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Can additional N fertiliser ameliorate the elevated CO₂-induced depression in grain and tissue N concentrations of wheat on a high soil N background?

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- 1 Can additional N fertiliser ameliorate the elevated CO₂-induced depression in
- 2 grain and tissue N concentrations of wheat on a high soil N background?
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27 Abstract

28	Elevated CO_2 stimulates crop yields but leads to lower tissue and grain nitrogen
29	concentrations [N], raising concerns about grain quality in cereals. To test whether
30	N fertiliser application above optimum growth requirements can alleviate the
31	decline in tissue [N], wheat was grown in a Free Air CO_2 Enrichment facility in a low
32	rainfall cropping system on high soil N. Crops were grown with and without
33	addition of 50-60 kg N ha ⁻¹ in 12 growing environments created by supplemental
34	irrigation and two sowing dates over three years.
35	Elevated CO_2 increased yield and biomass (on average by 25%), and decreased
36	biomass [N] (3-9%) and grain [N] (5%). Nitrogen uptake was greater (20%) in
37	elevated CO_2 grown crops. Additional N supply had no effect on yield and biomass,
38	confirming high soil N. Small increases in [N] with N addition were insufficient to
39	offset declines in grain [N] under elevated CO2. Instead, N application increased the
40	[N] in straw and decreased N harvest index.
41	The results suggest that conventional addition of N does not mitigate grain [N]
42	depression under elevated CO_2 , and lend support to hypotheses that link decreases
43	in crop [N] with biochemical limitations rather than N supply.

44

45 Introduction

46	Atmospheric CO_2 concentration ([CO_2]) has been increasing since the industrial
47	revolution and is predicted to reach 550 $\mu\text{L}~\text{L}^{\text{-1}}$ or more by 2050, that is a 35%
48	change from the current (400 $\mu L \ L^{\text{-1}}$ in 2016) concentration (Stocker et al. 2013).
49	Because CO_2 is the main substrate for photosynthesis, such a large increase will
50	affect all plants and ecosystems (Ziska 2008). Many studies demonstrated that, at
51	least in C3 plants, elevated $[CO_2]$ (e $[CO_2]$) stimulates photosynthesis and
52	subsequently growth and yield (Kimball et al. 2002, Ainsworth and Long 2005)
53	through the so-called ' CO_2 fertilisation effect'.
54	While the 'CO ₂ fertilisation effect' may result in greater crop yields and help offset
55	some of the negative effects of climate change on food production (Hatfield et al.
56	2011), concerns have been raised about reductions in mineral nutrients and grain
57	quality (Högy and Fangmeier 2008, Myers et al. 2014). It is well established that
58	growth under $e[CO_2]$ changes the stoichiometry of plants, whereby the
59	concentration of many minerals, especially nitrogen (N), in plant tissues decreases
60	(Loladze 2002). Because photosynthetic N use efficiency (the photosynthetic carbon
61	fixation rate per g leaf N) increases under $e[CO_2]$, the critical tissue N concentration,
62	i. e. the leaf N concentration ([N]) that is necessary for optimum growth,
63	consequently decreases under $e[CO_2]$ (Conroy and Hocking 1993, Seneweera and
64	Norton 2011, Tausz-Posch et al. 2014). Despite lower tissue [N], the greater
65	biomass reported under $e[CO_2]$ may contain more N per ground surface area than
66	biomass under ambient [CO $_2$], hence N uptake of the crop may be greater (Tausz-
67	Posch et al. 2014, Lam et al. 2012b). In natural ecosystems, where N is often

limiting, the CO₂-stimulation on growth often decreases over time because
available N in the soil becomes depleted, a phenomenon termed 'progressive N
limitation' (Oren et al. 2001, Luo et al. 2004). In N-managed agro-ecosystems
progressive N limitation may not be relevant or immediately apparent, but some
experiments have shown that growth stimulation by e[CO₂] is less under low than
high N supply (Stitt and Krapp 1999).

