

1 Original Article

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3 Elevated CO₂ causes large changes to morphology of perennial ryegrass (*Lolium perenne*)

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10 Running Head: Elevated CO₂, water supply and ryegrass morphology

11 Summary Text: Plant morphology is particularly important for agricultural species since
12 economically important traits are linked to simple features such as blade length and plant
13 height. We tested the combined effects of elevated CO₂ and water supply on morphological
14 traits of *Lolium perenne*. Size of leaves and individual tillers were dramatically smaller in
15 plants grown at elevated CO₂ concentrations, and this was independent of water supply.
16 This will have important implications for selective breeding and pasture productivity under
17 future conditions.

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20 **Abstract**

21 Plant morphology and architecture are essential characteristics for all plants but perhaps
22 most importantly for agricultural species since economic traits are linked to simple features
23 like blade length and plant height. It is likely that key morphological traits respond to CO₂
24 concentration ([CO₂]) and the degree of this response could be influenced by water
25 availability, but this has been comparatively little studied. This study aimed to determine
26 the impacts of [CO₂] on gross morphology of the most widespread temperate pasture
27 species, perennial ryegrass (*Lolium perenne*), and whether these impacts are influenced by
28 water availability. We grew perennial ryegrass in a well-fertilised Free-Air Carbon dioxide
29 Enrichment (FACE) experiment in southern Tasmania. Plants were exposed to one of three
30 CO₂ concentrations; ambient (~400 μmol mol⁻¹), 475 μmol mol⁻¹ and 550 μmol mol⁻¹ at
31 each of three watering treatments (adequate, limited and excess). We measured shoot dry
32 weight, height, leaf area, leaf blade separation, leaf size, relative water content and specific
33 leaf area. We also measured shoot density per unit area as a measure of tillering. Perennial
34 ryegrass morphology responded dramatically to elevated [CO₂], with plants being smaller,
35 with shorter leaf blade separation lengths and smaller leaves than in ambient plots. Elevated
36 [CO₂] increased tillering but did not substantially affect relative water content or specific
37 leaf area. Water supply did not affect any traits measured nor the response to elevated
38 [CO₂]. The impacts of elevated [CO₂] on the morphology of a globally-important forage
39 crop that we observed have profound implications for pasture productivity. The reduction
40 in plant and leaf size were consistent across a range of soil water availability, indicating
41 that they are likely to be uniform. Clearly, elucidating the mechanisms driving these
42 responses will be essential in improving predictability of these changes and potentially for
43 breeding varieties suited to future conditions.

44

45 Keywords: Elevated CO₂, FACE, climate change, perennial ryegrass, temperate pasture,
46 plant morphology.

47 **Introduction**

48 The effects of elevated CO₂ concentration ([CO₂]) on plant physiology have been
49 extensively studied. It is well known that as [CO₂] increases, carbon assimilation increases
50 and stomatal conductance to CO₂ decreases (Morison 1987; Ainsworth and Long 2005).
51 These responses mean that growth and biomass production is nearly always stimulated
52 under elevated [CO₂] (Leakey *et al.* 2009). There has been comparatively little research into
53 the effects of elevated [CO₂] on plant traits and morphology, but this is something worthy
54 of attention, as changes in morphology could affect the efficacy of crucial physiological
55 functions such as light interception and gas exchange. This would have important
56 implications for future crop production, as changes to these physiological functions would
57 influence productivity and water use. Furthermore, specific morphological traits that have
58 been intentionally selected in breeding programs to maximise the suitability of crops may
59 be altered by [CO₂], interfering with efforts to optimise production.

60 Natural variation in morphological traits have been shown to affect physiological functions.
61 Leaf size, leaf arrangement, internode length and branching angle have been shown to
62 affect carbon assimilation, as they affect the degree of self-shading and therefore the light
63 interception efficiency (Honda and Fisher 1978; Falster and Westoby 2003; Kern *et al.*
64 2004). For example, the degree of self-shading and therefore light interception and carbon
65 gain by a tropical pioneer species can largely be explained by petiole length and angle
66 (Yamada *et al.* 2000). Brites and Valladares (2005) showed that Mediterranean shrubs with
67 an opposite leaf arrangement could increase light interception efficiency by optimising
68 other morphological traits such as internode elongation and leaf elevation angle. When light
69 is limited to a plant, self-shading can be detrimental as light interception efficiency is
70 reduced, but when light is non-limiting, alterations to morphological traits which increase
71 self-shading can protect the plant from photoinhibition (Werner *et al.* 2001; Kern *et al.*

72 2004). This has been demonstrated in grasses, for example, Setter *et al.* (1997) found that
73 self-shading in rice due to lodging reduced canopy photosynthesis which had consequences
74 for grain yield. Several simulation studies on grasses have found that increased self-shading
75 due to steeper leaf angles can limit photo-oxidative damage (Ryel and Beyschlag 1995;
76 Valladares and Pugnaire 1999). Therefore, morphological traits influence self-shading,
77 which may have either positive or negative consequences for photosynthesis.

