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11 **Can additional N fertiliser ameliorate the elevated CO<sub>2</sub>-induced depression in**  
12 **grain and tissue N concentrations of wheat on a high soil N background?**

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36 Contains 1 Table and 3 Figures

37 **Abstract**

38 Elevated CO<sub>2</sub> stimulates crop yields but leads to lower tissue and grain nitrogen  
39 concentrations [N], raising concerns about grain quality in cereals. To test whether  
40 N fertiliser application above optimum growth requirements can alleviate the  
41 decline in tissue [N], wheat was grown in a Free Air CO<sub>2</sub> Enrichment facility in a low  
42 rainfall cropping system on high soil N. Crops were grown with and without  
43 addition of 50-60 kg N ha<sup>-1</sup> in 12 growing environments created by supplemental  
44 irrigation and two sowing dates over three years.

45 Elevated CO<sub>2</sub> increased yield and biomass (on average by 25%), and decreased  
46 biomass [N] (3-9%) and grain [N] (5%). Nitrogen uptake was greater (20%) in  
47 elevated CO<sub>2</sub> grown crops. Additional N supply had no effect on yield and biomass,  
48 confirming high soil N. Small increases in [N] with N addition were insufficient to  
49 offset declines in grain [N] under elevated CO<sub>2</sub>. Instead, N application increased the  
50 [N] in straw and decreased N harvest index.

51 The results suggest that conventional addition of N does not mitigate grain [N]  
52 depression under elevated CO<sub>2</sub>, and lend support to hypotheses that link decreases  
53 in crop [N] with biochemical limitations rather than N supply.

54

55 **Introduction**

56 Atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) has been increasing since the industrial  
57 revolution and is predicted to reach 550 μL L<sup>-1</sup> or more by 2050, that is a 35%  
58 change from the current (400 μL L<sup>-1</sup> in 2016) concentration (Stocker et al. 2013).  
59 Because CO<sub>2</sub> is the main substrate for photosynthesis, such a large increase will  
60 affect all plants and ecosystems (Ziska 2008). Many studies demonstrated that, at  
61 least in C3 plants, elevated [CO<sub>2</sub>] (e[CO<sub>2</sub>]) stimulates photosynthesis and  
62 subsequently growth and yield (Kimball et al. 2002, Ainsworth and Long 2005)  
63 through the so-called 'CO<sub>2</sub> fertilisation effect'.

64 While the 'CO<sub>2</sub> fertilisation effect' may result in greater crop yields and help offset  
65 some of the negative effects of climate change on food production (Hatfield et al.  
66 2011), concerns have been raised about reductions in mineral nutrients and grain  
67 quality (Högy and Fangmeier 2008, Myers et al. 2014). It is well established that  
68 growth under e[CO<sub>2</sub>] changes the stoichiometry of plants, whereby the  
69 concentration of many minerals, especially nitrogen (N), in plant tissues decreases  
70 (Loladze 2002). Because photosynthetic N use efficiency (the photosynthetic carbon  
71 fixation rate per g leaf N) increases under e[CO<sub>2</sub>], the critical tissue N  
72 concentration, i. e. the leaf N concentration ([N]) that is necessary for optimum  
73 growth, consequently decreases under e[CO<sub>2</sub>] (Conroy and Hocking 1993,  
74 Seneweera and Norton 2011, Tausz-Posch et al. 2014). Despite lower tissue [N], the  
75 greater biomass reported under e[CO<sub>2</sub>] may contain more N per ground surface  
76 area than biomass under ambient [CO<sub>2</sub>], hence N uptake of the crop may be  
77 greater (Tausz-Posch et al. 2014, Lam et al. 2012b). In natural ecosystems, where N  
78 is often limiting, the CO<sub>2</sub>-stimulation on growth often decreases over time because  
79 available N in the soil becomes depleted, a phenomenon termed 'progressive N  
80 limitation' (Oren et al. 2001, Luo et al. 2004). In N-managed agro-ecosystems  
81 progressive N limitation may not be relevant or immediately apparent, but some  
82 experiments have shown that growth stimulation by e[CO<sub>2</sub>] is less under low than  
83 high N supply (Stitt and Krapp 1999).

84 For non-legume food and fodder crops, decreases in tissue [N] are particularly  
85 concerning because they translate to lower protein concentrations, thus lowering  
86 food and feed quality as shown in wheat (Högy et al. 2013; Wroblewitz et al. 2013).  
87 Grain protein concentration is also an important determinant of baking quality and  
88 market value of wheat. Dough and baking quality of wheat was shown to  
89 deteriorate under e[CO<sub>2</sub>] (Panozzo et al. 2014). Synthesis papers report around 5-  
90 10% reduction in grain protein concentration in wheat (Högy and Fangmeier 2008;  
91 Lam et al. 2012b, Wang et al. 2013), and about 10% for leaf [N] (Ainsworth and  
92 Long 2005; Wang et al. 2013).

93 The exact mechanism for the decrease in [N] is unclear, and a number of not  
94 mutually exclusive hypotheses have been proposed (Tausz-Posch et al. 2014). The  
95 most straightforward one contends that soil N supply does not keep up with  
96 increased demand by e[CO<sub>2</sub>]-stimulated biomass growth, leading to a 'dilution' of N  
97 in tissue biomass and N-limitation to biochemistry and growth (Taub and Wang  
98 2008). Evidence for this hypothesis comes from experiments where leaf [N]  
99 decreased upon e[CO<sub>2</sub>] exposure at low, but not at high soil N supply (Stitt and  
100 Krapp 1999, Sinclair et al. 2000). If limited N availability leads to decreases in tissue  
101 [N], it could be hypothesised that reductions in biomass tissue [N] in managed agro-  
102 ecosystems could be reversed by additional soil N inputs.

