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Effect of elevated carbon dioxide on plant biomass and grain protein concentration differs across bread, durum and synthetic hexaploid wheat genotypes

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PII: S0733-5210(18)30601-5

DOI: https://doi.org/10.1016/j.jcs.2019.03.007

Reference: YJCRS 2732

To appear in: Journal of Cereal Science

Received Date: 3 August 2018

Revised Date: 4 March 2019

Accepted Date: 9 March 2019

Please cite this article as: Thompson, M., Gamage, D., Ratnasekera, D., Perera, A., Martin, A., Seneweera, S., Effect of elevated carbon dioxide on plant biomass and grain protein concentration differs across bread, durum and synthetic hexaploid wheat genotypes, *Journal of Cereal Science* (2019), doi: https://doi.org/10.1016/j.jcs.2019.03.007.

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1	Effect of elevated carbon dioxide on plant biomass and grain protein concentration
2	differs across bread, durum and synthetic hexaploid wheat genotypes
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9	Keywords: future climate; total grain protein; climate stress; shoot biomass.
10	Abbreviations: e[CO2], elevated carbon dioxide concentration; a[CO2], ambient carbon
11	dioxide concentration; HI, harvest index; GPC, grain protein concentration; GPY, grain
12	protein yield
13	

#### 14 Abstract

Atmospheric carbon dioxide conditions predicted for future climates cause increases in wheat 15 16 biomass, but also decreases wheat grain protein concentration. We investigated the response of grain protein concentration of wheat to elevated carbon dioxide in nineteen wheat 17 genotypes, including five tetraploid, eleven hexaploid and three synthetic hexaploid 18 genotypes to test whether decreased grain protein is genotype dependent and whether it is 19 caused by biomass dilution. These were grown in ambient and elevated carbon dioxide 20 conditions simultaneously. Shoot biomass and grain samples were taken at maturity. The 21 22 grain protein concentration, grain biomass, shoot biomass and harvest index were analysed for each genotype. Despite most genotypes increasing in total grain protein (g), the majority 23 of genotypes decreased in grain protein concentration (%) under elevated carbon dioxide. 24 25 Elevated carbon dioxide caused an increase in grain biomass for all genotypes and total shoot biomass for most genotypes, with harvest index increasing for all genotypes except the two 26 synthetic hexaploids CPI133814 and CPI133811. Most of the differences between wheat 27 types were not statistically significant, suggesting that the individual genotype of wheat 28 plants determines the response to elevated carbon dioxide rather than the wheat type. 29

#### 31 **1. Introduction**

One of the main components of global climate change is the increasing concentration of 32 33 carbon dioxide  $(CO_2)$  in the atmosphere. Under future climates, the increased atmospheric  $CO_2$  concentration ([ $CO_2$ ]) will directly affect the yield, growth and development of crop 34 plants (Ainsworth and Long 2005; Leakey et al. 2009). For wheat (Triticum aestivum), 35 although elevated [CO<sub>2</sub>] (e[CO<sub>2</sub>]) usually improves plant biomass and grain yield 36 (Thilakarathne et al. 2013), the nutritional aspects of the grain suffer the opposite effect, 37 where the concentration of protein and many macro and micronutrients declines (Fernando et 38 al. 2012). With the global human population expected to increase, there will be a greater 39 demand on food production. As such, the effect of climate change on food crops is of great 40 41 concern.

42 Wheat is one of the most important food crops in the world, accounting for nearly a third of the global cereal production in the 2015/2016 season (FAO 2017). Wheat species typically 43 belong to three different ploidy levels, consisting of diploids (2n = 2x = 14), tetraploids 44 4x = 28) and hexaploids (2n = 6x = 42). The hexaploid wheat genome is comprised of seven 45 pairs of chromosomes each in three genomes, called the A, B and D genomes. Hexaploid 46 wheat was created from the hybridisation of the tetraploid T. turgidum (containing the A and 47 B genomes) with the D donor Aegilops tauschii (Matsuoka, 2011). Synthetic hexaploid wheat 48 is created by hybridising these two species, followed by amphidiploidisation (Yang et al. 49 2009). With this method, breeders are able to develop synthetic hexaploid wheat genotypes 50 which incorporate genes from T. turgidum and Ae. tauschii that were not maintained during 51 hexaploid wheat evolution, including traits such as drought tolerance (Reynolds et al. 2007), 52 increased nutrient uptake (Calderini and Ortiz-Monasterio 2003) and pathogen resistance 53 (Wang et al. 2016). These synthetic hexaploids can then be crossed with bread wheat 54

cultivars to transfer across the elite genes and improve upon the bread wheat cultivar (Li *et al.* 2014).

Growth under e[CO<sub>2</sub>] causes increased yields in wheat (Amthor 2001; Högy *et al.* 2009), but
many studies have shown that it also causes a decline in nitrogen stored in the grain at
maturity (Taub *et al.* 2008; Högy *et al.* 2013; Fernando *et al.* 2015). Protein composition of
wheat grain grown under e[CO<sub>2</sub>] is also affected, resulting in lower bread making quality in
some cultivars (Fernando *et al.* 2015). Of the proteins in the grain, storage proteins (glutens),
rather than structural or metabolic proteins, appear to be the most affected by e[CO<sub>2</sub>] (S.
Arachchige *et al.* 2017).

