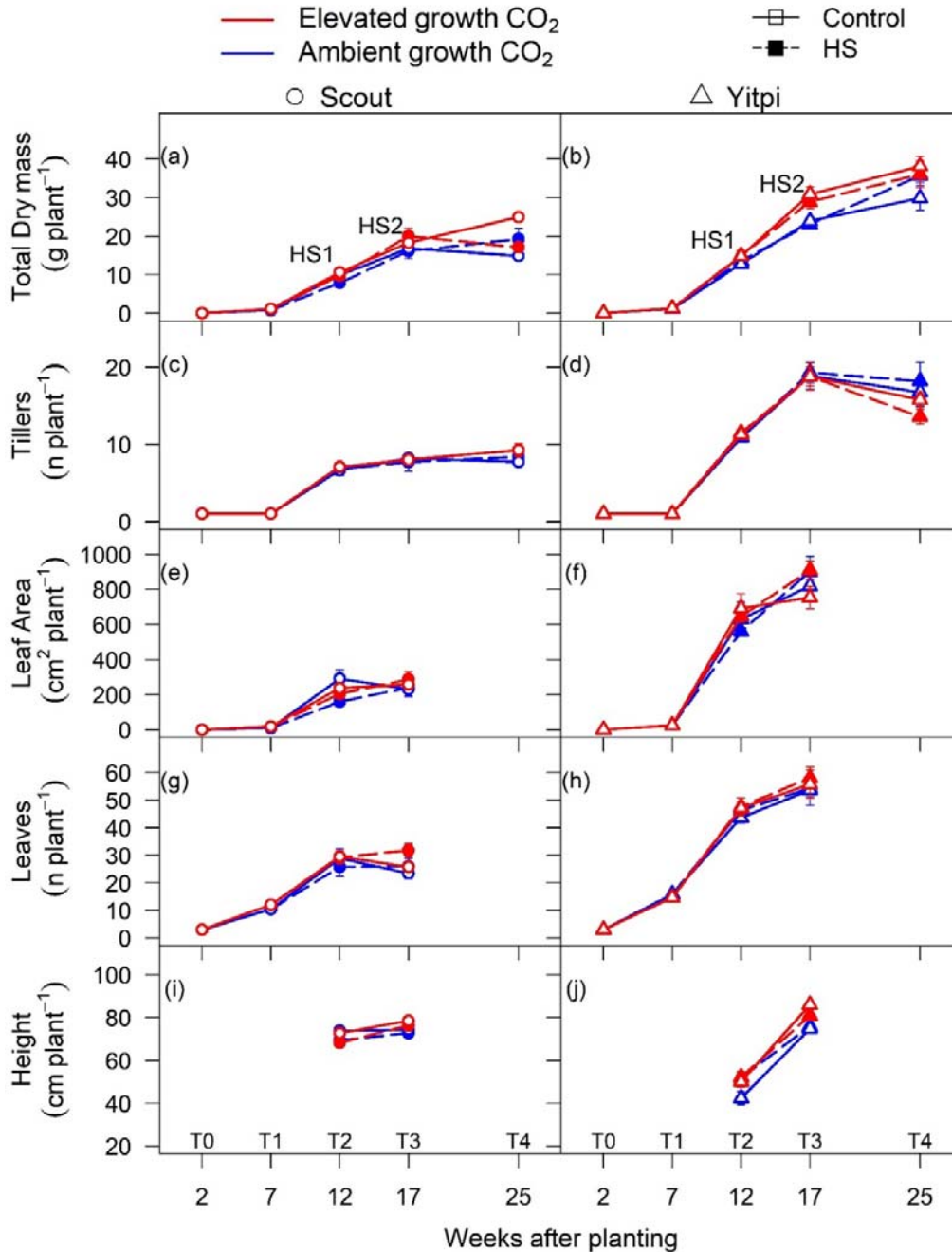


Figure 6 Response of plant growth and morphological traits to elevated CO₂ and HS: Total dry mass (a, b), tillers or number of tillers (c, d), leaf area (e, f), leaf number (g, h) and height (i, j) were measured at different time points across the life cycle of wheat cultivars Scout (Circles) and Yitpi (Triangles). Ambient and elevated CO₂ are depicted in blue and red color, respectively. Open symbols connected with solid cultivars and closed symbols connected with dashed cultivars represent control and HS plants, respectively. HS1 and HS2 depict the timing of HS applied at 10 and 15 weeks after planting respectively.