103 Alternative hypotheses for the decline in tissue [N] under e[CO<sub>2</sub>], such as nutrient  
104 uptake limitations by reduced transpiration flow (Conroy and Hocking 1993,  
105 McGrath and Lobell 2013), or decreased rates of nitrate reduction (Bloom et al.  
106 2010; Bloom et al. 2014), do not suggest that an increase in soil N supply would  
107 restore tissue [N]. For example, in one study conducted in a high yielding, irrigated  
108 wheat cropping system under ample N supply, the deleterious effect of e[CO<sub>2</sub>] on  
109 grain protein concentration was partially alleviated but was still present, even if  
110 very small (Kimball et al. 2001). This would imply that insufficient soil N supply is  
111 not the only mechanism contributing to decreased [N]. Recent meta-analyses  
112 suggested that high soil N supply cannot fully restore, but at best only moderates  
113 the negative effect of e[CO<sub>2</sub>] on tissue and grain [N] (Lam et al 2012b, Wang et al.

114 2013). It is not clear under what conditions, and to which extent, additional N  
115 application can restore leaf and grain [N] (and protein) under e[CO<sub>2</sub>].

116 In cereals such as wheat, N requirement for growth and grain yield is generally  
117 satisfied before that of increased grain protein, so that grain protein concentration  
118 can be increased by N application above the level needed for growth and yield  
119 responses (Fowler 2003; Hooper et al. 2015). To understand whether N supply  
120 beyond the demand for growth and yield can restore grain protein under e[CO<sub>2</sub>] to  
121 that achieved under ambient CO<sub>2</sub>, it would be important to investigate an agro-  
122 ecosystem that has adequate N supply for growth and yield. Under these  
123 conditions, added N would not promote additional growth or yield, but the  
124 additional N may meet the protein synthesis demands in the grain. Previous e[CO<sub>2</sub>]  
125 studies (Sinclair et al. 2000, Kimball et al. 2001) have compared adequate with  
126 deficient soil N-supply and were conducted in high rainfall or well irrigated agro-  
127 ecosystems where growth and yield was most likely limited without fertiliser N  
128 application. Conversely, in some rainfed Mediterranean and semiarid agro-  
129 ecosystems yield and growth is primarily limited by water availability. These are  
130 also relatively low yielding systems, so that N demand of crops is low by global  
131 standards, and in some cases crop N requirements can even be met by soil supply  
132 without the need for fertilisation (Angus 2001).

133 The relationship between plant demand for N and its supply from soil and fertiliser  
134 is a function of interactions between a range of plant and soil processes and the  
135 environment (Angus 2001). Consequently, it is important to investigate crop-level  
136 system responses to e[CO<sub>2</sub>] in a realistic field setting, where these environmental  
137 and physiological interactions are present. Free Air CO<sub>2</sub> Enrichment (FACE)  
138 technology provides a platform to investigate crop growth under e[CO<sub>2</sub>] without  
139 potential large artefacts on irradiance and canopy and root microclimate common  
140 to chamber systems (Ainsworth et al. 2008). Importantly, undisturbed soil  
141 processes are present in such a system. The Australian Grains Free Air CO<sub>2</sub>  
142 Enrichment (AGFACE) facility is globally unique in that it operates in a water-limited  
143 wheat cropping agro-ecosystem and is on a site where the soil N supply is generally  
144 high and adequate to meet demand for crop yield (Fitzgerald et al. 2016). This

145 enabled the present study to address the question whether N application above the  
146 level normally recommended for growth and yield response in current CO<sub>2</sub>  
147 environments can ameliorate the e[CO<sub>2</sub>]-related decline in biomass and grain [N] of  
148 wheat.

## 149 **Materials and Methods**

### 150 *Site*

151 The Australian Grains Free Air CO<sub>2</sub> Enrichment (AGFACE) facility is located on an  
152 experimental farm managed by the Victorian State Government, near Horsham,  
153 Victoria, Australia (36°45'07"S, 142°06'52"E, 127 m above sea level), and described  
154 in detail in previous papers (Mollah et al. 2009, Fitzgerald et al. 2016). In brief: The  
155 experimental plots are on a 7.5 ha field on heavy Vertosol clay soil (~35% clay at  
156 the surface and 60% in 1.4 m depth). Long term (30-year) average annual rainfall is  
157 435 mm with 274 mm typically falling during the growing season of winter wheat  
158 (May – Nov). Typical commercial wheat yields under rainfed conditions and local  
159 agronomic practice are 3-4 t ha<sup>-1</sup>, but range from 1 to 6 t ha<sup>-1</sup>. Mean annual growing  
160 season temperature is 16.5 °C and the mean annual evaporation rate is around  
161 1500 mm. Detailed weather data for the seasons in question (2007-2009 growing  
162 seasons) were recorded by an on-site automatic weather station and are given in  
163 Fitzgerald et al. (2016).

### 164 *Plant Material and Experimental Design*

165 All measurements were done on a popular local bread wheat cultivar (*Triticum*  
166 *aestivum* L. cv. 'Yitpi'). The experiment comprised a factorial combination of two  
167 levels of [CO<sub>2</sub>] (elevated e[CO<sub>2</sub>] viz. target 550 μmol mol<sup>-1</sup> air and ambient a[CO<sub>2</sub>]  
168 viz. approximately 370 μmol mol<sup>-1</sup> air; daytime medians for 2007-2009) that were  
169 each split for two levels of N application (N-sufficient N0 and N addition N+) in four  
170 replicates (plots or 'rings'), fully repeated in 12 different growing environments  
171 created by various combinations of water supply (rainfed or supplemented  
172 irrigation), and sowing times. The irrigation treatments were not designed to create  
173 non-limiting conditions but to create conditions within the site that are typical of

174 the multiseason rainfall variability. This approach provides a range of crop yields  
175 that are realistic in the region. Two sowing times per year (TOS1 according to local  
176 practice and TOS2 late sowing) were used so that the later sowing moved the crop  
177 growing season towards hotter and drier conditions. These treatments were  
178 repeated over three growing seasons (2007, 2008, 2009). Sowing dates and an  
179 overview over the growing environments investigated in this study is given in Table  
180 1.