78 There is some evidence that exposure to elevated [CO₂] can alter important morphological
79 traits. For example, a meta-analysis of FACE experiments found that elevated [CO₂]
80 increases plant height by 14% and leaf number by 8% on average (Ainsworth and Long
81 2005). Total leaf area and leaf size are often larger under elevated CO₂ than in control
82 conditions (Pritchard *et al.* 1999; Ainsworth and Long 2005), while specific leaf area (the
83 ratio of leaf area to leaf dry mass; SLA) often decreases (Poorter and Navas 2003;
84 Ainsworth and Long 2005; Temme *et al.* 2015). Some studies on crop species have noted
85 longer internodes and altered branching patterns in response to elevated [CO₂] (Jitla *et al.*
86 1997; Slafer and Rawson 1997). For example, Jitla *et al.* (1997) found an increase in
87 internode length in rice of 25% under a doubling of [CO₂], and tillering is promoted by
88 elevated [CO₂] in several species including rice (Jitla *et al.* 1997), wheat (McMaster *et al.*
89 1999; Houshmandfar *et al.* 2016) and perennial ryegrass (Schapendonk *et al.* 1997).

90 CO₂-induced morphological changes have important consequences for physiological
91 processes. Increased leaf area under elevated [CO₂] increases the area available for both
92 light capture light capture and transpiration, therefore influencing photosynthesis and plant
93 water balance (Prior *et al.* 2011; Fatichi *et al.* 2016). Increased tillering under elevated
94 [CO₂] has been shown to influence reproduction in wheat as more fertile tillers are
95 produced (Tausz-Posch *et al.* 2015), and increased branching under elevated [CO₂]

96 increased sink strength and consequently promoted growth in a biofuel crop (Kumar *et al.*
97 2014). Thilakarathne *et al.* (2013) demonstrated that when specific leaf area (SLA) is
98 decreased by elevated [CO₂] in wheat, more nitrogen is able to be stored in leaf blades,
99 which may increase photosynthetic rate and slow leaf senescence. Conversely, a decrease in
100 SLA under elevated [CO₂] may reflect the accumulation of non-structural carbohydrates,
101 which may inhibit photosynthesis (Pritchard *et al.* 1999). Therefore, morphological changes
102 caused by elevated [CO₂] may influence physiological processes in a variety of ways.

103 Perennial ryegrass (*Lolium perenne*) is the most widely-grown forage crop in temperate
104 zones, and therefore has enormous agricultural significance (Wilkins 1991). It exhibits
105 strong clonal growth, with a basal stem consisting of usually short internodes with
106 continual growth at shoot apices forming a dense cluster of shoots. The continual decay of
107 portions of the basal stem and hence fragmentation of plants results in stable size
108 distributions within populations, and new shoots are also formed by elongation of
109 internodes to form stolons (Brock *et al.* 1996). Perennial ryegrass is fast growing, and has a
110 high specific leaf area and leaf water content (Tsialtas *et al.* 2004). Morphological traits
111 that are important for the success of this species in agriculture are those which allow
112 grazing intake to be maximised, namely leaf blade length (Barre *et al.* 2006), leaf mass
113 (Gautier *et al.* 1999), sward height and bulk density (Griffiths and Gordon 2003). Ryegrass
114 morphology is sensitive to changes in many environmental variables (Brock *et al.* 1996;
115 Erley *et al.* 2001; Cereti *et al.* 2004; Tozer *et al.* 2017). Therefore, assessing changes to
116 ryegrass morphology under elevated [CO₂] will be important for its efficacy as a pasture
117 species in the future.

118 Perennial ryegrass is responsive to changes in [CO₂], with many studies reporting changes
119 to physiological functions in response to elevated [CO₂] (Ainsworth *et al.* 2003; Ainsworth

120 and Long 2005). Research into elevated [CO₂]-induced changes in morphology has been
121 minimal, but glasshouse studies have shown a doubling in leaf size (Ferris *et al.* 1996), and
122 decrease in specific leaf area (Morison and Gifford 1984; Schapendonk *et al.* 1997) in
123 response to elevated [CO₂]. Most studies that have investigated plant morphological
124 responses to elevated [CO₂] have only exposed plants to two levels of [CO₂]. It is possible
125 that responses may be non-linear and therefore only testing two levels might lead to over-
126 estimates of plant responses to the rising [CO₂]. We aim to test the hypothesis that the
127 impact of elevated [CO₂] on the morphology of perennial ryegrass is dependent on water
128 availability in a Free Air Carbon dioxide Enrichment experimental system in Tasmania,
129 Australia. Here, we quantitatively describe morphological changes in detail, as a foundation
130 to investigate the mechanisms behind these changes and determine how these changes
131 might influence physiological processes.

132 **Materials and Methods**

133 *Field site*

134 All field measurements were taken at the TasFACE2 experimental site, which was
135 established at the University of Tasmania Farm in Cambridge, Tasmania (42°48'S,
136 147°25'E; 50 m a.s.l.) in September 2015. The site has a Mediterranean climate with a
137 mean annual temperature of 12.1°C and a mean annual rainfall of 598 mm (BOM 2017.
138 'Climate statistics for Australian locations' (Bureau of Meteorology: Canberra).). The soil
139 is strongly duplex, consisting of a grey-brown sandy loam to 30 cm on sandy medium to
140 heavy clay (Sinclair 2016).

