Genotypic response of wheat under semi-arid conditions showed no specific responsive traits when grown under elevated CO₂

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ABSTRACT
Atmospheric carbon dioxide (CO₂) is predicted to reach 550 µmol mol⁻¹ by 2050, increasing from the current ~410 µmol mol⁻¹ concentration, and this will have an impact on wheat production and grain quality. Genetic differences in response to future CO₂ levels, which might be exploited for higher yield and sustainable grain quality, were investigated. Twelve diverse genotypes (11 wheat lines and 1 triticale cultivar) were grown in the Australian Grains Free-Air CO₂ Enrichment facility under ambient CO₂ (~400 µmol mol⁻¹) and elevated CO₂ (eCO₂) concentrations (550 µmol mol⁻¹) in 2014 and 2015 to test for different responses to CO₂. Genotype response to eCO₂ for the parameters measured showed strong linear relationships. eCO₂ increased plant height (11%), aboveground biomass (31%) and grain yield (32%) as means across all genotypes. Yield response to eCO₂ was driven by increases in spike number and weight. The increase in CO₂ caused a mean 10% decrease in grain nitrogen content and increased grain weight by 7%. Measures of bread dough quality decreased due to eCO₂. Genotypes with large yield response did not show larger than mean reductions in grain %N. The apparent near-universal decline in grain %N under eCO₂ might be compensated for by selection of genotypes that are highly responsive to increasing yields but resist dramatic declines in grain %N. Selection for responsiveness to eCO₂ for yield and grain %N are likely to involve a range of co-related characteristics that balance sink and source relationships.

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Introduction

Atmospheric carbon dioxide ($\text{CO}_2$) is an important environmental factor influencing crop production and is vital for plant photosynthesis. Atmospheric $\text{CO}_2$ levels are predicted to continue to increase from the current concentration of $\approx 410$ to $550$ µmol mol$^{-1}$ by the year 2050 (IPCC, 2013). Future food production might benefit from increasing levels of atmospheric $\text{CO}_2$ if it can capitalize from the additional $\text{CO}_2$. Since phenology, physiological responses, biomass accumulation, yield and processing quality are dependent on genetic factors, environmental variations and their interactions, identifying $\text{CO}_2$-responsive traits would provide plant breeders with information to target traits to maximize the positive effects of elevated $\text{CO}_2$ ($\text{eCO}_2$), such as yield increases, and to minimize the negative impacts such as reduced grain protein concentrations.

$\text{eCO}_2$ results in higher photosynthetic rates leading to the accumulation of carbohydrates, more tillers, greater leaf area index, increased aboveground and belowground biomass, spike number and yield, though different genotypes respond differently (Bourgault et al., 2013; Dias de Oliveira et al., 2015; Franzaring et al., 2013; Kant et al., 2012; Thilakarathne et al., 2013; Ziska et al., 2004). $\text{eCO}_2$ also increases intrinsic water-use efficiency by closing stomata (Leakey et al., 2009), and because of increased early season leaf growth, it can conserve water loss from soil evaporation (Christy et al., 2018). In addition, it also causes a shift in the carbon-to-nitrogen ratio in plants and reduces grain N and protein concentration in grains (Leakey et al., 2009). Understanding these changes creates opportunities to select for traits to maximize yields while slowing the reduction in grain protein.

Some traits that have been studied for response to $\text{eCO}_2$ include transpiration efficiency (TE) (Tausz-Posch et al., 2012) and tillering (Tausz-Posch et al., 2015) in wheat and yield components in rice (Hasegawa et al., 2013). For example, Tausz-Posch et al. (2012) and Christy et al. (2018) found that a TE trait bred into wheat increased yield more under $\text{eCO}_2$ than current $\text{CO}_2$ concentrations. Bourgault et al. (2013) observed that there were no differential genotypic responses between high tillering and restricted tillering wheat lines grown in the glasshouse. However, in a field study, yield response under $\text{eCO}_2$ of two wheat genotypes with different high and restricted tillering showed different mechanisms related to source and sink in a semi-arid environment, indicating potential to manipulate yield components through breeding (Tausz-Posch et al., 2015). In rice, there was a $\text{CO}_2$-by-genotype interaction for brown rice yield and sink-related yield components, indicating potential for active selection of greater sink size to increase yields under $\text{eCO}_2$ more than through passive selection to increasing $\text{CO}_2$ concentrations (Hasegawa et al., 2013).

In semi-arid environments (such as SE Australia), early season vigorous growth followed by late season drought during grain fill can lead to a condition termed ‘hay-off’ (van Herwaarden et al., 1998), where carbon supply to developing grains is reduced, leading to greater biomass relative to grain and often farmers are forced to then cut the crop as hay if yields are very low. Because higher levels of $\text{CO}_2$ allow plants to accumulate more biomass early in the season, if late season available soil water cannot sustain the crop during grain fill, it would result in lower yields and a lower harvest index (HI) (yield/biomass). This has been noted in other studies from the Australian Grains Free-Air $\text{CO}_2$ Enrichment (AGFACE) facility (Fitzgerald et al., 2016) and was also described in a modelling study by Nuttall et al. (2012).

