

***CARBON SOURCE-SINK LIMITATIONS DIFFER BETWEEN TWO
SPECIES WITH CONTRASTING GROWTH STRATEGIE***

Burnett, A., Rogers, A., Rees, M., and Osborne, C. P.

*Accepted for publication in
Plant, Cell and Environment*

July 2016

**Environmental & Climate Science Dept.
Brookhaven National Laboratory**

**U.S. Department of Energy
DOE Office of Science**

Notice: This manuscript has been authored by employees of Brookhaven Science Associates, LLC under Contract No. DE-SC0012704 with the U.S. Department of Energy. The publisher by accepting the manuscript for publication acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, world-wide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes.

This preprint is intended for publication in a journal or proceedings. Since changes may be made before publication, it may not be cited or reproduced without the author's permission.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or any third party's use or the results of such use of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof or its contractors or subcontractors. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23

Title:

**CARBON SOURCE-SINK LIMITATIONS DIFFER BETWEEN
TWO SPECIES WITH CONTRASTING GROWTH
STRATEGIES**

Running title:

Source-sink limitations vary with growth strategy

Authors

Angela C Burnett¹, Alistair Rogers², Mark Rees¹, Colin P Osborne¹

¹ Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10
2TN, UK

² Environmental and Climate Sciences Department, Brookhaven National Laboratory,
Upton, NY 11973, USA

Corresponding Author

Colin P Osborne

Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN,
UK

Email: c.p.osborne@sheffield.ac.uk

24 **Keyword index:**

25 Crop yield, source, sink, barley, CO₂, carbon, nitrogen, photosynthesis, allocation,

26 growth

27 **ABSTRACT**

28

29 Understanding how carbon source and sink strengths limit plant growth is a critical
30 knowledge gap that hinders efforts to maximise crop yield. We investigated how
31 differences in growth rate arise from source-sink limitations, using a model system
32 comparing a fast-growing domesticated annual barley (*Hordeum vulgare* cv. NFC
33 Tipple) with a slow-growing wild perennial relative (*Hordeum bulbosum*). Source
34 strength was manipulated by growing plants at sub-ambient and elevated CO₂
35 concentrations ([CO₂]). Limitations on vegetative growth imposed by source and sink
36 were diagnosed by measuring relative growth rate, developmental plasticity,
37 photosynthesis and major carbon and nitrogen metabolite pools. Growth was sink
38 limited in the annual but source limited in the perennial. RGR and carbon acquisition
39 were higher in the annual, but photosynthesis responded weakly to elevated [CO₂]
40 indicating that source strength was near maximal at current [CO₂]. In contrast,
41 photosynthetic rate and sink development responded strongly to elevated [CO₂] in
42 the perennial, indicating significant source limitation. Sink limitation was avoided in
43 the perennial by high sink plasticity: a marked increase in tillering and root:shoot ratio
44 at elevated [CO₂], and lower non-structural carbohydrate accumulation. Alleviating
45 sink limitation during vegetative development could be important for maximising
46 growth of elite cereals under future elevated [CO₂].

47 INTRODUCTION

48

49 Global population growth, economic development and climate change are exerting
50 increasing pressure on our global food supply, raising demand that must be met, in
51 part, by improving crop yields (Ainsworth *et al.*, 2008b; Godfray *et al.*, 2010; Foley *et al.*,
52 2011; von Caemmerer *et al.*, 2012; Reynolds *et al.*, 2012; FAO *et al.*, 2014; Ort *et al.*,
53 2015). Increasing yield depends critically on a firm understanding of plant growth,
54 which is in turn underpinned by the interactions between carbon and nitrogen
55 sources and sinks (White *et al.*, 2016). Sources provide net uptake of resources from
56 the external environment whilst sinks cause a net internal drawdown of these
57 resources. For carbon, mature leaves are sources and roots are sinks, and the
58 balance between them is achieved by well-characterised molecular crosstalk
59 mechanisms (Smith and Stitt, 2007; Lawlor and Paul, 2014; White *et al.*, 2016).
60 Decades of research into the effects of elevated CO₂ have demonstrated that
61 increasing source activity through a stimulation of photosynthesis often does not
62 translate into corresponding yield increases (Long *et al.*, 2006; Ainsworth *et al.*,
63 2008a; Leakey *et al.*, 2009), although this depends on the species (Yamori *et al.*,
64 2016). Similarly, increasing sink capacity does not always translate into greater yield
65 under field conditions (Weichert *et al.*, 2010). A holistic approach to growth and yield
66 considering both source and sink capacities is therefore essential for developing
67 higher yielding crop varieties (White *et al.*, 2016). In this context, source strength is
68 the product of source activity and size, with an equivalent definition for sinks (Geiger
69 and Shieh, 1993; White *et al.*, 2016).

70

71 One strategy for understanding the fundamental limitations on growth is to
72 investigate the natural diversity of growth rates in wild plants. In wild species, one of
73 the major causes of growth rate variation is life-history (Grime and Hunt, 1975;
74 Garnier, 1992). Annual and perennial growth strategies enable plants to allocate
75 resources in a way that is appropriate for their environment: annuals grow quickly
76 and invest everything in reproduction in the first year before they die, whilst
77 perennials grow more slowly and conserve resources for the following season
78 (Garnier, 1992; Iwasa, 2000; Bennett *et al.*, 2012). Annuals are typically seen as
79 having flexible growth strategies for exploiting fluctuating environments, whereas
80 perennials have more conservative growth strategies – i.e. lower allocation to
81 reproduction and slower growth (Atkinson *et al.*, 2012, 2014). Although perennials
82 with large storage organs may never be sink limited, annuals generally transition
83 from sink to source limitation during development when they switch from vegetative
84 to reproductive growth (Arp, 1991), and perennials lacking large storage organs are
85 likely to undergo this transition as well. Because perennials grow more slowly than
86 annuals and transition to the reproductive growth stage later, they are therefore likely
87 to be sink limited for a longer period of time.

88

89 Despite this well developed ecological theory, we do not currently know the extent to
90 which slower growth in perennials than annuals arises from greater source or sink
91 limitation. Experimental manipulations of the source:sink ratio provide insights into
92 the relative contributions of source and sink processes to growth rate, and may be
93 achieved through a variety of techniques including: sink removal (Arp, 1991); genetic
94 modification (Ainsworth *et al.*, 2004; Weichert *et al.*, 2010; Zuther *et al.*, 2011);
95 source removal (von Caemmerer and Farquhar, 1984; Bryant *et al.*, 1998; Rogers *et*

96 al., 1998; Eyles et al., 2013); inhibiting resource export from the source (Ainsworth
97 and Bush, 2011); and increasing source activity using elevated CO₂ (Kinsman *et al.*,
98 1997; Masle, 2000), reviewed by White et al. (2016). Here, we alter the atmospheric
99 CO₂ concentration ([CO₂]) to non-invasively manipulate the source:sink ratio in barley
100 – elevated [CO₂] to increase the source strength and sub-ambient [CO₂] to decrease
101 it – with current [CO₂] as a reference against which to compare the source
102 manipulations. This approach enables analysis of source and sink limitation under
103 current [CO₂], and strong CO₂ treatments are applied in order to produce marked
104 perturbations of the system. In C₃ plants, [CO₂] affects carbon source strength
105 directly through one well-understood process i.e. carbon assimilation by Rubisco,
106 and therefore avoids wounding responses and other confounding effects, which may
107 arise from alternative approaches for source:sink manipulation. We took a holistic
108 approach to investigating source-sink interactions, measuring the responses of
109 development, growth, allocation, photosynthesis and key carbon and nitrogen
110 metabolite pools on the same plants. Together, these simultaneous measurements
111 of growth, carbon uptake and carbon utilization allowed us to diagnose source and
112 sink limitation in our model system. For example, a high concentration of free amino
113 acids indicates carbon source limitation (Paul and Driscoll, 1997; Stitt and Krapp,
114 1999; Isopp *et al.*, 2000; Rogers *et al.*, 2006), whilst a build-up of non-structural
115 carbohydrates in leaves indicates carbon sink limitation (Rogers and Ainsworth,
116 2006; Ainsworth and Bush, 2011).

117

118 In order to elucidate physiological mechanisms underpinning differences in growth
119 rate, this study compared domesticated annual barley (*Hordeum vulgare* cv. NFC
120 Tipple) and a wild perennial relative (*Hordeum bulbosum*). Annual barley is sink

121 limited during grain filling (Schnyder, 1993; Bingham *et al.*, 2007; Serrago *et al.*,
122 2013), yet to our knowledge no study of source- and sink limitation during the
123 vegetative growth stage has been made in this species. The annual barley used
124 here is an elite agricultural spring barley from the HGCA recommended list (HGCA,
125 2014) and has a fast-growing life-history strategy. The perennial is a wild species
126 from Turkey, which is able to grow in diverse habitats but generally occupies nutrient-
127 rich environments (von Bothmer, 1996). CO₂ treatments were applied at germination
128 and maintained until harvest, which occurred during the vegetative growth phase of
129 the life cycle. We predicted that annual barley, which grows faster than perennial
130 barley, would be sink limited during vegetative growth, and the perennial would be
131 more strongly sink limited (Jaikumar *et al.*, 2014). Based on this hypothesis we would
132 expect the fast-growing annual to show a greater increase in growth and
133 photosynthesis in response to elevated [CO₂] than the perennial (Poorter, 1993;
134 Roumet and Roy, 1996). This is because the elevated [CO₂] alleviates source
135 limitation and will therefore have a greater effect in the plants which are less sink
136 limited (Bryant *et al.*, 1998; Rogers *et al.*, 1998; Ainsworth *et al.*, 2003). In contrast,
137 we expected that the more strongly sink limited, slower-growing perennial would
138 show a greater increase in the storage of carbon-rich metabolites under elevated
139 [CO₂].

140 MATERIALS AND METHODS

141

142 Plant material and growth conditions

143

144 Seeds of *Hordeum vulgare* cv. NFC Tipple and *Hordeum bulbosum* (Accessions
145 GRA1031 and GRA947) were obtained from Syngenta and IPK Gatersleben
146 respectively. Seeds were germinated on wet filter paper and transplanted to 4-litre
147 pots filled with 1:10 sand:vermiculite and topped with an additional layer of sand to
148 aid root development of the seedlings. Plants were grown in controlled environment
149 growth chambers (BDR 16, Conviron, Isleham, UK) at the University of Sheffield, two
150 of which had been modified to scrub CO₂ using soda lime. Plants were grown in three
151 chambers with fixed CO₂ levels of 180 μmol mol⁻¹, 400 μmol mol⁻¹ and 1500 μmol
152 mol⁻¹ for 61 days. 180 and 1500 μmol mol⁻¹ were chosen in order to impose strong
153 carbon source and sink manipulations. All chambers had a 12-hour photoperiod with
154 day/night temperatures of 20/18°C, 65% humidity, and daytime light levels of 600
155 μmol photons m⁻² s⁻¹ at plant height resulting in a daily light integral of 25.92 mols m⁻²
156 day⁻¹. Plants were kept adequately watered with 20% Long Ashton's nutrient solution.
157 During seedling establishment, plants were watered daily – with 150ml Reverse
158 Osmosis water for 8 days and with 150ml Long Ashton's solution thereafter. After 17
159 days, plants were watered three times per week with 150ml Long Ashton's solution
160 until 29 days old, 225ml until 45 days old, and 450ml thereafter.

161

162 Photosynthesis measurements and metabolite harvests were carried out three times
163 in consecutive weeks, between 46 and 61 days after germination (DAG). In each of
164 these harvest weeks, six annuals from each CO₂ level were harvested (three at dawn

165 and three at dusk), giving a total of 54 individuals across three weeks. In the first and
166 third of these harvest weeks, six perennials from each CO₂ level were harvested
167 (three at dawn and three at dusk), giving a total of 36 individuals.