181 Plots were re-established each season, so that wheat was not grown consecutively  
182 to avoid soil-borne disease carry over and residual treatment effects from the  
183 previous season. In 2007, plots were split in half and each (East or West) half  
184 randomly assigned to one of two time of sowing (TOS1 and TOS2) treatments, while  
185 in 2008 and 2009, each plot was randomly split to irrigation. A plastic barrier buried  
186 to 0.8 m depth ensured hydraulic separation between half-plots. In 2007, the  
187 experiment was replicated for water supply treatment (separate plots), in 2008 and  
188 2009 for time of sowing. In 2007 and 2008 the plots ('rings') were 12 m in diameter  
189 and in 2009, 16 m diameter. Details on the FACE system and its performance are  
190 given in (Mollah et al. 2009), and more details on experimental design, agronomic  
191 treatments, and weather data in (Fitzgerald et al. 2016).

192 Within each half-plot, two N-treatments were allocated to sub-plots, each 1.4 m x 4  
193 m with 8 rows of wheat sown in a north-south direction. Rows were spaced either  
194 0.214 m (2007, 2008) or 0.195 m (2009) and samples were collected from middle  
195 rows, leaving the outside rows as buffers. Plant counts about three weeks after  
196 emergence reported an average 120 plants m<sup>-2</sup>.

197 Pre-sowing soil test results from the sites showed a total soil N of 0.14% (0-10 cm)  
198 and mineral N in 0-50 cm depth of 145±50 kg N ha<sup>-1</sup> in 2007, 233±114 kg ha<sup>-1</sup> in  
199 2008, and 164±98 kg ha<sup>-1</sup> in 2009. The N sufficient treatment (N0) did not receive  
200 any N fertiliser, and the N+ treatment received 50-60 kg N ha<sup>-1</sup> as urea top dressing:  
201 50 kg ha<sup>-1</sup> before growth stage DC30 (decimal code according to Zadoks et al. 1974)  
202 in 2008 and 2009, and split in two times 30 kg ha<sup>-1</sup> between after sowing and DC31  
203 in 2007.

204 *Biomass and N measurements*

205 Biomass samples were taken at stem elongation (DC31), anthesis (DC65) and  
206 maturity (DC90) from three pre-determined sample areas in 2008 and 2009, and  
207 from sub-plot random row lengths in 2007. The areas sampled were 0.43 m<sup>2</sup> in  
208 2007 and 2008 for DC31 and DC65, 1.28 m<sup>2</sup> for DC90 in 2007, 0.86 m<sup>2</sup> for DC90 in  
209 2008 and, in 2009 0.4 m<sup>2</sup> for DC31 and DC65, and 0.78 m<sup>2</sup> for DC90. At DC31,  
210 samples were separated into leaf blades (cut off at the ligule) and stems (including  
211 leaf sheaths) and at DC65 samples were separated into leaves (cut off at the ligule),  
212 stems (including leaf sheaths) and heads and then oven dried at 70°C. At DC90, dry  
213 samples were separated into heads and straw (stems and leaves together), the  
214 heads threshed to separate grains and chaff, and the chaff combined with the  
215 straw. All biomass and grain yield are expressed on a dry weight basis.

216 Biomass [N] was analysed on dried and ground tissue aliquots by Dumas  
217 combustion in an elemental analyser (LECO, TruMac, MI), and grain [N] by near-  
218 infrared (NIR) spectrometry calibrated against the elemental analyser method. N  
219 content of biomass fractions (leaves, stems, heads) was calculated as: [N] in the  
220 fraction x biomass of that fraction expressed on a m<sup>2</sup> ground area basis. N content  
221 of biomass fractions were summed for total biomass N content at each sampled  
222 growth stage. Nitrogen uptake was calculated as the difference in biomass N  
223 content between two sampled growth stages. Post-anthesis N remobilisation from  
224 stems and leaves was calculated as the difference between maturity (DC90) and  
225 anthesis (DC65) of the products of average [N] in vegetative biomass and that  
226 vegetative biomass. As straw samples at maturity (DC90) were not separated into  
227 leaves and stems, 'vegetative biomass' refers to stems and leaves taken together.  
228 Nitrogen utilisation efficiency (NutE) was defined as the ratio of grain yield over  
229 total N in biomass (at DC90), and N harvest index (NHI) as the proportion of N  
230 content in grains in total N in biomass at DC90.

231 *Statistical evaluation*

232 This present study addresses potential interactions of N and [CO<sub>2</sub>]. Growing year,  
233 time of sowing, and water supply were therefore combined into a factor

234 'environment', resulting in 12 different environments (Table 1). Data analysis was  
235 performed in the software R (version 3.13, R Core Team 2015). The statistical  
236 evaluation was done with a linear mixed-effect model using the default REML  
237 method (R package nlme, version 3.1-120, Pinheiro et al. 2016) with [CO<sub>2</sub>] and  
238 environment as main plots, and N-treatment as split-plot.

## 239 **Results**

240 The grain yield of wheat under a[CO<sub>2</sub>] in each environment (combination of  
241 irrigation x TOS x season) ranged from just over 1.0 t ha<sup>-1</sup> to just below 3.5 t ha<sup>-1</sup>  
242 (Table 1). Yields were relatively low, but are typical of the district averages for those  
243 years. Not surprisingly across such a wide range of yields, the factor environment  
244 had a significant effect on most investigated variables. Because environmental  
245 effects on wheat growth, grain yield and grain protein are well studied and data on  
246 the NO treatment only were included in analyses in previous papers (yield and yield  
247 components in Fitzgerald et al. 2016, and grain protein in Fernando et al. 2014),  
248 environment effects will only be considered in this present study where there were  
249 significant interactions with N or CO<sub>2</sub> treatments. Across all environments, e[CO<sub>2</sub>]  
250 stimulated biomass at maturity and grain yield by about 25%: Biomass at maturity  
251 increased from 6.89 (±0.25) to 8.70 (±0.34) t ha<sup>-1</sup> and grain yield increased from  
252 2.30 (±0.09) to 2.87 (±0.13) t ha<sup>-1</sup> (means (SE) of n = 96 from 24 N x environment  
253 combinations). Addition of 50-60 kg ha<sup>-1</sup> fertiliser N had no significant effect on  
254 either biomass or grain yield.