In addition to affecting agronomic and physiological traits, atmospheric $\text{CO}_2$ levels can potentially influence grain quality, in particular, grain protein content and composition, grain size, dough rheology and bread quality (Fernando et al., 2014a; Högy et al., 2009; Panozzo et al., 2014). These changes may occur due to changes in the uptake or remobilization of nitrogen (N) into the grain. The decrease in grain protein content is commonly observed, and sometimes an increase in grain size under $\text{eCO}_2$ is primarily due to a shift in the carbon-to-nitrogen ratio. This would result in possible adverse effects on grain characteristics and ultimately on processing quality traits such as dough rheology (water absorption, development time, stability and extensibility) which are heavily reliant on grain protein content. Some of the changes might be caused by changes in grain protein composition or depend on interaction with other environmental factors (Fernando et al., 2014a, 2014b; Panozzo et al., 2014).

The overall effect of $\text{CO}_2$ on grain yield and quality varies with genotype and environment, and although there is evidence that water-use-efficient lines can contribute to the $\text{CO}_2$ response through stomatal control (Tausz-Posch et al., 2012), mechanisms associated with sink and source allocation (e.g. including tillering, grain number and size) are still unclear (see review by Tausz et al., 2013). For example, it has been suggested that older wheat varieties might have greater response to $\text{eCO}_2$ than more recently released varieties (Tausz et al., 2013; Ziska, 2008) because of more plasticity in tiller formation compared to modern cultivars. Whether there are cultivars that can reverse or slow the decline of grain N concentration is currently unclear. The hypothesis was that genotypes with potentially different source and sink characteristics for C and N would respond differently to $\text{eCO}_2$. This formed the rationale for choosing a broad range of genotypes described in the ‘Material and methods’ section.
Material and methods

Plant material and experimental conditions

Twelve wheat genotypes were used in this study: C342-74, Cudesnaja IW860, EGA Gregory (pedigree Pelsart/2°Batavia doubled haploid line), Excalibur (pedigree RAC177(Sr26)/Uniculm492/RAC311S), Federation (pedigree Purple Straw/Fife/Etawah/Yandilla), Fusion (pedigree Everest/Stylet), WB4-1-12, WB4-1-16, Westonia (pedigree Everest/Stylet), WB4-1-12, WB4-1-16, Westonia (pedigree Everest/Stylet), Y334-05 and Yitpi (pedigree Chamlein*8156)*(Mengavi*Siete Corros) (Chamlein*8156)*Hron*(Mengavi*Siete Corros)*Fame/Yitpi. These were chosen based on their diverse characteristics of sink, source and potential water- and nitrogen-use efficiencies (Table 1). The breeding lines WB4-1-12 and WB4-1-16 are a pair of backcross-derived lines from self-pollinated progeny of a BC4 progeny plant (WB4-1) with Wyalkatchem as its recurrent parent and Burnside as its donor parent. In the Gpc-B1 region, WB4-1-12 (Gpc-B1+) carries donor- parent alleles and WB4-1-16 (Gpc-B1-) carries recurrent- parent alleles (Eagles et al., 2014). The paired spikelet lines, Cudesnaja and IW860, were experimental lines with multiple heads and were hypothesized to provide different sink potentials. Fusion, a triticale, was included because it tends not to fill all seed locations and it was hypothesized that CO2 might enable this process more than under current ambient CO2 (aCO2) conditions. The other genotypes are described in Table 1.

The experiment was a split-plot design with two levels of CO2 as the main factor and 12 genotypes nested within the CO2, replicated four times. Each experimental plot consisted of six rows with each genotype sown as a single 1.8 m row, at a target 160 plants per m² density and 0.225 m spacing between rows. Genotypes were randomly assigned to row positions each season.

Table 1. Genotypes tested under ambient and elevated CO2 field conditions with putative traits.

| Genotype       | Characteristic trait                        |
|----------------|--------------------------------------------|
| Y334-05        | Dwarf, resource partitioning               |
| C342-74        | Dwarf, resource partitioning               |
| Cudesnaja      | Paired spikelet, resource partitioning     |
| IW860          | Paired spikelet, resource partitioning     |
| EGA Gregory    | Nitrogen use efficiency                    |
| Excalibur      | Nitrogen use efficiency                    |
| WB4-1-12       | Grain protein content positive for ‘grain protein content’ gene (Gpc-B1) |
| WB4-1-16       | Grain protein content negative for ‘grain protein content’ gene (Gpc-B1) |
| Federation     | Australian-released variety in 1901, potential difference in sink (tillers and spikes) |
| Fusion         | Triticale (potential difference in sink)    |
| Westonia       | Water-use efficiency                       |
| Yitpi          | Widely grown variety (reference check, less water-use efficient than Westonia) |

The field experiment was conducted at the AGFACE facility in Horsham, Victoria, Australia (36°75’S, 142°11’E; 128 m above sea level) during the 2014 and 2015 seasons. The AGFACE facility is described in detail in earlier studies (Fitzgerald et al., 2016; Mollah et al., 2009; Mollah et al., 2011). The experiment involved exposure to aCO2 (~400 µmol mol⁻¹) and eCO2 (~550 µmol mol⁻¹). There were eight plots per genotype, four for aCO2 and four for eCO2. Exposure to CO2 commenced a week after sowing and the horizontal emission pipes were continually raised during the season to maintain height about 10–15 cm above the canopy. Horsham is a semi-arid grain-production region with a ‘Mediterranean’-type climate. Long-term (1981–2010) mean annual rainfall is 435 mm, with 274 mm typically falling during the growing season (June–November), long-term average minimum temperature is 8.2°C and long-term average maximum temperature is 21.5°C (Australian Bureau of Meteorology). Plants were irrigated when required, to prevent crop loss during these extremely dry years. Water inputs (rainfall and irrigation) from sowing to harvest were 115 and 94 mm in 2014 and 129 and 96 mm in 2015, respectively. In mid-April each year, there were preseason irrigations of 34 mm (2014) and 33 mm (2015). Even counting preseason irrigations, water inputs were below the long-term mean. Bourgault et al. (2017) published soil water data from the same site, years and CO2 levels for a complementary experiment in lentils showing that 2015 had lower plant available water and was about 50% of that in 2014, before later season irrigations were applied.