168

169 **Relative growth rate, root:shoot ratio and tillering**

170

171 RGR was calculated based on the plant mass estimated from weekly imaging of
172 above ground biomass, beginning when plants were two weeks old. Plants were
173 photographed (PowerShot G9, Canon, Tokyo, Japan) six times, from the side against
174 a white background, with the plant rotated 60 degrees between successive
175 photographs. A scale bar of known length was included for calibration. Leaf area in
176 pixels was obtained for each photograph using Image J (U. S. National Institutes of
177 Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>), and converted to mm²
178 using the area of the scale bar. A batch of 29 additional plants, not used in the main
179 study, was also photographed weekly. At nine time points between 33 and 60 DAG,
180 individuals from this batch were separated into leaf, leaf sheath and root, and oven-
181 dried to provide a calibration curve for leaf area and dry mass for each species.
182 These curves were then used to predict dry mass for each plant for each set of six
183 photographs (Fig. S1; $r^2 = 0.97$ for species-based calibration). Individual growth
184 curves showing predicted dry mass over time were obtained for each plant using a
185 nonlinear mixed-effects model obtained by stepwise selection and used to estimate
186 RGR at multiple timepoints by differentiation, where more than three timepoints had
187 been measured.

188

189 The calibration was also used to predict root:shoot ratio for the plants in the main
190 study. For each of the oven-dried individuals, the relative contribution of shoot and
191 root to whole plant mass was recorded and the mean fraction of root and shoot was
192 calculated. This was then applied to the plant mass predicted for the plants in the
193 main study using calibration of image data, to give an estimate of root:shoot ratio for
194 each individual.

195

196 Tillers were counted the day before metabolite harvests were carried out.

197

198 **Photosynthesis**

199

200 Diurnal measurements of photosynthesis were made the day before plants were
201 harvested for metabolite assays. 51 annual and 26 perennial individuals were
202 measured (the remaining plants were too small for gas exchange measurements to
203 be performed). Instantaneous net photosynthetic rate was measured every 3.5 hours
204 between 30 minutes after dawn and 30 minutes before dusk, using the LI-6400XT
205 Portable Photosynthesis System (LI-COR Biosciences, Lincoln, NE, USA). Net
206 photosynthetic rate was measured *in situ* within growth chambers, under the ambient
207 environmental conditions of each chamber described above. These measurements
208 were used to obtain a curve of photosynthesis during the photoperiod for each plant,
209 and the area underneath was integrated to give a daily rate of net carbon fixation per
210 unit area – i.e. the carbon source activity. This value was multiplied by the projected
211 shoot area to estimate the total daily photosynthesis in the whole shoot – i.e. the
212 carbon source strength.

213

214 **Metabolites**

215

216 Plants were harvested from 47 to 61 DAG, within one hour before dawn and one
217 hour before dusk. Samples were flash frozen in liquid nitrogen, stored at -80°C, and
218 freeze-dried prior to analysis. For small plants, the entire plant was harvested; for
219 larger plants, representative samples of leaf, leaf sheath and root from both young
220 and old tissue were harvested. In the first week, plants were harvested at 47, 48 and
221 49 DAG. In the second week, plants were harvested at 52, 53 and 54 DAG. In the
222 third week, plants were harvested at 59, 60 and 61 DAG. Three replicates from each
223 species from one chamber were harvested on each date, with the exception of the
224 second week when only annuals were harvested. The order of chambers was
225 randomised in each of the three harvest weeks.

226

227 Metabolite analysis was carried out at Brookhaven National Laboratory. Metabolites
228 were extracted from the freeze-dried ground tissue using sequential ethanol
229 extractions.

230 Ethanol soluble carbohydrates (glucose, fructose, sucrose, low degree of
231 polymerisation (LDP) fructan) were analysed using a continuous enzymatic substrate
232 assay as described previously (Ainsworth *et al.*, 2007) adapted for measuring
233 sucrose in the presence of LDP fructans (Harrison *et al.*, 1997). All biochemical
234 analysis was conducted in standard 96-well microplates (Microtest Plate 96-Well Flat
235 Bottom, Sarstedt, Nümbrecht, Germany), using a robotic liquid handling system
236 (Evolution P³ Precision Pipetting Platform, Perkin Elmer, Waltham, MA, USA).

237

238 The pellets from the ethanol extraction were heated to 95°C in 0.1M NaOH, to
239 solubilise protein. A commercially available protein assay kit (Pierce BCA protein
240 assay kit, ThermoScientific, Rockford, IL, USA) based on the Lowry method was used
241 to measure protein content (Lowry et al., 1951) using BSA as a standard. Following
242 the protein assay, samples were neutralised with HCl.

243

244 For the starch and high degree of polymerisation (HDP) fructan assay, starch and
245 HDP fructans from 40 µl aliquots of the suspended pellet material were digested
246 using enzymes in 60 µl 0.05M acetate buffer as follows. Starch: 0.17U well⁻¹
247 amyloglucosidase (EC 3.2.1.3) and 0.1U well⁻¹ α-amylase (EC 3.2.1.1); starch and
248 HDP fructan: 0.1U well⁻¹ exo-inulinase (EC 3.2.1.80), 0.1U well⁻¹ endo-inulinase (EC
249 3.2.1.7), 0.17U well⁻¹ amyloglucosidase and 0.1U well⁻¹ α-amylase. Plates were
250 incubated overnight at 37°C. 40 µl of the supernatant from the overnight digest was
251 transferred to each well of a 96-well microplate. 262µg ATP well⁻¹, 349µg NADP well⁻¹
252 and 3.6U well⁻¹ glucose-6-phosphate dehydrogenase (EC 1.1.1.49, grade II) were
253 added in a buffer of 0.1M HEPES/KOH, 3mM MgCl₂, pH7.0 to initiate the reaction.
254 Microplates were centrifuged for 1 minute to remove bubbles then inserted into a
255 plate reader and the NADPH associated with the carbohydrates in the sample was
256 measured at A₃₄₀ (ELx808, BioTek, Winooski, VT, USA).

257

258 Starch and HDP fructans were assayed by sequentially adding 1U enzyme in HEPES
259 buffer to each well as follows, using the rationale for the soluble carbohydrates assay
260 (Ainsworth *et al.*, 2007). Starch: hexokinase (EC 2.7.1.1); starch and HDP fructan:
261 hexokinase, phosphoglucose-isomerase (EC 5.3.1.9). HDP fructan values were
262 obtained by subtracting the starch assay values for starch from the starch and fructan

263 values for combined starch and fructan. This approach was necessary because
264 preliminary recovery experiments had shown that digesting fructan using
265 exoinulinase and endoinulinase also degraded a small amount of starch, leading to
266 an artificially high value for fructan content which was corrected using the approach
267 described here. Starch and HDP fructan content was measured as nmol hexose
268 equivalents using a standard glucose curve loaded on each plate.

269 Total free amino acids were quantified using fluorescamine. 15µl 0.1M sodium borate
270 buffer, 90µl fluorescamine and 100µl water were combined with 2µl of ethanol extract
271 in a black 96-well microplate (Nunc MicroWell, Thermo Fisher Scientific, Waltham,
272 MA, USA). Following a 5 minute dark incubation, fluorescence (360nm excitation,
273 460nm emission, 40 nm bandwidth) was measured (Synergy HT, BioTex, Winooski,
274 VT, USA) and converted into nmol amino groups using a standard glutamate curve
275 loaded on each plate.

276 The Griess reaction was used to quantify free nitrate. First, 0.005U well⁻¹ nitrate
277 reductase (EC 1.7.1.3) and 50 nmol NADPH in 0.11M potassium phosphate buffer
278 were added to a 10µl aliquot of the ethanol extract. Each microplate was shaken.
279 Following a 30 minute incubation (dark, room temperature), 20µl 0.25mM phenazine
280 methosulfate was added to each well. Plates were shaken again and incubated for a
281 further 20 minutes. 45µl 1% w/v sulfanilamide in 5% phosphoric acid followed by
282 45µl 0.02% N(1-Naphthyl)ethylenediamine dihydrochloride was added to each well.
283 Following another shake and a 5 minute incubation, A₅₄₀ was measured (ELx808,
284 BioTex, Winooski, VT, USA) and converted into nmol nitrate using a standard nitrate
285 curve loaded on each plate.

286

287 Metabolite data were expressed per g carbohydrate-corrected dry weight, obtained
288 by subtracting the mass of total non-structural carbohydrate (the sum of glucose,
289 fructose, sucrose, LDP and HDP fructan, and starch) from the dry mass of each
290 sample. Technical and analytical replicates were run for all assays (Ferne *et al.*,
291 2011).

292

293 **Statistical Methods**

294

295 Analysis was performed using R (2015). Analysis of variance models incorporating
296 error terms reflecting the split-split plot design of the experiment were carried out for
297 each variable measured. Logarithmic transformations were performed on all data
298 prior to analysis to improve the fit of the models.

299

300 For photosynthesis, TNC, amino acids and amino acid:sucrose (Figs. 2, 4, 6, 7),
301 small error bars are present but are obscured by symbols.

302

303

304

305

306

307

308

309

310 **RESULTS**

311

312 **Perennial barley shows greater developmental plasticity in response to**
313 **elevated [CO₂] than the annual species**

314

315 Relative growth rate (RGR; the efficiency of whole plant dry mass increase obtained
316 from calibration of shoot area, and measured in $\text{g g}^{-1} \text{ day}^{-1}$) was obtained from
317 individual growth curves by differentiation, and represents the sink activity of plant
318 growth. RGR was higher in the annual than perennial plants, and greater at higher
319 CO₂ levels (Fig. 1). Stepwise model selection was used to choose fixed effects, and
320 the effects of species and [CO₂] on the maximum plant size and the time to reach half
321 size were each highly significant ($p < 0.001$) – although these are additive effects with
322 no significant interaction between [CO₂] and species. In both species, the increase in
323 RGR was greater between 180 and 400 $\mu\text{mol mol}^{-1}$ CO₂ than between 400 and 1500
324 $\mu\text{mol mol}^{-1}$ CO₂. Peak RGR in the annual increased by 17.1% between 180 and 400
325 $\mu\text{mol mol}^{-1}$ CO₂, but only 5.0% between 400 and 1500 $\mu\text{mol mol}^{-1}$ CO₂. Peak RGR in
326 the perennial increased by 20.5% between 180 and 400 $\mu\text{mol mol}^{-1}$ CO₂, but only
327 6.0% between 400 and 1500 $\mu\text{mol mol}^{-1}$ CO₂. However the difference between
328 annual and perennial remained relatively consistent: peak RGR in the annual was
329 21.1%, 17.7% and 16.7% higher than in the perennial, at 180, 400 and 1500 μmol
330 mol^{-1} CO₂ respectively.

331

332 The modular nature of plant body plans means that, in order for RGR to increase,
333 plants must either increase the biomass of existing organs, or initiate new structures
334 through branching (tillering, in the case of grasses). Tillering in the perennial

335 increased by 163% between 180 and 1500 $\mu\text{mol mol}^{-1}$ CO_2 , whereas the number of
336 tillers in the annual increased by just 15% across the same CO_2 range (Table 1). This
337 highly significant species x $[\text{CO}_2]$ interaction ($F_{(1,80)} = 56$, $p < 0.001$) indicates greater
338 developmental plasticity in the perennial.

339

340 Root:shoot ratio also showed a larger response to increasing $[\text{CO}_2]$ in perennial than
341 annual barley. In the annual, the root:shoot ratio increased by only 2.8% between
342 180 and 400 $\mu\text{mol mol}^{-1}$ CO_2 , whilst in the perennial it increased 11.6% between 180
343 and 400 $\mu\text{mol mol}^{-1}$, and 4.2% between 400 and 1500 $\mu\text{mol mol}^{-1}$ (Table 1). This
344 highly significant species x $[\text{CO}_2]$ interaction ($F_{(1,77)} = 24$, $p < 0.001$) provides further
345 evidence of greater developmental plasticity in the perennial.