255 Elevated [CO<sub>2</sub>] depressed [N] in grains on average by 5%. Nitrogen concentrations  
256 in the leaves decreased on average by only 3% at stem elongation, but by 9% at  
257 anthesis. The [CO<sub>2</sub>] effect was similar for stems at anthesis (9% decrease), but not  
258 significant for stems at stem elongation, or for heads at anthesis. Elevated [CO<sub>2</sub>] did  
259 not significantly affect N concentrations in straw and chaff at maturity (Figure 1).

260 Despite these decreases in tissue [N], N uptake into aboveground biomass was  
261 significantly greater under e[CO<sub>2</sub>]. By the time of stem elongation, e[CO<sub>2</sub>] grown  
262 crops had accumulated about 20% more N per unit area than a[CO<sub>2</sub>] grown ones,  
263 by anthesis 17% more, and by harvest 20% more (Figure 2). Because all crops were

264 on similar soil and had similar N availability, the increase in N uptake increased N  
265 uptake efficiency by the same proportion. N utilisation efficiency was however not  
266 significantly affected by  $e[\text{CO}_2]$  (Figure 3).

267 On average, only a very small fraction of the total N content was taken up after  
268 anthesis. Up to 50% of total N was taken up during the early vegetative phase (up  
269 to stem elongation; DC31; Figure 2), and about 90% by anthesis (DC65; Figure 2).  
270 Even under the assumption that all N taken up after anthesis went into the grains,  
271 only about 10% of N recovered in the grain could have come from post-anthesis  
272 uptake. These fractions were not significantly affected by either  $[\text{CO}_2]$  or N  
273 treatments.

274 There was no significant increase under  $e[\text{CO}_2]$  in the amount of N remobilised  
275 from stems and leaves post-anthesis (Figure 3), and the proportion of this  
276 remobilised N in grain N remained unchanged under  $e[\text{CO}_2]$  (between 60-65%).

277 Compared to N-sufficient (N0) treatment, additional N had little effect on leaf or  
278 stem [N] at stem elongation, but significantly increased [N] in leaves at anthesis,  
279 and more so under  $e[\text{CO}_2]$  (significant interaction). The N treatment also  
280 significantly increased grain [N], but this was independent of the  $[\text{CO}_2]$ -treatment  
281 (no significant interaction) and not sufficient to completely restore grain [N] to  
282 a  $[\text{CO}_2]$  values. However, N treatment increased [N] by about 9% in straw, where [N]  
283 was not significantly affected by  $e[\text{CO}_2]$  (all Figure 1). This led to a significantly  
284 lower N harvest index (NHI) when N fertiliser was applied (by on average about 5%),  
285 and a significant negative effect of N application on N utilisation efficiency,  
286 independently of  $[\text{CO}_2]$ -treatments (Figure 3). N remobilisation remained  
287 unaffected by N application (Figure 3).

288 Despite small increases in [N] in some tissues upon N treatment, total N uptake was  
289 on average not significantly increased by the additional fertiliser application (Figure  
290 2).

291 **Discussion**

292 The lack of yield response to additional application of 50-60 kg ha<sup>-1</sup> N confirmed  
293 that the experimental plots had sufficient soil N for the prevailing growing  
294 conditions, especially the range of soil water supply. Yields in water-limited  
295 Australian wheat crops are low by global standards (Angus 2001) so that N  
296 demands are modest compared to higher yielding production regions in Australia  
297 and internationally. However, unlike many other cropping lands, the site used in  
298 this present study has high organic N and mineral N concentrations, probably a  
299 consequence of prior land use of growing lucerne for 5 years, a faba bean crop in  
300 2006, and years of irrigation with communal effluent.

301 Tuohy and Robson (1980), working on the same soils as our site, proposed that  
302 grain yield was not increased by N fertiliser in any season where total soil N (0-15  
303 cm) was greater than 0.11%, compared to 0.14% (albeit for 0-10 cm) at our site.  
304 Adequate mineral N concentration in the top 60 cm for a 5 t ha<sup>-1</sup> crop yield  
305 potential has been reported at 110 kg N ha<sup>-1</sup> (Bell et al. 2013), compared to a  
306 minimum of 145 kg N ha<sup>-1</sup> (in 2007) at our site. Using both metrics, the site was  
307 more than adequately supplied with N.

308 Average grain [N] in the present study was high, translating to between 14 and 15%  
309 average grain protein, thereby apparently exceeding most standards. For example,  
310 in Australian wheat classification protein concentrations above 13% represent the  
311 highest wheat quality classes (Blakeney et al. 2009). The high grain N result reflects  
312 that half of the growing environments were achieved by delaying sowing of the  
313 crops (Table 1), effectively reducing the length of the growing season and moving  
314 the grain filling phase towards hotter and drier conditions. Grain yields, already  
315 comparatively low in the three seasons investigated here, were even lower under  
316 these conditions (Table 1, TOS2 environments 3, 4, 7, 8, 11, 12). For wheat grain,  
317 conditions that lower maximum yield often lead to greater protein concentrations  
318 (Blumenthal et al. 1993; Fowler 2003), because of the typical inverse relationship  
319 between yield and grain protein (Simmonds 1995). Significant interactions with the  
320 growing environment of [CO<sub>2</sub>] and N were only found for two parameters (leaf [N]

321 at DC65 and stem [N] at DC31); all other reported average trends remain therefore  
322 valid across all investigated environments.

323 Studies undertaken in more humid temperate or continuously irrigated  
324 environments have reported greater yield stimulation by e[CO<sub>2</sub>] under high than  
325 under low N conditions (Stitt and Krapp 1999). Other FACE studies showed no such  
326 interaction between [CO<sub>2</sub>] and N supply, suggesting that growth and yield  
327 responses were of similar magnitudes under low and high N (Weigel and  
328 Manderscheid 2012). Those studies were designed to address N limitation and  
329 therefore compared sufficient with inadequate N supply levels. In our study, N  
330 supply was non-limiting for growth and yield in all treatments and the e[CO<sub>2</sub>]-  
331 stimulation of growth and yield was similar under both N treatments.