The AGFACE site was on a Murtoa Clay, which has ~35% clay at the surface increasing to 60% at 1.4 m depth and the soil is a Vertosol according to the Australian Soil Classification (Isbell, 2002). In 2014, the experiment was sown on 29 May and harvested between 26 November and 5 December, and in 2015, it was sown on 26 May and harvested from 1 to 9 December. Harvesting was done over a period of time because of differing maturity dates. Temperature and rainfall data during the growing season were collected from an on-site weather station.

Phenological measurements

Phenological measurements included days to heading, days to flag leaf senescence and days to physiological maturity. Heading date was recorded when 50% of the plants in a plot had fully emerged spikes. Flag leaf senescence was recorded as the date in which 90% of the flag leaves in a plot had completely turned yellow. Physiological maturity was recorded as the date at which 90% of the spikes in a plot had completely turned yellow.
**Measurements at physiological maturity**

Measured biomass parameters included plant height (cm) at heading (DC65 according to Zadoks et al., 1974), tiller weight (g m\(^{-2}\)), spike weight (g m\(^{-2}\)), total aboveground biomass (g m\(^{-2}\)) and HI (= grain yield/aboveground biomass). Plant height was measured from the soil surface to the tip of the spike (excluding awns) in three plants and then averaged. Plants were manually harvested from a 1.2 m row length, excluding 30 cm from both ends of the row. Total aboveground biomass (g m\(^{-2}\)) was measured as the total weight of tillers including leaves (dried at 70°C for 72 h) plus the total weight of spikes (dried at 40°C for 120 h). Spikes were threshed with a Kimseed Multi-Thresher CW08 (Kimseed Engineering, Wangara, WA, Australia) and aspirated with a vacuum separator (Kimseed Engineering). Yield component measures included spikes m\(^{-2}\), grains/spike, grains m\(^{-2}\) and grain yield (g m\(^{-2}\)).

**Grain quality traits**

Grain nitrogen was estimated by near-infrared reflectance (NIR) (RACI-CCD 11-01, 2010) using a Foss XDS NIR Rapid content analyser instrument (FOSS ANA, Denmark). The NIR prediction is based on calibration to Leco Dumas N combustion method (RACI-CCD 02-03, 2010). Grain size distribution was measured through shaking and fractionating the sample in screens of sizes >2.8 mm, between 2.5–2.8, 2.0–2.5 and <2.0 mm (hereby referred to as percentage screenings) using a Sortimat (TypeK3, Pfeuffer, Germany). Thousand grain weight (TGW, g) was measured by weighing 1000 grains. Grain hardness was measured using the Single Kernel Characterisation System (SKCS) (Perten Instruments, Springfield, MO, USA) according to the AACC method AACC-55-31 (2000). Flour milling yield was determined using the Quadrumat Jr. laboratory mill according to AACC-26-50.01 (2000).

**Grain and dough rheology traits parameters**

Dough rheology measurements were derived from Buhler-milled flour. Measure for flour water absorption (%), dough development time (min) and dough stability (BU) were obtained from the Brabender farinograph using AACC-54-21 (2000). Dough extensibility (cm) and dough maximum resistance (Rmax, measured in BU) were determined using the Brabender extensograph (AACC54-10, 2000).

**Data analysis**

Statistical analyses were performed on four replicates per treatment in a combined 2-year (2014 and 2015) analysis. Analysis of variance (ANOVA) was done using the REML algorithm GenStat 18th Edition (Payne et al., 2009) to understand year, CO\(_2\) and genotype main effects, as well as their interactions. Data for all genotypes are presented in Supplemental tables. Individual genotypic responses were compared using Fisher’s unprotected least significant difference test. The CO\(_2\) response ratio (slope of the regression) was obtained by plotting eCO\(_2\) against aCO\(_2\) with the slope of the regression being the CO\(_2\) response and the difference of the slope from one was tested using REML. To understand genotypic response to eCO\(_2\) and normalize environmental differences, the response factor (eCO\(_2\)/aCO\(_2\)) for each genotype was normalized by the 2-year mean response factor ([eCO\(_2\)/aCO\(_2\)]/[2-year mean response factor for all genotypes]) for yield and grain %N as these are generally the most important factors for wheat production. Those with values greater than 1 maintained yield above the mean for the 2-year measurement period, allowing comparison and ranking of responses across the seasons.

**Results**

The effect of eCO\(_2\) across all the measured parameters showed a significant interaction for year in only a few parameters (Table 2). Results are presented for the 2-year analysis with data for individual years presented in the Supplemental Tables (S1–S5).

**Phenology traits**

Number of days to heading was on average 1.2 days less under eCO\(_2\) (Table 2) as a mean across years. The effect of eCO\(_2\) was significant (P < 0.01) for days to maturity in 2014, with crops taking on average 2.3 days longer to mature than under aCO\(_2\) but showed no effect of CO\(_2\) as a mean across years. In 2015, days to flag leaf senescence occurred 3.5 days earlier under eCO\(_2\) than aCO\(_2\) with no effect across years. There were year-by-genotype interactions for the three phenological traits (Table 2).