346

347 **Perennial barley also shows a greater photosynthetic response to elevated**
348 **$[\text{CO}_2]$ than the annual species**

349

350 Annual barley generally has a higher photosynthetic rate than the perennial, but the
351 photosynthetic rate in the perennial shows a much stronger response to $[\text{CO}_2]$ (Fig.
352 2B). In the annual plants, the daily photosynthetic rate increased by 87% between
353 180 and 400 $\mu\text{mol mol}^{-1}$ CO_2 , but only by 13% between 400 and 1500 $\mu\text{mol mol}^{-1}$. In
354 contrast, in the perennial it increased 58% between 180 and 400 $\mu\text{mol mol}^{-1}$, but 75%
355 between 400 and 1500 $\mu\text{mol mol}^{-1}$. This led to a significant species x $[\text{CO}_2]$
356 interaction: $F_{(1,67)} = 4.9$, $p < 0.05$; Fig. 2B. Because the annual is a larger plant than
357 the perennial, the difference in whole shoot photosynthetic rate, i.e. carbon source
358 strength (Fig. 2C) is greater than the difference in the rate per unit area, i.e. carbon
359 source activity (Fig. 2B). In the annual, the whole shoot daily photosynthetic rate

360 increased by 177% between 180 and 400 $\mu\text{mol mol}^{-1}$ CO_2 , but only 25% between
361 400 and 1500 $\mu\text{mol mol}^{-1}$. In contrast, in the perennial it increased 528% between
362 180 and 400 $\mu\text{mol mol}^{-1}$, and 123% between 400 and 1500 $\mu\text{mol mol}^{-1}$. There was a
363 highly significant species x $[\text{CO}_2]$ interaction: $F_{(1,66)} = 18$, $p < 0.001$; Fig. 2C.

364

365 The ratio of photosynthesis to growth is higher in the annual than the perennial (Fig.
366 3), seen in the plots of individuals (Fig. 3A,B) and means (Fig. 3C,D) with a highly
367 significant effect of species: $F_{(1,61)} = 25$, $p < 0.001$. When expressed in $\text{g C g}^{-1} \text{ day}^{-1}$
368 (Fig. 3A), growth shows three clusters corresponding to the decreasing values of
369 RGR as time progresses over the three harvests. When expressed in g C plant^{-1}
370 day^{-1} (Fig. 3B), these clusters are no longer present, and a positive correlation
371 between photosynthesis and growth is seen. The ratio increases with $[\text{CO}_2]$ (Fig. 3C)
372 and with plant age at harvest (Fig. 3D). There was a highly significant interaction of
373 the harvest week x $[\text{CO}_2]$ ($F_{(4,61)} = 16$, $p < 0.001$), such that the photosynthesis:growth
374 ratio is greater at higher $[\text{CO}_2]$, but this trend becomes less pronounced at later
375 harvests.

376

377 **Annual barley accumulates more non-structural carbohydrates than the**
378 **perennial species**

379

380 Pre-dawn measurements indicate the basal level of carbohydrates in plant organs,
381 when metabolites accumulated during the previous photoperiod have been utilised
382 for respiration, exported or consumed by growth at night. Before dawn, annual barley
383 had a higher concentration of total non-structural carbohydrates (TNC, the sum of
384 glucose, fructose, sucrose, fructan and starch) than the perennial, and showed a

385 greater accumulation of TNC in leaf sheaths and roots when [CO₂] was increased
386 from 180 to 1500 μmol mol⁻¹ (Fig. 4). However, in the leaves, perennial barley
387 showed a stronger TNC response than the annual when [CO₂] was increased from
388 180 to 1500 μmol mol⁻¹ (Fig. 4). Across all TNC data, there was a significant organ
389 type x time of day x species interaction ($F_{(2,120)} = 9.2$, $p < 0.001$); a significant organ
390 type x time of day x [CO₂] interaction ($F_{(4,120)} = 18$, $p < 0.001$); a significant organ x
391 species x [CO₂] interaction ($F_{(4,120)} = 22$, $p < 0.001$); and a significant organ x harvest
392 week x [CO₂] interaction ($F_{(8,120)} = 4.5$, $p < 0.001$). In the leaf, TNC was 114% greater
393 in the annual than the perennial at 180 μmol mol⁻¹ CO₂, 57% greater in the annual at
394 400 μmol mol⁻¹ CO₂, but approximately equal at 1500 μmol mol⁻¹ CO₂ (Fig. 4A). In
395 the leaf sheath, TNC was 29% greater in the annual than the perennial at 180 μmol
396 mol⁻¹ CO₂, 57% greater in the annual at 400 μmol mol⁻¹ CO₂, and 25% greater in the
397 annual at 1500 μmol mol⁻¹ CO₂ (Fig. 4B). In the root, TNC was 35% greater in the
398 annual than the perennial at 180 μmol mol⁻¹ CO₂, 56% greater in the annual at 400
399 μmol mol⁻¹ CO₂, and 97% greater in the annual at 1500 μmol mol⁻¹ CO₂ (Fig. 4C). In
400 both species, TNC concentration is highest at 1500 μmol mol⁻¹ CO₂ suggesting that
401 sinks are replete under these conditions.

402

403 Subtracting the mean pre-dawn values from the mean pre-dusk values provides a
404 differential of TNC (Fig. 5), which represents the amount of carbon accumulated
405 during the photoperiod, and is equivalent to the amount of carbon available for
406 respiration, export or growth at night. These differentials are much greater in the leaf
407 than in leaf sheath or root (Fig. 5), since diurnal fluctuations in leaves are more tightly
408 coupled to the diurnal activity of photosynthesis than the distal sinks of leaf sheath
409 and root. The perennial shows a greater TNC differential than the annual in leaves at

410 400 and 1500 $\mu\text{mol mol}^{-1}$ CO_2 , yet there is little difference in TNC differentials in leaf
411 sheath and root, across the CO_2 concentrations (Fig. 5). Therefore, whilst the basal
412 pre-dawn level of TNC is higher in annuals (Fig. 4), the diurnal accumulation of TNC
413 is greater in perennials for leaves at 400 and 1500 $\mu\text{mol mol}^{-1}$ CO_2 (Fig. 5).

414

415 **Perennial barley accumulates more free amino acids than the annual species**

416

417 Free amino acids are an indicator of source limitation (Paul and Driscoll, 1997; Stitt
418 and Krapp, 1999; Isopp *et al.*, 2000). A high free amino acid concentration or high
419 amino acid:sucrose ratio reflects a surplus of available nitrogen for biosynthesis,
420 since source limited plants lack sufficient carbon to use along with this nitrogen for
421 growth and development. The perennial has a higher concentration of free amino
422 acids than the annual (Fig. 6). In both annual and perennial, free amino acid
423 concentration is highest at 180 $\mu\text{mol mol}^{-1}$ CO_2 , which implies a carbon source
424 limitation, and decreases as $[\text{CO}_2]$ increases (Fig. 6). Before dawn, amino acid
425 concentration is 41% greater in the perennial than the annual at 180 $\mu\text{mol mol}^{-1}$ CO_2 ,
426 127% greater in the perennial at 400 $\mu\text{mol mol}^{-1}$ CO_2 , and 12% greater in the
427 perennial at 1500 $\mu\text{mol mol}^{-1}$ CO_2 (Fig. 6A). Before dusk, amino acid concentration is
428 67% greater in the perennial than the annual at 180 $\mu\text{mol mol}^{-1}$ CO_2 , 47% greater in
429 the perennial at 400 $\mu\text{mol mol}^{-1}$ CO_2 , and 64% greater in the perennial at 1500 μmol
430 mol^{-1} CO_2 (Fig. 6B).

431

432 There was a highly significant organ x species x $[\text{CO}_2]$ interaction for free amino acid
433 concentration: $F_{(4,117)} = 9.3$, $p < 0.001$. A similar trend for the two species and three
434 CO_2 levels is seen for free nitrate (data shown in summary form in Fig. 8) and there

435 was also a significant organ x species x [CO₂] interaction for these data: $F_{(4,116)} = 6.9$,
436 $p < 0.001$. The perennial also has a higher free amino acid:sucrose ratio than the
437 annual (Fig. 7), indicative of carbon source limitation. This ratio is higher pre-dawn
438 since sucrose accumulates during the day, and decreases with [CO₂]; for leaves,
439 there is a significant species x [CO₂] x time of day interaction: $F_{(1,70)} = 13$, $p < 0.001$.

440

441 **Metabolite data reveal source limitation in the perennial and sink limitation in**
442 **the annual**

443

444 Figure 8 synthesises the metabolite data, expressed as ratios relative to 400 μmol
445 mol^{-1} CO₂, in each compartment (leaf, sheath and root), for each species and time of
446 day. In general, the amount of each non-structural carbohydrate was lower at 180
447 $\mu\text{mol mol}^{-1}$ and higher at 1500 $\mu\text{mol mol}^{-1}$, compared to 400 $\mu\text{mol mol}^{-1}$ CO₂ (Fig. 8),
448 with short- and long-chain fructans representing the major stores for carbon at
449 elevated CO₂ (Fig. 8). In contrast, free nitrate, free amino acid and protein levels
450 tended to show the opposite trend (especially for the annual, Fig. 8A,B). At 400 μmol
451 mol^{-1} , growth in the annual shows strong evidence of sink limitation, shown by a high
452 rate of photosynthesis (Fig. 2), high TNC accumulation (Fig. 4) and low amino acid
453 concentration and amino acid:sucrose ratio – indicating that sufficient carbon
454 skeletons are available for utilising available amino acids (Fig. 6, 7). At 180 μmol
455 mol^{-1} CO₂, growth becomes more source limited, with lower carbohydrate and higher
456 nitrate and amino acid concentrations compared to 400 $\mu\text{mol mol}^{-1}$ (Figs. 4, 6, 8A,B),
457 whilst at 1500 $\mu\text{mol mol}^{-1}$ CO₂, growth becomes more sink limited, with higher
458 carbohydrate and lower nitrate and lower amino acid concentrations (Figs. 4, 6,
459 8A,B). This trend is seen at both times of day, but is most pronounced before dawn

460 (Figs. 8A,B), since carbon skeletons and reductants from photosynthesis are
461 required to incorporate free nitrate into amino acids and to assimilate amino acids
462 into proteins. As a result, the levels of these metabolites decrease during the day as
463 carbohydrates build up. Although this trend is seen in all organ types, it is most
464 pronounced in leaves, where photosynthesis is strongly coupled to changes in
465 carbon and nitrogen metabolism.

466

467 In contrast to the annual, at $400 \mu\text{mol mol}^{-1}$ the perennial shows strong evidence of
468 source limitation, having a lower rate of photosynthesis than the annual (Fig. 2), low
469 TNC accumulation (Fig. 4) and high amino acid concentrations and amino
470 acid:sucrose ratio (Figs. 6, 7). At $180 \mu\text{mol mol}^{-1} \text{CO}_2$, the perennial remains source
471 limited, so levels of free nitrate and amino acids generally do not increase relative to
472 $400 \mu\text{mol mol}^{-1}$ (Fig. 8C,D). Just as the perennial shows a greater response of
473 tillering and root allocation (Table 1) and photosynthesis (Fig. 2B) than the annual
474 between 400 and $1500 \mu\text{mol mol}^{-1} \text{CO}_2$, as this alleviates source limitation, it also
475 shows a more dramatic decrease in free amino acids and amino acid:sucrose (Figs.
476 6, 7) as it is better able than the annual to pair additional sugars from photosynthesis
477 with existing free amino acids to bring about a growth response. However at 1500
478 $\mu\text{mol mol}^{-1}$, growth in the perennial transitions to become sink limited, and the plants
479 have a high carbohydrate content, and low nitrate and low amino acid concentrations
480 (Figs. 4, 6, 8A,B). Thus the treatments imposed are sufficiently strong that even the
481 annual becomes more source limited at low $[\text{CO}_2]$, and even the perennial becomes
482 more sink limited at elevated $[\text{CO}_2]$.