332 In previous FACE experiments, e[CO<sub>2</sub>] decreased wheat grain protein by an average  
333 of about 5-10% (Högy and Fangmeier 2008, Taub et al. 2008, Lam et al. 2012b), and  
334 results from AGFACE were of similar magnitude (Fernando et al. 2014). Interactions  
335 (or lack of interactions) of e[CO<sub>2</sub>] with environmental conditions on grain protein  
336 concentrations were reported elsewhere for AGFACE (Fernando et al. 2014).

337 In the present study we focused on the question whether additional N application  
338 mitigates the deleterious effect of e[CO<sub>2</sub>] on grain [N]. The observations from most  
339 FACE studies show a decline in grain protein concentration under e[CO<sub>2</sub>], but the  
340 depression seen varies possibly due to the relative soil and fertiliser N supply and  
341 the demand by the crop. Where N supply was relatively low, e[CO<sub>2</sub>] reduced grain  
342 protein, but this reduction was very small with adequate N fertiliser (Kimball et al.  
343 2001). In other experiments, grain protein concentrations were significantly  
344 depressed by both e[CO<sub>2</sub>] and low N, and no interaction between N supply and  
345 eCO<sub>2</sub> was reported (Erbs et al. 2010, Wroblewitz et al. 2013). The “high N” rates in  
346 those studies were considered sufficient according to local agronomic practice,  
347 although it was not specifically demonstrated that N was not limiting for yield. The  
348 “low N” rates referred to half the normal fertiliser application, and growth and  
349 yields were lower than under “high N”. Furthermore, these studies were conducted  
350 in high yielding, high input agro-ecosystems, either under continuous irrigation

351 (Kimball et al. 2001), or in high rainfall temperate climates (Erbs et al. 2010;  
352 Wroblewitz et al. 2013). In contrast, in our study, N was added to a cropping system  
353 with adequate soil N for yield that was largely limited by water availability (Table 1).

354 N application above the requirement for growth and yield generally increases grain  
355 protein concentrations further (Fowler 2003). Such an effect of N treatment on  
356 grain [N], albeit small, was observed in our study, even though N application that  
357 targets grain protein would ideally be applied later in the season (Hooper et al.  
358 2015). However, there was no interaction between N application and [CO<sub>2</sub>] effect  
359 on grain [N], indicating that crops grown under e[CO<sub>2</sub>] suffered a grain protein  
360 penalty compared to those grown under a[CO<sub>2</sub>] irrespective of N supply.  
361 Furthermore, additional N was not able to compensate for this decline under  
362 e[CO<sub>2</sub>].

363 Grain N in cereals is supplied by root uptake during grain filling or by translocation  
364 of N previously accumulated in the biomass. In agro-ecosystems where cereals  
365 ripen under terminal drought conditions that largely inhibit further N uptake, N  
366 remobilisation from vegetative biomass (stems and leaves) contributes a large  
367 proportion to grain N (Palta et al. 1994; Buchner et al. 2015), placing particular  
368 importance on leaf [N]. In our study, N uptake after anthesis (calculated as the  
369 difference between maturity and anthesis of the products of N concentration in  
370 biomass and biomass; cf. Figure 2) could contribute only about 10% of grain N, and  
371 post-anthesis N remobilisation from stems and leaves contributed around 60-65%  
372 of grain N (the rest was already in heads at anthesis). These figures are broadly  
373 consistent with earlier reports in environments with terminal drought (Palta et al.  
374 1994).

375 Whilst this underlines the importance of post-anthesis remobilisation of nitrogen  
376 from vegetative biomass into grains under the prevailing environmental conditions,  
377 there was no indication that e[CO<sub>2</sub>] changed the extent of that remobilisation.

378 Nitrogen harvest index (NHI), the proportion of grain N in total amount of N in  
379 biomass at maturity, results from the sum of N translocation from vegetative  
380 biomass, pre-anthesis N uptake into developing heads, and post-anthesis N uptake

381 into grains, and remained unaffected by  $e[\text{CO}_2]$ (Figure 3). Similarly, post-anthesis N  
382 remobilisation from vegetative organs was not affected by elevated  $e[\text{CO}_2]$  (Figure  
383 3).

384 Decreases in [N] in vegetative plant parts, which are well documented under  $e[\text{CO}_2]$   
385 (Stitt and Krapp 1999, Tausz-Posch et al. 2014), could therefore be directly related  
386 to decreases in grain [N], because proportionally less N is available for  
387 remobilisation per each g grain yield. At anthesis, leaf [N] in our study averaged 9%  
388 lower under  $e[\text{CO}_2]$ . This is comparable with Buchner et al. (2015) who reported  
389 that N concentrations were reduced by about 8% in flag leaves or 9% in second  
390 leaves under  $e[\text{CO}_2]$  as compared to  $a[\text{CO}_2]$ . Additionally, Wang et al. (2013)  
391 reported an average 9% decrease in their meta-analysis for wheat. Averages for  
392 multiple species under FACE conditions were of similar magnitude (Ainsworth and  
393 Long 2005, Tausz-Posch et al. 2014). The relative decrease in leaf [N] under  $e[\text{CO}_2]$   
394 was less, albeit still significant, at the vegetative growth stage.

