1 # Warming differentially influences the effects of drought on stoichiometry and metabolomics in shoots and roots

4 **Albert Gargallo-Garriga[1,2,3*], Jordi Sardans[1,2], Míriam Pérez-Trujillo[3], Michal Oravec[4], Otmar Urban[4], Anke Jentsch[5], Juergen Kreyling[6], Carl Beierkuhnlein[6], Teodor Parella[3], Josep Peñuelas[1,2].**

7 [1]CSIC, Global Ecology Unit CREAF-CSIC-UAB, Cerdanyola del vallès, 08193, Catalonia, Spain. [2]CREAF, Cerdanyola del vallès, 08193, Catalonia, Spain. [3]Service of Nuclear Magnetic Resonance and Chemistry Department, Faculty of Sciences and Biosciences, Universitat Autònoma de Barcelona, Bellaterra, 08913, Catalonia, Spain. [4]Global Change Research Centre, Academy of Sciences of the Czech Republic, Belidla 4a, CZ-60300 Brno, Czech Republic. [5]Disturbance Ecology and Vegetation Dynamics, University of Bayreuth, D-95440 Bayreuth, Germany. [6]Department of Biogeography, University of Bayreuth, D-95440 Bayreuth, Germany.

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15 Author for correspondence: Jordi Sardans, Tel: +34 581 46 73 email: j.sardans@creaf.uab.cat

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21 ## Summary

22 · Plants in natural environments are increasingly subjected to a combination of abiotic
23 stresses such as drought and warming in many regions. The effects of each stress and
24 the combination of stresses on shoots and roots functioning have been studied
25 extensively, but little is known about the simultaneous metabolome responses of the
26 different organs of the plant to different stresses acting at once.

27 · We studied the shift in metabolism and elemental composition of shoots and roots of
28 two perennial grasses, *Holcus lanatus* and *Alopecurus pratensis*, in response to
29 simultaneous drought and warming.

30 · These species responded differently to individual and to simultaneous stresses. These
31 responses were even opposite in roots and shoots. In plants exposed to simultaneous
32 drought and warming, terpenes, catechin, and indole acetic acid accumulated in
33 shoots, while amino acids, quinic acid, nitrogenous bases, the osmoprotectants choline
34 and glycine-betaine, and elements involved in growth (N, P, and K) accumulated in
35 roots. Under drought, warming further increased the allocation of primary metabolic
36 activity to roots and changed the composition of secondary metabolites in shoots.

37 · These results highlight the plasticity of plant metabolomes and stoichiometry and the
38 different complementary responses of shoots and roots to complex environmental
39 conditions.

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41 **Key words: Climate change, drought, warming, HPLC-MS, NMR, metabolomics, N:P,
42 stoichiometry.**

43 ## Introduction

44 Predictions of climate change project that different stresses will occur simultaneously in many
45 regions. The combination of drought and warming and its high impact on aridity are of great
46 concern (Mittler *et al.*, 2001; Moffat, 2002; Rizhsky *et al.*, 2004). Regions such as the
47 Mediterranean Basin or the Sahel are already affected and may become increasingly affected
48 (IPCC, 2012). In other regions such as central Europe, extreme events such as winter warming
49 and summer drought are likely to occur together with higher frequency (Jentsch *et al*., 2011;
50 IPCC, 2012). Some experimental approaches, simulating the impact of extremes in
51 precipitation (periods of drought or heavy rain) and warming, have been tested in various
52 ecosystems (Beier *et al.*, 2004; Peñuelas *et al.*, 2004, 2007, 2013b; Fay *et al.*, 2008; Smith,
53 2011). A combination of stresses from drought and warming alters the physiological status of
54 grasses and other plants, inhibiting photosynthesis and accumulating products of lipid
55 peroxidation (Jianga & Huang; Perdomo *et al.*, 1996; Jagtap *et al.*, 1998). Some of these studies
56 suggest a molecular response of the plants to the combined effects of drought and warming
57 different from those caused by the single stresses (Rizhsky *et al.*, 2002), but we know little of
58 the metabolomic response at a whole-plant level and of the relationships between the overall
59 use of nutrients by plants, and the elemental stoichiometric composition, and shifts in
60 metabolites.

61 Also Central Europe is affected by extreme events, such as the 2003 heat wave (Schär
62 & Jendritzky, 2004 Jentsch *et al*., 2007). Numerous studies have observed changes in extremes,
63 for example centennial increases in frequency of heavy precipitation (10-30%) in Switzerland
64 (Schmidli & Frei, 2005) and increases in duration of both extremely wet conditions in winter
65 (Schonwiese *et al*., 2003) and of unusually dry periods in summer in whole Europe (Beck *et al.*,

66 2001). The heat wave of 2003 has convincingly been associated with anthropogenically forced
67 global warming (Schär & Jendritzky, 2004). Both drought and warming can thus increase their
68 frequency and intensity (IPCC, 2013), which can affect plants in different seasons, e.g. extreme
69 heat waves in winter and summer (during the growing season) and droughts in summer
70 (Jentsch *et al.*, 2007). These climatic events are expected to have a large impact on plants and
71 ecosystems, to the point of surpassing the thresholds of resistance of ecosystems (Gutschick &
72 BassiriRad, 2003; Schär & Jendritzky, 2004; Reusch *et al.*, 2005; Knapp *et al.*, 2008; Jentsch &
73 Beierkuhnlein, 2008; Jentsch *et al.*, 2011; Smith, 2011). Improving tolerance to these events
74 will be a target for ongoing and future agricultural and nature-conservation programmes.
75 Species of fundamental importance in nature conservation, such as the grasses *Holcus lanatus*
76 and *Alopecurus pratensis* (Beierkuhnlein *et al.*, 2011), can be affected by these extreme events.
77 These species are interesting subjects for studying the impacts of droughts and warming on
78 the metabolomic and stoichiometric shifts in roots and shoots.