For non-legume food and fodder crops, decreases in tissue [N] are particularly 74 75 concerning because they translate to lower protein concentrations, thus lowering 76 food and feed quality as shown in wheat (Högy et al. 2013; Wroblewitz et al. 2013). 77 Grain protein concentration is also an important determinant of baking quality and 78 market value of wheat. Dough and baking quality of wheat was shown to 79 deteriorate under $e[CO_2]$ (Panozzo et al. 2014). Synthesis papers report around 5-10% reduction in grain protein concentration in wheat (Högy and Fangmeier 2008; 80 Lam et al. 2012b, Wang et al. 2013), and about 10% for leaf [N] (Ainsworth and 81 82 Long 2005; Wang et al. 2013).

83 The exact mechanism for the decrease in [N] is unclear, and a number of not mutually exclusive hypotheses have been proposed (Tausz-Posch et al. 2014). The 84 most straightforward one contends that soil N supply does not keep up with 85 increased demand by e[CO₂]-stimulated biomass growth, leading to a 'dilution' of N 86 in tissue biomass and N-limitation to biochemistry and growth (Taub and Wang 87 2008). Evidence for this hypothesis comes from experiments where leaf [N] 88 decreased upon e[CO₂] exposure at low, but not at high soil N supply (Stitt and 89 90 Krapp 1999, Sinclair et al. 2000). If limited N availability leads to decreases in tissue

91 [N], it could be hypothesised that reductions in biomass tissue [N] in managed agro92 ecosystems could be reversed by additional soil N inputs.

93 Alternative hypotheses for the decline in tissue [N] under $e[CO_2]$, such as nutrient uptake limitations by reduced transpiration flow (Conroy and Hocking 1993, 94 95 McGrath and Lobell 2013), or decreased rates of nitrate reduction (Bloom et al. 96 2010; Bloom et al. 2014), do not suggest that an increase in soil N supply would 97 restore tissue [N]. For example, in one study conducted in a high yielding, irrigated 98 wheat cropping system under ample N supply, the deleterious effect of $e[CO_2]$ on 99 grain protein concentration was partially alleviated but was still present, even if very small (Kimball et al. 2001). This would imply that insufficient soil N supply is 100 101 not the only mechanism contributing to decreased [N]. Recent meta-analyses 102 suggested that high soil N supply cannot fully restore, but at best only moderates 103 the negative effect of e[CO₂] on tissue and grain [N] (Lam et al 2012b, Wang et al. 104 2013). It is not clear under what conditions, and to which extent, additional N 105 application can restore leaf and grain [N] (and protein) under $e[CO_2]$.

106 In cereals such as wheat, N requirement for growth and grain yield is generally 107 satisfied before that of increased grain protein, so that grain protein concentration can be increased by N application above the level needed for growth and yield 108 109 responses (Fowler 2003; Hooper et al. 2015). To understand whether N supply 110 beyond the demand for growth and yield can restore grain protein under $e[CO_2]$ to 111 that achieved under ambient CO_2 , it would be important to investigate an agro-112 ecosystem that has adequate N supply for growth and yield. Under these 113 conditions, added N would not promote additional growth or yield, but the

114 additional N may meet the protein synthesis demands in the grain. Previous e[CO₂] 115 studies (Sinclair et al. 2000, Kimball et al. 2001) have compared adequate with 116 deficient soil N-supply and were conducted in high rainfall or well irrigated agro-117 ecosystems where growth and yield was most likely limited without fertiliser N 118 application. Conversely, in some rainfed Mediterranean and semiarid agro-119 ecosystems yield and growth is primarily limited by water availability. These are 120 also relatively low yielding systems, so that N demand of crops is low by global 121 standards, and in some cases crop N requirements can even be met by soil supply 122 without the need for fertilisation (Angus 2001).