395 Some previous FACE investigations on wheat found that decreases in leaf [N] under  
396  $e[\text{CO}_2]$  were less pronounced with adequate N nutrition than under N deficit  
397 (Sinclair et al. 2000, Weigel and Manderscheid 2012), and photosynthetic  
398 downward acclimation, a response to  $e[\text{CO}_2]$  commonly linked to decreases in leaf  
399 [N], was less pronounced under high N (Stitt and Krapp 1999). At the earlier  
400 vegetative growth stage in our study, the additional N application had no effect on  
401 leaf [N], and leaf [N] was decreased by  $e[\text{CO}_2]$  regardless of N application. This is in  
402 agreement with the FACE results reported by (Sinclair et al. 2000) who found no  
403 effect of soil fertility (*viz.* soil N supply) on leaf [N] early in the season, but  $e[\text{CO}_2]$   
404 decreased leaf [N] regardless of N supply at that stage. At anthesis, our results did  
405 indicate some attenuation of the decrease in leaf [N] by additional N supply, as  
406 shown by the interaction between  $[\text{CO}_2] \times \text{N}$  (Figure 1). However, supplying  
407 additional N during the vegetative growth phase did not restore leaf [N] under  
408  $e[\text{CO}_2]$ . In contrast, in Sinclair et al. (2000), leaf [N] depression by  $e[\text{CO}_2]$  at anthesis  
409 was only evident in plants where insufficient N was supplied but not in those  
410 adequately supplied with N. In contrast, but in agreement with Wang et al. (2013),  
411 our results suggest that increased N supply can moderate the effect of  $e[\text{CO}_2]$  on

412 leaf [N] to some extent, but not restore leaf [N] under e[CO<sub>2</sub>]. Insufficient N supply  
413 will amplify the effect of e[CO<sub>2</sub>] on leaf [N], but is not the sole cause for decreased  
414 leaf [N]. The attenuation effect of the additional N application on leaf [N] at  
415 anthesis could represent the mitigation of a short-term supply deficit, because in  
416 dryland agro-ecosystems (such as the one investigated here) even soils with high N  
417 status can leave the crop with insufficient mineral N supply during certain stages,  
418 because mineralisation rates and crop demand can be temporarily mismatched  
419 (Angus 2001). This effect does however not explain the full extent of leaf [N]  
420 depression under e[CO<sub>2</sub>], and alternative mechanisms, such as a direct limitation to  
421 nitrate assimilation (Bloom et al. 2014, Bloom 2015), decreased N allocation to the  
422 photosynthetic machinery due to downward acclimation of photosynthesis or  
423 increased leaf area index (leading to denser canopies), or changes in N mass flow  
424 related to changes in transpiration, are very likely (for review see Tausz-Posch et al.  
425 2014).

426 Despite these evident decreases in grain and biomass [N], overall N uptake of the  
427 crops averaged 20% (or around 30 kg ha<sup>-1</sup>) greater under e[CO<sub>2</sub>]. Whilst N supply by  
428 mineralisation was sufficient to meet additional crop demand at this high N  
429 experimental site in the short term, this may not be sustainable and in the mid to  
430 long-term this N will have to be provided by additional inputs. Where biomass and  
431 yield stimulation are relatively greater than the decrease in biomass [N], crops will  
432 have greater N demands under CO<sub>2</sub>-enrichment. This seems to be the case in many,  
433 but not all reported analyses (Lam et al. 2012a, b, Chen et al. 2012, Wang et al.  
434 2013; Tausz-Posch et al. 2014).

435 The N source and management methods to meet additional crop demands for N in  
436 a high [CO<sub>2</sub>] atmosphere need to be carefully considered (Carlisle et al. 2012,  
437 Bloom 2015), because N fertiliser can have large negative impacts on the  
438 environment (Robertson and Vitousek 2009) and already constitutes a relatively  
439 costly and risky farm input in these cropping systems primarily limited by low and  
440 unreliable rainfall (Angus 2001). Probably even more important than such  
441 quantitative considerations are qualitative aspects of nitrogen management (Bloom  
442 2015), such as selection of nitrogen form to promote uptake of reduced nitrogen so

443 that biochemical limitations to nitrate assimilation can be circumvented (Carlisle et  
444 al. 2012, Bloom et al. 2014), or appropriate timing of N applications to target grain  
445 N (Hooper et al. 2015).

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624 Ziska, L. H., 2008: Rising atmospheric carbon dioxide and plant biology: The  
625 overlooked paradigm. *DNA and Cell Biol.* **27**, 165-172.

626 Table 1. Summary of growing conditions and wheat (*Triticum aestivum* L. cv Yitpi) grain yields and grain [N] across the 12 growing  
 627 environments (numbered in the first column) investigated in this study. Grain yields are averages for ambient [CO<sub>2</sub>] and two N regimes (N0  
 628 and N+), because N treatment had no significant effect on yield (n=8 in each environment). Grain [N] data are averages for ambient [CO<sub>2</sub>] and  
 629 N0 (without additional N), because N treatment significantly affected grain [N]. Time of sowing (TOS) at local practice (TOS1), late (TOS2), in-  
 630 season water rainfall only (Rain) or rainfall plus supplemental irrigation (Sup) treatments, replicated four times (n=4) in each environment  
 631 (Env). Temperatures are reported as seasonal means, mean daily minima and maxima for the growing season from sowing to harvest for each  
 632 year. Sowing dates, water inputs, temperatures and days to harvest were previously reported in Fitzgerald et al. (2016).