Previous studies have looked at the effect of e[CO<sub>2</sub>] across diploid, tetraploid and hexaploid 64 wheat species (Sinha et al. 2009; Uprety et al. 2009). Uprety et al. (2009) observed that the 65 responses of each species to e[CO<sub>2</sub>] was different depending on the physiological variable 66 measured. For example, variables such as photosynthesis, leaf area, dry weight, grain yield 67 and harvest index (HI) had a greater response to  $e[CO_2]$  in hexaploids and tetraploids than 68 diploids. Sinha et al. (2009) also found differing responses of each ploidy level for their 69 variables studied. Protein concentration in grains decreased for all ploidy levels, though the 70 71 decrease was lowest in tetraploids and highest in hexaploids. How synthetic wheat responds to  $e[CO_2]$  has not previously been determined. 72

A major goal for wheat breeders has been to develop cultivars with improved HI. As such, identifying wheat with a high HI is important for the continual improvement of commercial wheat cultivars. Elevated [CO<sub>2</sub>] increases both the grain yield (Amthor 2001) and shoot biomass (Kimball 2016) of wheat, with the ratio of these two components determining the plant's HI. The stimulation of both biomass and yield at the same magnitude can lead to no change in HI, which has been seen in both hexaploid bread wheat and tetraploid durum wheat

79 (Wang et al. 2013; Aranjuelo et al. 2015; Fitzgerald et al. 2016). Furthermore, some studies have shown HI to both increase and decrease in some wheat cultivars (Uddling et al. 2008; 80 Wang et al. 2013). Thilakarathne et al. (2013) found that increases in grain yield are 81 82 associated with increases to leaf mass area due to  $e[CO_2]$ . As such, the degree that  $e[CO_2]$ increases grain yield, and in turn HI, may rely partly on how leaf mass area is affected. 83 Increased HI, however, may lead to decreased grain protein concentration (GPC) in wheat 84 due to dilution of N with increased carbohydrates (Taub et al. 2008). 85 In this study, we aimed to identify whether the effect of  $e[CO_2]$  on wheat GPC is dependent 86 87 on wheat type and whether GPC decline is affected by HI and/or biomass dilution. We also investigated how e[CO<sub>2</sub>] affects the GPC of synthetic hexaploid wheats. To achieve these 88 aims we grew nineteen wheat cultivars under e[CO<sub>2</sub>] and a[CO<sub>2</sub>], consisting of five 89 90 tetraploid, eleven hexaploid and three synthetic hexaploid genotypes, and analysed their biomass and protein content. One-Way ANOVA analysis was used to determine the 91 significance of  $[CO_2]$  on the traits measured in the study. 92

#### 93 2. Materials and Methods

#### 94 2.1 Plant material and growth conditions

Nineteen wheat genotypes were grown in an environmentally controlled glasshouse at the 95 University of Southern Queensland, Toowoomba, Queensland, Australia, in 2014. The 96 genotypes consisted of five tetraploid durum wheats (Tjilkuri, WID802, Hyperno, Jandaroi 97 and Caparoi), eleven hexaploid bread wheats (Hartog, Sunbri, Longreach Dart, Sunvale, 98 Longreach Crusader, Aus29259, LRC2010-157, Longreach Scout, Longreach Lincoln, 99 Sunguard and Longreach Spitfire) and three synthetic hexaploid wheats (CPI133814, 100 CPI133811 and CPI133898) (Supplementary Table 1). All three synthetic hexaploid 101 genotypes share a similar pedigree, with CPI133814 and CPI133811 being derived from the 102

103	same parental cultivars. The number of genotypes used for each wheat type was limited by
104	the availability of genotypes at the time of the experiment and therefore, is not consistent
105	among wheat types.

106	The average day/night temperatures of the glasshouse chambers were maintained at $20 \pm 2^{\circ}$ C
107	and $17 \pm 2^{\circ}$ C, respectively, with 60-70% relative humidity. During the experiment, the light
108	inside the glasshouse ranged from $700 - 1000 \ \mu mol \ m^{-2} \ s^{-1}$ during midday. A large
109	compartmented glasshouse was used, where ambient $[CO_2]$ (a $[CO_2]$ ) (~389 µmol mol <sup>-1</sup> ) and
110	$e[CO_2]$ (~700± 20 µmol mol <sup>-1</sup> ) were maintained in their respective treatments. All nineteen
111	genotypes were grown in both $CO_2$ conditions (a[ $CO_2$ ] & e[ $CO_2$ ]) at the same time. For each
112	CO <sub>2</sub> treatment all genotypes were grown as four replicates within the same glasshouse
113	chamber, where each replicate consisted of one pot containing four plants. Seeds were pre-
114	germinated and planted into pots containing 2.5kg top soil. Each pot had a diameter of 175
115	mm and were placed with 15 cm between each pot, resulting in a plant density of 36 plants m
116	$^{2}$ . All pots were randomized and rearranged weekly to eliminate chamber effects.

#### 117 2.2 Biomass analysis

Plants were sampled at physiological maturity and separated into leaf blades, stems
(including sheaths) and heads, and dried at 60°C for 48 hours. Heads were hand threshed to
obtain the grain. The grains were weighed to obtain total seed weight. Plant tissues were
weighed to obtain total shoot biomass. Grain number per plant was counted.

122 2.3 Nitrogen analysis

Grain from each genotype was ground using a Millser IFM-800DG grinder (Iwatani, Japan).
A 100mg sample of the ground grain was analysed for its nitrogen concentration using a CN
analyser (LECO CN628 analyser, Michigan, USA). The total GPC was calculated by

- multiplying the total N concentration of grain by the conversion factor of 5.7. Protein contentwas analysed by multiplying the GPC by total grain biomass.
- 128 2.4 Harvest Index
- 129 Harvest index was calculated by dividing the total grain biomass by the total plant biomass
- 130 (grain biomass + shoot biomass):