123 The relationship between plant demand for N and its supply from soil and fertiliser 124 is a function of interactions between a range of plant and soil processes and the 125 environment (Angus 2001). Consequently, it is important to investigate crop-level system responses to e[CO₂] in a realistic field setting, where these environmental 126 127 and physiological interactions are present. Free Air CO₂ Enrichment (FACE) 128 technology provides a platform to investigate crop growth under $e[CO_2]$ without 129 potential large artefacts on irradiance and canopy and root microclimate common 130 to chamber systems (Ainsworth et al. 2008). Importantly, undisturbed soil 131 processes are present in such a system. The Australian Grains Free Air CO₂ 132 Enrichment (AGFACE) facility is globally unique in that it operates in a water-limited 133 wheat cropping agro-ecosystem and is on a site where the soil N supply is generally 134 high and adequate to meet demand for crop yield (Fitzgerald et al. 2016). This 135 enabled the present study to address the question whether N application above the 136 level normally recommended for growth and yield response in current CO₂

environments can ameliorate the e[CO₂]-related decline in biomass and grain [N] ofwheat.

139 Materials and Methods

140 *Site*

141 The Australian Grains Free Air CO₂ Enrichment (AGFACE) facility is located on an 142 experimental farm managed by the Victorian State Government, near Horsham, Victoria, Australia (36°45'07"S, 142°06'52"E, 127 m above sea level), and described 143 144 in detail in previous papers (Mollah et al. 2009, Fitzgerald et al. 2016). In brief: The 145 experimental plots are on a 7.5 ha field on heavy Vertosol clay soil (~35% clay at 146 the surface and 60% in 1.4 m depth). Long term (30-year) average annual rainfall is 147 435 mm with 274 mm typically falling during the growing season of winter wheat (May – Nov). Typical commercial wheat yields under rainfed conditions and local 148 agronomic practice are 3-4 t ha⁻¹, but range from 1 to 6 t ha⁻¹. Mean annual growing 149 150 season temperature is 16.5 °C and the mean annual evaporation rate is around 151 1500 mm. Detailed weather data for the seasons in question (2007-2009 growing seasons) were recorded by an on-site automatic weather station and are given in 152 153 Fitzgerald et al. (2016).

154 Plant Material and Experimental Design

All measurements were done on a popular local bread wheat cultivar (*Triticum aestivum* L. cv. 'Yitpi'). The experiment comprised a factorial combination of two
levels of [CO₂] (elevated e[CO₂] *viz*. target 550 µmol mol⁻¹ air and ambient a[CO₂] *viz.* approximately 370 µmol mol⁻¹ air; daytime medians for 2007-2009) that were

each split for two levels of N application (N-sufficient NO and N addition N+) in four 159 160 replicates (plots or 'rings'), fully repeated in 12 different growing environments created by various combinations of water supply (rainfed or supplemented 161 162 irrigation), and sowing times. The irrigation treatments were not designed to create 163 non-limiting conditions but to create conditions within the site that are typical of 164 the multiseason rainfall variability. This approach provides a range of crop yields 165 that are realistic in the region. Two sowing times per year (TOS1 according to local 166 practice and TOS2 late sowing) were used so that the later sowing moved the crop growing season towards hotter and drier conditions. These treatments were 167 168 repeated over three growing seasons (2007, 2008, 2009). Sowing dates and an 169 overview over the growing environments investigated in this study is given in Table 170 1.

Plots were re-established each season, so that wheat was not grown consecutively 171 172 to avoid soil-borne disease carry over and residual treatment effects from the 173 previous season. In 2007, plots were split in half and each (East or West) half randomly assigned to one of two time of sowing (TOS1 and TOS2) treatments, while 174 175 in 2008 and 2009, each plot was randomly split to irrigation. A plastic barrier buried 176 to 0.8 m depth ensured hydraulic separation between half-plots. In 2007, the 177 experiment was replicated for water supply treatment (separate plots), in 2008 and 2009 for time of sowing. In 2007 and 2008 the plots ('rings') were 12 m in diameter 178 179 and in 2009, 16 m diameter. Details on the FACE system and its performance are 180 given in (Mollah et al. 2009), and more details on experimental design, agronomic 181 treatments, and weather data in (Fitzgerald et al. 2016).