483

484

485 **DISCUSSION**

486

487 **Developmental plasticity in the perennial enables extra CO₂ to be utilised in** 488 **growth, suggesting source limitation**

489

490 Increasing [CO₂] increases the availability of photosynthetic substrate and
491 suppresses photorespiration (Farquhar *et al.*, 1980). This increases the potential
492 rate of carbon uptake into the plant, increasing source strength, alleviating source
493 limitation, and increasing the source:sink ratio. Conversely, decreasing [CO₂] has the
494 opposite effects. The stronger photosynthetic, tillering and root partitioning responses
495 of perennial than annual barley to increasing [CO₂] (Table 1; Fig. 2) suggest that the
496 source is more limiting for growth than the sink in this species during the vegetative
497 stage. This response is not seen to such a great extent in the annual, suggesting that
498 its growth is primarily sink limited and constrained by developmental potential; as a
499 consequence the annual is operating at near-maximum source activity under current
500 ambient conditions (400 µmol mol⁻¹ CO₂). The ratio of photosynthesis to growth is
501 higher in annual barley (Fig. 3), a further indication of sink limitation, and increases at
502 higher [CO₂] and as plants become older and leave the exponential phase of growth.
503 Furthermore, the developmental plasticity seen in the perennial, via its ability to
504 increase tillering and root partitioning in response to greater carbon source strength,
505 suggests that it is better able than the domesticated annual crop to adapt to
506 fluctuating environmental conditions. In general, selective breeding of crops has
507 resulted in plants with fewer tillers because, although additional non-flowering tillers
508 provide a selective advantage through competition in wild plants, they reduce the
509 yield of crop stands by diverting resources away from flowering tillers. To an extent,

510 domesticated barley has retained its tillering capacity (Doust, 2007; Sang, 2009).
511 However, under experimental conditions, the perennial barley was far readier to
512 increase tillering in response to increased [CO₂] than the annual crop.

513

514 Altering the root:shoot ratio enables plants to increase access to the most limiting
515 resources by adjusting allocation to nitrogen- or carbon-acquiring tissues (Stitt and
516 Krapp, 1999; Freschet *et al.*, 2015). Under elevated [CO₂], nitrogen becomes more
517 limiting for growth, making an increase in root:shoot ratio advantageous. The
518 perennial was better able to make this plastic adjustment to growth (Table 1).
519 However, its greater relative increase in allocation to roots (Table 1) would have also
520 tended to offset its growth response, since roots are heterotrophic and root
521 respiration represents a significant carbon sink. This greater allocation to a
522 respiratory carbon sink may explain why the perennial still showed a similar increase
523 in RGR to the annual at higher CO₂ levels (Fig. 1). In combination, these results
524 suggest that the combined response of sink strength (growth and respiration) to
525 [CO₂] was stronger in the perennial than annual. Increasing root allocation enabled
526 the perennial to take up more nitrogen, further increasing its ability to match carbon
527 skeletons with amino acids for growth.

528

529 Our findings suggest a more opportunistic growth strategy in the perennial than
530 annual, whereby the use of additional resources is maximised via partitioning into
531 more branches above ground and roots below ground. In contrast, the annual
532 appears to be highly constrained in its ability to develop larger sinks at 400 μmol mol⁻¹
533 CO₂ (Table 1; Fig. 2), and unable to increase these to the same extent as the
534 perennial. It thus seems that the strategy of the annual is for maximal growth under

535 current [CO₂] – and as a result it is sink limited. The annual has been subjected to
536 intense selective breeding that has maximised growth under current ambient CO₂
537 conditions, but suppressed its developmental plasticity, and growth during the
538 vegetative phase is largely unresponsive to increased [CO₂].

539

540 **The annual accumulates carbohydrates whilst having low amino acids,**
541 **suggesting carbon sink limitation**

542

543 The metabolite data reinforce the pattern of source limitation in the perennial and
544 sink limitation in the annual seen in the growth and photosynthesis data. The annual
545 has higher TNC concentration, and lower amino acid concentration and amino
546 acid:sucrose ratio than the perennial, indicating an excess of carbon that cannot be
547 invested in growth (Figs. 4, 6, 7, 8). **Although many studies into the relationship**
548 **between amino acid accumulation and carbon source limitation have focused on a**
549 **single species (Paul and Driscoll, 1997; Isopp *et al.*, 2000), the use of the amino**
550 **acid:sucrose ratio, which is a more robust measurement, confirms the trend seen for**
551 **free amino acids, and is one of several lines of evidence pointing towards greater**
552 **carbon source limitation in the perennial.** The lower basal level of TNC in the
553 perennial (Fig. 4) suggests that this species is highly efficient at utilising the carbon
554 acquired each day – by developing new sinks or enlarging existing ones, seen in the
555 strong tillering response to elevated [CO₂] (Table 1), or by increasing TNC storage in
556 the leaf sheath (Fig. 4). Developing new sinks such as tillers increases sink size,
557 whilst increasing storage in existing sink organs increases sink activity; both enable
558 the plant to upregulate its sink capacity (Geiger and Shieh, 1993; White *et al.*, 2016).
559 The high rate of tillering and root allocation in the perennial translates to a higher sink

560 capacity and high demand for photosynthate which could explain the high
561 accumulation of carbohydrates in these organs. As a consequence, the large
562 quantity of leaf carbohydrates accumulated during the day are likely to be exported to
563 developing tillers or other sinks, in addition to the carbon sink of maintenance
564 respiration at night; in future work the use of isotopic CO₂ in a series of staged
565 harvests could enable diurnal carbon utilisation to be tracked (e.g. Ferrieri *et al.*,
566 2013).

567

568 Both species are carbon sink limited at elevated [CO₂]; leaf sucrose is a key driver of
569 phloem loading for photosynthate export (Ainsworth and Bush, 2011), yet the
570 increase in TNC at elevated [CO₂] seen here is primarily driven by increases in
571 storage carbohydrates (fructans and starch, Fig. 8) and not transport carbohydrates
572 (sucrose). This provides evidence that the carbohydrate accumulation at elevated
573 [CO₂] arises from sink limitation rather than reflecting the increased phloem loading
574 of recent photosynthate. Indeed, the increased accumulation of carbohydrates will
575 feed back on phloem transport throughout the plant and phloem loading in the leaf
576 (Ainsworth and Bush, 2011), and high foliar TNC concentration is thus an indicator of
577 sink limitation in both species. The fact that TNC does not accumulate in roots of the
578 perennial under elevated CO₂ suggests that carbon transport may be more limiting in
579 this species.

580

581 It is interesting to note that the negative correlation between starch and biomass
582 observed in a range of accessions of *Arabidopsis* (Sulpice *et al.*, 2009) is not borne
583 out by the data of this study – rather, the fast-growing annual species has a higher
584 rate of carbohydrate accumulation despite having greater biomass. However, the

585 physiology and metabolism of *Arabidopsis* do not always map onto those of crop
586 plants (White *et al.*, 2016), for example the relationship between protein and starch
587 found by Sulpice *et al.* (2009) was uncoupled in these data. Growth in plants with
588 different life forms and life histories may be subject to different constraints; in slow
589 growing *Arabidopsis* accessions, growth is slow because it is sink limited, whereas in
590 perennial barley, growth is slower than the annual because it is source limited and
591 therefore uncorrelated with carbohydrate content.

592

593 **Ecological strategies and intrinsic limits to growth**

594

595 The typical growth strategy of wild annual plants can be caricatured as 'live fast, die
596 young', leading to the expectation of a growth strategy that is primarily source limited
597 during the lifetime of the plant, and that enables the annual to maximise the use of
598 available CO₂ for growth. We therefore expected the annual to be less sink limited
599 than the perennial during vegetative growth, especially since it is adapted for
600 fertilised soils. In contrast, we expected the perennial to have a more conservative
601 growth habit, 'live slow, live long', which limits photosynthesis and growth but is
602 opportunistic, being better adapted for the possibility of low nutrients in a variable
603 environment yet able to capitalise on rising [CO₂] by increasing storage when
604 substrates are available. Although plants are typically sink limited during the
605 vegetative stage and transition to source limitation at reproduction (Arp, 1991), many
606 crops are co-limited by sinks and sources during grain-filling (Álvarez *et al.*, 2008;
607 Acreche and Slafer, 2009; Peterhansel and Offermann, 2012; Slewinski, 2012). We
608 anticipated that during the vegetative stage, the 'live slow' perennial would be more

609 sink limited than the 'live fast' annual (Jaikumar *et al.*, 2014). The results confounded
610 these expectations.

611

612 The perennial adopts more of a 'live fast' strategy than anticipated; perennials
613 generally store carbon for future use (Atkinson *et al.*, 2012), yet here the perennial
614 showed a dramatic increase in growth under elevated [CO₂] rather than an increase
615 in storage, indicating source limitation. Coming from a fluctuating natural
616 environment, and being able to grow in a variety of habitats including roadsides,
617 ditches and rich grassy meadows and at varying altitudes (von Bothmer, 1996), this
618 species has the plasticity to maximise growth when CO₂ is abundant. However, a
619 perennial confined to unproductive habitats might be expected to display slower
620 growth and greater sink limitation.

621

622 Although the perennial displays a greater response to [CO₂] for photosynthetic rate
623 per unit leaf area and leaf TNC concentration, even the maximal values at 1500 μmol
624 mol⁻¹ CO₂ never exceed those of the annual, implying intrinsic physiological or
625 developmental limits that are common to both species. The annual is unable to
626 utilise more photosynthate than it acquires at 400 μmol mol⁻¹ CO₂ by increasing
627 partitioning to tillers and roots; the perennial has greater developmental flexibility and
628 is able to utilise the additional photosynthate acquired at the highest CO₂
629 concentration, but never exceeds the maximum rates of growth and photosynthesis
630 seen in the annual (Figs. 1, 2).

631

632 The developmental plasticity of the annual species appears to have been altered
633 through selective breeding such that it cannot adapt to live faster when conditions

634 allow, and the results of this study show that it is sink limited during vegetative growth
635 even under elevated [CO₂], in addition to being sink limited during reproduction
636 (Schnyder, 1993; Bingham *et al.*, 2007; Serrago *et al.*, 2013). It thus seems that the
637 sink strength of barley will limit yield of this important crop in the current global
638 context of rising atmospheric [CO₂], and a concerted effort to increase sink strength
639 would be a vital part of breeding programmes in order to increase yield.

640

641 **CONCLUSIONS**

642

643 Contrary to expectations these results indicate that annual barley is more sink limited
644 and perennial barley is more source limited during the vegetative growth stage. Our
645 findings show that annual barley germplasm is optimised for growth at current [CO₂]
646 and that future elevated [CO₂] may be unlikely to facilitate yield increases in this
647 species; the lack of developmental plasticity in the annual means that new sinks are
648 not readily initiated, which could result in a critical lack of flexibility for developing
649 additional grain sinks and thus increasing yield under elevated [CO₂]. The holistic
650 approach taken here enables a broad view of source-sink balance to be taken,
651 encompassing measurements of resource acquisition, storage, allocation to growth,
652 and plant development, in a model system of congeneric species. In order to draw
653 firm conclusions of agricultural relevance, it will be vital to extend such research:
654 including nitrogen as well as carbon source-sink manipulations, following source-sink
655 processes throughout crop development to their impact on yield; investigating these
656 processes in a wider range of cereal varieties and wild species; and carrying out
657 agronomically relevant experiments in the field.

658

659 **ACKNOWLEDGEMENTS**

660 This research was supported by a PhD studentship from the Society for Experimental
661 Biology (SEB) awarded to ACW. AR was supported by the United States
662 Department of Energy contract No. DE-SC00112704 to Brookhaven National
663 Laboratory.

664 We thank Tiffany Bowman (Brookhaven National Laboratory) for assistance with
665 graphic design.

666 **REFERENCES**

- 667 **Acreche MM, Slafer GA.** 2009. Grain weight, radiation interception and use
668 efficiency as affected by sink-strength in Mediterranean wheats released from 1940
669 to 2005. *Field Crops Research* **110**, 98–105.
- 670 **Ainsworth EA, Bush DR.** 2011. Carbohydrate export from the leaf: a highly
671 regulated process and target to enhance photosynthesis and productivity. *Plant*
672 *Physiology* **155**, 64–9.
- 673 **Ainsworth EA, Davey PA, Hymus GJ, Osborne CP, Rogers A, Blum H,**
674 **Nösberger J.** 2003. Is stimulation of leaf photosynthesis by elevated carbon dioxide
675 concentration maintained in the long term? A test with *Lolium perenne* grown for 10
676 years at two nitrogen fertilization levels under Free Air CO₂ Enrichment (FACE).
677 *Plant, Cell & Environment* **26**, 705–714.
- 678 **Ainsworth EA, Leakey ADB, Ort DR, Long SP.** 2008a. FACE-ing the facts:
679 inconsistencies and interdependence among field, chamber and modeling studies of
680 elevated [CO₂] impacts on crop yield and food supply. *New Phytologist* **179**, 5–9.
- 681 **Ainsworth EA, Rogers A, Leakey ADB.** 2008b. Targets for crop biotechnology in a
682 future high-CO₂ and high-O₃ world. *Plant Physiology* **147**, 13–9.
- 683 **Ainsworth EA, Rogers A, Leakey ADB, Heady LE, Gibon Y, Stitt M, Schurr U.**
684 2007. Does elevated atmospheric [CO₂] alter diurnal C uptake and the balance of C
685 and N metabolites in growing and fully expanded soybean leaves? *Journal of*
686 *Experimental Botany* **58**, 579–91.
- 687 **Ainsworth EA, Rogers A, Nelson R, Long SP.** 2004. Testing the ‘source–sink’
688 hypothesis of down-regulation of photosynthesis in elevated [CO₂] in the field with
689 single gene substitutions in *Glycine max*. *Agricultural and Forest Meteorology* **122**,
690 85–94.

