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Tausz, Michael; Norton, Robert; Tausz-Posch, Sabine; Loew, Markus; Seneweera, Saman; O'Leary, Garry; Armstrong, Roger; Fitzgerald, Glenn

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Michael Tausz[1) 3)*], Robert M Norton[1) 4)], Sabine Tausz-Posch[1)], Markus Löw[1)], Saman Seneweera[1)5)], Garry O'Leary[2)], Roger Armstrong[2)], Glenn J Fitzgerald[2)]

[1)]Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Creswick Victoria 3363, Australia

[2)]Agriculture Victoria, Victoria State Department of Economic Development, Jobs, Transport and Resources, Horsham Victoria 3400, Australia

[3)]Department of Ecosystem and Forest Sciences, The University of Melbourne, Creswick Victoria 3363, Australia

[4)]present affiliation: International Plant Nutrition Institute, Horsham Victoria 3400, Australia

[5)]present affiliation: Centre for Crop Health, University of Southern Queensland, Toowoomba Queensland 4350, Australia

*correspondence:
Michael.tausz@unimelb.edu.au
Department of Ecosystem and Forest Sciences
The University of Melbourne
4 Water Street, Creswick Victoria 3363
Australia

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Abstract

Elevated CO_2 stimulates crop yields but leads to lower tissue and grain nitrogen concentrations [N], raising concerns about grain quality in cereals. To test whether N fertiliser application above optimum growth requirements can alleviate the decline in tissue [N], wheat was grown in a Free Air CO_2 Enrichment facility in a low rainfall cropping system on high soil N. Crops were grown with and without addition of 50-60 kg N ha^{-1} in 12 growing environments created by supplemental irrigation and two sowing dates over three years.

Elevated CO_2 increased yield and biomass (on average by 25%), and decreased biomass [N] (3-9%) and grain [N] (5%). Nitrogen uptake was greater (20%) in elevated CO_2 grown crops. Additional N supply had no effect on yield and biomass, confirming high soil N. Small increases in [N] with N addition were insufficient to offset declines in grain [N] under elevated CO_2. Instead, N application increased the [N] in straw and decreased N harvest index.

The results suggest that conventional addition of N does not mitigate grain [N] depression under elevated CO_2, and lend support to hypotheses that link decreases in crop [N] with biochemical limitations rather than N supply.

Introduction

Atmospheric CO_2 concentration ($[CO_2]$) has been increasing since the industrial revolution and is predicted to reach 550 $\mu L\ L^{-1}$ or more by 2050, that is a 35% change from the current (400 $\mu L\ L^{-1}$ in 2016) concentration (Stocker et al. 2013). Because CO_2 is the main substrate for photosynthesis, such a large increase will affect all plants and ecosystems (Ziska 2008). Many studies demonstrated that, at least in C3 plants, elevated $[CO_2]$ ($e[CO_2]$) stimulates photosynthesis and subsequently growth and yield (Kimball et al. 2002, Ainsworth and Long 2005) through the so-called 'CO_2 fertilisation effect'.

While the 'CO_2 fertilisation effect' may result in greater crop yields and help offset some of the negative effects of climate change on food production (Hatfield et al. 2011), concerns have been raised about reductions in mineral nutrients and grain quality (Högy and Fangmeier 2008, Myers et al. 2014). It is well established that growth under $e[CO_2]$ changes the stoichiometry of plants, whereby the concentration of many minerals, especially nitrogen (N), in plant tissues decreases (Loladze 2002). Because photosynthetic N use efficiency (the photosynthetic carbon fixation rate per g leaf N) increases under $e[CO_2]$, the critical tissue N concentration, i. e. the leaf N concentration ([N]) that is necessary for optimum growth, consequently decreases under $e[CO_2]$ (Conroy and Hocking 1993, Seneweera and Norton 2011, Tausz-Posch et al. 2014). Despite lower tissue [N], the greater biomass reported under $e[CO_2]$ may contain more N per ground surface area than biomass under ambient $[CO_2]$, hence N uptake of the crop may be greater (Tausz-Posch et al. 2014, Lam et al. 2012b). In natural ecosystems, where N is often

68 limiting, the CO_2-stimulation on growth often decreases over time because
69 available N in the soil becomes depleted, a phenomenon termed ‘progressive N
70 limitation‘ (Oren et al. 2001, Luo et al. 2004). In N-managed agro-ecosystems
71 progressive N limitation may not be relevant or immediately apparent, but some
72 experiments have shown that growth stimulation by e[CO_2] is less under low than
73 high N supply (Stitt and Krapp 1999).