**Biomass and related traits**

Plants were significantly taller at DC65 (11% or 7.5 cm) under eCO\(_2\) for each year and as a mean across both years (Table 2). Tiller weight and aboveground biomass increased under eCO\(_2\) (P < 0.01; Table 2) by about 30% as a mean across both seasons. There was no main effect of eCO\(_2\) on HI, but there was a significant interaction between CO\(_2\) and genotype in 2015 (P < 0.01).
Yield and yield-related traits

eCO₂ increased 2-year pooled mean spike number m⁻², grain number m⁻² and yield (Table 2) by 35%, 28% and 32%, respectively. Grains/spike did not show a significant CO₂ effect across years. When four outliers were removed (see below), eCO₂ stimulated spike weight (Figure 1(b,c)) more than spike number (43% vs. 23%). While overall yield response to spike weight response showed a highly linear ($r^2 = 0.94$) relationship (Figure 2(a)), the actual increase in yield response due to the spike weight response was only 6% (Figure 2(a), slope of regression). Yield response increased much more when spike number response increased (Figure 2(b), although the relationship was more variable ($r^2 = 0.75$). For yield and spike weight in 2015, four lines (C342-74, EGA Gregory, Excalibur, Fusion) did not show a response to eCO₂ (Figure 1). When these were excluded, the response for the remaining genotypes was a 44% and 43% increase for yield and spike weight due to eCO₂ (Figure 1(a,b)) pooled across years, respectively. Spike number (m⁻²) showed a similar effect from these four genotypes (Figure 1(c)). These outliers showed very high leverage in an ANOVA, which did not fit the regression model and the residuals did not appear to be random, indicating a very distinct relationship to yield. When response to eCO₂ was ranked as shown in Table 3, Westonia and the WB4-1-16 lines ranked above 1 for yield.

Grain and dough quality characteristics

eCO₂ had no effect on grain size distribution measured as screenings above 2 mm (Table 2), TGW was increased by 7% and grain %N concentration was reduced by 10% (Table 2). Milling yield was not affected as a mean across years, but there was a significant interaction with CO₂ and year (for 2015) with slight (1–2%) changes and a CO₂-by-genotype (Table 2) interaction. Grain hardness showed a CO₂-by-year interaction (Table 2). Water absorption, dough development time, dough stability and dough extensibility were reduced by 2%, 16%, 15% and 5%, respectively, due to eCO₂ and dough Rmax was increased (Table 2) in 2015 by 5%. Genotypes WB4-1-12, Y334-05, Excalibur, Fusion and IWA860 maintained grain %N response above the mean (Table 3). The genotypes

Table 2. Combined 2-year data set showing ambient (aCO₂) and elevated (eCO₂) means, significance of experimental year, CO₂ main effects and their interaction on different traits.

| Trait type and trait | Mean aCO₂ | Mean eCO₂ | Response (eCO₂/aCO₂) | Year CO₂ | Year × Genotype | Year × CO₂ | Genotype × CO₂ |
|---------------------|-----------|-----------|----------------------|----------|----------------|-----------|---------------|
| **Phenology**       |           |           |                      |          |                |           |               |
| Days to heading     | 132 (1.0) | 130.8 (1.3) | 0.99 *** * ***     | NS       | NS             | NS        | NS            |
| Days to maturity    | 167.5 (1.0) | 168.0 (0.9) | 1.00 NS NS ***     | *** NS   | ***            | NS        | NS            |
| Days to flag leaf senescence | 161.4 (1.6) | 160.2 (1.8) | 0.99 NS NS **      | *        | NS             | NS        | NS            |
| **Biomass**         |           |           |                      |          |                |           |               |
| Plant height (cm)   | 64.5 (1.7) | 72.0 (1.8) | 1.11 ** ** **       | NS       | NS             | NS        | NS            |
| Tiller weight (g m⁻²) | 324 (29) | 423 (30) | 1.30 NS ** *        | NS       | NS             | NS        | NS            |
| Aboveground biomass (g m⁻²) | 645 (57) | 845 (58) | 1.31 NS ** **       | NS       | NS             | NS        | NS            |
| Harvest index       | 0.31 (0.01) | 0.32 (0.01) | 1.03 NS NS ***     | NS       | NS             | NS        | NS            |
| **Yield and components** |           |           |                      |          |                |           |               |
| Spikes (m⁻²)        | 294 (26) | 396 (25) | 1.35 * *** ***     | NS       | NS             | NS        | NS            |
| Spike weight (g m⁻²) | 321 (29) | 423 (30) | 1.32 * ** **       | NS       | NS             | NS        | NS            |
| Grains (spike⁻¹)    | 18.7 (1.1) | 20.2 (1.3) | 1.08 NS NS NS       | NS       | NS             | NS        | NS            |
| Grains (m⁻²)        | 5121 (563) | 6569 (621) | 1.28 NS ** **       | NS       | NS             | NS        | NS            |
| Yield (g m⁻²)       | 219 (21) | 288 (22) | 1.32 NS NS NS       | NS       | NS             | NS        | NS            |
| **Grain quality**   |           |           |                      |          |                |           |               |
| Screen below 2 mm (%) | 1.46 (0.34) | 1.33 (0.21) | 0.91 * NS NS        | NS       | NS             | NS        | NS            |
| Screen 2.0–2.5 mm (%) | 9.9 (2.0) | 7.4 (2.2) | 0.75 NS NS NS       | NS       | NS             | NS        | NS            |
| Screen 2.5–2.8 mm (%) | 29.5 (2.2) | 25.8 (2.8) | 0.87 NS NS NS       | NS       | NS             | NS        | NS            |
| Screen above 2.8 mm (%) | 58.4 (4.0) | 64.4 (5.0) | 1.10 NS NS NS       | NS       | NS             | NS        | NS            |
| Thousand grain weight (g) | 37.9 (1.0) | 40.4 (1.2) | 1.07 NS NS NS       | NS       | NS             | NS        | NS            |
| Grain N concentration (%) | 2.78 (0.14) | 2.49 (0.13) | 0.90 * ** ***       | NS       | NS             | NS        | NS            |
| Grain hardness (SKCS units) | 67.9 (1.8) | 66.4 (2.1) | 0.98 NS NS NS       | NS       | NS             | NS        | NS            |
| Milling yield (%)    | 70.6 (0.6) | 70.7 (0.6) | 1.00 NS NS NS       | NS       | NS             | NS        | NS            |
| **Dough quality**   |           |           |                      |          |                |           |               |
| Water absorbance (%) | 62.7 (0.7) | 61.2 (0.7) | 0.98 NS NS NS       | NS       | NS             | NS        | NS            |
| Dough development time (min) | 6.3 (0.5) | 5.2 (0.5) | 0.84 NS NS NS       | NS       | NS             | NS        | NS            |
| Dough stability (BU) | 7.9 (0.5) | 6.7 (0.6) | 0.85 NS NS NS       | NS       | NS             | NS        | NS            |
| Dough extensibility (cm) | 20.6 (0.5) | 19.6 (0.5) | 0.95 NS NS NS       | NS       | NS             | NS        | NS            |
| Dough Rmax (BU)      | 475 (15) | 476 (13) | 1.00 NS NS NS       | NS       | NS             | NS        | NS            |