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

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

194 Biomass and N measurements

195 Biomass samples were taken at stem elongation (DC31), anthesis (DC65) and 196 maturity (DC90) from three pre-determined sample areas in 2008 and 2009, and from sub-plot random row lengths in 2007. The areas sampled were 0.43 m² in 197 2007 and 2008 for DC31 and DC65, 1.28 m² for DC90 in 2007, 0.86 m² for DC90 in 198 2008 and, in 2009 0.4 m² for DC31 and DC65, and 0.78 m² for DC90. At DC31, 199 200 samples were separated into leaf blades (cut off at the ligule) and stems (including 201 leaf sheaths) and at DC65 samples were separated into leaves (cut off at the ligule), 202 stems (including leaf sheaths) and heads and then oven dried at 70°C. At DC90, dry samples were separated into heads and straw (stems and leaves together), the 203

heads threshed to separate grains and chaff, and the chaff combined with the
straw. All biomass and grain yield are expressed on a dry weight basis.

206 Biomass [N] was analysed on dried and ground tissue aliquots by Dumas 207 combustion in an elemental analyser (LECO, TruMac, MI), and grain [N] by near-208 infrared (NIR) spectrometry calibrated against the elemental analyser method. N 209 content of biomass fractions (leaves, stems, heads) was calculated as: [N] in the fraction x biomass of that fraction expressed on a m² ground area basis. N content 210 of biomass fractions were summed for total biomass N content at each sampled 211 212 growth stage. Nitrogen uptake was calculated as the difference in biomass N 213 content between two sampled growth stages. Post-anthesis N remobilisation from 214 stems and leaves was calculated as the difference between maturity (DC90) and 215 anthesis (DC65) of the products of average [N] in vegetative biomass and that 216 vegetative biomass. As straw samples at maturity (DC90) were not separated into 217 leaves and stems, 'vegetative biomass' refers to stems and leaves taken together. 218 Nitrogen utilisation efficiency (NutE) was defined as the ratio of grain yield over total N in biomass (at DC90), and N harvest index (NHI) as the proportion of N 219 220 content in grains in total N in biomass at DC90.

221 Statistical evaluation

This present study addresses potential interactions of N and [CO₂]. Growing year,
time of sowing, and water supply were therefore combined into a factor
'environment', resulting in 12 different environments (Table 1). Data analysis was
performed in the software R (version 3.13, R Core Team 2015). The statistical
evaluation was done with a linear mixed-effect model using the default REML

method (R package nlme, version 3.1-120, Pinheiro et al. 2016) with [CO₂] and
environment as main plots, and N-treatment as split-plot.

229 Results

230 The grain yield of wheat under a[CO₂] in each environment (combination of irrigation x TOS x season) ranged from just over 1.0 t ha⁻¹ to just below 3.5 t ha⁻¹ 231 (Table 1). Yields were relatively low, but are typical of the district averages for those 232 233 years. Not surprisingly across such a wide range of yields, the factor environment 234 had a significant effect on most investigated variables. Because environmental 235 effects on wheat growth, grain yield and grain protein are well studied and data on 236 the NO treatment only were included in analyses in previous papers (yield and yield 237 components in Fitzgerald et al. 2016, and grain protein in Fernando et al. 2014), 238 environment effects will only be considered in this present study where there were significant interactions with N or CO₂ treatments. Across all environments, e[CO₂] 239 240 stimulated biomass at maturity and grain yield by about 25%: Biomass at maturity increased from 6.89 (\pm 0.25) to 8.70 (\pm 0.34) t ha⁻¹ and grain yield increased from 241 2.30 (\pm 0.09) to 2.87 (\pm 0.13) t ha⁻¹ (means (SE) of n = 96 from 24 N x environment 242 combinations). Addition of 50-60 kg ha⁻¹ fertiliser N had no significant effect on 243 either biomass or grain yield. 244

Elevated [CO₂] depressed [N] in grains on average by 5%. Nitrogen concentrations in the leaves decreased on average by only 3% at stem elongation, but by 9% at anthesis. The [CO₂] effect was similar for stems at anthesis (9% decrease), but not significant for stems at stem elongation, or for heads at anthesis. Elevated [CO₂] did not significantly affect N concentrations in straw and chaff at maturity (Figure 1). Despite these decreases in tissue [N], N uptake into aboveground biomass was
significantly greater under e[CO₂]. By the time of stem elongation, e[CO₂] grown
crops had accumulated about 20% more N per unit area than a[CO₂] grown ones, by
anthesis 17% more, and by harvest 20% more (Figure 2). Because all crops were on
similar soil and had similar N availability, the increase in N uptake increased N
uptake efficiency by the same proportion. N utilisation efficiency was however not
significantly affected by e[CO₂] (Figure 3).