79 Metabolomics is a powerful tool for improving our understanding of the changes in
80 metabolism and biochemical composition of organisms, i.e. the ultimate phenotypic response
81 to environmental changes (Fiehn *et al.*, 2000; Weckwerth *et al.*, 2004; Peñuelas & Sardans,
82 2009; Sardans *et al.*, 2011). It is increasingly applied to ecological studies in what has been
83 called ecometabolomics (Peñuelas & Sardans, 2009; Bundy *et al.*, 2009; Sardans *et al.*, 2011;
84 Rivas-ubach *et al.*, 2014). Ecometabolomics can explore the effects of the ecological organism-
85 environment interaction by detecting the final phenotypic response of the organism and by
86 detecting the metabolic pathways that are up- and down-regulated in response to
87 environmental changes.

88 Ecometabolomics has recently been used to monitor the phenotypic changes of a
89 particular genotype in response to the drivers of global change, particularly shifts in
90 temperature (Pinheiro *et al.*, 2004; Michaud & Denlinger, 2007; Michaud *et al.*, 2008; Charlton
91 *et al.*, 2008; Lugan *et al.*, 2009; Fumagalli *et al.*, 2009; Sardans *et al.*, 2011; Rivas-ubach *et al.*,

92 2012, 2014). The effects of drought and warming on metabolomes have been widely studied
93 separately (Cramer *et al.*, 2007; Michaud *et al.*, 2008; Lugan *et al.*, 2009; Fumagalli *et al.*, 2009;
94 Sardans *et al.*, 2011; Rivas-ubach *et al.*, 2012, 2014), but less is known about their combined
95 effect in plants (Rizhsky *et al.*, 2002, 2004). The majority of studies have focused on a single
96 stress treatment applied to plants under controlled conditions, mostly only the effect on
97 photosynthetic tissues. Real field conditions, however, involve different stresses occurring
98 simultaneously, and various plant organs can respond differently to these changes. Roots and
99 shoots can respond asymmetrically, as has been observed at the morphological level, e.g.
100 shifts in the shoot/root biomass and growth-rate ratios occur when the availability of soil
101 water changes (Jefferies, 1993; Guenni & Mar, 2002; García *et al*., 2007; Cordoba-Rodriguez,
102 2011).

103 Exposure to drought has led to the accumulation of fructans, several amino acids, and
104 GABA (Alvarez *et al*., 2008; Charlton *et al*., 2008; Fumagalli *et al*., 2009; Rizhsky *et al*., 2004;
105 Sardans *et al*., 2011). Gargallo-Garriga *et al*. (2014), in the first study of the metabolomic
106 response to drought in whole plants (shots and roots), observed that the metabolomic
107 response differed between and was nearly opposite in shoots and roots. Metabolomic studies
108 of warming stress have observed increases in the concentrations of saturated fatty acids
109 (Horváth *et al.*, 1989; Allakhverdiev *et al.*, 1999) in the thylakoid (Vigh *et al.*, 1989) and plasma
110 membranes (Vigh *et al.*, 1993). As observed for the drought conditions (Gargallo-Garriga *et al.*,
111 2014), plants respond differently to warming at the shoot and root levels. Under elevate
112 temperature, the level of saturation of membrane lipids extracted from the leaves of creeping
113 bentgrass increased, whereas no change in membrane lipids was observed in root tissues
114 (Larkindale & Huang, 2004). Warming has also increased biomass production in several
115 ecosystems (Rustad *et al.*, 2001). Warming can have a positive effect on growth and biological
116 activity when water is not limited, but it can also negatively affect plant growth and primary
117 productivity in other ecosystems, mostly due to lower water availability. Hence we

118 investigated the effects of warming and drought on plant metabolomics in different organs
119 (shoots and roots) simultaneously.

120 Metabolomic changes can imply shifts in the proportional use of various nutrients and
121 the consequent changes in elemental composition and stoichiometry. Rivas-Ubach *et al*. (2012,
122 2014) have recently reported foliar metabolomic changes associated with changes in foliar
123 elemental composition and stoichiometry in response to abiotic (climatic) and biotic (herbivory)
124 factors. These elemental changes are of great ecological importance because they may lead to
125 changes in the species composition of communities and in ecosystem function (Sterner & Elser,
126 2002; Sardans *et al.*, 2012; Peñuelas *et al.*, 2013a). The relationships of the metabolomes and
127 stoichiometries of whole plants (roots and shoots) in response to simultaneous conditions of
128 drought and warming, however, have not received much attention.

129 We investigated the impact of water availability and warming in factorial combination
130 on the elemental composition and stoichiometry and the metabolomic structure of above- and
131 belowground organs (shoots and roots) of *H. lanatus* and *A. pratensis* in different seasons. We
132 tested the hypothesis that warming differentially influences the effects of drought on
133 stoichiometry and metabolomics in shoots and roots.

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141 ## Materials and methods

142 ### Study site

143 The sampling was part of the EVENT II experiment, described in detail by Walter *et al.* (2013)
144 and Gargallo-Garriga *et al.* (2014), where precipitation patterns have been experimentally
145 modified in a semi-natural, extensively managed grassland in the Ecological-Botanical Garden
146 of the University of Bayreuth, Germany (49°55'19''N, 11°34'55''E, 365 m a.s.l.). The climate is
147 temperate and moderately continental with a mean annual temperature of 8.2 °C and a mean
148 annual precipitation of 724 mm (1971-2000, data from the German Weather Service).

149

150 ### Experimental design

151 The field experiment had a two-factorial design manipulating (1) drought (irrigated control,
152 ambient control, and drought) and (2) warming (ambient, winter warming, and summer
153 warming). The design consisted of 45 plots, each 1.5 × 1.5 m in size, with five replications of all
154 factorial combinations (Figure S1). The warmed and unwarmed plots were blocked and
155 randomly assigned within each manipulation of the precipitation. The treatments are
156 described in detail in the supplementary material of Gargallo-Garriga *et al.* (2014).

