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**Highlighting the threat from current and near-future ozone pollution to clover in pasture.**

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1 **Highlighting the threat from current and near-future ozone pollution to clover in**  
2 **pasture**

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12

13 **Full research paper**

14  
15 **Abstract**

16 Globally, the legume-rhizobia symbiosis, contained within specialised organs called  
17 root nodules, is thought to add at least 30 Tg N annually to agricultural land. The  
18 growth and functioning of a modern white clover (*Trifolium repens* cv. Crusader) and  
19 red clover (*T. pratense* cv. Merviot) cultivar were investigated in current and future  
20 ozone scenarios in solardomes. Both cultivars developed leaf injury and had significant  
21 reductions in root biomass and root nodule number in response to ozone, with Crusader  
22 also displaying a reduced size and mass of nodules. In-situ measurements of N-fixation  
23 in Crusader by acetylene reduction assay revealed reduced N-fixation rates in a future  
24 scenario with an increased background and moderate peaks of ozone. The implications  
25 for the sustainability of temperate pasture are discussed.  
26

27 **Capsule:** ozone effects on the growth and functioning of clover cultivars

28 **Keywords:** clover; nodulation; ozone; nitrogen fixation; pasture; background ozone

29 **Introduction**

30 Nitrogen (N) fixation by legumes (Fabaceae) is of vital agronomic importance. On a  
31 global scale, the legume-rhizobia symbiosis, contained within specialised organs called  
32 root nodules, is thought to add at least 30 Tg N annually to agricultural land (Herridge

33 *et al.* 2008). At present, legume crops account for ~15% of utilised arable land area  
34 (FAO, 2013), constituting the primary source of dietary protein for a substantial  
35 proportion of the human population. Legumes are also an essential component of many  
36 pasture systems; improving the protein content, nutritional value and uptake of forage,  
37 as well as providing ancillary benefits to the structure and long-term fertility of soils  
38 (Parsons & Chapman, 1999). In temperate regions of Europe, Oceania and the  
39 Americas, clovers (*Trifolium* spp.) are the most important pasture legume. Surprisingly,  
40 given the agricultural importance of clover, little attention has been paid in recent  
41 decades to the fact that *Trifolium* spp. are amongst the most sensitive known to ground-  
42 level ozone pollution (*e.g.* Hayes *et al.* 2007). Worryingly, concentrations of  
43 tropospheric ozone have risen in that time over arguably all of the clover-growing  
44 regions of the world (The Royal Society, 2008). The potential for losses in quantity and  
45 quality of pasture forage, with a concurrent need for increased usage of artificial  
46 fertiliser in current and near-future ozone regimes, formed the motivation for this study.

47 At present, background levels of tropospheric ozone are high enough to damage  
48 sensitive crops across the Northern Hemisphere (Mills *et al.* 2011a), with a mean  
49 concentration of 30-40ppb representing a doubling of the pre-industrial background  
50 (Vingarzan, 2004). In respect of its threat to agricultural production and food security,  
51 tropospheric ozone is the most important air pollutant (Avnery *et al.* 2011; Mills *et al.*  
52 2011a; Wilkinson *et al.* 2011). Ozone damage occurs in plants via the induction of  
53 oxidative stress, leading to foliar injury, impacts on gas exchange, photosynthesis,  
54 growth and eventual yield (Wilkinson *et al.* 2011).

55 Grassland systems and constituent species have been identified as particularly  
56 sensitive to ozone pollution (*e.g.* Hayes *et al.* 2007; Mills *et al.* 2007). Indeed,  
57 numerous studies have highlighted the complex response of managed grasslands to  
58 ozone (for reviews see Bassin *et al.* 2007 & Fuhrer, 2009), with pasture forage

59 susceptible to reductions in quality and yield, as well as shifts in species composition,  
60 with uncertain effects upon the carbon (C) sink strength of grassland systems (see Mills  
61 *et al.* 2012). Most previous experiments on ozone effects on clover were conducted in  
62 the 1970s and mid-1990s, usually with ozone profiles exhibiting high peaks and a low  
63 baseline concentration, no longer representative of current ambient conditions in  
64 Europe. Due to the improved control of precursor emissions, local peak concentrations  
65 of ozone have decreased in Europe in the last 20 years, whilst the baseline has steadily  
66 risen, in part due to the hemispheric transport of ozone precursors from other regions  
67 (Parrish *et al.* 2012). Furthermore, previous studies often used relatively high ozone  
68 concentrations, delivering unrealistically acute dosages (*e.g.* Letchworth & Blum, 1976;  
69 Blum *et al.* 1983). Results from studies with mixed-species swards are highly complex  
70 and range from a gradual reduction in yield of the *Trifolium* fraction to no overall effect  
71 on botanical composition (*e.g.* Blum *et al.* 1983; Rebbeck *et al.* 1988; Heagle *et al.*  
72 1989; Fuhrer *et al.* 1994; Ashmore & Ainsworth, 1995; Pleijel *et al.* 1996; Nussbaum *et*  
73 *al.* 1995; Wilbourn *et al.* 1995; Gonzalez-Fernandez *et al.* 2008; Hayes *et al.* 2009).  
74 Differential sensitivity to ozone induced foliar injury within *Trifolium* spp. lends utility  
75 for their use as ozone biomonitors (Mills *et al.* 2011b).