131 2.5 Statistical analysis

- 132 Statistical analysis to determine significant difference between means of the dependent
- 133 variables (Grain biomass, total shoot biomass, GPC and HI) was performed using Compare
- 134 Means in IBM SPSS Statistics ver. 23 (IBM Corp., 2015, Armonk, NY). Statistical
- 135 significance was determined using a One-Way ANOVA with the wheat type (tetraploid,
- hexaploid and synthetic hexaploid) as the independent factor and CO<sub>2</sub> response (GPC, grain
- 137 biomass, total shoot biomass and HI) as the dependent variable. CO<sub>2</sub> response was calculated
- as the difference between the  $e[CO_2]$  data and the  $a[CO_2]$  data. Results were regarded as
- 139 significant at  $P \le 0.05$ .
- 140 IBM SPSS Statistics ver. 23 was also used to perform a Pearson product-moment correlation
- 141 test using Correlate to analyse any correlation between grain biomass and total grain protein.
- 142 Statistical significance was determined with Bivariate Correlations using grain biomass and
- total grain protein as the variables. Results were regarded as significant at  $P \le 0.01$ .
- 144 **3. Results**
- 145 *3.1 Grain protein concentration and total grain protein*
- Of the 19 genotypes tested thirteen had a lower GPC under e[CO<sub>2</sub>] compared to a[CO<sub>2</sub>] (Fig.
  1, Table 1). Among the tetraploid wheat genotypes, Caparoi, Jandaroi, WID802 and Hyperno
- had a lower GPC in plants grown under e[CO<sub>2</sub>]. Similarly, of the hexaploid genotypes, GPC

declined in Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout and Sunvale, while it also
declined in the synthetic hexaploid genotypes CPI133814 and CPI133898 under e[CO<sub>2</sub>] (Fig.
1). Despite the majority of genotypes decreasing in GPC under e[CO<sub>2</sub>], however, a pairedsamples t test found no significant difference between e[CO<sub>2</sub>] and a[CO<sub>2</sub>] grown wheats for
any of the wheat types.

Tetraploids showed the largest variation of GPC between the least and most responsive 154 genotypes in response to  $e[CO_2]$  between the three wheat types, with Caparoi incurring the 155 greatest reduction of GPC among tetraploids. The largest decrease in GPC occurred in the 156 hexaploid genotype Sunbri where the GPC at  $e[CO_2]$  was 5.04% lower than at  $a[CO_2]$ . On 157 average, synthetic hexaploids had the least decline in GPC under  $e[CO_2]$ , while hexaploids 158 incurred the greatest reduction of GPC. On the other hand, some genotypes in each wheat 159 160 type increased in GPC. For both tetraploids and synthetic hexaploids only one genotype was found to increase in GPC under e[CO<sub>2</sub>], while there were four hexaploid genotypes. 161 Statistical analysis by One-Way ANOVA showed that the effect of e[CO<sub>2</sub>] on GPC was not 162 significantly different between the tetraploid, hexaploid and synthetic hexaploid wheat 163 genotypes examined in this study. 164

165 Despite the majority of genotypes decreasing in GPC in response to e[CO<sub>2</sub>], the total grain protein content increased in 18 out of 19 genotypes when grown under e[CO<sub>2</sub>] (Fig. 1, Table 166 1). Only Lincoln (a hexaploid genotype) decreased in total grain protein content. Similarly to 167 GPC response, Tjilkuri increased the most in total grain protein content when grown in 168  $e[CO_2]$  (Fig. 1). For those genotypes which increased in protein in response to  $e[CO_2]$ , total 169 grain protein content of tetraploids increased on average by 1.03g and by 0.64g and 0.55g for 170 hexaploids and synthetic hexaploids, respectively. There was no significant difference 171 between wheat types for total grain protein, however, a pairwise t test showed that [CO<sub>2</sub>] 172 173 significantly affected the tetraploids (p = 0.033) and hexaploids (p = 0.001).

#### 174 *3.2 Grain biomass and number*

Total grain biomass increased in all genotypes grown under e[CO<sub>2</sub>] compared to a[CO<sub>2</sub>] (Fig. 175 2, Table 2). In addition, the grain number per plant also increased in all genotypes (Table 2). 176 On average, e[CO<sub>2</sub>] stimulated the greatest increase in grain biomass for tetraploid genotypes 177 compared to both hexaploid and synthetic hexaploid genotypes, with the increase in 178 tetraploids averaging about 67% compared to 44% for hexaploids and 34% for synthetic 179 hexaploids. Tetraploids also had, on average, a greater response of grain number. Both of the 180 genotypes which displayed the greatest and least increase in grain biomass, respectively, were 181 Sunbri and Lincoln, both of which are hexaploids (Fig. 2). Despite the differences in average 182 grain biomass response to  $e[CO_2]$ , there was no significant difference between the three 183 groups. On the other hand, the three synthetic hexaploid genotypes were significantly 184 185 different in grain number compared to both tetraploids and hexaploids. A paired-samples t test revealed that the effect of  $e[CO_2]$  on grain biomass was significant for all three wheat 186 types (tetraploids, p < 0.001; hexaploids, p < 0.001; synthetic hexaploids, p = 0.012). This 187 was also the case for grain number (tetraploids, p = 0.043; hexaploids, p < 0.001; synthetic 188 hexaploids, p = 0.002). Pearson product-moment correlation analysis revealed that there was 189 190 a moderately positive correlation between grain biomass and total grain protein (r = 0.584, n = 19, p = 0.009). Grain number had a strong positive correlation with both grain biomass (r = 191 0.820, n = 19, p < 0.001) and HI (r = 0.698, n = 19, p = 0.001), while there was a moderately 192 193 negative correlation with shoot biomass (r = -0.494, n = 19, p = 0.032).

194 *3.3 Total shoot biomass* 

195 Total shoot biomass increased in all genotypes grown under e[CO<sub>2</sub>] compared to a[CO<sub>2</sub>]

196 except for the tetraploid wheat Jandaroi (Fig. 3, Table 3). Synthetic hexaploid genotypes had

the greatest increase in total shoot biomass under  $e[CO_2]$ , with an average increase of 71.1%,

compared to tetraploids and hexaploids, which increased on average by 17.4 (excluding Jandaroi) and 14.9%, respectively. Among the synthetic hexaploids CPI133811 increased the most under  $e[CO_2]$ . Statistical analysis by One-Way ANOVA found that synthetic hexaploids were significantly different from hexaploids (P = 0.001) and tetraploids (P = 0.002), however, no significant difference was found between tetraploids and hexaploids. A paired-samples t test found that the effect of  $e[CO_2]$  on total shoot biomass was only significant for hexaploids (p < 0.001).