157

158 ### Target species

159 Two C3 grasses were selected as the target species for this study: *A. pratensis* L. and *H. lanatus*
160 L. Both species were selected based on their high frequency in the experimental plots and their
161 importance in semi-natural grasslands across Central Europe. *A. pratensis* is the dominant
162 species at the experimental site, producing about 18% of the annual aboveground biomass. It
163 is a tall (up to 110 cm) and productive crop of agricultural importance in moist and nutrient-
164 rich meadows. *H. lanatus* is also common at the site but is less productive (3% of the annual

165 aboveground biomass). It occurs in semi-natural grasslands throughout Europe, Asia, and
166 North Africa and is invasive in North America and Australia. It tolerates a wide range of
167 conditions but prefers moist conditions.
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169 ## Collection and preparation of tissue samples

170 Samples were collected at the end of the drought manipulation before irrigation in July and
171 again at the end of the growing season in September. Above- and belowground 360 tissue
172 samples were collected (2 species × 2 organs (leaf blades and fine roots) × 2 sampling dates × 3
173 precipitation manipulations × 15 plots). The procedure for sample preparation is described in
174 detail by Rivas-Ubach *et al*. (2013). Briefly, the frozen samples were lyophilized and stored in
175 plastic cans at -80 °C. Soil contamination was removed from the root samples. Finally, the
176 samples were ground with a ball mill (Mikrodismembrator-U, B. Braun Biotech International,
177 Melsungen, Germany) at 1700 rpm for 4 min, producing a fine powder that was stored at -
178 80 °C until the extraction of the metabolites. See the supplementary material of Gargallo-
179 Garriga *et al.* (2014) for details.
180

181 ## Elemental analysis

182 C and N concentrations were determined from 1.5 mg of each powdered sample by
183 combustion coupled to gas chromatography using a CHNS-O Elemental Analyser (EuroVector,
184 Milan, Italy).

185 P, K, Fe, Mn, Mg, Ca, and S concentrations were determined by extraction by acid
186 digestion in a MARS Xpress microwave reaction system (CEM, Mattheus, USA) under high
187 pressure and temperature. Briefly, 250 mg of dry sample powder were added to 5 mL of nitric

188 acid and 2 mL of H_2O_2 in a Teflon tube. The digested material was transferred to 50-mL flasks
189 and resuspended in Milli-Q water to a final volume of 50 mL. The elemental concentrations
190 were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma)
191 (Perkin-Elmer Corporation, Norwalk, USA).

192

193 Extraction of metabolites

194 Two sets of 50-mL centrifuge tubes were labelled/ set A for analysis by liquid chromatography-
195 mass spectrometry (LC-MS) and set B for analysis by nuclear magnetic resonance (NMR). Each
196 tube of set A received 150 mg of a powdered sample and 6 mL of water/methanol (1/1), and
197 the samples were vortexed for 15 s and then sonicated for 2 min at room temperature. All
198 tubes were centrifuged at 1100 × g for 15 min. Next, 4 mL of each tube of set A were
199 transferred to its corresponding tube of set B. This procedure was repeated for two extractions
200 of the same sample. The resulting extracts were used for metabolomic analysis.

201

202 Preparation of extracts for LC-MS and NMR analyses

203 Two millilitres of the supernatants of each tube of set A were collected using crystal syringes,
204 filtered through 0.22-µm microfilters, and transferred to a labelled set of LC vials. The vials
205 were stored at -80 °C until the LC-MS analysis.

206 Eight millilitres of the extracts were resuspended in water to reduce the proportion of
207 methanol (<15%). The solutions were lyophilised, and 4 mL of water were added to each tube,
208 which was vortexed and centrifuged at 23 000 × g for 3 min. The samples were frozen at -80 °C
209 and lyophilised again. Finally, 1 mL of KD_2PO_4-buffered D_2O solution containing 0.01% TSP
210 (trimethylsilyl propionic acid sodium salt) (pH 6.0) was added to each dried fraction. TSP was

211 used as the internal standard for the NMR experiments. The solutions were transferred to 2-
212 mL centrifuge tubes with a micropipette and centrifuged at 23 000 × *g* for 3 min, and 0.6 mL of
213 the supernatants were transferred to the NMR sample tubes. The procedure for the extraction
214 of the metabolites is described in detail by Rivas-Ubach *et al.* (2013).

215

216 LC-MS analysis

217 LC-MS chromatograms were obtained with a Dionex Ultimate 3000 HPLC system (Thermo
218 Fisher Scientific/Dionex RSLC, Dionex, Waltham USA) coupled to an LTQ Orbitrap XL high-
219 resolution mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with an HESI
220 II (heated electrospray ionisation) source. Chromatography was performed on a reversed-
221 phase C18 Hypersil gold column (150 × 2.1 mm, 3-µ particle size; Thermo Scientific, Waltham,
222 USA) at 30 °C. The mobile phases consisted of acetonitrile (A) and water (0.1% acetic acid) (B).
223 Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath prior to use.
224 The elution gradient, at a flow rate of 0.3 mL per minute, began at 10% A (90% B) and was
225 maintained for 5 min, then to 10% B (90% A) for the next 20 min. The initial proportions (10%
226 A and 90% B) were gradually recovered over the next 5 min, and the column was then washed
227 and stabilised for 5 min before the next sample was injected. The injection volume of the
228 samples was 5 µL. HESI was used for MS detection. All samples were injected twice, once with
229 the ESI operating in negative ionisation mode (-H) and once in positive ionisation mode (+H).
230 The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry)
231 full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60 000). The
232 resolution and sensitivity of the spectrometer were monitored by injecting a standard of
233 caffeine after every 10 samples, and the resolution was further monitored with lock masses
234 (phthalates). Blank samples were also analysed during the sequence. The assignment of the

235 metabolites was based on the standards, with the retention time and mass of the assigned
236 metabolites in both positive and negative ionisation modes (Table S1).