Numbers in parentheses are standard error of the means (n = 12) of the genotypes.

* P < 0.05; ** P < 0.01; *** P < 0.001; NS, not significant.

a BU Brabender Units (see text)

Genotype: All parameters were significant (P < 0.001).

Year × Genotype × CO₂: There were no significant three-way interactions for any parameter.
Y334-05 and IWA860 showed very low yields which may have allowed them to maintain higher grain %N levels due to lack of N dilution known to occur at higher yields (Oury et al., 2003; Oury & Godin, 2007).

Individual genotype responses to eCO$_2$

Fisher's unprotected least significant difference test ($P < 0.05$) was performed on individual genotypes (Table 4) comparing CO$_2$ response across the pooled 2 years between aCO$_2$ and eCO$_2$ to test whether the different strategies used to breed for these traits were effective in imparting a response in the parameters measured under eCO$_2$. There were no significant individual genotypic responses to eCO$_2$ for grains/spike, days to maturity, days to flag leaf senescence, screenings above 2 mm, dough Rmax or dough hardness.

Among the genotypes compared in this study, there were two dwarf genotypes (C342-74 and Y334-05). For Y334-05 yield, HI, plant height and other parameters were extremely low indicating it did not perform well in this dry environment. However, it showed increased response to CO$_2$ for spikes m$^{-2}$ (32%), TGW (11%) and milling yield (5%) as well as reduction in grain %N (12%) and two dough quality parameters (Table 4). C342-74 showed increased responses to eCO$_2$ for TGW (9%) and reductions in grain %N (12%) and two of the dough rheology measures. It maintained yields in the general range of the other genotypes and was among the highest yielding in 2015 (Table S3b). The normalized response of C342-72 to eCO$_2$ (Table 3) was among the lowest, indicating that in this semi-arid environment it did not have characteristics lending it to CO$_2$ responsiveness and in 2015 was one of four genotypes that did not respond to eCO$_2$ (Figure 1). C342-74 ranked below the mean for grain %N response.

The paired spikelet genotypes (Cudesnaja and IWA860) were hypothesized to have more sink capacity compared to more traditional genotypes due to increased number of spikelets for higher grain number. They increased tiller weight under eCO$_2$ (45 and 54%, respectively, Table 4) and height (20 and 11%) but otherwise showed different responses to other parameters (Table 4).

Comparing the two contrasting sister lines, WB4-1-12 and WB4-1-16, which were chosen due to differences in the ‘GPC’ (Grain Protein Content) gene (Table 1), both showed significant increases in spike weight, above-ground biomass and dough stability. Each showed differences in other parameters (Table 4). Grain %N was greater for WB4-1-12 (with the gene maintaining grain protein) than WB4-1-16, as would be expected, and WB4-1-12 did not show a significant decline due to eCO$_2$, while WB4-1-16 did. Across all genotypes there was a reduction in grain %N, and in 2014, these two lines were slightly below the mean, but in 2015 and as a mean across both years, these ‘GPC’ lines were at or above the mean response (Table 3).

EGA Gregory and Excalibur are putatively more nitrogen efficient and the hypothesis was that these genotypes might not be as affected by the decrease in grain %N under eCO$_2$; however; both showed reductions in grain %N, albeit Excalibur only 8% compared to EGA Gregory of 13%. eCO$_2$ increased plant height by 10% for EGA Gregory and TGW for both (Table 4). Excalibur showed a better than mean response for grain %N when normalized across both years (Table 3).
Federation, a tall genotype released in 1901, was included as there are reports of greater yield responses for older varieties of wheat compared to modern ones (Ziska, 2008) but greater yield response from this older genotype was not observed in this study. Federation responded to eCO$_2$ by increasing tiller weight (26%), TGW (10%) and height (14%) as well as reducing grain %N (by 11%) and dough quality parameters (Table 4). The normalized yield and grain %N response for Federation equalled the mean (0.99) of other genotypes (Table 3) indicating it did not have traits that were as responsive to eCO$_2$ in this environment.