691 **Álvaro F, Royo C, García del Moral LF, Villegas D.** 2008. Grain filling and dry
692 matter translocation responses to source–sink modifications in a historical series of
693 durum wheat. *Crop Science* **48**, 1523.

694 **Arp WJ.** 1991. Effects of source-sink relations on photosynthetic acclimation to
695 elevated CO₂. *Plant, Cell & Environment* **14**, 869–875.

696 **Atkinson RRL, Burrell MM, Osborne CP, Rose KE, Rees M.** 2012. A non-targeted
697 metabolomics approach to quantifying differences in root storage between fast- and
698 slow-growing plants. *New Phytologist* **196**, 200–11.

699 **Atkinson RRL, Burrell MM, Rose KE, Osborne CP, Rees M.** 2014. The dynamics
700 of recovery and growth: how defoliation affects stored resources. *Proceedings of the*
701 *Royal Society B* **281**, 20133355.

702 **Bennett E, Roberts JA, Wagstaff C.** 2012. Manipulating resource allocation in
703 plants. *Journal of Experimental Botany* **63**, 3391–400.

704 **Bingham IJ, Blake J, Foulkes MJ, Spink J.** 2007. Is barley yield in the UK sink
705 limited? *Field Crops Research* **101**, 198–211.

706 **von Bothmer R.** 1996. Distribution and habitat preferences in the genus *Hordeum* in
707 Iran and Turkey. *Annalen des Naturhistorischen Museums in Wien, Serie B* **98-**
708 **Suppl.**, 107–116.

709 **Bryant J, Taylor G, Frehner M.** 1998. Photosynthetic acclimation to elevated CO₂ is
710 modified by source:sink balance in three component species of chalk grassland
711 swards grown in a free air carbon dioxide enrichment (FACE) experiment. *Plant, Cell*
712 *& Environment* **21**, 159–168.

713 **von Caemmerer S, Farquhar GD.** 1984. Effects of partial defoliation, changes of
714 irradiance during growth, short-term water stress and growth at enhanced p(CO₂) on
715 the photosynthetic capacity of leaves of *Phaseolus vulgaris* L. *Planta* **160**, 320–329.

716 **von Caemmerer S, Quick WP, Furbank RT.** 2012. The development of C4 rice:
717 current progress and future challenges. *Science* **336**, 1671–2.

718 **Doust A.** 2007. Architectural evolution and its implications for domestication in
719 grasses. *Annals of Botany* **100**, 941–950.

720 **Eyles A, Pinkard EA, Davies NW, Corkrey R, Churchill K, O’Grady AP, Sands P,**
721 **Mohammed C.** 2013. Whole-plant- versus leaf-level regulation of photosynthetic
722 responses after partial defoliation in *Eucalyptus globulus* saplings. *Journal of*
723 *Experimental Botany* **64**, 1625–1636.

724 **FAO, IFAD, WFP.** 2014. *The State of Food Insecurity in the World. Strengthening the*
725 *enabling environment for food security and nutrition.* Rome, FAO.

726 **Farquhar GD, Caemmerer S, Berry JA.** 1980. A biochemical model of
727 photosynthetic CO₂ assimilation in leaves of C3 species. *Planta* **149**, 78–90.

728 **Fernie AR, Aharoni A, Willmitzer L, Stitt M, Tohge T, Kopka J, Carroll AJ, Saito**
729 **K, Fraser PD, DeLuca V.** 2011. Recommendations for reporting metabolite data.
730 *The Plant Cell* **23**, 2477–82.

731 **Ferrieri A, Agtuca B, Appel H, Ferrieri R, Schultz J.** 2013. Temporal Changes in
732 Allocation and Partitioning of New Carbon as ¹¹C Elicited by Simulated Herbivory
733 Suggest that Roots Shape Aboveground Responses in *Arabidopsis*. *Plant Physiology*
734 **161**, 692-704.

735 **Foley JA, Ramankutty N, Brauman KA, et al.** 2011. Solutions for a cultivated
736 planet. *Nature* **478**, 337–342.

737 **Freschet GT, Sward EM, Cornelissen JHC.** 2015. Integrated plant phenotypic
738 responses to contrasting above- and below-ground resources: key roles of specific
739 leaf area and root mass fraction. *New Phytologist* **206**, 1247–1260.

740 **Garnier E.** 1992. Growth analysis of congeneric annual and perennial grass species.

741 Journal of Ecology **80**, 665–675.

742 **Geiger DR, Shieh W.** 1993. Sink strength: learning to measure, measuring to learn.
743 Plant, Cell & Environment **16**, 1017–1018.

744 **Godfray H, Beddington J, Crute I, Haddad L, Lawrence D, Muir J, Pretty J,**
745 **Robinson S, Thomas S, Toulmin C.** 2010. The Challenge of Food Security.
746 Science **327**, 812–818.

747 **Grime JP, Hunt R.** 1975. Relative Growth-Rate: Its range and adaptive significance
748 in a local flora. Journal of Ecology **63**, 393–422.

749 **Harrison J, Gallagher JA, Pollock CJ.** 1997. A simple and rapid method for the
750 analysis of water-soluble carbohydrates from small segments of cereal leaf tissue.
751 Journal of Plant Physiology **151**, 654–659.

752 **HGCA.** 2014. *HGCA Recommended List Spring barley 2014.*

753 **Isopp H, Frehner M, Long SP, Nösberger J.** 2000. Sucrose-phosphate synthase
754 responds differently to source-sink relations and to photosynthetic rates: *Lolium*
755 *perenne* L. growing at elevated $p(\text{CO}_2)$ in the field. Plant, Cell and Environment **23**,
756 597–607.

757 **Iwasa Y.** 2000. Dynamic optimization of plant growth. Evolutionary Ecology
758 Research **2**, 437–455.

759 **Jaikumar NS, Snapp SS, Flore JA, Loescher W.** 2014. Photosynthetic Responses
760 in Annual Rye, Perennial Wheat, and Perennial Rye Subjected to Modest Source:
761 Sink Ratio Changes. Crop Science **54**, 274–283.

762 **Kinsman EA, Lewis C, Davies MS, Young JE, Francis D, Vilhar B, Ougham HJ.**
763 1997. Elevated CO_2 stimulates cells to divide in grass meristems: a differential effect
764 in two natural populations of *Dactylis glomerata*. Plant, Cell & Environment **20**, 1309–
765 1316.

766 **Lawlor DW, Paul MJ.** 2014. Source/sink interactions underpin crop yield: the case
767 for trehalose 6-phosphate/SnRK1 in improvement of wheat. *Frontiers in Plant*
768 *Science* **5**, 418.

769 **Leakey ADB, Ainsworth EA, Bernacchi CJ, Rogers A, Long SP, Ort DR.** 2009.
770 Elevated CO₂ effects on plant carbon, nitrogen, and water relations: six important
771 lessons from FACE. *Journal of Experimental Botany* **60**, 2859–76.

772 **Long SP, Ainsworth EA, Leakey ADB, Nösberger J, Ort DR.** 2006. Food for
773 thought: lower-than-expected crop yield stimulation with rising CO₂ concentrations.
774 *Science* **312**, 1918–21.

775 **Lowry O, Rosebrough N, Farr A, Randall R.** 1951. Protein measurement with the
776 folin phenol reagent. *The Journal of Biological Chemistry* **193**, 265–275.

777 **Masle J.** 2000. The effects of elevated CO₂ concentrations on cell division rates,
778 growth patterns, and blade anatomy in young wheat plants are modulated by factors
779 related to leaf position, vernalization, and genotype. *Plant Physiology* **122**, 1399–415.

780 **Ort DR, Merchant SS, Alric J, et al.** 2015. Redesigning photosynthesis to
781 sustainably meet global food and bioenergy demand. *Proceedings of the National*
782 *Academy of Sciences* **112**, 8529–8536.

783 **Paul MJ, Driscoll SP.** 1997. Sugar repression of photosynthesis: the role of
784 carbohydrates in signalling nitrogen deficiency through source:sink imbalance. *Plant,*
785 *Cell & Environment* **20**, 110–116.

786 **Peterhansel C, Offermann S.** 2012. Re-engineering of carbon fixation in plants -
787 challenges for plant biotechnology to improve yields in a high-CO₂ world. *Current*
788 *Opinion in Biotechnology* **23**, 204–8.

789 **Poorter H.** 1993. Interspecific variation in the growth response of plants to an
790 elevated ambient CO₂ concentration. *Vegetatio* **104**, 77–97.

791 **R Core Team. 2015.** R: A language and environment for statistical computing. R
792 Foundation for Statistical Computing, Vienna, Austria. URL <http://www.R-project.org/>.

793 **Reynolds M, Foulkes J, Furbank R, Griffiths S, King J, Murchie E, Parry M,**
794 **Slafer G. 2012.** Achieving yield gains in wheat. *Plant, Cell & Environment* **35**, 1799–
795 1823.

796 **Rogers A, Ainsworth EA. 2006.** The response of foliar carbohydrates to elevated
797 [CO₂]. In: Nosberger J,, In: Long S,, In: Blum H,, In: Norby R,, In: Hendrey G,, In:
798 Stitt M, eds. *Managed ecosystems and CO₂ case studies processes and*
799 *perspectives.* Springer, 293–308.

800 **Rogers A, Fischer B, Bryant J, Frehner M, Blum H, Raines C, Long S. 1998.**
801 Acclimation of photosynthesis to elevated CO₂ under low-nitrogen nutrition is
802 affected by the capacity for assimilate utilization. *Perennial ryegrass under free-Air*
803 *CO₂ enrichment.* *Plant Physiology* **118**, 683–9.

804 **Rogers A, Gibon Y, Stitt M, Morgan PB, Bernacchi CJ, Ort DR, Long SP. 2006.**
805 Increased C availability at elevated carbon dioxide concentration improves N
806 assimilation in a legume. *Plant, Cell and Environment* **29**, 1651–1658.

807 **Roumet C, Roy J. 1996.** Prediction of the growth response to elevated CO₂: A
808 search for physiological criteria in closely related grass species. *New Phytologist*
809 **134**, 615–621.

810 **Sang T. 2009.** Genes and mutations underlying domestication transitions in grasses.
811 *Plant Physiology* **149**, 63–70.

812 **Schnyder H. 1993.** The role of carbohydrate storage and redistribution in the source-
813 sink relations of wheat and barley during grain filling - a review. *New Phytologist* **123**,
814 233–245.

815 **Serrago RA, Alzueta I, Savin R, Slafer GA. 2013.** Understanding grain yield

816 responses to source–sink ratios during grain filling in wheat and barley under
817 contrasting environments. *Field Crops Research* **150**, 42–51.

818 **Slewinski TL**. 2012. Non-structural carbohydrate partitioning in grass stems: a target
819 to increase yield stability, stress tolerance, and biofuel production. *Journal of*
820 *Experimental Botany* **63**, 4647–70.

821 **Smith AM, Stitt M**. 2007. Coordination of carbon supply and plant growth. *Plant, Cell*
822 *& Environment* **30**, 1126–49.

823 **Stitt M, Krapp A**. 1999. The interaction between elevated carbon dioxide and
824 nitrogen nutrition: the physiological and molecular background. *Plant, Cell &*
825 *Environment* **22**, 583–621.