237

238 NMR analysis

239 ^{1}H NMR-based fingerprints were obtained for all samples. One-dimensional (1D) ^{1}H NMR
240 spectra were acquired with suppression of the residual water resonance. The water-resonance
241 signal was presaturated using a power level of 55 dB during a relaxation delay of 2 sec. Each
242 spectrum acquired 32 k data points over a spectral width of 16 ppm as the sum of 128
243 transients and with an acquisition time of 1.7 sec. The total experimental time was ~8 min per
244 sample. All ^{1}H NMR spectra were phased and baseline corrected and referenced to the
245 resonance of the internal standard (TSP) at δ 0.00 ppm using TOPSPIN 3.1 software (Bruker
246 BioSpin, Rheinstetten, Germany). See Rivas-Ubach *et al*. (2013) for more details of the
247 sampling and NMR determination. The data were subsequently used for the statistical analysis.
248 A variable-size bucketing, where buckets were scaled relative to the internal standard (TSP),
249 was applied to all ^{1}H NMR spectra using AMIX software (Bruker BioSpin, Rheinstetten,
250 Germany). The output was a data set containing the integral values for each assigned ^{1}H NMR
251 spectral peak in the described pattern. The buckets corresponding to the same molecular
252 compound were summed.

253 For the assignment of the fingerprint peaks (i.e. identification of the metabolites), 2D
254 NMR experiments on selected representative samples were carried out using the NMR
255 equipment and software previously described. The probe temperature was set to 298.0 K. 1D
256 ^{1}H NMR, 2D ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy
257 (TOCSY), ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC), and ^{1}H-^{13}C heteronuclear
258 multiple-bond correlation (HMBC) were acquired using standard Bruker pulse sequences and

259 routine conditions (see Method S1 of the Supporting Information for details) (Rivas-Ubach *et*
260 *al.*, 2013).

261 Processing of LC-MS and NMR data

262 The LC-MS raw data files were processed using MZMINE 2.10 (Pluskal et al., 2010) (see Table
263 S1 of the Supporting Information for details). Before the numerical database was exported in
264 "csv" format, the chromatograms were base-line-corrected, deconvoluted, aligned and filtered.
265 Metabolites were assigned by comparison with the analyses of the standards (retention time
266 and mass spectrometry) (see Table S2 of the Supporting Information for details). Assigned
267 variables corresponding to the same molecular compounds were summed. The LC-MS data for
268 the statistical analyses corresponds to the absolute peak area at each retention time (RT). The
269 area of a peak is directly proportional to the concentration (i.e. µg/mL) of its corresponding
270 (assigned) metabolite in the sample. Thus, a change in the area of a peak will mean a change in
271 the concentration of its assigned metabolite.

272 The procedure followed for the processing of the ^{1}H NMR spectra and for the
273 assignment of the NMR peaks to their corresponding metabolite is detailed in Rivas-Ubach et
274 al. (2013). Briefly, for the statistical analysis, before the exportation of the ^{1}H NMR numerical
275 databases, all spectra were phased, baseline-corrected and referenced to the resonance of the
276 internal standard TSP (trimethylsilyl propionic acid sodium salt) at δ 0.00 ppm with TOPSPIN
277 3.1 (Bruker Biospin). A variable-size bucketing was thus applied to all the ^{1}H NMR spectra using
278 AMIX software (Bruker Biospin) and the buckets were scaled relative to the internal standard
279 (TSP). The output was a data set containing the integral values for each assigned ^{1}H NMR
280 spectral peak in the described pattern. The buckets corresponding to the same molecular
281 compound were summed.

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284 ## Statistical analyses

285 HPLC-MS and NMR-based fingerprinting and stoichiometric data were analysed by univariate
286 and multivariate statistical analyses. We conducted permutational multivariate analyses of
287 variance (PERMANOVAs) (Anderson *et al*., 2008) using the Euclidean distance, with season
288 (July and September), water-availability (control, drought, and irrigation), warming treatment
289 (control, winter warming, and summer warming), and plant organ (shoots and roots) as fixed
290 factors and individuals as random factors. Multivariate ordination principal component
291 analyses (PCAs) (based on correlations) and partial least squares discriminant analyses (PLS-
292 DAs) were also performed to detect patterns of sample ordination in the metabolomic and
293 stoichiometric variables. The PCAs were initially constructed from the HPLC-MS analysis and
294 the NMR data and included as variables the metabolic profiles and elemental concentrations
295 and ratios of shoots and roots in the different seasons to enable the identification of clusters,
296 groups, and outliers (Sandasi *et al*., 2011) (Fig. 1). The profiles of shoots and roots from July
297 and September were additionally submitted to separate PCAs (Fig. 1). The PC scores of the
298 cases were subjected to one-way ANOVAs to determine the statistical differences among
299 groups with different levels of the categorical independent variables studied (season, species,
300 plant organ, and climatic treatment). The PERMANOVAs, PCAs, PLS analyses, and clustered
301 image maps were conducted by the *mixOmics* package of R software (R Development Core
302 Team 2008). The Kolmogorov-Smirnov (KS) test was performed on each variable to test for
303 normality. All assigned and identified metabolites were normally distributed, and any
304 unidentified metabolomic variable that was not normally distributed was removed from the
305 data set. Statistica v8.0 was used to perform the ANOVAs, post hoc tests, and KS tests.

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309 Results

310 General results

311 Plant shoots and roots had different overall metabolisms (PERMANOVA pseudo-F = 162; P <
312 0.001). The overall metabolisms and elemental concentrations and stoichiometries were also
313 significantly affected by species (pseudo-F = 53.7; P < 0.001), season (pseudo-F = 45.5; P <
314 0.001), drought (pseudo-F = 5.29; P < 0.001), and warming (pseudo-F = 5.53; P < 0.001). Some
315 two-level interactions between factors were also significant season with organ (pseudo-F =
316 13.4; P < 0.001), season with species (pseudo-F = 12.02; P < 0.001), season with drought
317 (pseudo-F = 2.44; P < 0.05), species with drought (pseudo-F = 2.56; P < 0.05), species with
318 warming (pseudo-F = 2.07; P < 0.05), organ with drought (pseudo-F = 3.23; P < 0.001), organ
319 with warming (pseudo-F = 2.50; P < 0.01), and drought with warming (pseudo-F = 1.98; P <
320 0.05). The interaction of season with warming was not significant (pseudo-F = 1.26; P > 0.05).
321 More metabolites were found in the shoots than in the roots. In total 850 metabolic variables
322 were detected, 729 were found in the shoots and 577 in the roots. Shoots and roots shared
323 456 metabolites, 273 compounds were detected in shoots but not in roots, and 121
324 metabolites in roots but not in shoots.