205 *3.4 Harvest Index* 

Harvest index increased in all plants when grown under  $e[CO_2]$  compared to  $a[CO_2]$ , except 206 for the two synthetic hexaploid genotypes CPI133814 and CPI133811 (Fig. 3, Table 3). 207 These synthetic hexaploid genotypes share the same pedigree. The genotype with the greatest 208 increase in HI under e[CO<sub>2</sub>] was the tetraploid genotype WID802, with an increase of 209 26.28%. Of the two genotypes which declined in response to  $e[CO_2]$ , CPI133814 declined the 210 most, decreasing in HI by 19.25%. The tetraploids had an average increase in HI of 12.84%, 211 while hexaploids increased on average by 6.23%. On the other hand the synthetic hexaploids 212 had an average decrease of 15.51%, excluding CPI133898, which increased in HI in response 213 214 to e[CO<sub>2</sub>] by 1.20%. Like total shoot biomass, statistical analysis by One-Way ANOVA found that synthetic hexaploids were significantly different from hexaploids (P = 0.007) and 215 tetraploids (P = 0.001), however, no significant difference was found between tetraploids and 216 hexaploids. Paired-samples t tests were carried out for each wheat type, which revealed the 217 effect of  $e[CO_2]$  to be significant for both tetraploids (p = 0.022) and hexaploids (p = 0.001), 218 but not for synthetic hexaploids (p = 0.219). In addition, we also analysed the correlation 219 between HI and GPC. Statistical analysis by Pearson product-moment correlation found there 220 was no significant correlation between these two variables. 221

#### 222 **4. Discussion**

It is currently unclear to which extent  $e[CO_2]$  affects different types of wheat, and thus our 223 research aimed to investigate how the GPC of wheat is affected by e[CO<sub>2</sub>] across three 224 different wheat types: tetraploid, hexaploid and synthetic hexaploid. In addition, we aimed to 225 investigate the relationship between GPC, HI and biomass of each wheat type, in order to 226 elucidate the mechanism behind GPC decline under  $e[CO_2]$ . Our results suggest that rather 227 than the wheat type determining GPC, it is specific genotypes within and between wheat 228 types that determine GPC. On the other hand, we found that the HI of the studied wheat 229 230 genotypes was significantly different between tetraploids and synthetic hexaploids, showing that while GPC may not be affected by type specific differences, wheat type may affect HI. 231 Our results did not show any significant link between GPC and HI. As the number of 232 genotypes differed between each wheat type, these results may have varied if further 233 genotypes were available for analysis. In particular, the similar pedigree shared by the 234 synthetic hexaploids limits the applicability of these results for other genotypes, which may 235 show a larger variability in the traits studied. 236

We examined the response of GPC, grain biomass, total shoot biomass and HI to e[CO<sub>2</sub>] for 237 238 19 wheat genotypes, consisting of five tetraploids, eleven hexaploids and three synthetic hexaploids. Overall, the majority of genotypes decreased in GPC. This is a typical response 239 of bread wheat to e[CO<sub>2</sub>] (Taub *et al.* 2008) and along with lower pasta quality, GPC has also 240 been found to decrease in tetraploid wheat under e[CO<sub>2</sub>] (Fares *et al.* 2016). However, GPC 241 response to e[CO<sub>2</sub>] is scarcely studied in tetraploid wheats, and to our knowledge, this is the 242 first study to observe the effect of  $e[CO_2]$  on the GPC of synthetic hexaploids. The synthetic 243 hexaploids in this study, however, share similar pedigrees, which may not represent other 244 synthetic hexaploids of more diverse origins. The effect of e[CO<sub>2</sub>] on GPC was not consistent 245 246 within each wheat type. While most genotypes decreased in GPC, at least one genotype of

247 each type increased in GPC. Tjilkuri was the only tetraploid genotype to increase in GPC and although it had the greatest increase of all genotypes, there were more hexaploid genotypes 248 than tetraploid for which we observed an overall increase in GPC. As such, our study can't 249 250 confirm the results of Sinha et al. (2009), who found tetraploid wheat had the lowest decline in GPC compared to hexaploid and diploid wheats, however, these differences can likely be 251 attributed in part to the variation in number of genotypes studied. When looking solely at the 252 genotypes which decreased in GPC, we found that the decrease in GPC for hexaploids on 253 average was greater than for tetraploids, thus supporting Sinha et al. (2009). This study was 254 limited to the availability of genotypes and experimental conditions. For a more 255 comprehensive picture, more genotypes need to be studied in addition to a greater number of 256 replicates. Furthermore, repeating this experiment using Free-air CO<sub>2</sub> Enrichment (FACE) 257 facilities would allow the comparison of glasshouse experiments with field data. 258 Despite the amount of genotypes with lower GPC under e[CO<sub>2</sub>], nearly all genotypes (16 of 259 19) increased in total grain protein (g) per plant. We found that there was a significant 260 correlation between grain biomass and total grain protein. This suggests that as the grain 261 biomass increases due to the greater carbon availability from e[CO<sub>2</sub>], the plant remobilizes or 262 takes up greater amounts of nitrogen to the grain. However, even though most genotypes 263 have greater grain protein and biomass under  $e[CO_2]$ , there is a wide variation in GPC. Most 264 genotypes have a lower GPC under  $e[CO_2]$ , which means that while more protein is being 265 stored in grains of plants grown under  $e[CO_2]$  than  $a[CO_2]$ , the stimulation of grain biomass 266 is too great for nitrogen uptake, transport or assimilation to keep up with. A study by Pleijel 267 & Uddling (2011) observed a growth dilution effect on grain protein accumulation due to 268 269 e[CO<sub>2</sub>], where the increase in grain protein yield (GPY; the amount of grain protein accumulated per unit area) was lower than the increase in grain yield. Furthermore, GPC also 270 declined as grain yield increased. An important observation, however, was that the decline in 271