826 **Sulpice R, Pyl E, Ishihara H, Trenkamp S, Steinfath M, Witucka-wall H, Korff M**
827 **Von, Caroline M, Gibon Y, Stitt M**. 2009. Starch as a major integrator in the
828 regulation of plant growth. *Proceedings of the National Academy of Sciences of the*
829 *United States of America* **106**, 10348–10353.

830 **Weichert N, Saalbach I, Weichert H, et al**. 2010. Increasing sucrose uptake
831 capacity of wheat grains stimulates storage protein synthesis. *Plant Physiology* **152**,
832 698–710.

833 **White AC, Rogers A, Rees M, Osborne CP**. 2016. How can we make plants grow
834 faster? A source–sink perspective on growth rate. *Journal of Experimental Botany*
835 **67**, 31–45.

836 **Yamori W, Kondo E, Sugiura D, Terashima I, Suzuki Y, Makino A**. 2016.
837 Enhanced leaf photosynthesis as a target to increase grain yield: Insights from
838 transgenic rice lines with variable Rieske FeS protein content in the Cytochrome b6 /f
839 complex. *Plant, Cell & Environment* **39**, 80–87.

840 **Zuther E, Hoermiller II, Heyer AG**. 2011. Evidence against sink limitation by the

841 sucrose-to-starch route in potato plants expressing fructosyltransferases. *Physiologia*
842 *Plantarum* **143**, 115–25.

843

844 **FIGURE LEGENDS & TABLE**

845

846 **Figure 1.** Relative growth rate is higher in annual (solid line) than perennial (dashed
847 line) barley, and greater at higher [CO₂]. Relative growth rate (RGR) is daily gain in
848 dry mass relative to whole plant dry mass, g g⁻¹ day⁻¹. A, elevated [CO₂]: 1500 μmol
849 mol⁻¹; B, current [CO₂]: 400 μmol mol⁻¹; C, sub-ambient [CO₂]: 180 μmol mol⁻¹.

850

851 **Figure 2.** Perennial barley (dashed line) has a more pronounced photosynthetic
852 response to elevated [CO₂] than annual barley (solid line). A, diurnal timecourse of
853 net leaf photosynthesis in annuals and perennials grown at 400 μmol mol⁻¹ CO₂. B,
854 daily rate of net leaf photosynthesis per unit area obtained from integrating curves
855 (e.g. A); C, total daily photosynthesis in the whole shoot, obtained by multiplying the
856 daily rate (B) by projected shoot area. Data show mean ± SE (A: annual n=18,
857 perennial n=9; B: at 180, 400, 1500 μmol mol⁻¹ CO₂, annual n=15, 18, 18, perennial
858 n=5, 9, 12; C: at 180, 400, 1500 μmol mol⁻¹ CO₂, annual n=15, 18, 18, perennial n=4,
859 9, 12).

860

861 **Figure 3.** The ratio of photosynthesis to growth is higher in annual than perennial
862 barley and greater at higher [CO₂] and in older plants. A, source activity vs sink
863 activity, plotted as photosynthesis and growth for individual plants at all times and
864 CO₂ levels, expressed in g C g⁻¹ day⁻¹, showing three clusters along the x-axis
865 corresponding to the three harvest times with RGR decreasing as time progresses;

866 B, source strength vs sink strength, plotted as photosynthesis and growth for
867 individual plants at all times and CO₂ levels expressed in g C plant⁻¹ day⁻¹; C,
868 photosynthesis:growth ratio in the three [CO₂] treatments; D, changes in the
869 photosynthesis:growth ratio with respect to the mean plant age at harvest. Data
870 show mean ± SE (C and D: at 180, 400, 1500 μmol mol⁻¹ CO₂, annual n=15, 18, 18,
871 perennial n=4, 9, 12).

872

873 **Figure 4.** Pre-dawn concentrations of total non-structural carbohydrates (TNC) are
874 higher in the annual (solid line) than perennial (dashed line) barley. A, leaf; B, leaf
875 sheath; C, root. The overall CO₂ response is greater for perennials in the leaf, but
876 greater for annuals in the leaf sheath and root. Data are expressed in μmol glucose
877 equivalents per g carbohydrate-corrected dry weight (CCDW). Data show mean ±
878 SE (annual n=9, perennial n=6).

879

880 **Figure 5.** The diurnal accumulation of total non-structural carbohydrates (TNC),
881 equivalent to the carbon pool available for nocturnal use or export in the leaf, leaf
882 sheath and root, in annual barley (solid line) and perennial barley (dashed line). Data
883 show the mean pre-dawn concentrations subtracted from mean pre-dusk
884 concentrations, expressed in μmol glucose equivalents per g carbohydrate-corrected
885 dry weight (CCDW). Different plants were harvested at dawn and dusk, so standard
886 errors cannot be calculated for these data (raw data presented in Table S1).

887

888 **Figure 6.** Free amino acid concentration is higher in perennial barley (dashed line)
889 than annual barley (solid line). A, pre-dawn; B, pre-dusk. Data are expressed in

890 μmol amino groups per g carbohydrate-corrected dry weight (CCDW). Data show
891 mean \pm SE (annual $n=9$, perennial $n=6$).

892

893 **Figure 7.** Ratio of free amino acids to free sucrose is higher in perennial barley
894 (dashed line) than annual barley (solid line) at 180 and 400 $\mu\text{mol mol}^{-1}$ CO_2 in leaves
895 pre-dawn. This is an indicator of carbon source limitation. Metabolites are
896 expressed in μmol amino groups and μmol sucrose per g carbohydrate-corrected dry
897 weight (CCDW), respectively. The ratio is lower at higher $[\text{CO}_2]$. Data show mean \pm
898 SE (annual $n=9$, perennial $n=6$).

899

900 **Figure 8.** Carbon- and nitrogen-based metabolites in annual and perennial barley at
901 pre-dawn and pre-dusk harvests. Data are ratios of metabolite concentrations in leaf,
902 leaf sheath and root of annual and perennial barley at 180 relative to 400 $\mu\text{mol mol}^{-1}$
903 CO_2 , and 1500 relative to 400 $\mu\text{mol mol}^{-1}$ CO_2 . Blue denotes a decrease and
904 orange/red an increase compared to 400 $\mu\text{mol mol}^{-1}$, according to the colour scale on
905 the left; a ratio of 1 signifies no change. Low and high DP refer to the degree of
906 polymerisation in short- and long-chain fructans respectively. A, Annual pre-dawn; B,
907 Annual pre-dusk; C, Perennial pre-dawn; D, Perennial pre-dusk. Exceptionally high
908 ratios, indicated by asterisks, are as follows: annual pre-dawn (A) at 180 $\mu\text{mol mol}^{-1}$
909 CO_2 , nitrate in leaf is 6.5x concentration at 400 $\mu\text{mol mol}^{-1}$, nitrate in leaf sheath is
910 7.7x concentration at 400 $\mu\text{mol mol}^{-1}$ and nitrate in root is 5.5x concentration at 400
911 $\mu\text{mol mol}^{-1}$; perennial pre-dawn (C), in leaf at 1500 $\mu\text{mol mol}^{-1}$ CO_2 , low DP fructan is
912 23.4x concentration at 400 $\mu\text{mol mol}^{-1}$ and high DP fructan is 6.3x concentration at
913 400 $\mu\text{mol mol}^{-1}$; perennial pre-dusk (D), in leaf at 1500 $\mu\text{mol mol}^{-1}$ CO_2 , low DP
914 fructan is 4.8x concentration at 400 $\mu\text{mol mol}^{-1}$.

915 **Table 1.** Responses of tillering and root:shoot ratio to increasing [CO₂]. Annual
 916 barley shows very limited tillering and root:shoot ratio responses to increasing CO₂
 917 concentration, whilst the perennial shows a dramatic increase in tillering and a
 918 significant increase in root:shoot ratio. Data shown are obtained from 54 annual and
 919 36 perennial individuals across the three treatments. Tillers were counted directly,
 920 whilst root:shoot ratio was estimated non-destructively using imaging. Means and
 921 their associated standard errors (S.E.) are reported to three significant figures
 922 (annual n=18, perennial n=12).
 923

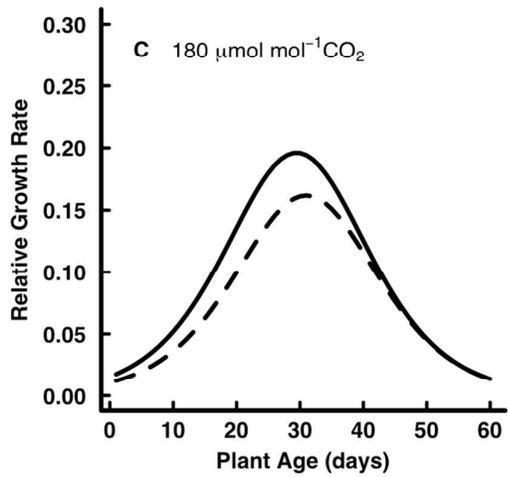
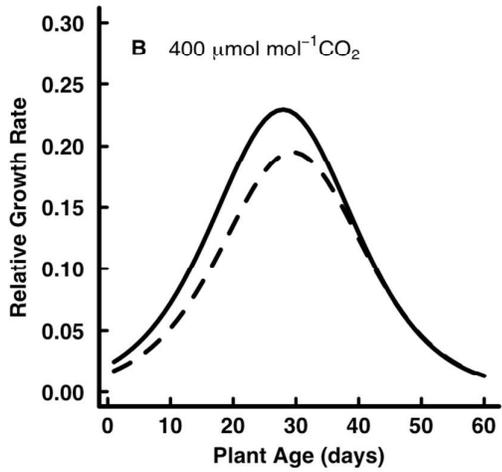
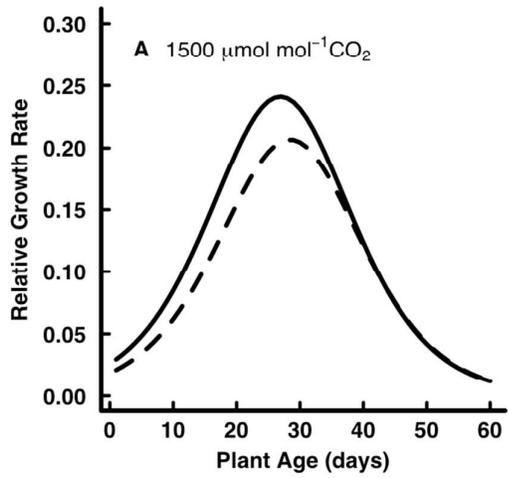
	Annual, 180 μmol mol ⁻¹ CO ₂	Annual, 400 μmol mol ⁻¹ CO ₂	Annual, 1500 μmol mol ⁻¹ CO ₂	Perennial, 180 μmol mol ⁻¹ CO ₂	Perennial, 400 μmol mol ⁻¹ CO ₂	Perennial, 1500 μmol mol ⁻¹ CO ₂
Tillers (mean)	13.3	13.4	15.3	12.4	17.4	32.6
Tillers (S.E.)	0.753	0.506	0.676	1.275	1.341	2.464
Root:Shoot Ratio (mean)	0.529	0.544	0.547	0.466	0.520	0.542
Root:Shoot Ratio (S.E.)	0.00321	0.00190	0.00187	0.0174	0.00768	0.00309