141 The TasFACE2 experiment is a Free-Air CO₂ Enrichment (FACE) experiment in which
142 CO₂ concentration [CO₂] is manipulated by injection of pure CO₂ in 1.8 m diameter “rings”
143 in order to achieve three CO₂ levels – ambient (~400 μmol mol⁻¹), 475 μmol mol⁻¹ and 550

144 $\mu\text{mol mol}^{-1}$. These levels are consistent with other current elevated $[\text{CO}_2]$ studies (Kimball
145 2016; Pazzagli *et al.* 2016; Roy *et al.* 2016), and are levels likely to be reached in the next
146 50-100 years (IPCC 2001). The facility is a redeployment of the TasFACE experiment
147 (Hovenden 2006), with modification to provide three rather than two different CO_2 levels.
148 The $[\text{CO}_2]$ in the centre of each elevated ring is constantly monitored by an infra-red gas
149 analyser and the CO_2 supply rate adjusted by proportional valves controlled via a central
150 processing unit running a proportional integration algorithm (Hovenden 2006). The
151 experiment is a random block design with the three CO_2 treatments replicated four times,
152 giving a total of 12 rings.

153 Each of the 12 rings is divided into five 0.5 m^2 sectors, three of which were used in this
154 study. Each sector either received adequate (soil moisture maintained at field capacity),
155 limit (-40%) or excess (+20%) water, with water delivered to each sector twice-weekly by
156 five evenly spaced dripper heads connected to automated irrigation controllers. Irrigation
157 amounts were altered monthly and adjusted such that the sectors receiving adequate
158 moisture were returned to field capacity upon each watering event. During the winter, all
159 sectors received the same irrigation, being equivalent to the adequate treatment. The
160 segments were separated from each other and from surrounding soil by plastic sheeting
161 buried into the ground to a depth of approximately 1 m and extending 10 mm above ground
162 level. Each experimental ring was covered by 1.5 m high sloping rain exclusion shelters
163 made from UV-transparent polycarbonate sheets to ensure plants were only exposed to the
164 irrigation given by the experimental treatment, without limiting light exposure.

165 Soil volumetric water content (SWC) was logged continuously with underground time-
166 domain reflectometry (TDR) sensors (CS616, Campbell Scientific Australia Pty Ltd, QLD)
167 installed horizontally at a depth of 15 cm. Data were logged hourly on a CR1000 datalogger

168 (Campbell Scientific Australia Pty Ltd, QLD), and daily averages of the hourly values used
169 thereafter.

170 Vegetation at the site is a monoculture of perennial ryegrass (*Lolium perenne* L.) cultivar
171 Base AR37, which was sown at a density of 20 g seed m⁻² in June (winter) 2015 by direct
172 drilling. A combined nitrogen and phosphorus fertiliser (Nutriphos N:P:K 16:20:0,
173 Hellagrolip, Athens) was applied three weeks after sowing at a rate of 30 kg N ha⁻¹. At the
174 beginning of the first summer (December 2015) phosphorus was applied as superphosphate
175 at a rate of 1,150 kg ha⁻¹ followed by subsequent applications of 250 kg ha⁻¹ every six
176 months to prevent P-limitation (Gourley *et al.* 2007). Nitrogen was applied as urea
177 immediately following each harvest at a rate of 2.3 kg N ha⁻¹ day⁻¹, which is approximately
178 the application rate at which N-saturation of growth occurs in perennial ryegrass in
179 Tasmania under well-watered conditions (Rawnsley *et al.* 2014). These values were chosen
180 in order to supply sufficient N and P to reduce the likelihood of nutrient limitation of
181 growth.

182 Above-ground biomass was removed from the plots at the three-leaf stage to maintain
183 vegetative growth and to prevent flowering. Plants were clipped to a height of
184 approximately 30 mm using handheld grass shears. During the experimental period of this
185 study, a full year after sowing, aboveground biomass was harvested on 4th August 2016, 19
186 September 2016, 17 October 2016, 29 November 2016, 11 January 2017 and 20 February
187 2017.

188 *Morphological measurements*

189 One shoot from three plants from each sector in each ring were cut at ground level and
190 collected before each harvest in August, September, October and February. These plants

191 were immediately weighed, scanned on a flat-bed scanner (Canon iR-ADV C5250/5255
192 UFR II) and oven-dried at 60°C for several days before being weighed again. From the
193 scanned images, morphological measurements were taken using the program ImageJ
194 (National Institute of Health, MD), and various ratios were calculated from these
195 measurements (Table 1).

196 *Shoot density*

197 In order to determine shoot density, the number of shoots per sector in each plot was
198 estimated following the harvest in February. All green shoots were counted in four
199 randomly-placed, circular quadrats (area 87 cm² each) per sector in all 12 plots, and this
200 was extrapolated to give an estimate of total shoots per sector.

201 *Statistical analysis*

202 All statistical analyses were carried out in R version 1.1.383 (R Core Team 2014). All
203 measured variables were analysed by analysis of variance (ANOVA). Box-Cox plots and
204 diagnostic plots (Venebles and Ripley 2002) were used to check all data for
205 heteroscedasticity and normality, and data were transformed where necessary to avoid
206 violation of the assumptions of the ANOVA model used. Where significant treatment
207 effects were detected, means were compared using Tukey's HSD *post hoc* comparisons.

208 Shoot density counts were analysed via a two-way ANOVA with CO₂ and watering
209 treatments as fixed factors. Morphology data were analysed by repeated measures ANOVA
210 with CO₂ and watering treatments as fixed factors and sample date the repeated measure.