On average, only a very small fraction of the total N content was taken up after
anthesis. Up to 50% of total N was taken up during the early vegetative phase (up
to stem elongation; DC31; Figure 2), and about 90% by anthesis (DC65; Figure 2).
Even under the assumption that all N taken up after anthesis went into the grains,
only about 10% of N recovered in the grain could have come from post-anthesis
uptake. These fractions were not significantly affected by either [CO₂] or N
treatments.

There was no significant increase under e[CO₂] in the amount of N remobilised from stems and leaves post-anthesis (Figure 3), and the proportion of this remobilised N in grain N remained unchanged under e[CO₂] (between 60-65%).

267 Compared to N-sufficient (N0) treatment, additional N had little effect on leaf or

stem [N] at stem elongation, but significantly increased [N] in leaves at anthesis,

and more so under e[CO₂] (significant interaction). The N treatment also

significantly increased grain [N], but this was independent of the [CO₂]-treatment

271 (no significant interaction) and not sufficient to completely restore grain [N] to

a[CO₂] values. However, N treatment increased [N] by about 9% in straw, where [N]

was not significantly affected by e[CO₂] (all Figure 1). This led to a significantly lower
N harvest index (NHI) when N fertiliser was applied (by on average about 5%), and a
significant negative effect of N application on N utilisation efficiency, independently
of [CO₂]-treatments (Figure 3). N remobilisation remained unaffected by N
application (Figure 3).

Despite small increases in [N] in some tissues upon N treatment, total N uptake was
on average not significantly increased by the additional fertiliser application (Figure
280 2).

281 Discussion

The lack of yield response to additional application of 50-60 kg ha⁻¹ N confirmed 282 283 that the experimental plots had sufficient soil N for the prevailing growing 284 conditions, especially the range of soil water supply. Yields in water-limited 285 Australian wheat crops are low by global standards (Angus 2001) so that N 286 demands are modest compared to higher yielding production regions in Australia 287 and internationally. However, unlike many other cropping lands, the site used in this present study has high organic N and mineral N concentrations, probably a 288 289 consequence of prior land use of growing lucerne for 5 years, a faba bean crop in 290 2006, and years of irrigation with communal effluent.

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

Average grain [N] in the present study was high, translating to between 14 and 15% 298 299 average grain protein, thereby apparently exceeding most standards. For example, 300 in Australian wheat classification protein concentrations above 13% represent the 301 highest wheat quality classes (Blakeney et al. 2009). The high grain N result reflects 302 that half of the growing environments were achieved by delaying sowing of the 303 crops (Table 1), effectively reducing the length of the growing season and moving 304 the grain filling phase towards hotter and drier conditions. Grain yields, already 305 comparatively low in the three seasons investigated here, were even lower under 306 these conditions (Table 1, TOS2 environments 3, 4, 7, 8, 11, 12). For wheat grain, 307 conditions that lower maximum yield often lead to greater protein concentrations 308 (Blumenthal et al. 1993; Fowler 2003), because of the typical inverse relationship 309 between yield and grain protein (Simmonds 1995). Significant interactions with the growing environment of [CO₂] and N were only found for two parameters (leaf [N] 310 311 at DC65 and stem [N] at DC31); all other reported average trends remain therefore 312 valid across all investigated environments.

313 Studies undertaken in more humid temperate or continuously irrigated

environments have reported greater yield stimulation by e[CO₂] under high than
under low N conditions (Stitt and Krapp 1999). Other FACE studies showed no such
interaction between [CO₂] and N supply, suggesting that growth and yield
responses were of similar magnitudes under low and high N (Weigel and

318 Manderscheid 2012). Those studies were designed to address N limitation and

319 therefore compared sufficient with inadequate N supply levels. In our study, N

320 supply was non-limiting for growth and yield in all treatments and the e[CO₂]-

321 stimulation of growth and yield was similar under both N treatments.