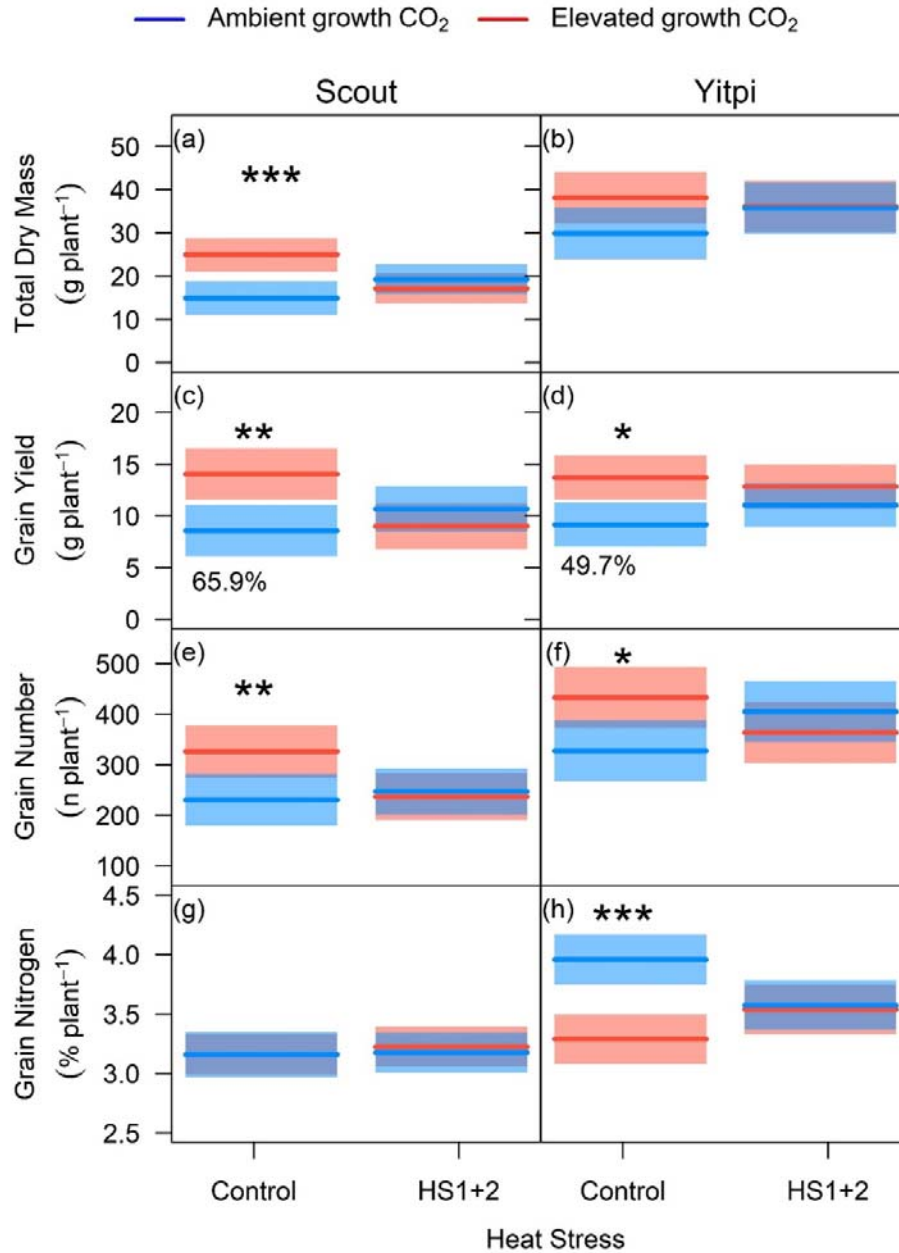


Figure 7 Response of total plant dry mass and grain parameters to growth at eCO₂ and HS at maturity (T4): Total dry mass (a, b), grain dry mass (c, d), grain number (e, f) and grain nitrogen (g, h) were measured at the final harvest. Cultivars indicate means and shaded region is 95% confidence interval. Ambient and elevated CO₂ are depicted in blue and red color respectively. Heat stress levels include plants not exposed to any heat stress (control) and both heat stresses (HS1+2). Statistical significance levels (t-test) for the growth condition within each cultivar is shown and they are: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.