grain protein could not be completely explained by growth dilution, as e[CO<sub>2</sub>] negatively
affected GPY and GPC in plants with no increase in grain yield. For our study, we
acknowledge the variation in number of genotypes per wheat type in this experiment and as
such, a larger number of cultivars is needed to further support this conclusion. Repeating this
experiment under FACE conditions with larger numbers of plants per genotype would give
more conclusive data relevant to plants grown under field conditions.

Synthetic hexaploids differ from other wheats in that they are derived from crosses between 278 Ae. tauschii and T. turgidum. The resulting GPC of each synthetic hexaploid then, is likely 279 dependant on the responsiveness of both parent genotypes to  $e[CO_2]$ . For instance, the 280 increased GPC of CPI133811 is likely a trait inherited from either its Ae. tauschii parent or 281 the T. turgidum parent. As it shares the same T. turgidum parent as the other synthetic 282 hexaploids in this study, it is likely to be a trait derived from its Ae. tauschii parent. As such, 283 identifying the GPC response to  $e[CO_2]$  for the parents of each synthetic hexaploid examined 284 in this study could explain the differences in CO<sub>2</sub> responsiveness of the synthetic hexaploids. 285 In order to develop more synthetic hexaploid cultivars with improved responsiveness to 286 e[CO<sub>2</sub>], it may be crucial to screen genotypes of *Ae. tauschii* and *T. turgidum* to identify 287 genotypes with high GPC responses to e[CO<sub>2</sub>] that could allow breeders to develop synthetic 288 hexaploid lines with a similar responsiveness. These elite synthetic hexaploid cultivars could 289 then be crossed with bread wheat cultivars to transfer the improved traits, as has been done 290 for other types of traits (Li et al. 2014). Synthetic hexaploids could be a solution for 291 improving the bread-making quality of bread wheat grown under e[CO<sub>2</sub>]. Ae. tauschii has 292 displayed a greater variety in high molecular weight and low molecular weight glutenin 293 294 subunits, encoded by the Glu- $D^t l$  and Glu- $D^t 3$  loci respectively, compared to the glutenin subunits of bread wheat (Pflüger et al. 2001). Within this variety of alleles in Ae. tauschii we 295

296 may find alleles capable of overcoming the poorer bread-making quality of wheat grown297 under e[CO<sub>2</sub>].

One of the main hypotheses explaining the decline in grain protein is the dilution hypothesis, 298 299 whereby e[CO<sub>2</sub>] causes greater biomass stimulation in wheat compared with that observed under a[CO<sub>2</sub>], and this increase is too great for the uptake and assimilation of N to keep up 300 with (Taub et al. 2008). We investigated whether HI, a measurement of biomass allocation, 301 was linked with GPC across tetraploid, hexaploid and synthetic hexaploid wheats. Harvest 302 index, like total grain protein, increased in the majority of genotypes (17 of 19). Unlike total 303 grain protein, however, where a tetraploid, hexaploid and synthetic hexaploid decreased 304 under e[CO<sub>2</sub>] (Jandaroi, Lincoln and CPI133811, respectively), the two genotypes which 305 declined in HI were both synthetic hexaploids (CPI133814 and CPI133811). This is likely 306 due to both genotypes sharing the same pedigree. We found that there was a significant 307 difference between tetraploids and synthetic hexaploids, however, hexaploids were not found 308 to be significantly different to either of the other wheat types. This may be influenced by the 309 lower number of genotypes for both the tetraploids and synthetic hexaploids than for the 310 hexaploids. Harvest index is determined by the total shoot biomass and total grain biomass of 311 the plant. These components are each affected by e[CO<sub>2</sub>], which means that HI will be 312 determined by the extent that either component is affected. For example, a genotype which is 313 greatly affected by e[CO<sub>2</sub>] in both grain biomass and total shoot biomass will have a much 314 different change in HI than a genotype which is mostly affected in grain biomass. Amthor 315 (2001) found the grain yield of wheat increased by 31% on average in response to  $e[CO_2]$ . 316 This would lead to greater harvest indices for plants with low total shoot biomass responses 317 318 to e[CO<sub>2</sub>]. However, as reported with the meta-analyses of Wang et al. (2013), HI remained the same because shoot biomass increased proportionally to the increase in grain biomass. In 319 our experiment, total shoot biomass and total grain biomass both increased for all genotypes 320