74 For non-legume food and fodder crops, decreases in tissue [N] are particularly
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-
80 10% reduction in grain protein concentration in wheat (Högy and Fangmeier 2008;
81 Lam et al. 2012b, Wang et al. 2013), and about 10% for leaf [N] (Ainsworth and
82 Long 2005; Wang et al. 2013).

83 The exact mechanism for the decrease in [N] is unclear, and a number of not
84 mutually exclusive hypotheses have been proposed (Tausz-Posch et al. 2014). The
85 most straightforward one contends that soil N supply does not keep up with
86 increased demand by e[CO_2]-stimulated biomass growth, leading to a ‘dilution’ of N
87 in tissue biomass and N-limitation to biochemistry and growth (Taub and Wang
88 2008). Evidence for this hypothesis comes from experiments where leaf [N]
89 decreased upon e[CO_2] exposure at low, but not at high soil N supply (Stitt and
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 agro-
92 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
94 uptake limitations by reduced transpiration flow (Conroy and Hocking 1993,
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
100 very small (Kimball et al. 2001). This would imply that insufficient soil N supply is
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[CO_2] 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
108 can be increased by N application above the level needed for growth and yield
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[CO_2]
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
126 system responses to e[CO_2] in a realistic field setting, where these environmental
127 and physiological interactions are present. Free Air CO_2 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 CO_2
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 CO_2

environments can ameliorate the e[CO_2]-related decline in biomass and grain [N] of wheat.

Materials and Methods

Site

The Australian Grains Free Air CO_2 Enrichment (AGFACE) facility is located on an 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 in detail in previous papers (Mollah et al. 2009, Fitzgerald et al. 2016). In brief: The experimental plots are on a 7.5 ha field on heavy Vertosol clay soil (~35% clay at the surface and 60% in 1.4 m depth). Long term (30-year) average annual rainfall is 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 agronomic practice are 3-4 t ha^{-1}, but range from 1 to 6 t ha^{-1}. Mean annual growing season temperature is 16.5 °C and the mean annual evaporation rate is around 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 Fitzgerald et al. (2016).

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 [CO_2] (elevated e[CO_2] *viz*. target 550 μmol mol^{-1} air and ambient a[CO_2] *viz.* approximately 370 μmol mol^{-1} air; daytime medians for 2007-2009) that were

each split for two levels of N application (N-sufficient N0 and N addition N+) in four replicates (plots or 'rings'), fully repeated in 12 different growing environments created by various combinations of water supply (rainfed or supplemented irrigation), and sowing times. The irrigation treatments were not designed to create non-limiting conditions but to create conditions within the site that are typical of the multiseason rainfall variability. This approach provides a range of crop yields that are realistic in the region. Two sowing times per year (TOS1 according to local 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 repeated over three growing seasons (2007, 2008, 2009). Sowing dates and an overview over the growing environments investigated in this study is given in Table 1.

Plots were re-established each season, so that wheat was not grown consecutively to avoid soil-borne disease carry over and residual treatment effects from the 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 in 2008 and 2009, each plot was randomly split to irrigation. A plastic barrier buried to 0.8 m depth ensured hydraulic separation between half-plots. In 2007, the 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 and in 2009, 16 m diameter. Details on the FACE system and its performance are given in (Mollah et al. 2009), and more details on experimental design, agronomic treatments, and weather data in (Fitzgerald et al. 2016).

182 Within each half-plot, two N-treatments were allocated to sub-plots, each 1.4 m x 4
183 m with 8 rows of wheat sown in a north-south direction. Rows were spaced either
184 0.214 m (2007, 2008) or 0.195 m (2009) and samples were collected from middle
185 rows, leaving the outside rows as buffers. Plant counts about three weeks after
186 emergence reported an average 120 plants m^{-2}.