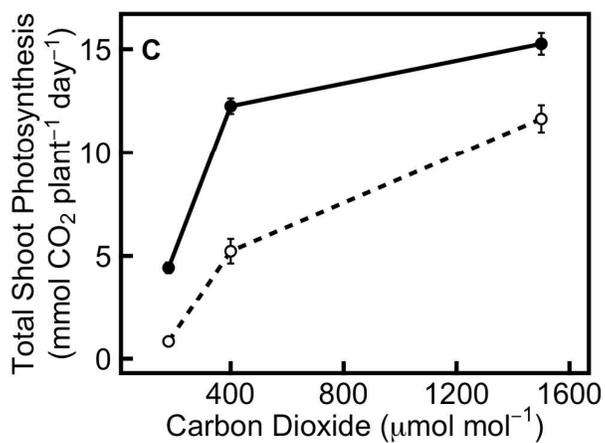
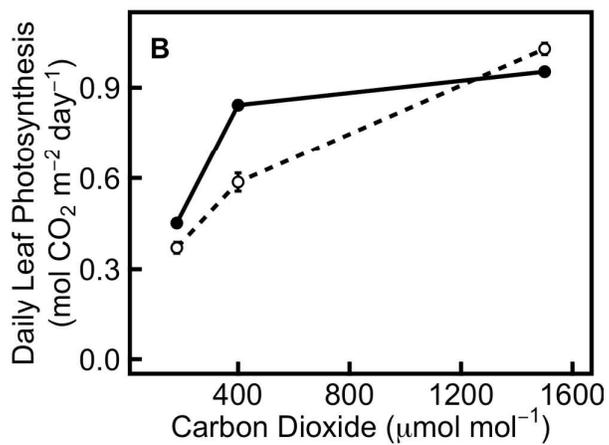
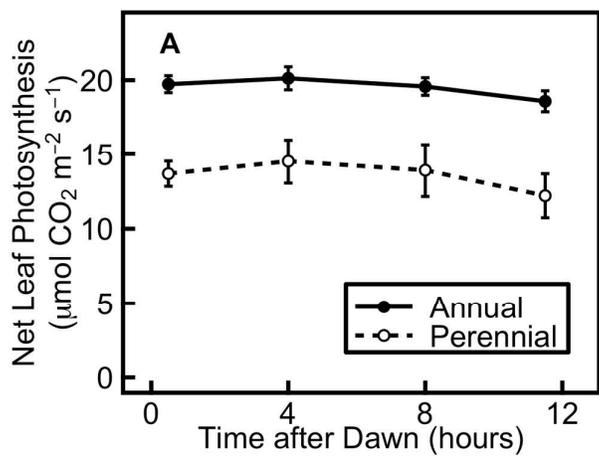
924

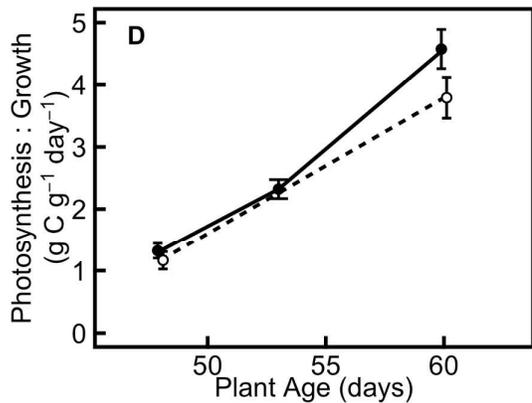
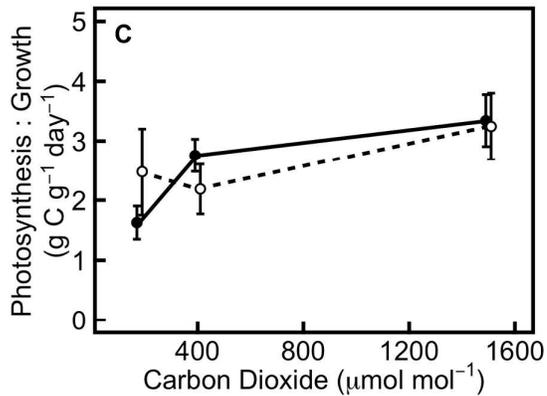
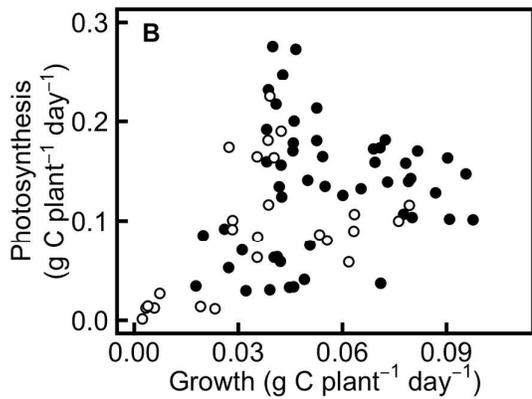
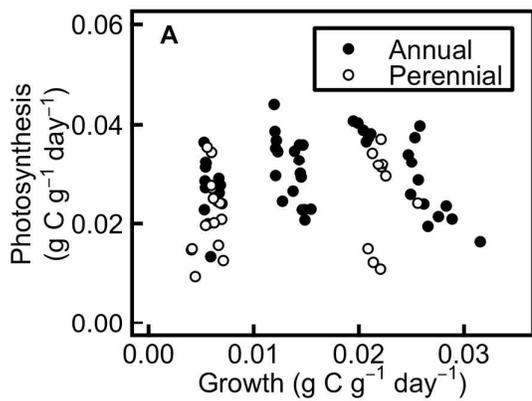
925

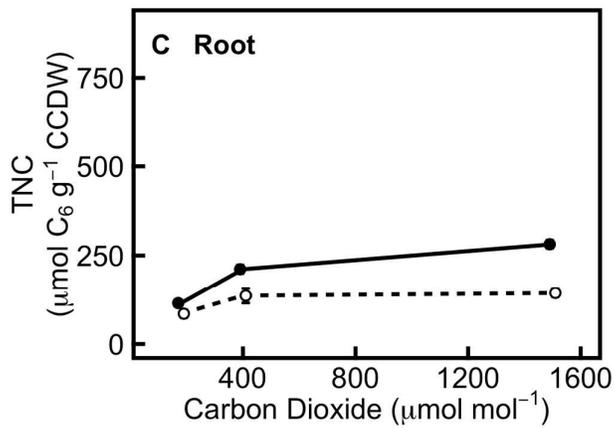
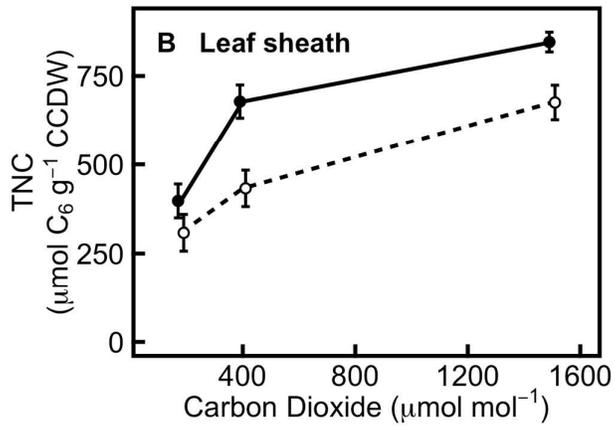
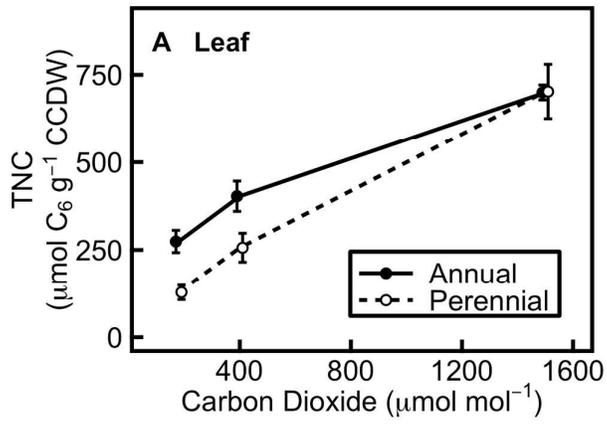
926

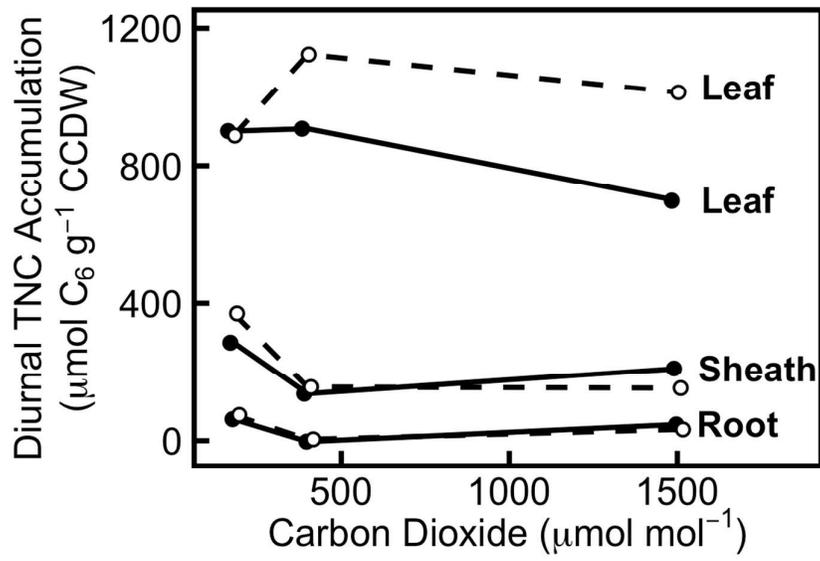
927

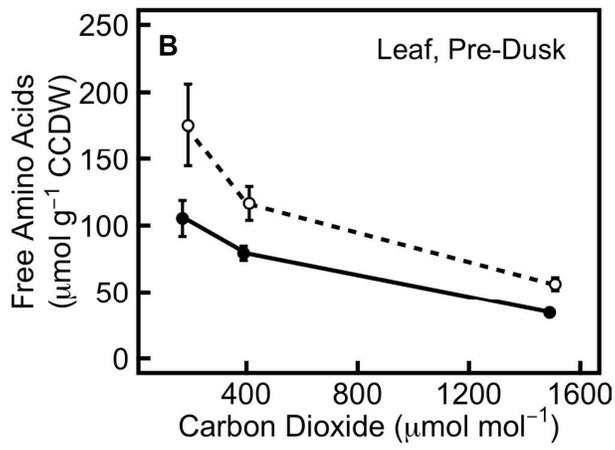
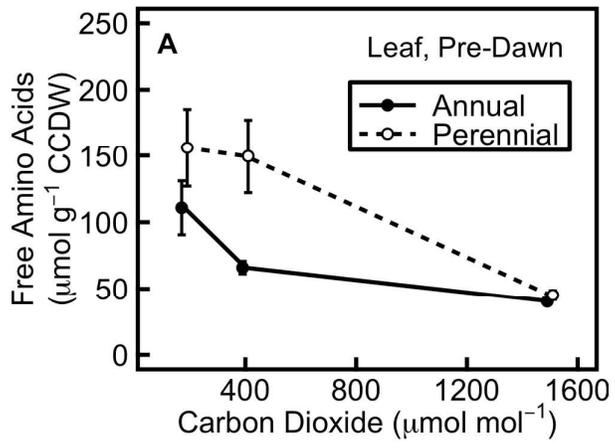