Fusion increased height by 12%. Fusion performed better than the normalized mean response for grain %N but was among the lowest in both years for yield response (Table 3). The lack of response in 2015 was noted as one of the five genotypes that did not respond to eCO$_2$ (Figure 1).

Westonia was selected for its purported water-use efficiency trait. Water-use efficiency is increased under eCO$_2$ (Leakey et al., 2009), and previous research in the AGFACE has shown that a transpiration efficient cultivar yielded better under eCO$_2$ than its less-efficient parent (Christy et al., 2018; Tausz-Posch et al., 2012). Westonia showed a small response to CO$_2$ in 2015, but in 2014, it had an 86% increase in yield (Table S3b) under eCO$_2$ compared to aCO$_2$ and therefore showed a better than mean response when normalized across the two years (Table 3) with a

**Table 3. Normalized yield response and yield means for 12 genotypes averaged across both years for yield and grain %N ranked in order of normalized value.**

| Genotype  | Normalized yield response | Yield (g m$^{-2}$) | Genotype  | Normalized grain N response | Grain N (%) |
|-----------|---------------------------|--------------------|-----------|-----------------------------|-------------|
| Fusion    | 0.85                      | 472                | Cudesnaja | 0.97                        | 2.81        |
| C342-74   | 0.89                      | 334                | Yipti     | 0.97                        | 2.31        |
| IWA860    | 0.90                      | 55                 | EGA Gregory | 0.98                        | 2.05        |
| EGA Gregory | 0.93                    | 358                | C342-74   | 0.98                        | 2.16        |
| Excellibur | 0.93                     | 383                | Westonia  | 0.99                        | 2.16        |
| WB4-1-12  | 0.94                      | 297                | Federation | 0.99                        | 2.36        |
| Federation | 0.96                     | 261                | Yipti     | 0.99                        | 2.58        |
| Westonia  | 1.05                      | 416                | Y334-05   | 1.02                        | 3.83        |
| WB4-1-16  | 1.16                      | 389                | Excellibur | 1.02                        | 2.27        |
| Cudesnaja | 1.29                      | 75                 | Fusion    | 1.03                        | 2.12        |
| Y334-05   | NA                        | 69                 | IWA860    | 1.04                        | 2.90        |

Values greater than 1 indicate mean yield or grain %N was better than the global 2-year mean. Yield and Grain N (%) values are those for elevated CO$_2$. NA: Not applicable (unrealistic response, see text).

**Table 4. Significant individual genotype response to eCO$_2$ (eCO$_2$ vs. aCO$_2$).**

| Genotype  | Spikes/ m$^{-2}$ | Spike wt (g/m$^{-2}$) | Tiller wt (g/m$^{-2}$) | Yield (g/m$^{-2}$) | AGB (g/m$^{-2}$) | TGW (g) | Screen (<2 mm) | Grains/m$^{2}$ | DC65 Height (cm) | N Grain (%) | Milling Yield (%) | DDT (min) | Stability (BU) | Water Abs (%) | Dough Ext (cm) |
|-----------|------------------|-----------------------|------------------------|-------------------|------------------|---------|---------------|---------------|------------------|------------|------------------|-----------|----------------|-------------|--------------|
| C342-74   | 1.45             | 1.09                  |                        |                   |                  |         |               |               |                  | 0.88       | 0.97             | 0.97      | 0.97          |              |              |
| Cudesnaja |                  |                       |                        |                   |                  |         |               |               |                  |            |                  |           |               |              |              |
| WB4-1-12  | 0.98             | 1.32                  | 1.30                   |                   |                  | 1.18    |               |               |                  | 0.90       | 0.84             | 0.77      | 0.97          |              |              |
| WB4-1-16  | 1.34             | 1.64                  | 1.55                   | 1.64              | 1.60             | 1.56    |               |               |                  | 0.90       | 0.84             | 0.77      | 0.97          |              |              |
| EGA Gregory | 1.08            | 1.10                  |                        |                   |                  |         |               |               |                  | 0.87       | 0.77             |           |               |              |              |
| Excellibur |                |                       |                        |                   |                  |         |               |               |                  | 0.92       |                  | 0.97      | 0.97          |              |              |
| Federation | 1.26             | 1.10                  |                        |                   |                  |         |               |               |                  | 0.89       | 0.72             | 0.97      | 0.97          |              |              |
| Fusion    |                  |                       |                        |                   |                  |         |               |               |                  |            |                  |           |               |              |              |
| IWA860    | 1.54             | 0.52                  | 1.11                   |                  |                  |         |               |               |                  |            |                  |           |               |              |              |
| Westonia  | 1.22             | 1.41                  | 1.35                   | 1.38              | 1.08             | 1.30    | 1.13           |               |                  | 0.88       |                  | 0.88      | 0.88          |              |              |
| Yipti     | 1.22             | 1.38                  | 1.35                   | 1.40              | 1.36             | 1.34    | 1.13           |               |                  | 0.87       | 0.81             | 0.81      | 0.97          |              |              |
| Y334-05   | 1.32             | 1.11                  |                        |                   |                  |         |               |               |                  | 0.88       | 1.05             | 0.82      | 0.85          |              |              |

Fisher’s unprotected least significant difference test was used to test differences between 2-year pooled mean values ($P < 0.05$). Only parameters that had significance are shown. DTH: days to heading; AGB: aboveground biomass; TGW: thousand grain weight; Screen: screenings; DDT: dough development time; Water Abs: water absorbance, Dough Ext: dough extensibility.
41% response to eCO₂ (Table 4). Other biomass and yield components increased correspondingly, ranging from 22% to 41%: spikes m⁻², spike weight, tiller weight, above-ground biomass and grains m⁻². Height increased by 13% and TGW by 8%. Grain %N was reduced by 12% but there was no effect to dough quality parameters. It was also among the highest yielding in both years (Table S3b).