325 Elemental, stoichiometric, and metabolomic shifts across shoots and roots, species,
326 and seasons

327 When all cases were analysed together, PC1 accounted for the differences between roots and
328 shoots, whereas PC2 separated species and seasons (Fig. 1). PCs 1 and 2 explained 26% of
329 variance in the PCA conducted with the shoot samples (including seasons, species, and
330 treatments). Post hoc analysis of the scores indicated that overall shoot metabolome and
331 stoichiometry differed significantly depending on species (PC1, P < 0.001) and season (PC2, P <

332 0.05). Species was thus the primary factor and seasonality the secondary factor for plant
333 shoots. N, P, and K concentrations and C/N, C/K, N/K, and K/P ratios also differed depending
334 on season (Fig. S2, Table S1). The highest P, N, and K concentration ratios were found in the
335 growing season (September sampling) (see Table S3-S10), while the concentration of C and the
336 C/P and N/P ratios were lower in September.

337 The shoot concentrations of amino acids, some related compounds of amino-acid and
338 sugar metabolism (RCAAS), and some sugars such as xylose and mannose were higher in
339 September than in July in both *H. lanatus* and *A. pratensis* (Fig. S2, Table S4).

340 The PCA conducted with all root samples (including species, seasons, and treatments)
341 and the elemental, stoichiometric, and metabolomic data indicated that 18% of the variance
342 was explained by the first and second PCs. A post hoc analysis of the score coordinates showed
343 that the stoichiometries and metabolomes for the seasons were differentiated in PC1 ($P <$
344 0.001) and those for the species in PC2 ($P < 0.05$). These results differed from those for shoots,
345 where the highest variance was explained first by species and secondly by season. Also, the
346 overall metabolism/stoichiometry of the roots did not differ between the two species in July
347 but did in September, when the plants were growing. The roots had the highest C, P, N, and K
348 concentrations in the growing season (September sampling) (Fig. S3 and Sardans *et al*., 2013b
349 for more details).

350 In September, the roots had higher concentrations of some amino acids, while in July
351 (mature plants) the roots had higher concentrations of some RCAAS and some sugars such as
352 pentoses and disaccharides, products directly related to growth (Tab. S3-S18). The shift in
353 metabolism/stoichiometry between seasons was very similar in the roots and shoots (Fig. S2
354 and Fig. S3).

355

356 ## Effects of drought on elemental, stoichiometric, and metabolomic structure in shoots
357 and roots

358 A PLS-DA indicated that both shoot and root samples corresponding to plants growing in both
359 season under the control temperature were separated by their different levels of water
360 availability (control, drought, and irrigation) across factor 1 (Figs. 2). The second cause of
361 variability (separated across PLS-DA factor 2) was plant organ, i.e. the differences between
362 shoots and roots. Species were also separated in the shoot samples. Secondary metabolites,
363 such as ocimene, α-terpinene, limonene, sabinene, and quercetin, had higher concentrations
364 in the drought treatment. The C/N, C/K, N/K, C/P, and N/P ratios were higher in the root
365 samples of the drought treatment than in those in the control and irrigated treatments. The
366 concentrations of C, N, P, K, and S in shoots were higher in the control and irrigated shoot
367 samples than in the drought samples.

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370 ## Effects of warming on elemental, stoichiometric, and metabolomic composition in
371 shoots and roots

372 The PLS-DA conducted only with samples of plants grown under control water conditions
373 showed that shoots and roots growing under different warming stresses tended to be
374 distributed in different directions with respect to axis 2 (Figs. 3, S4 and S5, Tables S3-S10),
375 coinciding with the PERMANOVA results, and also that the metabolomes of shoots and roots
376 differed. No differences among the three warming levels were observed throughout the
377 different seasons, consistent with the lack of significance in the interaction of these two
378 factors in the PERMANOVA. The PLS-DA indicated that the winter-warming and control
379 treatments did not differ, but warming had a higher impact on metabolomic and

380 stoichiometric composition under control water conditions when applied in summer than in
381 winter. The main effects of warming were an increase in the concentrations of most amino
382 acids (mainly in roots) and RCAAS (mainly in shoots) (Fig. 3).

383

384 Effects of the interaction between drought and warming on elemental concentrations, 385 stoichiometries, and metabolomes in shoots

386 Water availability separated shoot metabolomic-stoichiometric composition along factor 1 in
387 the PLS-DA, whereas warming separated plant metabolomic-stoichiometric structure along
388 factor 2 (Fig. 4, Table S3, S5, S7, S9). The metabolites involved in plant growth had higher
389 concentrations in the ambient control and irrigated samples in the water-manipulation
390 treatments, and at these two levels of water availability, warming enhanced the
391 concentrations of these metabolites very little. The PLS-DA thus indicated that warming
392 produced different effects depending on water availability, which was consistent with the
393 interaction between the drought and warming treatments identified by the PERMANOVA. The
394 warming treatments along factor 1 identified different metabolomic-stoichiometric
395 compositions only in the shoot samples of the drought-stressed plants. As previously indicated,
396 secondary metabolites (sabinene, ocimene, α-terpinene, and limonene) generally had higher
397 concentrations in the drought treatment. Catechin and indole acetic acid had higher
398 concentrations when drought coincided with summer warming. Moreover, the warming
399 treatment partially reduced the concentrations of some secondary metabolites, such as some
400 terpenes, and also reduced the C/nutrient and N/P ratios (Fig. 4 and S4, Tables S11, S12, S13,
401 S14). Warming thus moderated the differences of the overall shoot metabolomic-
402 stoichiometric composition of the drought-stressed plants relative to the control and irrigated
403 plants (Fig. 3). Simultaneous drought and warming had different consequences on the
404 metabolomic-stoichiometric composition in shoots than did drought and warming separately.

405 Warming partially diminished the effect of drought but increased the concentrations of some
406 secondary metabolites (catechin and acacetin) and elements (Ca, Mg, and Mn). These results
407 were supported by the ANOVAs of the univariant analyses (Tables. S11-S18).