76 Nodulation in legumes is primarily controlled by long distance root and shoot-  
77 derived signalling (termed autoregulation of nodulation (AON)) (Mortier *et al.* 2012). A  
78 complete understanding regarding the molecular nature of AON signalling, and more  
79 generally, the role of C and N supply in the determination of nodule number, remains  
80 obscure (*e.g.* Ludidi *et al.* 2007; Mortier *et al.* 2012). N-fixation is an energy-intensive  
81 process, and nodules in legumes are a strong sink for assimilates, such that root and  
82 shoot growth may be suppressed in hypernodulating mutants (*e.g.* Ito *et al.* 2007;  
83 Yoshida *et al.* 2010). Superfluous nodulation is regulated by a shoot-derived inhibitor  
84 (SDI), with the long-distance transport and differential concentration of auxin,

85 brassinosteroids and jasmonic acid (JA) suggested as likely candidates for the SDI  
86 signal (Mortier *et al.* 2012). Nodulation is also determined by local hormonal  
87 regulation, with JA, abscisic acid (ABA) and ethylene together acting as local negative  
88 regulators of nodule initiation (Mortier *et al.* 2012).

89 Ozone-impacts on nodulation or N-fixation have been shown in several legumes  
90 including soybean (Tingey & Blum, 1973; Reinhart & Weber, 1980; Jones *et al.* 1985;  
91 Pausch *et al.* 1996), peanut (Ensing *et al.* 1985; Cong *et al.* 2009) and beans (Manning  
92 *et al.* 1971; Blum & Heck, 1980). Research by Blum & Tingey (1977) does not support  
93 a significant direct influence of ozone on legume root nodules, with reduced  
94 photosynthate translocation suggested by this, and other studies, as the cause for a  
95 reduction in nodule growth (*e.g.* Tingey & Blum, 1973; Reinhart & Weber, 1980).  
96 Stable isotope studies by Pausch *et al.* (1996), and Cong *et al.* (2009), also attribute  
97 ozone impacts on N-fixation to a reduced availability of assimilate. However, relatively  
98 few studies have directly addressed the impacts of ozone on clover nodulation; still less  
99 having explored the mechanistic basis of these effects, and the potential impacts on  
100 pasture sustainability caused by the current and near-future concentrations of ozone.  
101 Letchworth & Blum (1976) reported a reduction in nodule growth in *T. repens* in  
102 response to acute exposure in closed chamber studies, although nitrogenase activity per  
103 nodule, and per plant, was not significantly altered. In contrast, Ensing *et al.* (1982), and  
104 Montes *et al.* (1983), in open-top-chamber studies, reported ozone-induced reductions  
105 in N-fixation in *T. pratense* and *T. repens* respectively. Further, ozone-induced  
106 reductions in total N or % N in *T. repens* biomass are reported by Letchworth & Blum  
107 (1976), Blum *et al.* (1983) and Montes *et al.* (1983), with some studies reporting some  
108 effect upon the crude protein content (*e.g.* Blum *et al.* 1983; Fuhrer *et al.* 1994; Sanz *et*  
109 *al.* 2005) and digestibility (*e.g.* Fuhrer *et al.* 1994; Sanz *et al.* 2005; Muntifering *et al.*  
110 2006; Gonzalez-Fernandez *et al.* 2008) of *Trifolium* forage. Ozone impacts may occur

111 in earliest root tip development in *Trifolium* spp. (Vollnes *et al.* 2010), whilst infection  
112 by rhizobia may afford some level of protection to ozone impacts on growth relative to  
113 non-inoculated controls (Miller *et al.* 1997).