187 Pre-sowing soil test results from the sites showed a total soil N of 0.14% (0-10 cm)
188 and mineral N in 0-50 cm depth of 145±50 kg N ha^{-1} in 2007, 233±114 kg ha^{-1} in
189 2008, and 164±98 kg ha^{-1} in 2009. The N sufficient treatment (N0) did not receive
190 any N fertiliser, and the N+ treatment received 50-60 kg N ha^{-1} as urea top dressing:
191 50 kg ha^{-1} before growth stage DC30 (decimal code according to Zadoks et al. 1974)
192 in 2008 and 2009, and split in two times 30 kg ha^{-1} between after sowing and DC31
193 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
197 from sub-plot random row lengths in 2007. The areas sampled were 0.43 m^2 in
198 2007 and 2008 for DC31 and DC65, 1.28 m^2 for DC90 in 2007, 0.86 m^2 for DC90 in
199 2008 and, in 2009 0.4 m^2 for DC31 and DC65, and 0.78 m^2 for DC90. At DC31,
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
203 samples were separated into heads and straw (stems and leaves together), the

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.

Biomass [N] was analysed on dried and ground tissue aliquots by Dumas combustion in an elemental analyser (LECO, TruMac, MI), and grain [N] by near-infrared (NIR) spectrometry calibrated against the elemental analyser method. N content of biomass fractions (leaves, stems, heads) was calculated as: [N] in the fraction x biomass of that fraction expressed on a m^2 ground area basis. N content of biomass fractions were summed for total biomass N content at each sampled growth stage. Nitrogen uptake was calculated as the difference in biomass N content between two sampled growth stages. Post-anthesis N remobilisation from stems and leaves was calculated as the difference between maturity (DC90) and anthesis (DC65) of the products of average [N] in vegetative biomass and that vegetative biomass. As straw samples at maturity (DC90) were not separated into leaves and stems, 'vegetative biomass' refers to stems and leaves taken together. 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 content in grains in total N in biomass at DC90.

Statistical evaluation

This present study addresses potential interactions of N and [CO_2]. 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

227 method (R package nlme, version 3.1-120, Pinheiro et al. 2016) with [CO_2] and
228 environment as main plots, and N-treatment as split-plot.

229 **Results**

230 The grain yield of wheat under a[CO_2] in each environment (combination of
231 irrigation x TOS x season) ranged from just over 1.0 t ha^{-1} to just below 3.5 t ha^{-1}
232 (Table 1). Yields were relatively low, but are typical of the district averages for those
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 N0 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
239 significant interactions with N or CO_2 treatments. Across all environments, e[CO_2]
240 stimulated biomass at maturity and grain yield by about 25%: Biomass at maturity
241 increased from 6.89 (±0.25) to 8.70 (±0.34) t ha^{-1} and grain yield increased from
242 2.30 (±0.09) to 2.87 (±0.13) t ha^{-1} (means (SE) of n = 96 from 24 N x environment
243 combinations). Addition of 50-60 kg ha^{-1} fertiliser N had no significant effect on
244 either biomass or grain yield.

245 Elevated [CO_2] depressed [N] in grains on average by 5%. Nitrogen concentrations
246 in the leaves decreased on average by only 3% at stem elongation, but by 9% at
247 anthesis. The [CO_2] effect was similar for stems at anthesis (9% decrease), but not
248 significant for stems at stem elongation, or for heads at anthesis. Elevated [CO_2] did
249 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[CO_2]. By the time of stem elongation, e[CO_2] grown crops had accumulated about 20% more N per unit area than a[CO_2] 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[CO_2] (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 [CO_2] or N treatments.

There was no significant increase under e[CO_2] 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[CO_2] (between 60-65%).

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[CO_2] (significant interaction). The N treatment also significantly increased grain [N], but this was independent of the [CO_2]-treatment (no significant interaction) and not sufficient to completely restore grain [N] to a[CO_2] values. However, N treatment increased [N] by about 9% in straw, where [N]

was not significantly affected by e[CO_2] (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 [CO_2]-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 2).

Discussion

The lack of yield response to additional application of 50-60 kg ha^{-1} N confirmed that the experimental plots had sufficient soil N for the prevailing growing conditions, especially the range of soil water supply. Yields in water-limited Australian wheat crops are low by global standards (Angus 2001) so that N demands are modest compared to higher yielding production regions in Australia 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 consequence of prior land use of growing lucerne for 5 years, a faba bean crop in 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

295 potential has been reported at 110 kg N ha^{-1} (Bell et al. 2013), compared to a
296 minimum of 145 kg N ha^{-1} (in 2007) at our site. Using both metrics, the site was
297 more than adequately supplied with N.