211 Where there were significant treatment × date interactions, treatment effects were analysed
212 separately for each date using a two factor ANOVA with CO₂ and watering treatment as
213 fixed factors. SWC data from each sector were averaged over each calendar month and then

214 analysed by repeated measures ANOVA, with watering treatment as a fixed factor and
215 month the repeated measure. Where there were significant treatment \times date interactions,
216 treatment effects were analysed separately for each date using a one way ANOVA with
217 watering treatment as a fixed factor.

218

219 **Results**

220 There were very distinct morphological differences between the plants grown under the
221 elevated [CO₂] treatments and those grown in the control plots (Figs. 1 and 2; Tables 2 and
222 3). In general, elevated [CO₂] caused a substantial reduction in plant size and a change from
223 a tall, slender, unbranched habit to one dense, branched and sprawling (Fig. 1). For all
224 variables that were significantly impacted by [CO₂], there was a large difference between
225 the control and elevated [CO₂] plots but little difference between the two different elevated
226 [CO₂] treatments (Fig. 2, Tables 2 and 3). In other words, increasing the [CO₂] from 400 to
227 475 $\mu\text{mol mol}^{-1}$ had a substantial impact on plant morphology but increasing [CO₂] further
228 to 550 $\mu\text{mol mol}^{-1}$ had no additional influence (Figs 1 and 2). The magnitude of the effect
229 of CO₂ treatment varied by month for most traits but overall the CO₂ effect on perennial
230 ryegrass morphology was consistent over time (Fig. 2, Tables 2 and 3). All of the
231 morphological traits showed no relationship with watering treatment (Table 3). the effect of
232 watering treatment on total leaf blade separation, leaf width and the average leaf blade
233 separation to height ratio were each depended upon the [CO₂], such that the relationship
234 between watering and these traits was different at each [CO₂] (Table 3).

235 *Plant size*

236 The mass of each individual shoot was significantly reduced by elevated [CO₂] whether
237 expressed as total fresh or dry weight; an effect that was consistent across all four sample
238 times (Table 3, Fig. 2A, tables 2 and 3). Plants growing in 475 and 550 $\mu\text{mol mol}^{-1}$ plots
239 were only 54% and 59% as large by dry weight, respectively, as those growing in plots at
240 400 $\mu\text{mol mol}^{-1}$ (Fig. 2A). The magnitude of the effect of elevated [CO₂] on shoot dry
241 weight was consistent over the four sampling periods, whereas the effect on fresh weight
242 varied by month but remained significant in each (Table 3). Elevated CO₂ (550 $\mu\text{mol mol}^{-1}$)

243 decreased shoot dry weight by 30%, 47%, 45% and 36% in August, September, October
244 and February respectively. Leaf area per shoot was also lower in plants growing in elevated
245 [CO₂] plots, being 41% lower in the 475 μmol mol⁻¹ plots than in the control plots and 35%
246 lower in the 550 μmol mol⁻¹ plots than in the control plots (Tables 2 and 3). Again, the
247 magnitude of this effect varied by month, but the effect was significant in each (Table 3).
248 Leaf area was reduced by 29% in August, 39% in September and February and 38% in
249 October by elevated [CO₂] (550 μmol mol⁻¹).

250 *Plant height*

251 Plants grew 52% taller in the ambient plots than in the 475 μmol mol⁻¹ plots, and 55% taller
252 than in 550 μmol mol⁻¹ plots. This effect varied by month, with 550 μmol mol⁻¹ CO₂
253 reducing plant height by 20% in August, 36% in September, 42% in October and 43% in
254 February (Table 2, Fig. 2B). Both average and total leaf blade separation were also greater
255 in control plots than elevated [CO₂] plots, with average leaf blade separation being 2.01 and
256 2.15 times as long in plants from control plots as in those from 475 and 550 μmol mol⁻¹
257 plots, respectively (Fig. 2C, Tables 2 and 3). Thus, leaf blade separation in control plants
258 was at least double that of plants grown at elevated [CO₂]. The effect of elevated [CO₂] on
259 both these traits varied by month but was always significant (Table 3). All impacts of
260 elevated [CO₂] on shoot size were unaffected by the watering treatment, indicating that the
261 elevated [CO₂] effect was independent of water supply (Table 3).

262 The ratio of leaf blade separation to plant height, which is a measurement of leaf crowding
263 relative to plant height, was reduced by elevated [CO₂] (Fig. 2E, Table 3). Thus for plants
264 of a given height, average leaf blade separation was longer in the plants grown in the
265 ambient plots than both of the elevated [CO₂] treatments, meaning that the leaves on these
266 plants were more spread out along the culm. The magnitude of this effect varied with time

267 but was significant in all months (Table 3). There was no difference in the ratio between
268 plant height and the length of the longest leaf between the three CO₂ treatments. Similarly,
269 there was no effect of watering treatment or a CO₂ x water interaction on this ratio.