| Env | Year | Sowing date                   | Days to harvest | In-season water [mm]    | Air Temperatures (mean, min, max) [°C] | Grain yield [t grain ha <sup>-1</sup> ] | Grain Protein [%] |
|-----|------|-------------------------------|-----------------|-------------------------|--|---|-------------------|
| 1   | 2007 | Local practice (18 Jun), TOS1 | 177             | 219 (Rain)              | 12.2, 5.2, 19.3                        | 2.8                                     | 14.0              |
| 2   | 2007 | Local practice (18 Jun), TOS1 | 177             | 267 (Sup)               | 12.2, 5.2, 19.3                        | 3.4                                     | 13.2              |
| 3   | 2007 | Late (23 Aug), TOS2           | 123             | 178 <sup>1</sup> (Rain) | 15.0, 6.9, 23.1                        | 2.1                                     | 14.1              |
| 4   | 2007 | Late (23 Aug), TOS2           | 123             | 226 <sup>2</sup> (Sup)  | 15.0, 6.9, 23.1                        | 2.2                                     | 14.0              |
| 5   | 2008 | Local practice (4 Jun), TOS1  | 187             | 178 (Rain)              | 11.1, 4.5, 17.7                        | 3.0                                     | 14.8              |
| 6   | 2008 | Local practice (4 Jun), TOS1  | 187             | 208 (Sup)               | 11.1, 4.5, 17.7                        | 3.3                                     | 16.3              |
| 7   | 2008 | Late (5 Aug), TOS2            | 132             | 109 (Rain)              | 12.5, 5.0, 19.9                        | 1.5                                     | 15.0              |
| 8   | 2008 | Late (5 Aug), TOS2            | 132             | 164 (Sup)               | 12.5, 5.0, 19.9                        | 1.8                                     | 15.5              |
| 9   | 2009 | Local practice (23 Jun), TOS1 | 164             | 223 (Rain)              | 12.8, 6.2, 19.4                        | 2.6                                     | 15.2              |

|    |      |                               |     |            |                 |     |      |
|----|------|-------------------------------|-----|------------|-----------------|-----|------|
| 10 | 2009 | Local practice (23 Jun), TOS1 | 164 | 293 (Sup)  | 12.8, 6.2, 19.4 | 2.5 | 15.3 |
| 11 | 2009 | Late (19 Aug), TOS2           | 116 | 170 (Rain) | 14.8, 7.3, 22.2 | 1.1 | 17.3 |
| 12 | 2009 | Late (19 Aug), TOS2           | 116 | 230 (Sup)  | 14.8, 7.3, 22.2 | 1.3 | 17.1 |

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633 <sup>1,2</sup> These data were reported as 159 and 207 mm of in-season rainfall, respectively in Fitzgerald et al. (2016) but are corrected here.

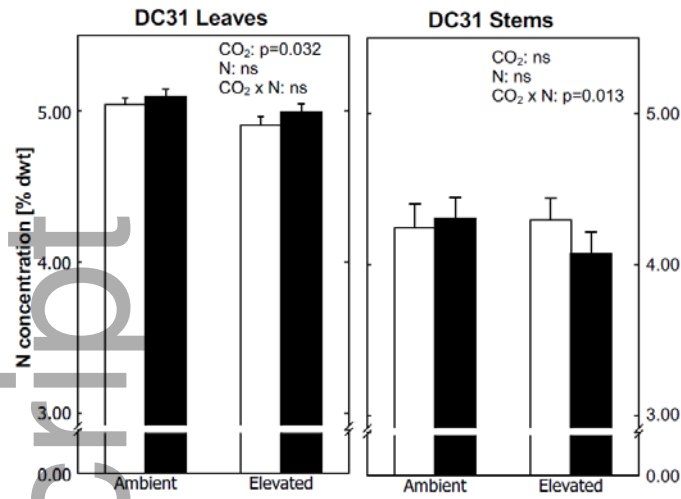
634 **Figure Legends**

635 Figure 1. Tissue concentrations of N [% of plant dry weight] of wheat grown in the  
636 Australian Grains Free Air CO<sub>2</sub> Enrichment (AGFACE) facility. White columns N0; no N  
637 addition. Black columns N+; 50-60 kg ha<sup>-1</sup> N added during vegetative growth (before  
638 DC30). Each data point represents the mean and SE of n=48 samples (4 replicates in  
639 each of 12 growing environments – Table 1). Ambient [CO<sub>2</sub>] at 370 μmol mol<sup>-1</sup> air<sup>-1</sup>;  
640 Elevated [CO<sub>2</sub>] at 550 μmol mol<sup>-1</sup> air<sup>-1</sup>. P-values for effects of CO<sub>2</sub>, N and CO<sub>2</sub> x N. ns  
641 P≥0.100.

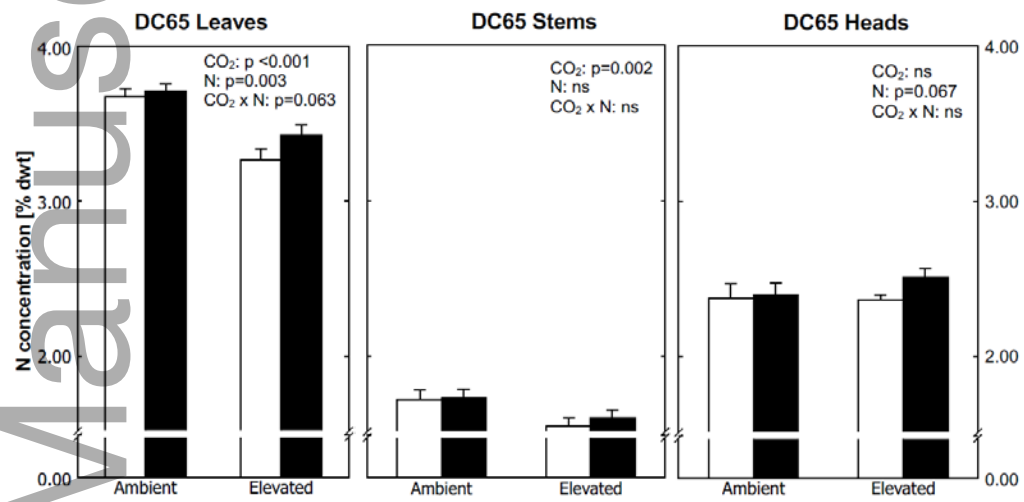
642 Figure 2. N content in aboveground biomass [g N m<sup>-2</sup> ground area] of wheat grown in  
643 the Australian Grains Free Air CO<sub>2</sub> Enrichment (AGFACE) facility. White columns N0; no  
644 N addition. Black columns N+; 50-60 kg ha<sup>-1</sup> N added during vegetative growth (before  
645 DC30). Each data point represents the mean and SE of n=48 samples (4 replicates in  
646 each of 12 growing environments – Table 1). Ambient [CO<sub>2</sub>] at 370 μmol mol<sup>-1</sup> air<sup>-1</sup>;  
647 Elevated [CO<sub>2</sub>] at 550 μmol mol<sup>-1</sup> air<sup>-1</sup>. P-values for effects of CO<sub>2</sub>, N and CO<sub>2</sub> x N. ns  
648 P≥0.100.