In previous FACE experiments, e[CO₂] decreased wheat grain protein by an average of about 5-10% (Högy and Fangmeier 2008, Taub et al. 2008, Lam et al. 2012b), and results from AGFACE were of similar magnitude (Fernando et al. 2014). Interactions (or lack of interactions) of e[CO₂] with environmental conditions on grain protein concentrations were reported elsewhere for AGFACE (Fernando et al. 2014).

327 In the present study we focused on the question whether additional N application 328 mitigates the deleterious effect of $e[CO_2]$ on grain [N]. The observations from most 329 FACE studies show a decline in grain protein concentration under $e[CO_2]$, but the 330 depression seen varies possibly due to the relative soil and fertiliser N supply and 331 the demand by the crop. Where N supply was relatively low, e[CO₂] reduced grain 332 protein, but this reduction was very small with adequate N fertiliser (Kimball et al. 333 2001). In other experiments, grain protein concentrations were significantly 334 depressed by both e[CO₂] and low N, and no interaction between N supply and eCO₂ was reported (Erbs et al. 2010, Wroblewitz et al. 2013). The "high N" rates in 335 those studies were considered sufficient according to local agronomic practice, 336 337 although it was not specifically demonstrated that N was not limiting for yield. The "low N" rates referred to half the normal fertiliser application, and growth and 338 339 yields were lower than under "high N". Furthermore, these studies were conducted in high yielding, high input agro-ecosystems, either under continuous irrigation 340

341 (Kimball et al. 2001), or in high rainfall temperate climates (Erbs et al. 2010;

342 Wroblewitz et al. 2013). In contrast, in our study, N was added to a cropping system

- 343 with adequate soil N for yield that was largely limited by water availability (Table 1).
- N application above the requirement for growth and yield generally increases grain
- 345 protein concentrations further (Fowler 2003). Such an effect of N treatment on
- 346 grain [N], albeit small, was observed in our study, even though N application that
- targets grain protein would ideally be applied later in the season (Hooper et al.
- 2015). However, there was no interaction between N application and [CO₂] effect
- on grain [N], indicating that crops grown under e[CO₂] suffered a grain protein
- 350 penalty compared to those grown under $a[CO_2]$ irrespective of N supply.
- Furthermore, additional N was not able to compensate for this decline undere[CO₂].

353 Grain N in cereals is supplied by root uptake during grain filling or by translocation of N previously accumulated in the biomass. In agro-ecosystems where cereals 354 355 ripen under terminal drought conditions that largely inhibit further N uptake, N 356 remobilisation from vegetative biomass (stems and leaves) contributes a large 357 proportion to grain N (Palta et al. 1994; Buchner et al. 2015), placing particular 358 importance on leaf [N]. In our study, N uptake after anthesis (calculated as the difference between maturity and anthesis of the products of N concentration in 359 biomass and biomass; cf. Figure 2) could contribute only about 10% of grain N, and 360 post-anthesis N remobilisation from stems and leaves contributed around 60-65% 361 362 of grain N (the rest was already in heads at anthesis). These figures are broadly

363 consistent with earlier reports in environments with terminal drought (Palta et al.364 1994).

365	Whilst this underlines the importance of post-anthesis remobilisation of nitrogen
366	from vegetative biomass into grains under the prevailing environmental conditions,
367	there was no indication that $e[CO_2]$ changed the extent of that remobilisation.
368	Nitrogen harvest index (NHI), the proportion of grain N in total amount of N in
369	biomass at maturity, results from the sum of N translocation from vegetative
370	biomass, pre-anthesis N uptake into developing heads, and post-anthesis N uptake
371	into grains, and remained unaffected by $e[CO_2]$ (Figure 3). Similarly, post-anthesis N
372	remobilisation from vegetative organs was not affected by elevated $e[CO_2]$ (Figure
373	3).