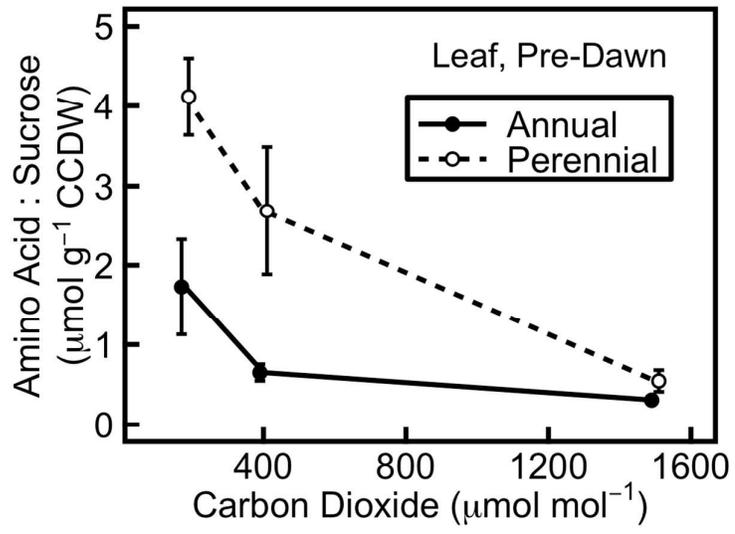












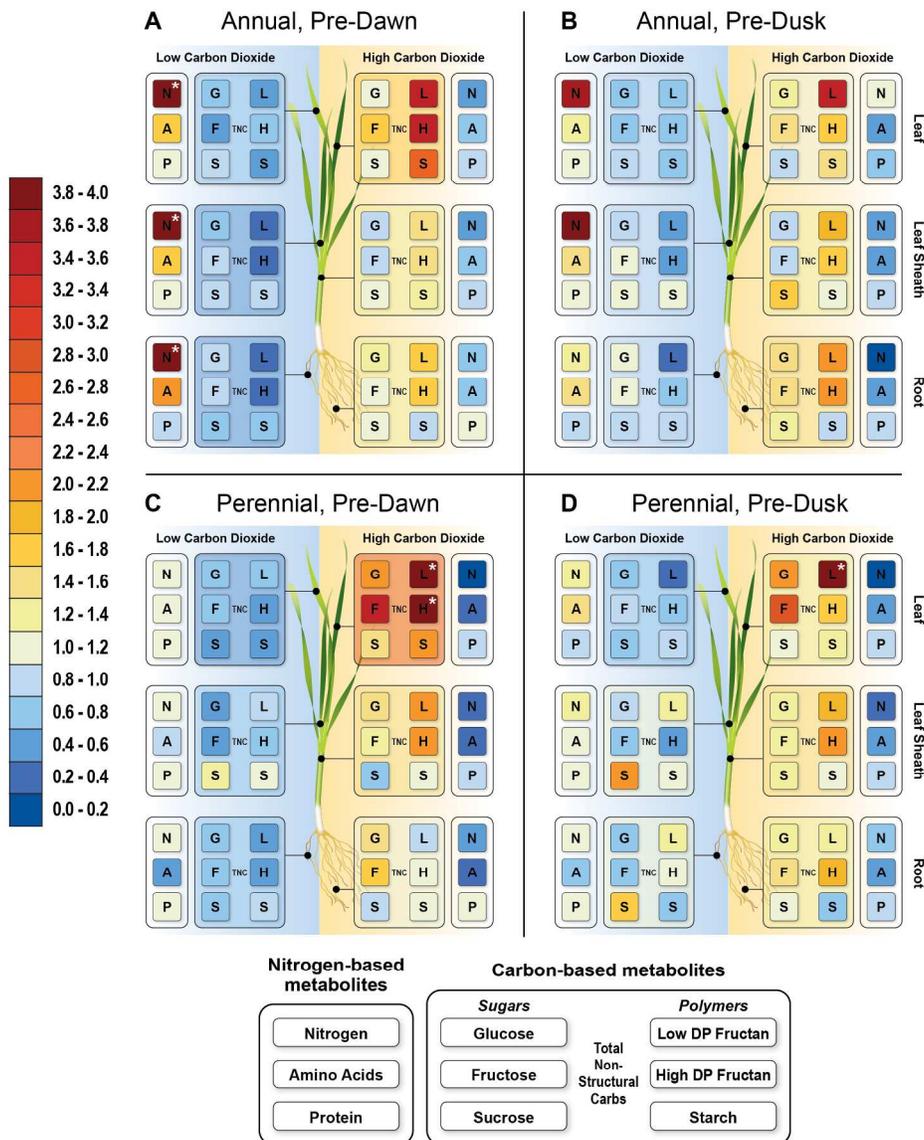
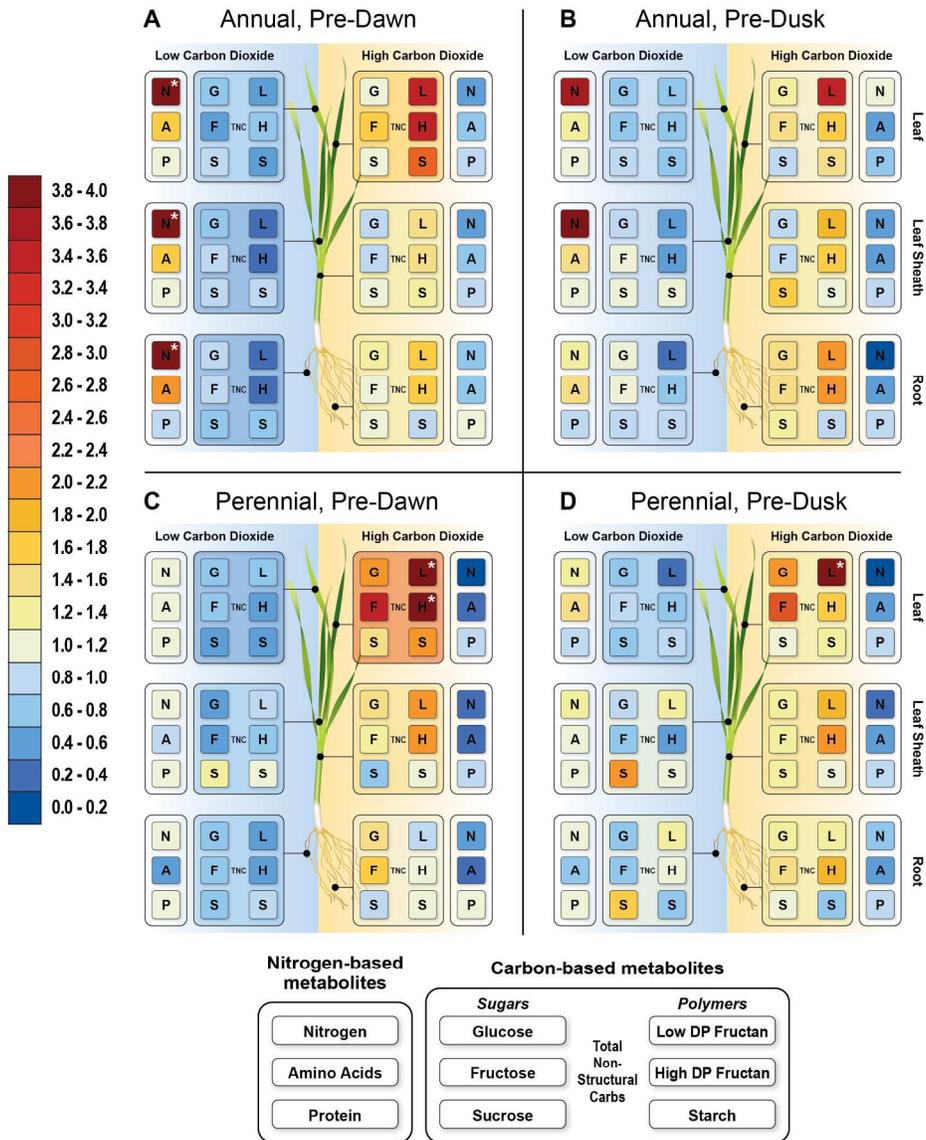


Figure 8. Carbon- and nitrogen-based metabolites in annual and perennial barley at pre-dawn and pre-dusk harvests. Data are ratios of metabolite concentrations in leaf, leaf sheath and root of annual and perennial barley at 180 relative to 400 $\mu\text{mol mol}^{-1}$ CO₂, and 1500 relative to 400 $\mu\text{mol mol}^{-1}$ CO₂. Blue denotes a decrease and orange/red an increase compared to 400 $\mu\text{mol mol}^{-1}$, according to the colour scale on the left; a ratio of 1 signifies no change. Low and high DP refer to the degree of polymerisation in short- and long-chain fructans respectively. A, Annual pre-dawn; B, Annual pre-dusk; C, Perennial pre-dawn; D, Perennial pre-dusk. Exceptionally high ratios, indicated by asterisks, are as follows: annual pre-dawn (A) at 180 $\mu\text{mol mol}^{-1}$ CO₂, nitrate in leaf is 6.5x concentration at 400 $\mu\text{mol mol}^{-1}$, nitrate in leaf sheath is 7.7x concentration at 400 $\mu\text{mol mol}^{-1}$ and nitrate in root is 5.5x concentration at 400 $\mu\text{mol mol}^{-1}$; perennial pre-dawn (C), in leaf at 1500 $\mu\text{mol mol}^{-1}$ CO₂, low DP fructan is 23.4x concentration at 400 $\mu\text{mol mol}^{-1}$ and high DP fructan is 6.3x concentration at 400 $\mu\text{mol mol}^{-1}$; perennial pre-dusk (D), in leaf at 1500 $\mu\text{mol mol}^{-1}$ CO₂, low DP fructan is 4.8x concentration at 400 $\mu\text{mol mol}^{-1}$.

203x241mm (300 x 300 DPI)



Patterns of metabolite accumulation in annual and perennial barley grown at sub-ambient and elevated carbon dioxide.

203x241mm (300 x 300 DPI)

Figure S1. Log mass against log area, for the additional subset of annual and perennial barley plants harvested throughout the duration of the study in order to establish a calibration between mass and area for the plants in the main study. $r^2 = 0.97$, meaning that differences in species and plant area account for 97% of variation in plant mass. Lines show linear regression of area and mass. Log mass = 1.53 log area – 13.11 (annual); log mass = 1.06 log area – 8.37 (perennial).

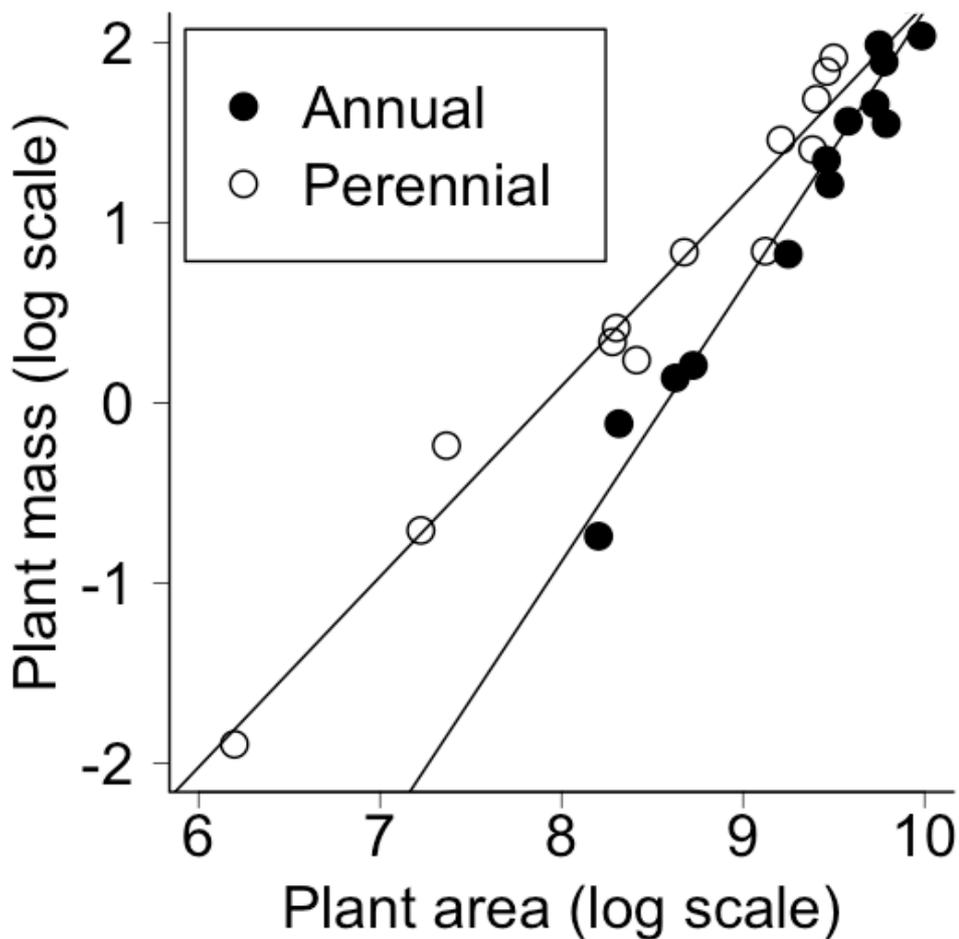


Figure S2. Growth curves for all individuals in the main study used to obtain relative growth rate. Log predicted mass, obtained using mass-area calibration from additional plants (shown in Fig. S1), is plotted against plant age. Blue lines show the overall mean using fixed effects from model; pink lines show growth curves for individual plants. Each plant has a unique ID: A denotes annual individuals and PM and PE denote the two accessions of perennial individuals; numbers were assigned to individuals at random.