270 *Leaf size*

271 Individual leaves were also significantly larger by weight and leaf area in the control plots
272 than in the elevated [CO₂] plots (Fig. 2D, Tables 2 and 3). The effect of elevated [CO₂] on
273 leaf area per leaf varied by month, but the effect on leaf weight was consistent over time
274 (Tables 2 and 3). The reduction in leaf area per leaf under elevated [CO₂] (550 μmol mol⁻¹)
275 varied from 29% in August to 42%, 43% and 40% in September, October and February,
276 respectively, but elevated [CO₂] reduced individual leaf area substantially in all months.
277 The average length of the longest leaf was 45% and 44% longer in control plots than in 475
278 μmol mol⁻¹ and 550 μmol mol⁻¹ plots respectively, and this effect was consistent over time
279 (Tables 2 and 3). The width of the widest leaf on each shoot was also reduced by elevated
280 [CO₂] (Tables 2 and 3), with leaves being 13% narrower on plants grown at 475 μmol mol⁻¹
281 and 550 μmol mol⁻¹ than on plants grown at 440 μmol mol⁻¹. Interestingly, the number of
282 leaves on each shoot was consistent across CO₂ treatments. At 400 μmol mol⁻¹ shoots had
283 3.53±1.32 leaves, while plants growing at 475 μmol mol⁻¹ had 3.45±0.97 leaves and plants
284 growing at 550 μmol mol⁻¹ had 3.58±0.98 leaves. Similarly, leaf number was not affected
285 by watering treatment or by a CO₂ x water interaction.

286 *Relative water content and specific leaf area*

287 Elevating CO₂ decreased relative water content (RWC) very slightly but significantly, with
288 plants in 475 and 550 μmol mol⁻¹ plots having on average 1.8% and 1.3% lower water
289 content, respectively, than plants from control plots (Fig. 2F). This difference was

290 significant in all months apart from September (Table 3), but was so slight as to be
291 negligible. There was no effect of CO₂ treatment on specific leaf area. Similarly, SLA was
292 not affected by watering treatment or a CO₂ x water interaction.

293 *Shoot density*

294 Both CO₂ and watering treatment affected the number of shoots per sector (CO₂ $F_{2,27} =$
295 8.66, $P < 0.002$; water $F_{2,27} = 18.97$, $P < 0.001$; Fig. 3) but there was no significant
296 interaction between the CO₂ and watering treatments. There were 22% more shoots in the
297 475 and 550 $\mu\text{mol mol}^{-1}$ plots than in the ambient control plots, but there was no significant
298 difference between the two elevated [CO₂] treatments. There were 26% fewer shoots per
299 sector in the limited water treatments than the control, but no significant difference between
300 the control and excess treatments.

301 **Discussion**

302 The promotion of plant growth by elevated [CO₂] has been widely documented and is well
303 understood, but comparatively little focus has been given to the growth responses of
304 individual plants to elevated [CO₂] under field conditions, particularly in grasslands where
305 plant size can be important for various community and ecosystem functions. The results
306 presented here show that the morphology of a commercial variety of the widely-used
307 temperate pasture grass *Lolium perenne* is sensitive to relatively small changes in [CO₂],
308 with ramifications for future pasture productivity.

309 *Plant size*

310 Generally, plant size increases under elevated [CO₂], with a meta-analysis finding that a
311 doubling of [CO₂] leads to an increase in individual plant biomass by an average of 54% in

312 herbaceous crop species (Poorter 1993). Similarly, elevated [CO₂] increases leaf area per
313 plant of crop species by an average of 37% (Pritchard *et al.* 1999). However, we found that
314 elevated [CO₂] reduced the overall size of each shoot of perennial ryegrass whether
315 measured in terms of fresh weight, dry weight, total leaf area or height (Table 2). Under
316 elevated [CO₂], plant growth is usually promoted because of a direct stimulation of
317 photosynthesis, and the subsequent water saving that occurs as stomatal aperture is reduced
318 at higher [CO₂] (Ainsworth and Long 2005), however this is clearly not occurring in this
319 case. Further, a study on perennial ryegrass by Ferris *et al.* (1996) found that elevated
320 [CO₂] increased shoot dry weight in spring, but decreased it in summer, suggesting that
321 elevated [CO₂] effects on plant growth can vary seasonally. However, this was not
322 supported by the results in this study as elevated [CO₂] affected plant size similarly in
323 winter, spring and summer. Clearly, the response of the Base AR37 cultivar of perennial
324 ryegrass is responding in an unusual manner to elevated [CO₂] in field conditions.

325 *Plant height*

326 Plant height is an important trait for forage crops, as it is a determinant of forage intake by
327 grazers. Changes to plant height and stem elongation under elevated [CO₂] also have the
328 potential to bring about secondary physiological changes, as the distance between leaves
329 influences light interception patterns and therefore photosynthesis (Brites and Valladares
330 2005). Usually, shoot height and leaf blade separation increase under elevated [CO₂]; a
331 meta-analysis of crop plants grown in FACE experiments found plant height to increase by
332 14% under elevated [CO₂] (Ainsworth and Long 2005), and studies have found an increase
333 in shoot elongation under elevated [CO₂], including in wheat (Slafer and Rawson 1997) and
334 rice (Jitla *et al.* 1997). In stark contrast, we found plant height and leaf blade separation
335 lengths to decrease by at least half under elevated [CO₂] (Fig. 2B). Interestingly, we also

336 found that the ratio of leaf blade separation length to shoot height was reduced by eCO₂
337 (fig. 2E), meaning that for a plant of a given height, distances between leaves were shorter,
338 and therefore leaves were closer together along the culm in plants grown at eCO₂ compared
339 to those grown in control plots. Natural variation in shoot elongation and leaf placement
340 have been shown to affect light exposure and therefore carbon assimilation (Kern *et al.*
341 2004), so it is possible that self-shading may be increased by the close placement of leaves,
342 as indicated by the small leaf blade separation length to height ratio observed at eCO₂. It
343 would be important to investigate light levels through the canopy and determine the rate at
344 which light increases from ground level to the top of the canopy, in order to understand
345 whether self-shading is occurring to a greater extent under eCO₂. Also, it would be
346 interesting to study the nature of leaf angles in relation to the culm, whether this trait
347 responds to [CO₂] and how this relates to light interception.