321 in response to e[CO<sub>2</sub>], except in Jandaroi, which declined in total shoot biomass. Synthetic hexaploids were significantly different from both tetraploids and hexaploids in total shoot 322 biomass response to  $e[CO_2]$ . While the differences between means of tetraploids, hexaploids 323 324 and synthetic hexaploids were not significant for total grain biomass, the synthetic hexaploids showed the lowest response to e[CO<sub>2</sub>]. It appears that the main factor contributing to the 325 lower HI of the synthetic hexaploids is the response of total shoot biomass to e[CO<sub>2</sub>] rather 326 than grain biomass. This indicates that the extra carbon being assimilated under  $e[CO_2]$  is 327 being stored largely in the shoot, compared to grain, of synthetic hexaploids. 328 The increase in biomass, both shoot and grain, can be explained by the effect of  $e[CO_2]$  on 329 photosynthesis. While the rate of photosynthesis was not measured for the plants in this 330 experiment, it is accepted that e[CO<sub>2</sub>] generally increases the photosynthetic rate of C3 plants 331 (Ainsworth and Long 2005). An increase in photosynthesis means there is greater carbon 332 fixation, resulting in increased biomass in the plant. Increasing the HI of a plant involves 333 increasing how much carbon is stored in the grain as opposed to the shoot, as can be seen in 334 this study's results when comparing the HI of each genotype with the two components of 335 grain biomass and total shoot biomass. This increase in carbon could partly explain the 336 decline in GPC for plants grown under e[CO<sub>2</sub>]. As previously mentioned, one of the main 337 hypotheses for the decline in protein under  $e[CO_2]$  is dilution by carbohydrates, where the 338 increase in biomass is greater than the increase in nitrogen (Loladze 2002; Taub and Wang 339 2008). However, as with Taub and Wang (2008), we argue that biomass dilution cannot be 340 the only explanation for the decline in GPC. Our results showed that the change in GPC did 341 not correlate with the change in grain biomass or HI. While some genotypes, such as Sunbri 342 343 and Hartog had a clear decline in GPC and increase in both grain biomass and HI, others were not as consistent. Some genotypes increased in GPC despite the stimulation of grain 344

345 biomass, most notably Tjilkuri, which had the greatest increase in GPC and the second

346 greatest increase in grain biomass. This means that there are factors that are affecting the GPC of each genotype other than carbon dilution itself. In addition, Lincoln declined in GPC 347 despite a small change to grain biomass and HI. As such, while dilution might explain part of 348 the decline in GPC of some genotypes, there are very likely other factors controlling the 349 protein response of wheat to  $e[CO_2]$ . Other explanations have been proposed, such as altered 350 nitrogen assimilation (Bloom et al. 2014). Our results also found some correlation between 351 grain biomass and total grain protein, suggesting that as e[CO<sub>2</sub>] stimulates grain biomass, it 352 also causes the plant to transport more nitrogen to the grain. This was not always the case, 353 354 however, as Jandaroi in particular put less nitrogen into grain under  $e[CO_2]$  despite its increase in grain biomass. This supports the idea that there are other mechanisms being 355 affected by e[CO<sub>2</sub>] which control the transport of protein. Lincoln decreased in total grain 356 protein despite the low stimulation of grain biomass. This further suggests that lower total 357 grain protein is not controlled by how great e[CO<sub>2</sub>] stimulates grain biomass. 358

In addition to a decline in protein concentration, the composition of protein is altered and 359 ultimately the baking quality of grain harvested from plants grown under e[CO<sub>2</sub>] is affected 360 (Fernando et al. 2014; Panozzo et al. 2014). Therefore, it is important to not only identify 361 hexaploid genotypes with greater responses to e[CO<sub>2</sub>] with regards to GPC, but also those 362 which will not have decreased baking quality. As previously mentioned it will be important 363 to screen a wider range of wheat genotypes for those which are highly responsive to  $e[CO_2]$ 364 365 for their GPCs, but to ensure the end product quality it will also be necessary to screen the highly responsive genotypes for baking quality. 366

367 Our results have identified a number of wheat genotypes that increased in both HI and GPC.

368 Repeated confirmation of these results could provide breeders with genotypes that would

369 benefit breeding programs for developing wheat cultivars capable of maintaining or

improving upon current GPCs and HIs for future  $CO_2$  conditions. Future studies would also

371 benefit from increasing the number of genotypes, particularly tetraploids and synthetic hexaploids, as well performing this experiment in FACE conditions to gain data from field 372 trials. The genotypes in the current study could be used in further research to investigate the 373 374 mechanisms of GPC decline by providing wheat with contrasting CO<sub>2</sub> responsiveness. The tetraploid genotype Tjilkuri, which increased in GPC in response to e[CO<sub>2</sub>], may be a 375 potential parent for generating synthetic hexaploid genotypes. However, in addition to the 376 generation of synthetic hexaploids, there exists another possibility for developing wheat 377 genotypes with improved GPC and HI under e[CO<sub>2</sub>]. While there are many barriers to 378 success, crossing tetraploid genotypes with hexaploids can result in pentaploid wheats 379 (Padmanaban et al. 2017). Pentaploid wheat can be a source of great genetic variability and 380 has shown promise for improving resistance to both biotic and abiotic stress (Padmanaban et 381 al. 2017). Crossing highly [CO<sub>2</sub>] responsive tetraploid and hexaploid genotypes together 382 could lead to pentaploid genotypes with improved GPC and HI under  $e[CO_2]$ . These 383 pentaploid genotypes could then be crossed into either tetraploid or hexaploid genotypes, thus 384 allowing the transfer of durum genes into bread wheat and vice versa. 385 Nitrogen is not the only nutrient which is affected in wheat grain. A meta-analysis by 386 Broberg *et al.* (2017) investigated the effect of  $e[CO_2]$  on grain mineral concentration and 387 found significantly reduced concentrations of Ca, Cd, Cu, Fe, Mg, Mn, P, S, and Zn. 388 Deficiencies in two of these minerals in particular, Fe and Zn, is a problem for a large portion 389 of the global population, where wheat is one of the main sources of these minerals in their 390 diets (Myers *et al.* 2014). As such, it is crucial to study the effect e[CO<sub>2</sub>] has on these 391 minerals in addition to nitrogen. Future studies could therefore identify how the effect of 392  $e[CO_2]$  on the concentrations of these minerals differs between the three wheat types. 393 In summary, our results suggest that wheat type is not a major factor for determining GPC or 394 HI response to  $e[CO_2]$ , although this could be due to the limitation on number of genotypes. 395