298 Average grain [N] in the present study was high, translating to between 14 and 15%
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
310 growing environment of [CO_2] and N were only found for two parameters (leaf [N]
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
314 environments have reported greater yield stimulation by e[CO_2] under high than
315 under low N conditions (Stitt and Krapp 1999). Other FACE studies showed no such
316 interaction between [CO_2] and N supply, suggesting that growth and yield
317 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[CO_2]-
321 stimulation of growth and yield was similar under both N treatments.

322 In previous FACE experiments, e[CO_2] decreased wheat grain protein by an average
323 of about 5-10% (Högy and Fangmeier 2008, Taub et al. 2008, Lam et al. 2012b), and
324 results from AGFACE were of similar magnitude (Fernando et al. 2014). Interactions
325 (or lack of interactions) of e[CO_2] with environmental conditions on grain protein
326 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[CO_2] 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[CO_2] and low N, and no interaction between N supply and
335 eCO_2 was reported (Erbs et al. 2010, Wroblewitz et al. 2013). The “high N” rates in
336 those studies were considered sufficient according to local agronomic practice,
337 although it was not specifically demonstrated that N was not limiting for yield. The
338 “low N” rates referred to half the normal fertiliser application, and growth and
339 yields were lower than under “high N”. Furthermore, these studies were conducted
340 in high yielding, high input agro-ecosystems, either under continuous irrigation

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).

344 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
347 targets grain protein would ideally be applied later in the season (Hooper et al.
348 2015). However, there was no interaction between N application and [CO_2] effect
349 on grain [N], indicating that crops grown under e[CO_2] suffered a grain protein
350 penalty compared to those grown under a[CO_2] irrespective of N supply.
351 Furthermore, additional N was not able to compensate for this decline under
352 e[CO_2].

353 Grain N in cereals is supplied by root uptake during grain filling or by translocation
354 of N previously accumulated in the biomass. In agro-ecosystems where cereals
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
359 difference between maturity and anthesis of the products of N concentration in
360 biomass and biomass; cf. Figure 2) could contribute only about 10% of grain N, and
361 post-anthesis N remobilisation from stems and leaves contributed around 60-65%
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[CO_2]
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[CO_2]. 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
380 leaves under e[CO_2] as compared to a[CO_2]. Additionally, Wang et al. (2013)
381 reported an average 9% decrease in their meta-analysis for wheat. Averages for
382 multiple species under FACE conditions were of similar magnitude (Ainsworth and
383 Long 2005, Tausz-Posch et al. 2014). The relative decrease in leaf [N] under e[CO_2]
384 was less, albeit still significant, at the vegetative growth stage.

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

409 (Angus 2001). This effect does however not explain the full extent of leaf [N]
410 depression under e[CO_2], and alternative mechanisms, such as a direct limitation to
411 nitrate assimilation (Bloom et al. 2014, Bloom 2015), decreased N allocation to the
412 photosynthetic machinery due to downward acclimation of photosynthesis or
413 increased leaf area index (leading to denser canopies), or changes in N mass flow
414 related to changes in transpiration, are very likely (for review see Tausz-Posch et al.
415 2014).

416 Despite these evident decreases in grain and biomass [N], overall N uptake of the
417 crops averaged 20% (or around 30 kg ha^{-1}) greater under e[CO_2]. Whilst N supply by
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 CO_2-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).

425 The N source and management methods to meet additional crop demands for N in
426 a high [CO_2] atmosphere need to be carefully considered (Carlisle et al. 2012, Bloom
427 2015), because N fertiliser can have large negative impacts on the environment
428 (Robertson and Vitousek 2009) and already constitutes a relatively costly and risky
429 farm input in these cropping systems primarily limited by low and unreliable rainfall
430 (Angus 2001). Probably even more important than such quantitative considerations
431 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 [CO_2] and two N regimes (N0 and N+), because N treatment had no significant effect on yield (n=8 in each environment). Grain [N] data are averages for ambient [CO_2] and N0 (without additional N), because N treatment significantly affected grain [N]. Time of sowing (TOS) at local practice (TOS1), late (TOS2), in-season 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).

Env	Year	Sowing date	Days to harvest	In-season water [mm]	Air Temperatures (mean, min, max) [°C]	Grain yield [t grain ha^{-1}]	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[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**

625 Figure 1. Tissue concentrations of N [% of plant dry weight] of wheat grown in the
626 Australian Grains Free Air CO_2 Enrichment (AGFACE) facility. White columns N0; no N
627 addition. Black columns N+; 50-60 kg ha^{-1} N added during vegetative growth (before
628 DC30). Each data point represents the mean and SE of n=48 samples (4 replicates in
629 each of 12 growing environments – Table 1). Ambient [CO_2] at 370 μmol mol^{-1} air^{-1};
630 Elevated [CO_2] at 550 μmol mol^{-1} air^{-1}. P-values for effects of CO_2, N and CO_2 x N. ns
631 P≥0.100.