348 *Leaf size*

349 Leaf size is an important morphological trait since it is influential for resource capture,
350 water-use efficiency and competitive ability (Gautier *et al.* 1999; Mencuccini and
351 Comstock 1999; Falster and Westoby 2003). Blade length is a particularly important
352 characteristic for this species, as blade length is correlated with intake by grazing animals
353 (Barre *et al.* 2006). Leaf expansion can be driven by increased cell elongation (Ferris and
354 Taylor 1994; Pritchard *et al.* 1999), which is related to turgor pressure and hence water
355 relations. It may also be driven by increased cell division, which is related to resource
356 availability (Domec *et al.* 2017). Generally, elevated [CO₂] is seen to increase leaf size
357 (Ferris *et al.* 1996; Pritchard *et al.* 1999; Lin *et al.* 2015), but here we found that leaves
358 were smaller under elevated [CO₂] in terms of dry weight, leaf area, blade length and leaf
359 width (Tables 2 and 3). Tsutsumi *et al.* (2014) also found a decrease in blade length and

360 width under elevated [CO₂] in rice plants, and they attributed this to the suppression of cell
361 division on the adaxial side of the leaf, and suppression of cell elongation on the abaxial
362 side. Normally, elevated [CO₂] causes an increase in water content, which could lead to
363 increased cell elongation and therefore larger leaves, but we found no substantial change in
364 water content, making it unlikely that cell elongation was stimulated by elevated [CO₂].
365 Hence, it is unlikely that a CO₂-related change in cell turgor explains the decrease in leaf
366 size. Similarly, Samarakoon and Gifford (1995) found no change in leaf size under elevated
367 [CO₂] in wheat and attributed this to the complete saturation of tillering potential. Some
368 studies have shown that the impact of elevated [CO₂] on leaf size decreases over time, with
369 early leaf expansion stimulated but the difference eventually becomes insignificant
370 (Pritchard *et al.* 1999). We found that traits related to leaf size were generally consistent
371 across the growing season, but since this effect might change in subsequent growing
372 seasons.

373 *Relative water content*

374 Relative Water Content (RWC) is a good indicator of plant hydration status and turgor
375 (Jones 2007). Usually, RWC increases under elevated [CO₂] (Marchi *et al.* 2004; Robredo
376 *et al.* 2007) because of decreased stomatal conductance and the subsequent reduction in soil
377 drying. Conversely, we found a decrease in RWC under elevated [CO₂] (Fig. 2F, Table 3),
378 but this change was so small as to be negligible. We also found no difference in stomatal
379 conductance (data not shown), which supports this lack of change in RWC. We found no
380 change in RWC between the different watering treatments, which was surprising since
381 RWC is normally affected by irrigation amount and hence water availability in the soil
382 (Ainsworth and Long 2005; Omae *et al.* 2007). Similarly, the watering treatments did not
383 affect any other of the morphological traits tested. Plants were exposed to various soil water

384 availabilities as given by the watering treatments [supplementary information Table S1],
385 but these differences did not translate into differences in plant water status, which is
386 perhaps what would drive any morphological changes rather than water availability in the
387 soil.

388 *Specific leaf area*

389 Plant structure and morphology may influence physiology through altering carbon storage
390 capacity or sink strength. Plants with greater sink strength respond more in terms of
391 biomass (Poorter 1993), and photosynthetic acclimation to elevated [CO₂] occurs more
392 when sink capacity is low (Ainsworth and Long 2005). Starch accumulation may inhibit
393 photosynthesis by altering the integrity of chloroplasts, by soluble sugars acting on gene
394 expression, or cause reductions in thylakoid stacking (Pritchard *et al.* 1999). Elevated CO₂
395 has been shown to decrease specific leaf area (SLA) in many species (Poorter and Navas
396 2003; Ainsworth and Long 2005) including perennial ryegrass (Casella *et al.* 1996;
397 Schapendonk *et al.* 1997; Ainsworth and Long 2005). This usual decrease in SLA under
398 elevated [CO₂] has been attributed to the accumulation of leaf total non-structural
399 carbohydrates that occurs when total carbon assimilation exceeds total carbon utilisation
400 (Pritchard *et al.* 1999). We found no difference in SLA between [CO₂] treatments. This
401 perennial ryegrass cultivar has been selectively bred for high growth rates, so it is plausible
402 that the lack of response in SLA may be attributed to a relative insensitivity to non-
403 structural carbohydrate accumulation.