649 Figure 3. Left panel: N harvest index (NHI; proportion of grain N in total above ground  
650 biomass N content at maturity). Mid panel: N utilisation efficiency (NutE; grain yield  
651 divided by total N in biomass at maturity). Right panel: Post-anthesis N remobilisation  
652 from stems and leaves (per m<sup>2</sup> ground area) of wheat grown in the Australian Grains  
653 Free Air CO<sub>2</sub> Enrichment (AGFACE) facility. Open symbols and columns N0; no N  
654 addition. Black symbols and columns N+; 50-60 kg ha<sup>-1</sup> N added during vegetative  
655 growth. Each data point represents the mean and SE of n=48 samples (4 replicates in  
656 each of 12 growing environments – Table 1). Ambient [CO<sub>2</sub>] at 370 μmol mol<sup>-1</sup> air<sup>-1</sup>;  
657 Elevated [CO<sub>2</sub>] at 550 μmol mol<sup>-1</sup> air<sup>-1</sup>. P-values for effects of CO<sub>2</sub>, N and CO<sub>2</sub> x N. ns  
658 P≥0.100.

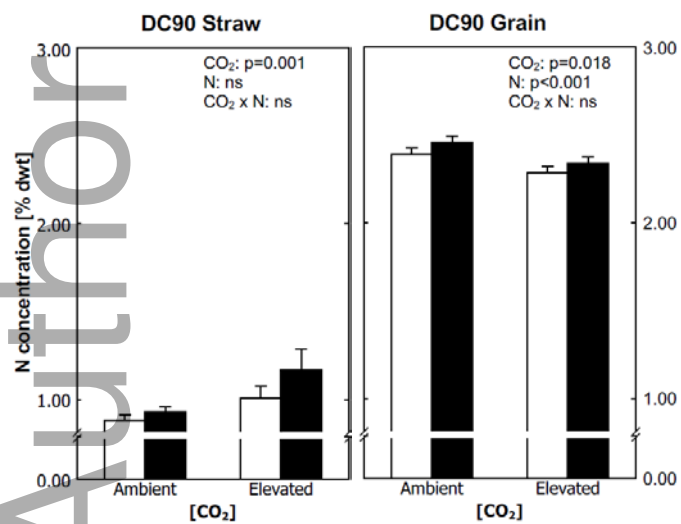
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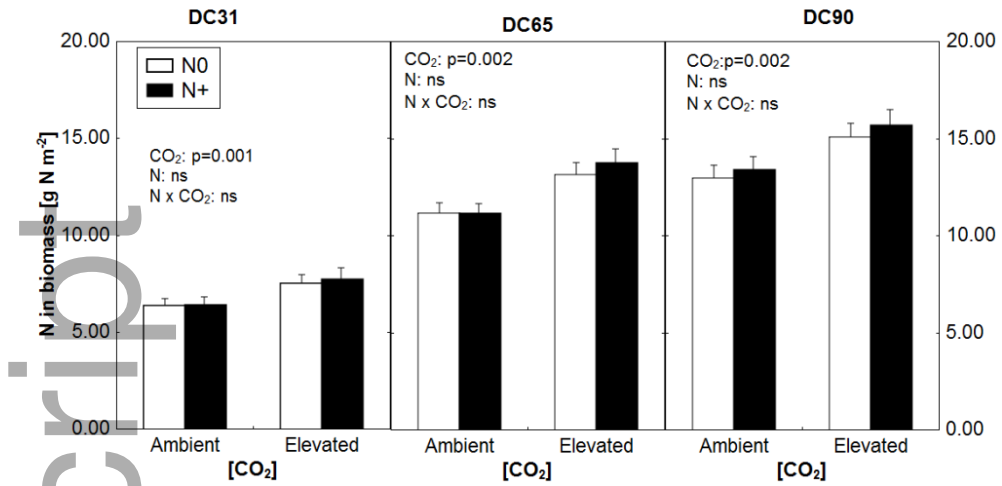
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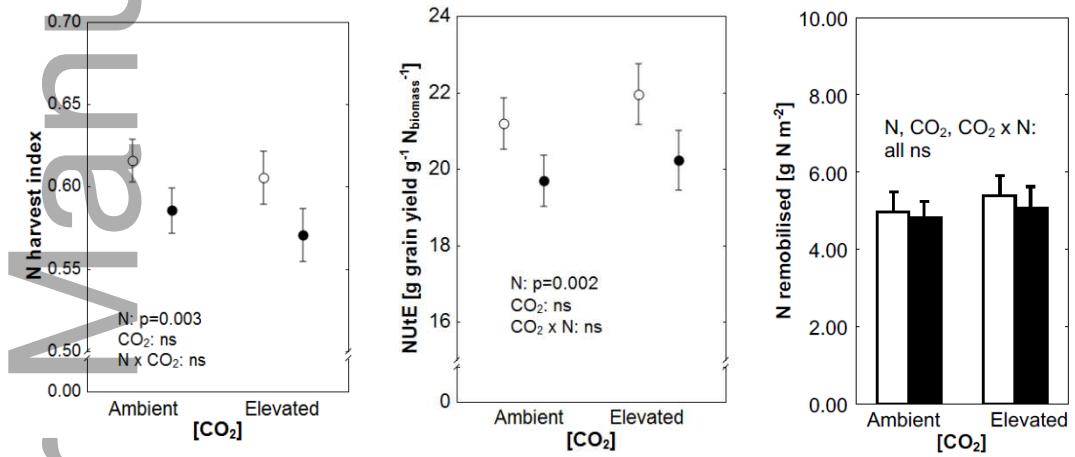
663 Figure 1.

664



665

666 Figure 2.



667

668 Figure 3.