396 GPC and HI both varied among the cultivars within each wheat type and no significant difference could be found between wheat types, except for the difference between the HI of 397 tetraploids and synthetic hexaploids. The difference in HI for the synthetic hexaploids was 398 due to the high response of total biomass to  $e[CO_2]$ , which itself was significantly different 399 from both tetraploids and hexaploids. However, more synthetic hexaploids with greater 400 genetic variation need to be studied to confirm this for other genotypes. There also does not 401 appear to be a strong connection between the CO<sub>2</sub> effects on GPC and HI regardless of wheat 402 type. Our results suggest that biomass dilution is not the sole cause of the decline in GPC 403 seen in this study. Ultimately, we think that the individual genotype is more important than 404 wheat type in determining the response of wheat GPC and HI to  $e[CO_2]$ , however, more 405 genotypes need to be studied to arrive at a definitive conclusion. 406

#### 407 **Conflict of interest**

408 The authors declare no conflicts of interest.

#### 409 Acknowledgements

410 This work was supported by the University of Southern Queensland and an Australian

411 Postgraduate Award. The genotype LRC2010-157 was obtained from Dr Cassy Percy from

412 the Centre for Crop Health, University of Southern Queensland.

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529 Table 1. Grain protein concentration (%) and total grain protein (g) of tetraploid, hexaploid and synthetic hexaploid genotypes in ambient [CO<sub>2</sub>]

and elevated [CO<sub>2</sub>]. ANOVA results show differences between each wheat type. Data represent values per plant. Abbreviations: P<sub>T</sub>, AVOVA

results against tetraploids; P<sub>H</sub>, ANOVA results against hexaploids; P<sub>S</sub>, ANOVA results against synthetic hexaploids; ns, not significant; \*,

532 P≤0.05; \*\*, P≤0.01.

			Grain	Protein Concentrati	on (%)					Total Grain Protein (g)				
		ANOVA												
Wheat type	Genotype	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ] – a[CO <sub>2</sub> ]	Ρ <sub>τ</sub>	P <sub>H</sub>	Ps	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ] – a[CO <sub>2</sub> ]	P <sub>T</sub>	P <sub>H</sub>	Ps	
	Caparoi	11.08	13.80	-2.72				1.67	1.40	0.27	-			
	Jandaroi	17.42	19.66	-2.24				3.22	2.12	1.10				
Tetraploid	WID802	11.86	13.16	-1.30	-	ns	ns	2.29	1.51	0.78		ns	ns	
	Hyperno	11.83	12.13	-0.30				2.33	1.53	0.80				
	Tjilkuri	18.49	13.89	4.61				3.64	1.44	2.20				
	Sunbri	9.68	14.72	-5.04				2.56	2.07	0.49				
	Spitfire	11.81	15.11	-3.30	0			2.08	1.78	0.29				
	Lincoln	12.11	15.33	-3.22				2.13	2.57	-0.44				
	Hartog	11.13	14.01	-2.89				2.30	1.78	0.52				
	Crusader	13.86	15.47	-1.61				2.98	2.15	0.83				
Hexaploid	Scout	11.90	13.40	-1.51	ns	-	ns	2.47	2.12	0.34	ns	-	ns	
	Sunvale	13.65	13.70	-0.05				2.43	1.53	0.89				
	LRC / 2010 / 157	13.29	12.78	0.51	) ′			2.69	2.00	0.69				
	Aus 29259	13.66	12.92	0.74				2.80	1.90	0.90				
	Dart	16.17	15.42	0.76				2.83	1.78	1.05				
	Sunguard	14.37	12.54	1.83				1.65	1.26	0.39				
	CPI 133814	14.53	14.55	-0.02				1.78	1.29	0.49				
Synthetic Hexaploid	CPI 133898	12.09	12.29	-0.20	ns	ns	-	1.02	0.74	0.28	ns	ns	-	
. in a profile	CPI 133811	19.62	15.54	4.08				2.46	1.56	0.90				

- Table 2. Grain biomass (g) and grain number of tetraploid, hexaploid and synthetic hexaploid genotypes in ambient [CO<sub>2</sub>] and elevated [CO<sub>2</sub>].
- 535 ANOVA results show differences between each wheat type. Data represent values per plant. Abbreviations: P<sub>T</sub>, AVOVA results against
- tetraploids;  $P_H$ , ANOVA results against hexaploids;  $P_S$ , ANOVA results against synthetic hexaploids; ns, not significant; \*, P $\leq 0.05$ ; \*\*, P $\leq 0.01$ .

				Grain Biomass (g)						Grain Number			
					А	NOV	Ά					ANOV	
Wheat									Č				
type	Genotype	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ] – a[CO <sub>2</sub> ]	Ρτ	Рн	Ps	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	e[CO <sub>2</sub> ] – a[CO <sub>2</sub> ]	PT	Рн	Ps
	Caparoi	15.07	10.15	4.92				262.00	182.75	79.25			
	Jandaroi	18.48	10.78	7.70		ns		296.00	221.75	74.25			
Tetraploid	WID802	19.35	11.50	7.85	-		ns	397.50	317.50	80.00	-	ns	ns
	Hyperno	19.70	12.65	7.05				417.25	308.75	108.50			
	Tjilkuri	19.70	10.40	9.30				388.75	239.50	149.25			
	Sunbri	26.43	14.07	12.36				639.50	491.67	147.83			
	Spitfire	17.58	11.80	5.78				356.00	288.00	68.00			
	Lincoln	17.60	16.75	0.85	ns			396.75	346.00	50.75			
	Hartog	20.68	12.73	7.95				391.75	292.50	99.25	ns		
	Crusader	21.50	13.90	7.60				456.50 360.75	325.50	131.00			
Hexaploid	Scout	20.73	15.83	4.90		-	ns		294.75	66.00		-	ns
	Sunvale	17.78	11.20	6.58			-	357.25	301.75	55.50	-		-
	LRC / 2010 /												
	157	20.23	15.67	4.56				422.00	381.75	40.25			
	Aus 29259	20.50	14.70	5.80				303.50	301.75	1.75			
	Dart	17.50	11.55	5.95				455.75	386.25	69.50			
	Sunguard	11.50	10.08	1.43				238.25	233.50	4.75			
Countly at it.	CPI 133814	12.23	8.88	3.35				198.75	186.25	12.50			
Synthetic Hexaploid	CPI 133898	12.52	10.03	2.50	ns	ns	-	308.00	307.00	1.00	ns	ns	-
пехаріоїи	CPI 133811	8.40	6.00	2.40				172.50	157.75	14.75			