404 *Shoot density*

405 Perennial ryegrass has a high capacity for tillering (Casler 2001). Tillering is important for
406 the rapid development of leaf area which increases resource capture (Schapendonk *et al.*

407 1997). Generally, elevated [CO₂] promotes tillering in grasses (Jitla *et al.* 1997;
408 Schapendonk *et al.* 1997; McMaster *et al.* 1999). As an equal amount of seed was sown
409 into each plot, shoot density can be used as a measure of the extent to which each plant
410 produces more tillers, or the degree of tillering. We found elevated [CO₂] to decrease shoot
411 size, increase shoot density (Fig. 3), but not change the number of leaves per shoot.
412 Therefore, since the plants at the different CO₂ concentrations were producing a similar
413 number of leaves per shoot per month, each plant grown at elevated [CO₂] was producing
414 more leaves but each leaf was smaller. Since we found elevated [CO₂] to stimulate
415 branching (Fig. 3), the smaller leaf size may be explained by the inverse relationship
416 between leaf size and leaf density that has been observed in grasses (Kemp and Culvenor
417 1994). Schapendonk *et al.* (1997) also found elevated [CO₂] to promote tillering in
418 perennial ryegrass, with 25% more shoots under doubled [CO₂]. However, this was
419 attributed to the strong correlation between biomass and tillering, which we did not
420 observe. In another study on perennial ryegrass, Daepf *et al.* (2001) found shoot number to
421 increase at elevated [CO₂] along with a small decrease in shoot dry mass, as was similar
422 with our results, but this only occurred under nitrogen-limited conditions.

423 *Conclusion*

424 The effects of elevated [CO₂] on plant morphology have received little attention in
425 comparison to physiological impacts. This study has shown that elevated [CO₂] can have
426 profound impacts on plant morphology. We found dramatic changes to individual plant
427 size, culm elongation and leaf characteristics. These changes to morphology were
428 consistent across a wide range of water availabilities. These changes were also relatively
429 consistent across the year, suggesting the impacts were largely independent of temperature.
430 The magnitude of the observed changes were large and they were in the opposite direction

431 to what is to be expected based on past studies. Perennial ryegrass Base AR37 is a highly
432 derived cultivar, so its response to elevated [CO₂] may be different from the natural
433 responses of plants that have not been subject so such intensive artificial selection. While
434 we have presented a detailed analysis of these changes, we have not been able to elucidate
435 the mechanisms driving these changes, which is something that warrants further study.
436 These changes may have been caused by elevated [CO₂] directly, or indirectly through
437 changes to other processes (e.g. nutrient relations; Drake *et al.* 1997; Ainsworth and Long
438 2005). Being such an important forage crop globally, it is essential that any changes to
439 perennial ryegrass morphology under future atmospheric conditions are well understood
440 and predictable, as their economic value has the potential to be altered both in terms of
441 changes to traits essential for grazing suitability, and secondary effects of these changes on
442 primary production.

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451 **Conflicts of Interest**

452 The authors declare no conflicts of interest.

453 Figure captions

454 **Fig. 1.** Growth habit and morphology of the perennial ryegrass plants (*Lolium perenne* cv.
455 Base AR37) grown at 400 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (A and C), 475 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (D) and 550
456 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (B and E). Growth habit of 475 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ closely resembled that of
457 the 550 $\mu\text{mol CO}_2 \text{ mol}^{-1}$. All images were taken on the same day, so differences are due to
458 $[\text{CO}_2]$ and not time since cutting.

459 **Fig. 2.** (A) Dry weight of plants grown at each CO_2 level across the four sample dates. (B)
460 Height of plants grown at each CO_2 level across the four sample dates. (C) Average leaf
461 blade separation of plants grown at each CO_2 level across the four sample dates. (D) Leaf
462 size of plants grown at each CO_2 level across the four sample dates. (E) Average leaf blade
463 separation to plant height ratio of plants grown at each CO_2 level across the four sample
464 dates (F) Relative Water Content (RWC) of plants grown at each CO_2 level across the four
465 sample dates. Error bars represent standard errors.

466 **Fig. 3.** Estimates of the number of shoots per sector in perennial ryegrass grown at three
467 CO_2 levels and three watering treatments. Lower case letters represent significant
468 differences between watering treatments within each CO_2 treatment level, and capital letters
469 represent significant differences between the CO_2 treatment levels.

470 **Table 1** Details of the morphological measurements and calculated ratios used in the study.

| Measurement/Ratio | Description |
|------------------------------------|---|
| Leaf area (mm ²) | Leaf area of whole shoot |
| Total leaf blade separation (mm) | Leaf blade separation was defined as the distance between the collars of two adjacent leaves. Total leaf blade separation was the sum of these distances from the collar of the oldest leaf to the collar of the newest leaf. This measurement was chosen over internode length as it gives a better estimation of self-shading, and is possible to measure in vegetative shoots. |
| Average leaf blade separation (mm) | Total leaf blade separation divided by (number of leaves -1). |
| Leaf weight (g) | Dry weight divided by the total number of leaves |
| Leaf size (mm ²) | Total leaf area divided by the number of leaves |

| | |
|---|---|
| Length of longest leaf (mm) | Measured from the collar to the tip. Newest leaf not counted as it was used in height measurement |
| Leaf width (mm) | Width at widest point on widest leaf blade |
| Average leaf blade separation : height | Average leaf blade separation / shoot height |
| Blade length : height | Length of longest leaf / shoot height |
| Relative water content (%) | Fresh weight - dry weight / fresh weight |
| Specific leaf area (mm ² /g) | Leaf area / dry weight |

471

472 **Table 2.** Means of morphological traits measured. Means for each month are given only if
 473 there was a significant CO₂ x month interaction. Superscript letters indicate which
 474 treatment means were significantly different (P=0.05).