374 Decreases in [N] in vegetative plant parts, which are well documented under e[CO₂] 375 (Stitt and Krapp 1999, Tausz-Posch et al. 2014), could therefore be directly related 376 to decreases in grain [N], because proportionally less N is available for 377 remobilisation per each g grain yield. At anthesis, leaf [N] in our study averaged 9% 378 lower under e[CO₂]. This is comparable with Buchner et al. (2015) who reported 379 that N concentrations were reduced by about 8% in flag leaves or 9% in second leaves under $e[CO_2]$ as compared to $a[CO_2]$. Additionally, Wang et al. (2013) 380 reported an average 9% decrease in their meta-analysis for wheat. Averages for 381 382 multiple species under FACE conditions were of similar magnitude (Ainsworth and Long 2005, Tausz-Posch et al. 2014). The relative decrease in leaf [N] under e[CO₂] 383 384 was less, albeit still significant, at the vegetative growth stage.

Some previous FACE investigations on wheat found that decreases in leaf [N] under 385 e[CO₂] were less pronounced with adequate N nutrition than under N deficit 386 (Sinclair et al. 2000, Weigel and Manderscheid 2012), and photosynthetic 387 downward acclimation, a response to e[CO₂] commonly linked to decreases in leaf 388 389 [N], was less pronounced under high N (Stitt and Krapp 1999). At the earlier vegetative growth stage in our study, the additional N application had no effect on 390 leaf [N], and leaf [N] was decreased by e[CO₂] regardless of N application. This is in 391 392 agreement with the FACE results reported by (Sinclair et al. 2000) who found no effect of soil fertility (viz. soil N supply) on leaf [N] early in the season, but $e[CO_2]$ 393 decreased leaf [N] regardless of N supply at that stage. At anthesis, our results did 394 395 indicate some attenuation of the decrease in leaf [N] by additional N supply, as 396 shown by the interaction between [CO₂] x N (Figure 1). However, supplying 397 additional N during the vegetative growth phase did not restore leaf [N] under 398 e[CO₂]. In contrast, in Sinclair et al. (2000), leaf [N] depression by e[CO₂] at anthesis was only evident in plants where insufficient N was supplied but not in those 399 adequately supplied with N. In contrast, but in agreement with Wang et al. (2013), 400 401 our results suggest that increased N supply can moderate the effect of e[CO₂] on 402 leaf [N] to some extent, but not restore leaf [N] under e[CO₂]. Insufficient N supply will amplify the effect of e[CO₂] on leaf [N], but is not the sole cause for decreased 403 leaf [N]. The attenuation effect of the additional N application on leaf [N] at 404 anthesis could represent the mitigation of a short-term supply deficit, because in 405 dryland agro-ecosystems (such as the one investigated here) even soils with high N 406 407 status can leave the crop with insufficient mineral N supply during certain stages, 408 because mineralisation rates and crop demand can be temporarily mismatched

(Angus 2001). This effect does however not explain the full extent of leaf [N]
depression under e[CO₂], and alternative mechanisms, such as a direct limitation to
nitrate assimilation (Bloom et al. 2014, Bloom 2015), decreased N allocation to the
photosynthetic machinery due to downward acclimation of photosynthesis or
increased leaf area index (leading to denser canopies), or changes in N mass flow
related to changes in transpiration, are very likely (for review see Tausz-Posch et al.
2014).

Despite these evident decreases in grain and biomass [N], overall N uptake of the 416 crops averaged 20% (or around 30 kg ha⁻¹) greater under e[CO₂]. Whilst N supply by 417 418 mineralisation was sufficient to meet additional crop demand at this high N 419 experimental site in the short term, this may not be sustainable and in the mid to 420 long-term this N will have to be provided by additional inputs. Where biomass and 421 yield stimulation are relatively greater than the decrease in biomass [N], crops will 422 have greater N demands under CO₂-enrichment. This seems to be the case in many, 423 but not all reported analyses (Lam et al. 2012a, b, Chen et al. 2012, Wang et al. 424 2013; Tausz-Posch et al. 2014).