408

409 Effects of the interaction between drought and warming on elemental concentrations, 410 stoichiometries, and metabolomes in roots

411 The PLS-DA identified the effect of the interaction of drought and warming on metabolomic-
412 stoichiometric structure in roots (Fig. 5 and S6, Table S15, S16, S17, S18). Warming had
413 different effects depending on the water availability of the plants, consistent with the
414 PERMANOVA results.

415 Warming had opposite effects in drought-stressed and irrigated roots. The interactive
416 effect in roots was completely different from that in shoots, where the warming treatment
417 partially negated the effects of drought on the metabolomic-stoichiometric structure relative
418 to the control and irrigated plants. Under drought, the warming treatments further increased
419 the concentrations of some secondary metabolites, such as GABA, choline, and glycine betaine,
420 and further decreased the C/nutrient ratio relative to the control and irrigated plants. The
421 effects of the warming treatments were the opposite under irrigation, leading to a minor
422 difference between the metabolomes and stoichiometries of the drought and control
423 plants/roots (Fig. 5). The concentrations of choline and glycine betaine, which are involved in
424 osmotic processes, were thus higher under drought and the combination of drought and
425 warming treatments. In summary, the overall effects of the warming treatment on
426 stoichiometry and metabolism in plant roots were dependent on water availability.

427

428

429 ## Discussion

430 ### Elemental, stoichiometric, and metabolomic shifts across shoots and roots, species,
431 and seasons

432 The metabolome and elemental stoichiometry of shoots differed more between species than
433 between seasons, whereas the opposite was observed for roots. Primary metabolic activity
434 was higher in September (the growing season) than in July for both shoots and roots. The
435 concentrations of amino acids directly linked to growth and pathways of energy metabolism
436 were higher in September shoots, when the plants were growing. The concentrations of sugars
437 were higher in shoots in July, when the plants were mature, likely due to their accumulation
438 during spring and/or to an increase in cellular osmotic potentials. On the other hand, the
439 shoots had the highest K/P ratio and the lowest N/K and C/K ratios in September, when the
440 plants were growing. K is involved in the plant-water relationship (Babita *et al.*, 2010) through
441 plant osmotic control (Sangakkara *et al.*, 2000; Babita *et al.*, 2010; Laus *et al.*, 2011) and
442 improvement in stomatal function (Farhad *et al.*, 2011).

443 The shoots and roots had higher concentrations of amino acids in September than in
444 July (Fig. 3b, Table S3-S10). The increase in the concentrations of primary metabolites
445 coincided with an increase in N and P concentrations. The increase in P concentration was
446 proportionally higher than the increase in N concentration, which led to lower N/P and C/P
447 content ratios and also coincided with the decrease in the concentration of some C-rich
448 secondary metabolites. These results are in agreement with the Growth Rate Hypothesis,
449 which relates high growth with high concentrations of P and N and low N/P ratios (Sterner &
450 Elser, 2002). High levels of these elements allow more synthesis of amino acids and proteins
451 (more N), which in turn requires more synthesis of RNA (more N and especially more P). The
452 decreasing N/P ratios during the growing season coupled to a shift towards primary metabolic
453 pathways related to growth and energy enhancement have also been found in other terrestrial

454 plants (Rivas-ubach *et al.*, 2012). Moreover, under these favourable conditions for growth, the
455 assimilated C is allocated more to growth and energy supply (more primary metabolism) than
456 to antistress or defensive mechanisms (less secondary metabolism). Higher levels of these
457 elements and also the higher concentrations of nitrogenous bases allow more synthesis of
458 amino acids and proteins (more N).

459

460 Effect of drought on elemental, stoichiometric, and metabolomic shifts in shoots and 461 roots

462 The metabolomes of shoots and roots under drought conditions generally shift in opposite
463 directions, although some metabolites change in the same direction (Gargallo-Garriga *et al*.,
464 2014). Plants accumulate a variety of compounds that function as osmoprotectants in shoots.
465 A moderate water stress may be accompanied by the accumulation of metabolites such as
466 proline and glycine betaine, whereas a severe water stress may be accompanied by the
467 accumulation of sugars such as sucrose (Bohnert, 2000; Hoekstra *et al.*, 2001). We observed
468 that shoots under drought conditions also accumulated other metabolites such as related
469 intermediate or derivative compounds of amino acids (RCAAS), osmoprotectants (glycine
470 betaine and choline), and hexoses.

471 Roots accumulate disaccharides and amino acids when exposed to drought conditions.
472 The accumulation of these metabolites has been described in leaves under different stresses
473 (Rizhsky *et al.*, 2004; Pinheiro *et al.*, 2004; Charlton *et al.*, 2008; Rivas-ubach *et al.*, 2012, 2014)
474 but have not been described in plant roots yet. The shoot samples of the drought-treated
475 plants from September and July had higher concentrations than control plants of metabolites
476 with an antioxidant function, such as some polyphenolic compounds, quinic acid, malic acid,
477 jasmonic acid, and sugars such as those of the family of hexoses and xylose (Fig. 4). Quinic acid

478 is a precursor in the shikimic acid pathway, a common metabolic pathway in the biosynthesis
479 of aromatic amino acids such as tyrosine, tryptophan, and phenylalanine (Draths *et al.*, 1999)
480 that are precursors of a large variety of secondary metabolites such as lignins, flavonoids,
481 alkaloids, and phytodexins (Herrmann, 1995).

482 These metabolic differences were accompanied by an increase in the concentration of
483 K, resulting in low C/K and N/K ratios and a high K/P ratio. The relationship between higher
484 concentrations of osmoprotective secondary metabolites and K concentrations in response to
485 drought has been also observed in the leaves of the Mediterranean shrub *Erica multiflora*
486 (Rivas-Ubach *et al*., 2012) and is related to the improvement in the control of water use
487 (Sangakkara *et al.*, 2000). In contrast, the production of other secondary metabolites related to
488 osmotic protection, such as choline and glycine betaine (McNeil *et al*., 2001), has not been
489 observed to be up-regulated in drought-stressed plants. These metabolic and stoichiometric
490 changes in plants under drought conditions are consistent with the increase in oxidative stress.