| Trait | Month | Mean | | |
|---------------------------------------|-----------|------------------------------|------------------------------|------------------------------|
| | | 400 $\mu\text{mol mol}^{-1}$ | 475 $\mu\text{mol mol}^{-1}$ | 550 $\mu\text{mol mol}^{-1}$ |
| Mean leaf weight (g) | - | 0.029 ^a | 0.017 ^b | 0.017 ^b |
| Length of longest leaf (mm) | - | 223.17 ^a | 153.71 ^b | 154.55 ^b |
| Leaf width (mm) | - | 3.44 ^a | 2.98 ^b | 2.99 ^b |
| Fresh weight (g) | August | 0.77 ^a | 0.38 ^c | 0.53 ^b |
| | September | 0.66 ^a | 0.38 ^b | 0.35 ^b |
| | October | 0.56 ^a | 0.26 ^b | 0.29 ^b |
| | February | 0.4 ^a | 0.25 ^b | 0.23 ^b |
| Total leaf area (mm ²) | August | 2356 ^a | 1215 ^c | 1673 ^b |
| | September | 1647 ^a | 1067 ^b | 1005 ^b |
| | October | 1379 ^a | 724 ^b | 853 ^b |
| | February | 1189 ^a | 882 ^b | 727 ^b |

| | | | | |
|----------------------------------|-----------|--------------------|--------------------|--------------------|
| Total leaf blade separation (mm) | August | 27.81 ^a | 14.74 ^b | 16.77 ^b |
| | September | 48.86 ^a | 24.99 ^b | 23.25 ^b |
| | October | 28.21 ^a | 9.41 ^c | 16.65 ^b |
| | February | 19.67 ^a | 15.19 ^a | 9.13 ^b |

475

476 **Table 3.** Summary statistics of traits measured, with *P* and *F* values from ANOVA.
 477 Statistics for each month are given only if there was a significant month interaction. *P*
 478 values in bold are significant, with the standard significance level of <0.05 used.

| Trait | Month | CO ₂ | | Water | | CO ₂ *Water | |
|------------------------------------|-----------|-----------------|--------|-------|------|------------------------|------|
| | | F | P | F | P | F | P |
| Shoot dry weight (g) | - | 77.63 | <0.001 | 0.35 | 0.71 | 0.99 | 0.43 |
| Mean leaf weight (g) | - | 81.17 | <0.001 | 0.53 | 0.6 | 1.06 | 0.4 |
| Length of longest leaf (mm) | - | 58.86 | <0.001 | 0.07 | 0.93 | 0.75 | 0.56 |
| Fresh weight (g) | August | 22.18 | <0.001 | 0.75 | 0.49 | 1.27 | 0.3 |
| | September | 27.44 | <0.001 | | | | |
| | October | 30.07 | <0.001 | | | | |
| | February | 16.8 | <0.001 | | | | |
| Total leaf area (mm ²) | August | 22.84 | <0.001 | 0.37 | 0.69 | 1.24 | 0.32 |
| | September | 23.98 | <0.001 | | | | |
| | October | 30.13 | <0.001 | | | | |

| | | | | | | | |
|------------------------------------|-----------|-------|--------|------|------|------|------|
| | February | 15.71 | <0.001 | | | | |
| Height (mm) | August | 8.628 | <0.001 | 1.81 | 0.18 | 0.74 | 0.57 |
| | September | 21.63 | <0.001 | | | | |
| | October | 34.91 | <0.001 | | | | |
| | February | 12.99 | <0.001 | | | | |
| Average leaf blade separation (mm) | August | 29.62 | <0.001 | 2.92 | 0.07 | 2 | 0.12 |
| | September | 38.52 | <0.001 | | | | |
| | October | 18.43 | <0.001 | | | | |
| | February | 19.98 | <0.001 | | | | |
| Total leaf blade separation (mm) | August | 14.92 | <0.001 | 2.98 | 0.07 | 2.94 | 0.04 |
| | September | 22 | <0.001 | | | | |
| | October | 15.9 | <0.001 | | | | |
| | February | 14.55 | <0.001 | | | | |
| Leaf size (mm ²) | August | 14.92 | <0.001 | 0.7 | 0.5 | 0.89 | 0.48 |
| | September | 31.96 | <0.001 | | | | |
| | October | 29.99 | <0.001 | | | | |
| | February | 15.92 | <0.001 | | | | |

| | | | | | | | |
|---------------------------------------|-----------|-------|--------|------|------|------|-------|
| Leaf width | August | 29.19 | <0.001 | 0.61 | 0.55 | 2.91 | 0.03 |
| | September | | | | | 3.32 | 0.01 |
| | October | | | | | 1.9 | 0.03 |
| | February | | | | | 0.5 | 0.7 |
| Average leaf blade separation:h eight | August | 26.06 | <0.001 | 2.27 | 0.12 | 4.95 | 0.004 |
| | September | 35.48 | <0.001 | | | | |
| | October | 22.19 | <0.001 | | | | |
| | February | 14.44 | <0.001 | | | | |
| RWC (%) | September | 35.48 | <0.001 | 0.5 | 0.61 | 0.5 | 0.7 |
| | October | 22.19 | <0.001 | | | | |
| | February | 14.44 | <0.001 | | | | |
| | February | 7.87 | <0.001 | | | | |

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