Discussion

The different yield component and yield responses in wheat genotypes between years seem to have been driven by differences in available water, affecting biomass, seed set and translocation during grain filling. The plant available water at this site was about 50% lower in 2015 than 2014 (Bourgault et al., 2017), before later season irrigations were applied. There has been a hypothesis that eCO₂ can stimulate yields proportionally more in dry years than wet (Kimball, 2016), and because plants under eCO₂ are more water use efficient (Leaky et al., 2009), this can potentially result in water savings that can be utilized later in the season. More recent evidence is emerging that this may not be the case (Bourgault et al., 2017; Christy et al., 2018; Gray et al., 2016), and in this study, the overall yield response to eCO₂ of 58% in 2014 (the wetter year) vs. 12% in 2015 supports this. Greater early season growth and canopy development before flowering may increase accumulation of reserves such as water-soluble carbohydrates for later season translocation (Gebbing & Schnyder, 1999; Zhu et al., 2010), although a recent paper by Macabuhay et al. (2017) in AGFACE showed that an eCO₂-induced increase in WSC may not translate into increased yields due to lack of available water under terminal drought conditions. There appears to be a more complex interaction between crop water-use efficiency under eCO₂, greater leaf growth and early season development and carbohydrate translocation between flowering and maturity that is still to be fully elucidated.

In 2015, four genotypes (C342-74, EGA Gregory, Excalibur, Fusion) showed no response to eCO₂ (slope = 0.96 for aCO₂ vs. eCO₂). Without these genotypes for yield, the eCO₂ response for all other treatments for both years was 44%, similar to values reported for previous years under hotter and drier conditions in the AGFACE (Fitzgerald et al., 2016) but much higher than other reports (Ainsworth & Long, 2005; Long et al., 2006; Wang et al., 2013). These genotypes had the highest biomass in 2015 and reduced response for these genotypes in 2015 might indicate hay-off, which can occur in environments that experience late season water stress and where the carbohydrate source is not transferred to the sink resulting in a large biomass, low yield and pinched grain (Nuttall et al., 2012; Passioura & Angus, 2010; van Herwaarden et al., 1998). Typically, hay-off is measured by the yield/biomass-at-anthesis ratio, and if this reduces, there is indication that translocation was reduced due to drought. Although biomass at anthesis was not measured in this study, biomass, weight of tillers, weight of spikes, spike and grain number followed the same low or non-responsive relationship for these genotypes for eCO₂ and so there was generally lower response for all factors that contribute to carbohydrate uptake. As was noted above, available soil water was lower in 2015 than 2014 and so plants may have run out of water during translocation in 2015. However, yields for the aCO₂ conditions for these genotypes were higher than for the others, and it was just the response to eCO₂ that was lacking. Hence, this may have been a hay-off effect, and the higher yields could not be sustained under eCO₂ under very dry conditions.

One hypothesis for this study was that genotypes with potentially different source and sink characteristics would respond differently to eCO₂, and this formed the rationale for choosing high grain protein sister lines, tall and dwarf, N and water use efficient, paired spikelets, triticale and the first Australian-released genotype, Federation, released in 1901. The hypothesis was that old and new genotypes might respond differently and that paired spikelets might have more sink capacity and be more responsive in terms of grains/spike and ultimately quality and yield, in line with that reported by Ziska (2008) who found older cultivars had greater response to eCO₂. Generally, the dwarf (C342-74, Y334-05) and paired spikelets (Cudesnaja, IWA860) did not respond well in this environment. C342-74 showed biomass, yield and quality-related values similar to others in this experiment, but the other three of this group had exceptionally low values for many key biomass and yield components. They did not respond differently to eCO₂, and particularly since yields and H₁ were so low, no clear conclusions can be made for responsiveness to eCO₂. Federation was developed for dryland conditions in Australia at the beginning of the twentieth century, and this is presumably adapted to conditions present in this study. However, it did not perform any better or worse than others in this cohort of genotypes, and thus, unlike earlier results (Ziska, 2008), this older cultivar did not show any advantage under eCO₂.

To take greatest advantage of the ‘CO₂ fertilization effect’, it is necessary to know which specific traits are most responsive to eCO₂ for selection. Shimono et al. (2009) concluded that spikelets and consequently spikes m⁻² was a major determinant of cultivar response to CO₂ in rice and hence could potentially be used to select cultivars at heading. This is in line with the
relationship found in this study for spikes m\(^{-2}\) and yield, albeit at maturity and across all genotypes. Spike weight was a strong determinant of yield across the different environments and genotypes, but there was a lower response to eCO\(_2\) than spikes m\(^{-2}\) pooled across all genotypes. For those specific genotypes with significant yield response (WB4-1-16, Westonia, Yitpi), spike number, spike weight, tiller weight and aboveground biomass were all significant indicating their potential role in driving yield increase response to eCO\(_2\). Thus, although traits tested in the experiment (dwarf, paired spikelets, etc.) did not show specific responses to eCO\(_2\), increased spike number and spike weight and aboveground biomass did appear to be general drivers for increasing the response of wheat to eCO\(_2\).