491

492 Effect of warming on elemental, stoichiometric, and metabolomic shifts in shoots and 493 roots

494 Warming under control conditions of water availability increased the concentrations of
495 primary metabolites mainly related to energy metabolism (RCAAS) in shoots but increased the
496 concentrations of amino acids in roots. Warming led to a general decrease in the
497 concentrations of several secondary metabolites in both shoots and roots. Plants in the
498 warming treatment did not have higher concentrations of metabolites related to the heat-
499 shock response, such as sucrose and glucose, or a coordinated increase in the pool sizes of
500 amino acids (asparagine, leucine, isoleucine, threonine, alanine, and valine), derivatives of
501 oxalacetate, and pyruvate (Kaplan *et al.*, 2004). The warming treatment applied in this study,

502 based on a realistic projection, thus apparently did not induce heat-shock metabolism. This
503 moderate warming, however, was associated with an increase in some primary metabolites
504 under the expected normal conditions of water availability.

505

506 Effects of the interaction between drought and warming on metabolomic and 507 stoichiometric shifts across shoots and roots

508 Shoots and roots subjected to simultaneous drought and warming responded differently than
509 when subjected to each treatment separately. Warming further increased the accumulation of
510 proline in shoots under a severe water stress. In contrast, the concentrations of osmolytes and
511 some compounds related to growth such as nitrogenous bases and some amino acids that help
512 to protect the root under water stress increased in roots when drought was applied together
513 with warming more than under drought alone. This increase was related to the higher
514 concentrations of C, N, P, and K observed under drought plus warming than under drought
515 alone, suggesting that the plants allocated more resources associated with growth and cellular
516 activity under drought and warming than solely under drought. Thus, in contrast to shoots,
517 roots had higher concentrations of metabolites linked to growth and energy in response to
518 warming applied together with drought than when submitted only to drought.

519 The literature on transcripts involved in the defence of plants against abiotic
520 conditions such as cold, drought, and salinity reports considerable common responses
521 (e.g.,Kreps *et al.*, 2002; Oztur *et al.*, 2002; Seki *et al.*, 2002; Sardans *et al.*, 2013). Other studies
522 have found that leaves respond to combined drought and warming stresses with a lower
523 suppression of primary metabolism, the production of some terpenes, and increases in
524 concentrations of other secondary metabolites such as catechin and indole acetic acid than do
525 plants growing under drought stress. Our results thus suggest that plants respond differently

526 under simultaneous drought and warming depending on the tissue. Shoots suppressed their
527 primary metabolism less and changed their anti-stress metabolic strategy less under combined
528 drought and warming conditions than under drought alone. Warming under drought enhanced
529 the concentrations of compounds in roots related to growth and energy metabolism more
530 than solely under drought. The combination of both stresses in this case likely enhanced the
531 effect of the drought by reducing the water availability due to the warming. Warming had a
532 stronger effect alone in roots than when applied with drought.

533 Changes in soil temperature not only influence the growth and development of roots,
534 but can also impact the root-shoot relationships. Gosselin & Trudel (1986) observed that
535 increasing the temperature of the root zone from 12 to 36 °C tended to increase the shoot dry
536 mass and the overall productivity of pepper (*Capsicum annum* L.). This higher activity of the
537 plant and the increase in primary elemental sources related with the growth of roots can
538 enhance the water-uptake capacity of plants. Other studies of *Arabidopsis* (Hellmann *et al.*,
539 2000) and *Nicotiana tabacum* (Rizhsky *et al.*, 2002) have suggested that this mode of defence
540 response is conserved among different plants subjected to the combination of warming and
541 drought.

542 The shift in metabolomic-stoichiometric composition in response to environmental
543 changes has thus been demonstrated to be very different in above-and belowground tissues of
544 the same plant. In response to drought, aboveground tissues had lower levels of metabolites
545 associated with energy and growth metabolism (sugars, amino acids, and nucleosides), lower
546 N, P, and K concentrations, and a higher C/N ratio. Belowground organs had the opposite
547 pattern.

548 In summary, the stoichiometric and metabolomic responses of plants to warming
549 strongly depend on water availability, and the response differs in shoots and roots. Warming
550 under drought conditions stimulates root primary metabolic activity more than drought alone.

551 Compared to drought alone, shoots under simultaneous warming and drought shifted their
552 osmoprotective and antis-tress strategies by down- and up-regulating the synthesis of various
553 secondary metabolites and by activating some primary metabolic pathways. Our results thus
554 demonstrated different metabolomic expressions in different parts of the plant and a large
555 plasticity in the responses to environmental changes.

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778 ## Supporting Information

779 Additional supporting information may be found in the online version of this article.

780 **Fig. S1** Experimental design.

781 **Fig. S2** PC1 versus PC2 of a seasonal PCA of *Holcus lanatus* and *Alopecurus pratensis* shoots.

782 **Fig. S3** PC1 versus PC2 of a seasonal PCA of *Holcus lanatus* and *Alopecurus pratensis* roots.

783 **Fig. S4** Component 1 vs component 2 of the PLS-DA of *Holcus lanatus* and *Alopecurus pratensis*
784 shoots in the warming plus drought (factorial) treatment.

785 **Fig. S5** Component 1 vs component 2 of the PLS-DA of *Holcus lanatus* and *Alopecurus pratensis*
786 roots in the warming plus drought (factorial) treatment.

787 **Fig. S6** Component 1 vs component 2 of the PLS-DA of *Holcus lanatus* and *Alopecurus pratensis*
788 shoots and roots in the warming plus drought (factorial) treatment.

789 **Table S1** Analytical technique (LC-MS and/or NMR) used for the identification of the
790 metabolites and their categorization in terms of biochemical group and metabolic pathway.

791 **Table S2** Processing parameters of LC-MS chromatograms

792 **Table S3** One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in July in
793 different warming treatments.

794 **Table S4** One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in July in different
795 warming treatments.

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797 **Table S5** One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in September in
798 different warming treatments.

799 **Table S6** One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in September in
800 different warming treatments.

801 **Table S7** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in July in
802 different warming treatments.

803 **Table S8** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in July in
804 different warming treatments.

805 **Table S9** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in
806 September in different warming treatments.

807 **Table S10** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in
808 September in different warming treatments.