- 537 Table 3. Total shoot biomass (g) and harvest index of tetraploid, hexaploid and synthetic hexaploid genotypes in ambient [CO<sub>2</sub>] and elevated
- 538 [CO<sub>2</sub>]. ANOVA results show differences between each wheat type. Data represent values per plant. Abbreviations: P<sub>T</sub>, AVOVA results against
- tetraploids;  $P_H$ , ANOVA results against hexaploids;  $P_S$ , ANOVA results against synthetic hexaploids; ns, not significant; \*, P $\leq 0.05$ ; \*\*, P $\leq 0.01$ .

			Tot	al Shoot Biomass	(g)					Harvest Index			
		ANOVA								ANOVA			
Wheat		100.1			_	_	_	100.1			_	_	_
туре	Genotype	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	$e[CO_2] - a[CO_2]$	Ρ	Рн	۲s	e[CO <sub>2</sub> ]	a[CO <sub>2</sub> ]	$e[CO_2] - a[CO_2]$	Ρ	Рн	Ps
	Caparoi	6.9	5.1	1.80				0.69	0.67	0.02			
	Jandaroi	6.0	5.1	0.90			**	0.75	0.68	0.08			
Tetraploid	WID802	6.1	7.6	-1.50	-	ns		0.76	0.60	0.16	-	ns	**
	Hyperno	6.3	5.5	0.80				0.76	0.70	0.06			
	Tjilkuri	4.2	4.1	0.10				0.82	0.72	0.11			
	Sunbri	6.6	6.1	0.50				0.80	0.70	0.10			
	Spitfire	6.4	5.3	1.10				0.73	0.69	0.04	ns		
	Lincoln	7.1	6.8	0.30				0.71	0.71	0.00			
	Hartog	7.0	6.1	0.90				0.75	0.68	0.07			
	Crusader	6.7	5.8	0.90				0.76	0.71	0.06			
Hexaploid	Scout	7.2	6.7	0.50	ns	_	**	0.74	0.70	0.04		-	**
•	Sunvale	7.0	6.3	0.70	$\mathcal{O}^{\prime}$			0.72	0.64	0.08			
	LRC / 2010 /												
	157	6.1	5.8	0.30				0.77	0.73	0.04			
	Aus 29259	7.3	5.3	2.00				0.74	0.74	0.00			
	Dart	5.5	4.2	1.30				0.76	0.73	0.03			
	Sunguard	5.3	4.9	0.40				0.68	0.67	0.01			
Curath atia	CPI 133814	8.5	4.4	4.10				0.59	0.67	-0.08			
Hexaploid	CPI 133898	9.6	7.9	1.70	**	**	-	0.57	0.56	0.01	**	**	-
пеларіон	CPI 133811	15.7	7.9	7.80				0.35	0.43	-0.08			

#### 540 Figure captions

- 541 Figure 1. Effect of e[CO<sub>2</sub>] on A) GPC and B) total grain protein. Data represents the
- 542 difference between  $a[CO_2]$  and  $e[CO_2]$  values. Positive values indicate greater values for
- 543 e[CO<sub>2</sub>] than a[CO<sub>2</sub>]. Genotypes are organised into tetraploids (Caparoi, Jandaroi, WID802,
- 544 Hyperno and Tjikuri), hexaploids (Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout,
- 545 Sunvale, LRC2010-157, Aus29259, Dart and Sungard) and synthetic hexaploids (CPI133814,
- 546 CPI133898, CPI133811).
- 547 Figure 2. Effect of e[CO<sub>2</sub>] on A) grain biomass and B) grain number. Data represents the
- 548 difference between a[CO2] and e[CO<sub>2</sub>] values. Positive values indicate greater values for
- 549 e[CO<sub>2</sub>] than a[CO<sub>2</sub>]. Genotypes are organised into tetraploids (Caparoi, Jandaroi, WID802,
- 550 Hyperno and Tjikuri), hexaploids (Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout,
- 551 Sunvale, LRC2010-157, Aus29259, Dart and Sungard) and synthetic hexaploids (CPI133814,
- 552 CPI133898, CPI133811).
- Figure 3. Effect of e[CO<sub>2</sub>] on A) total shoot biomass and C) HI. Data represents the
- 554 difference between a[CO2] and e[CO2] values. Positive values indicate greater values for
- e[CO2] than a[CO2]. Genotypes are organised into tetraploids (Caparoi, Jandaroi, WID802,
- 556 Hyperno and Tjikuri), hexaploids (Sunbri, Spitfire, Lincoln, Hartog, Crusader, Scout,
- Sunvale, LRC2010-157, Aus29259, Dart and Sungard) and synthetic hexaploids (CPI133814,
  CPI133898, CPI133811).

# 560 Figures





562

Figure 2 564







Genotype

566



567 Figure 3



Highlights

- Individual genotype is a greater determining factor for grain protein concentration decline under elevated CO<sub>2</sub> than wheat type.
- Most genotypes declined in grain protein concentration under elevated CO<sub>2</sub>, while total grain protein increased in most genotypes.
- Elevated CO<sub>2</sub> leads to a different response of harvest index between tetraploid and synthetic hexaploid genotypes.
- Biomass dilution is not the sole cause of grain protein concentration decline under elevated CO<sub>2</sub> in wheat.