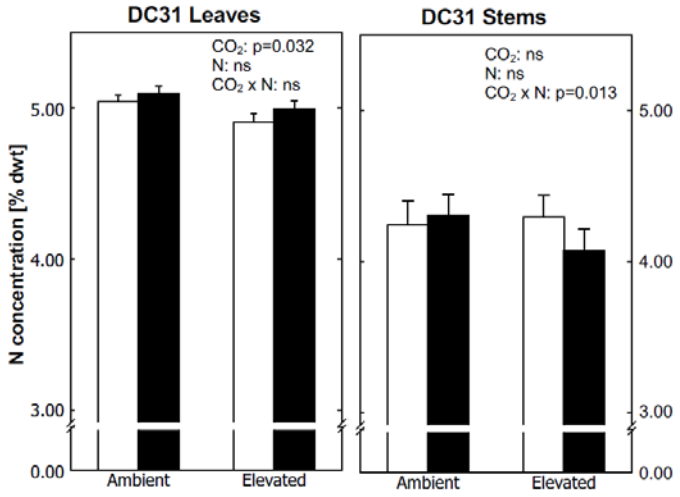
632 Figure 2. N content in aboveground biomass [g N m^{-2} ground area] of wheat grown in
633 the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility. White columns N0; no
634 N addition. Black columns N+; 50-60 kg ha^{-1} N added during vegetative growth (before
635 DC30). Each data point represents the mean and SE of n=48 samples (4 replicates in
636 each of 12 growing environments – Table 1). Ambient [CO_2] at 370 μmol mol^{-1} air^{-1};
637 Elevated [CO_2] at 550 μmol mol^{-1} air^{-1}. P-values for effects of CO_2, N and CO_2 x N. ns
638 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
642 from stems and leaves (per m^2 ground area) of wheat grown in the Australian Grains
643 Free Air CO_2 Enrichment (AGFACE) facility. Open symbols and columns N0; no N
644 addition. Black symbols and columns N+; 50-60 kg ha^{-1} N added during vegetative
645 growth. 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_2] at 370 μmol mol^{-1} air^{-1};

647 Elevated [CO_2] at 550 μmol mol^{-1} air^{-1}. P-values for effects of CO_2, N and CO_2 x N. ns

648 P≥0.100.

649



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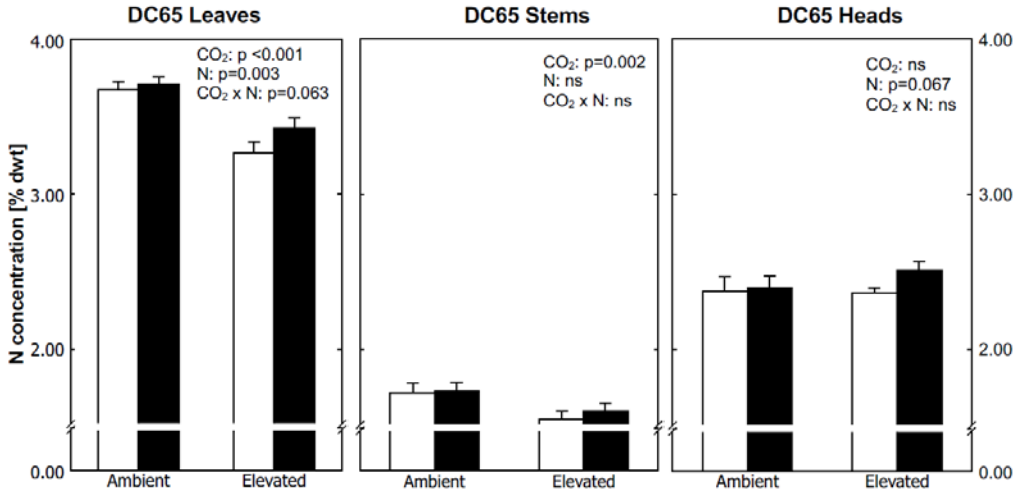