809 **Table S11** One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in July in
810 different warming treatments within different levels of water availability.

811 **Table S12** One-way ANOVAs of identified metabolites in *Holcus lanatus* shoots in September in
812 different warming treatments within different levels of water availability.

813 **Table S13** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in July in
814 different warming treatments within different levels of water availability.

815 **Table S14** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* shoots in
816 September in different warming treatments within different levels of water availability.

817 **Table S15** One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in July in
818 different warming treatments within different levels of water availability.

819 **Table S16** One-way ANOVAs of identified metabolites in *Holcus lanatus* roots in September in
820 different warming treatments within different levels of water availability.

821 **Table S17** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in July in
822 different warming treatments within different levels of water availability.

823 **Table S18** One-way ANOVAs of identified metabolites in *Alopecurus pratensis* roots in
824 September in different warming treatments within different levels of water availability.

825 **Methods S1** Details of NMR metabolite elucidation.

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Figure Captions

Figure 1. Plots of cases and variables in the PCA conducted with the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* using PC1 versus PC2. (A) The cases are categorized by season and organ. Seasons are indicated by different colours (green, September; red, July). The two species are indicated by geometric symbols (circles, *A. pratensis*; triangles, *H. lanatus*). Open symbols represent roots, and solid symbols represent shoots. (B) Loadings of the various elemental stoichiometric and metabolomic variables in PC1 and PC2. NMR variables are marked with inverted commas (‘) and LC-MS variables with asterisks (*). C, N, P, and K concentrations and ratios and Fe, Mn, Mg, Ca, and S concentrations are shown in red. The various metabolomic families are represented by colours; dark blue, sugars; green, amino acids; dark green, amino-acid derivats; yellow, compounds associated with the metabolism of amino acids and sugars; cyan, nucleotides; and brown, terpenes and phenolics. Metabolites; glycine-alanine‘ (Gly-Ala), valine* (Val.), tryptophan* (Try.), threonine* (Thr.), serine*‘ (Ser.), lysine* (Lys.), leucine* (Leu.), proline* (Pro.), phenylalanine* (Phe.), histidine* (Hys.), glycine* (Gly.), glutamine* (Gln.), asparagine* (Asn.), isoleucine* (Ile.), arginine* (Arg.), alanine* (Ala.), glutamic acid* (Glu.), aspartic acid* (Asp.), gamma-aminobutyric acid‘ (GABA), glycine betaine‘ (GB), choline‘ (choline), tartaric acid* (Tar.), pyruvate* (Pyr.), malic acid* (Mal.), jasmonic acid* (JA), indole acetic acid* (Indole acetic), caffeic acid* (Caff.), ascorbic acid* (Asco.), vanillic acid* (Vanillic acid), citric acid* (Cit.), α-ketoglutaric acid‘* (αKC), lactic acid* (Lac.), shikimic acid‘ (SA), quinic acid’* (QA), chlorogenic acid* (CGA), chinic acid* (Cin. acid), xylose* (Xyli.), hexose* (Hexose), mannose* (Man.), disaccharide*‘ (Dis.), adenine* (Adenine), uracil* (Uracil), thymine‘ (Thymine), uridine* (Uridine), acacetin* (Acace.), catechin* (Cate.), α-terpinene* (αTerpin.), sabinene* (Sabinene), resveratrol* (Resv.), quercetin* (Quer.), ocimene* (Ocimene), limonene* (Limonene), galangin* (Galangin), kaempferol* (Kamp.), phenolic group‘ (Phenol.). Unassigned metabolites are represented by small grey points.

Figure 2. Component 1 vs component 2 of the partial least squares discriminant analysis (PLS-DA) of the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons, shoots, and roots. (A) Samples categorised by shoots and roots in the drought treatment. Water availability is indicated by different colours (green, ambient control; red, drought; blue, irrigated). *Holcus lanatus* is represented by triangles and *Alopecurus pratensis* by circles. Shoots and roots are represented by solid and open symbols, respectively. (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Figure 1.

Figure 3. Component 1 vs component 2 of the partial least squares discriminant analysis (PLS-DA) of the elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons, shoots, and roots. (A) Samples categorised by shoots and roots in the warming treatment. Warming is indicated by different colours (green, ambient control; red, summer warming; blue, winter warming). *Holcus lanatus* is represented by triangles and *Alopecurus pratensis* by circles. Shoots and roots are represented by solid and open symbols, respectively. (B) Component 1 and component 2 of the stoichiometric and metabolomic variables. Metabolites as in Figure 1.

871 **Figure 4.** Component 1 vs component 2 of the partial least squares discriminant analysis of the
872 elemental, stoichiometric, and metabolomic variables in *Holcus lanatus* and *Alopecurus*
873 *pratensis* for data of both seasons, shoots, and roots. (A) Samples categorised scores (mean ±
874 S.E.) by shoots and roots in the warming plus drought (factorial) treatment. Drought is
875 indicated by different colours (green, ambient control; red, drought; blue, irrigated) and letters
876 (A, control ambient; D, drought; I, irrigated). Warming is indicated by letters (W_s, summer
877 warming; W_w, winter warming). (B) Component 1 and component 2 of the stoichiometric and
878 metabolomic variables. Metabolites as in Figure 1.

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880 **Figure 5.** Relevance networks of the elemental, stoichiometric, and metabolomic variables in
881 *Holcus lanatus* and *Alopecurus pratensis* for data of both seasons for warming plus drought
882 (factorial). Interaction networks between various environmental variables (root, shoots,
883 drought, summer warming, and winter warming) and analysed metabolites. This plot was
884 constructed after Sparse Partial Least Square (SPLS) analysis by differential metabolites among
885 provenances. Green (blue) indicates a high positive (negative) correlation. Shoots and roots
886 are represented by different letters (S, Shoots; R, Roots). Treatments are indicated by different
887 letters colours (green, ambient control; red, drought; blue, irrigated), letters (C, control
888 ambient; D, drought; I, irrigated) and circles. Warming is indicated by letters (sW, summer
889 warming; wW, winter warming). Stoichiometric and metabolomic variables are represented by
890 rectangle.

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