Response to CO\(_2\) varies by environment and cultivars (Högy et al., 2009; Kimball et al., 1995) and so comparison of relative response allows ranking of CO\(_2\) impacts to individual genotypes similar to that suggested by Kumagai et al. (2016). They suggested standardizing a regression coefficient response comparing rice and soya bean responses to eCO\(_2\). Here, the data set was much smaller, the species were similar (wheat and triticale) and the data were from the same experiment, and so standardizing to accommodate wider ranges in yield was not needed and comparison to the treatment means was deemed appropriate. Therefore, normalized values above 1 represented those with greater response to eCO\(_2\) than the mean of the data set. The highest value was noted for one of the lowest yielding genotypes, Cudesnaja. It is possible that the yield response was due to a small increase from a very low base resulting in relatively high responses. Ranking next after these for yield was WB4-1-16, the sister line to the Gpc-B1 gene WB4-1-12 followed by Westonia, the purported water-use-efficient genotype. If Y334-05 and Cudesnaja are excluded, then Yitpi showed a 2-year response that was greater than 1. The responses do not indicate a clear picture of the purported traits for these genotypes and any one strategy for the various genotypes to respond to eCO\(_2\). The high ranking and strong individual responses of the WB4-1-16 line suggests it may have been inadvertently selected for a greater sink capacity, certainly WB4-1-12, WB4-1-16, Westonia and Yitpi show significant and strong increases in spike weight due to eCO\(_2\), but it hints at the potential of selection of traits that optimize response to CO\(_2\). The response of these modern genotypes is consistent with what has been reported in various other studies that have used modern genotypes (Bourgault et al., 2013; Tausz-Posch et al., 2015; Thilakarathne et al., 2013).

HI has not been shown to be often affected by eCO\(_2\) (Wang et al., 2013), except under extreme dry or hot conditions at the AGFACE experimental site (Bourgault et al., 2017; Fitzgerald et al., 2016) and elsewhere (Kimball et al., 1995). In this current experiment, although HI showed an interaction between genotype and CO\(_2\), this was due to some genotypes increasing and others decreasing across both years and there were no outstanding patterns to indicate trends. If there are impacts of eCO\(_2\) on HI, there are likely environment-by-genotype interactions that would require targeted experiments across a range of controlled water and temperature conditions. The increase in yield or HI under eCO\(_2\) could also be due to non-significant but incremental increases in the various yield components.

The almost universal depression of grain %N due to eCO\(_2\) (Broberg et al., 2017; Fernando et al., 2014a; Högy et al., 2009) reduces grain quality, which translates to reductions in bread quality (Panozzo et al., 2014) and will work against breeding efforts to increase grain protein as yields and eCO\(_2\) increase in the coming decades. In a review of 25 studies conducted over a 10-year period, the role of the Gpc-B1 gene in the WB4-1-12 line in increasing protein content was demonstrated with little penalty to yield in various backgrounds and environments, in both tetraploid and hexaploid wheat (Tabbita et al., 2017). The values for grain %N in WB4-1-12 were higher than the WB4-1-16 line, consistent with the above-mentioned studies and they were statistically different in this environment but were reduced by eCO\(_2\) to a similar degree (~10%). It does not appear therefore that there is any inherent resistance to %N reduction per se due to eCO\(_2\). However, because both lines and the WB4-1-16 line, in particular, showed high yields under eCO\(_2\) and the analysis of the individual genotypes showed that multiple parameters were responsive to eCO\(_2\), it suggests that responsiveness to eCO\(_2\) under semi-arid conditions may involve selection of a range of co-related characteristics. The WB4-1-12 line maintained high yield response relative to other genotypes despite a similar drop in grain %N, suggesting that greater responsiveness of yield to eCO\(_2\) is not entirely linked to reduction in grain %N (i.e. ‘dilution’). Regardless, there does not appear to be any obvious reason why these lines, in particular, should have a more stable and high yield response to eCO\(_2\).

Although grain and dough quality characteristics were altered due to eCO\(_2\), there were clear differences across years or genotypes indicating that the environment plays a role in expression of eCO\(_2\) effects on quality. The interactions with CO\(_2\), such as for milling yield, did not indicate any patterns of significance for individual genotypes. The overall effect of eCO\(_2\) on grain and dough, when significant, was reduction in dough quality and grain %N but increases in TGW all of which are consistent with other reports (Fernando et al., 2014a, 2014b; Högy et al., 2009; Panozzo et al., 2014). In this
Conclusions

Although there was no clear response to eCO₂ based on specific traits tested, it does appear that yield increase was driven by responsiveness in spike number and weight, and tiller weight and aboveground biomass were also important for those genotypes with greatest response. This suggests that genotypes that can increase these yield components preferentially will confer better yield response under eCO₂. It may be that plant breeding will require simultaneously targeting small but interacting increases in these yield parameters that have a net positive feedback. Targeting specific physiological pathways that increase carbon mobilization to grain through setting up early season growth and more efficient translocation under drought conditions might allow greater utilization of CO₂ for yield in semi-arid conditions. However, it may be that without sufficient water, stimulation of growth by eCO₂ cannot be realized. In addition, it would be highly desirable to be able to reverse the decline in grain protein due to eCO₂, but there appears to be a universal reduction in grain N concentration. At this point, there does not appear to be any genetics to reverse this decline. Selection of genotypes that increase yields proportionally more than grain %N declines, would provide a net gain in yield per %N reduction.

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Disclosure statement

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