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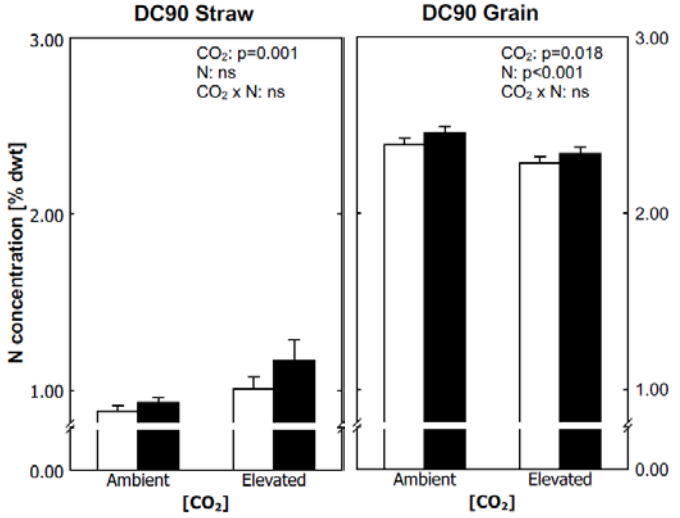
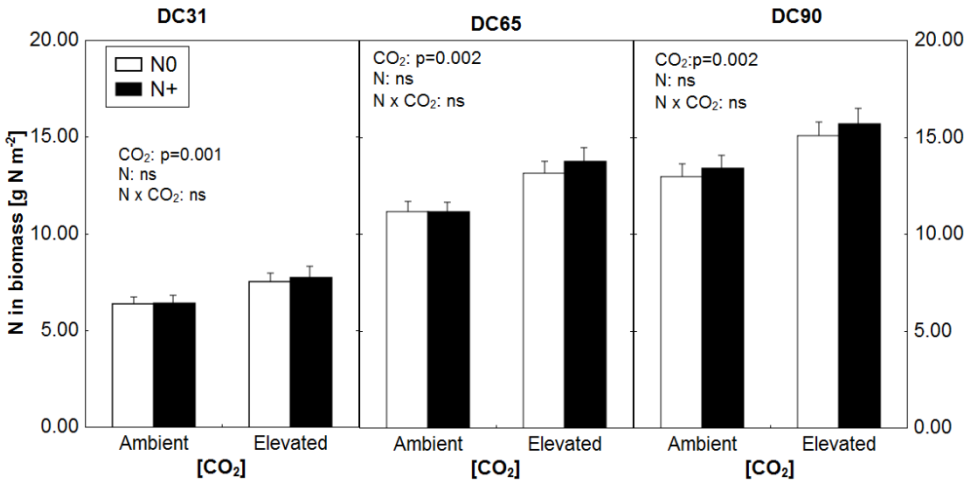


652

653 Figure 1.

654



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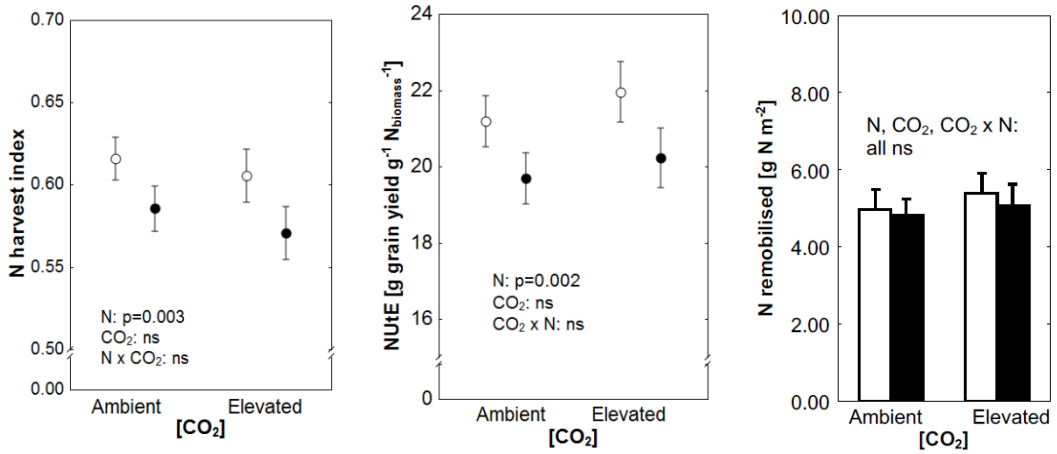
656 Figure 2.



657

658 Figure 3.

659