The N source and management methods to meet additional crop demands for N in a high [CO₂] atmosphere need to be carefully considered (Carlisle et al. 2012, Bloom 2015), because N fertiliser can have large negative impacts on the environment (Robertson and Vitousek 2009) and already constitutes a relatively costly and risky farm input in these cropping systems primarily limited by low and unreliable rainfall (Angus 2001). Probably even more important than such quantitative considerations are qualitative aspects of nitrogen management (Bloom 2015), such as selection of nitrogen form to promote uptake of reduced nitrogen so that biochemical
limitations to nitrate assimilation can be circumvented (Carlisle et al. 2012, Bloom
et al. 2014), or appropriate timing of N applications to target grain N (Hooper et al.
2015).

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Table 1. Summary of growing conditions and wheat (*Triticum aestivum* L. cv Yitpi) grain yields and grain [N] across the 12 growing
environments (numbered in the first column) investigated in this study. Grain yields are averages for ambient [CO₂] and two N regimes (NO and
N+), because N treatment had no significant effect on yield (n=8 in each environment). Grain [N] data are averages for ambient [CO₂] and NO
(without additional N), because N treatment significantly affected grain [N]. Time of sowing (TOS) at local practice (TOS1), late (TOS2), inseason water rainfall only (Rain) or rainfall plus supplemental irrigation (Sup) treatments, replicated four times (n=4) in each environment
(Env). Temperatures are reported as seasonal means, mean daily minima and maxima for the growing season from sowing to harvest for each
year. Sowing dates, water inputs, temperatures and days to harvest were previously reported in Fitzgerald et al. (2016).

			Days to	In-season	Air Temperatures	Grain yield	Grain Protein
Env	Year	Sowing date	harvest	water [mm]	(mean, min, max) [°C]	[t grain ha⁻¹]	[%]
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 ¹ (Rain)	15.0, 6.9, 23.1	2.1	14.1
4	2007	Late (23 Aug), TOS2	123	226 ² (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

^{1,2} These data were reported as 159 and 207 mm of in-season rainfall, respectively in Fitzgerald et al. (2016) but are corrected here.

624 Figure Legends

Figure 1. Tissue concentrations of N [% of plant dry weight] of wheat grown in the Australian Grains Free Air CO₂ Enrichment (AGFACE) facility. White columns NO; no N addition. Black columns N+; 50-60 kg ha⁻¹ N added during vegetative growth (before DC30). Each data point represents the mean and SE of n=48 samples (4 replicates in each of 12 growing environments – Table 1). Ambient [CO₂] at 370 µmol mol⁻¹ air⁻¹; Elevated [CO₂] at 550 µmol mol⁻¹ air⁻¹. P-values for effects of CO₂, N and CO₂ x N. ns P≥0.100.

Figure 2. N content in aboveground biomass [g N m⁻² ground area] of wheat grown in the Australian Grains Free Air CO₂ Enrichment (AGFACE) facility. White columns N0; no N addition. Black columns N+; 50-60 kg ha⁻¹ N added during vegetative growth (before DC30). Each data point represents the mean and SE of n=48 samples (4 replicates in each of 12 growing environments – Table 1). Ambient [CO₂] at 370 µmol mol⁻¹ air⁻¹; Elevated [CO₂] at 550 µmol mol⁻¹ air⁻¹. P-values for effects of CO₂, N and CO₂ x N. ns P≥0.100.

639 Figure 3. Left panel: N harvest index (NHI; proportion of grain N in total above ground 640 biomass N content at maturity). Mid panel: N utilisation efficiency (NutE; grain yield 641 divided by total N in biomass at maturity). Right panel: Post-anthesis N remobilisation from stems and leaves (per m² ground area) of wheat grown in the Australian Grains 642 Free Air CO₂ Enrichment (AGFACE) facility. Open symbols and columns N0; no N 643 addition. Black symbols and columns N+; 50-60 kg ha⁻¹ N added during vegetative 644 645 growth. Each data point represents the mean and SE of n=48 samples (4 replicates in each of 12 growing environments – Table 1). Ambient $[CO_2]$ at 370 μ mol mol⁻¹ air⁻¹; 646

Elevated $[CO_2]$ at 550 μ mol mol⁻¹ air⁻¹. P-values for effects of CO₂, N and CO₂ x N. ns

648 P≥0.100.













Figure 3.

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