114 Given the considerable agronomic importance of clover, there is a need to update and  
115 expand our understanding of the influence of ozone on nodulation and N-fixation in  
116 current clover cultivars. In this study, the effects of ozone on the injury, stomatal  
117 conductance (gs) and biomass accumulation of *T. repens* and *T. pratense* cultivars,  
118 recommended for general use in grazed leys (British Grassland Society, 2013) are  
119 assessed, with ozone exposure profiles representing a realistic range of reduced peak  
120 and increased baseline scenarios. The effect of ozone on the nitrogenase activity of the  
121 *T. repens* cultivar is also determined in-situ, and potential implications for the  
122 sustainability of temperate pasture are discussed.

123

## 124 **Materials and methods**

### 125 *Clover cultivars*

126 *T. repens* cv. Crusader, a medium-leaved cultivar used for frequent cutting and grazing,  
127 and *T. pratense* cv. Merviot, used for cutting and finishing autumn stock, (hereafter  
128 referred to as Crusader and Merviot) were sown as seeds into cell trays in compost  
129 (John Innes No. 2; J. Arthur Bowers, Lincoln, UK) in late spring 2012. Seeds were  
130 obtained from a commercial seed supplier, and originated from the UK (Wynnstay  
131 Seeds; UK). Plants were propagated in plug-plant trays in an unheated glass-house,  
132 watered by hand as necessary and thinned when appropriate to one seedling per cell.  
133 After 3 weeks of growth, seedlings of each cultivar were transferred into 5L plant pots  
134 (22cm diameter x 19.1cm depth), filled with sterile topsoil (Gravelmaster, UK), with 4  
135 seedlings arranged evenly in each pot. To introduce a soil microbe population, pots  
136 were inoculated with 200ml of a soil slurry mixture made from approximately 5kg of

137 soil from agricultural grassland (Abergwyngregyn, North Wales, UK, 53°14'N, 4°01'W)  
138 and 14L water. Seedlings were grown for a further 4 weeks. On 06/07/2012, 42 pots per  
139 cultivar, selected for consistent size, were then transferred to a series of 7 'solardomes'  
140 (hemispherical glasshouses; 3m diameter, 2.1m high) at the CEH solardome facility  
141 near Bangor, North Wales, with 6 pots of each cultivar per solardome.

142

143 *Ozone system and treatments.*

144 Plants were then exposed to a range of ozone treatments based on an episodic profile  
145 recorded at a rural ozone monitoring site (Aston Hill, Wales, UK, 52°50'N, 3°03'W)  
146 with a unique treatment in each solardome. Treatments were designed to reflect future  
147 ozone scenarios, with peak concentrations reduced by more than the background (Figure  
148 1). Treatments were applied to the solardomes randomly. Plants were exposed to the  
149 ozone treatments for a three-month period, starting 11/07/2012 and finishing  
150 03/10/2012.

151 Ozone was provided to the solardomes by a G11 ozone generator and a workhouse 8  
152 oxygen generator (Dryden Aqua, UK), with ozone added to charcoal-filtered air, and  
153 with concentration determined by a computer-controlled ozone injection system  
154 (LabVIEW version 8.6; National Instruments, Texas, US). Ozone was distributed to  
155 each solardome via PTFE tubing, with the concentration inside each solardome  
156 measured for 5 min every 30 minutes using two ozone analysers (400a, Enviro  
157 Technology Services, Stroud, UK) of matched calibration. In one solardome, ambient  
158 air temperature, photosynthetically active radiation (PAR) and vapour pressure deficit  
159 (VPD) were continuously monitored by an automatic weather station (Skye Instruments  
160 Ltd, Llandridod Wells, UK). Plants were rotated within each dome weekly and watered  
161 twice-weekly, with additional watering when necessary to maintain soil moisture  
162 content at or near field capacity.

163

164 *Ozone injury*

165 After 3 weeks exposure, visible ozone injury and senescence was scored for each  
166 cultivar across each ozone treatment. The number of injured leaves (ozone injury >25%  
167 leaflet area) in a representative quarter of each pot was recorded and expressed as a  
168 percentage of the total number of leaves.

169

170 *Stomatal conductance (gs)*

171 Stomatal conductance (gs) of both cultivars was determined at intervals throughout the  
172 growth season across all ozone treatments in naturally fluctuating climatic conditions.  
173 All measurements were made using a porometer (AP4, Delta T Devices, Cambridge,  
174 UK), between 10:00-16:00h, on the abaxial surface of leaves displaying <10% ozone  
175 injury and senescence. Solardomes were visited in random order, and measurements  
176 were made in the presence of ozone. Soil moisture content was determined after every  
177 measurement with a hand-held soil moisture probe and sensor (ML2x ThetaProbe, HH2  
178 Moisture Meter; Delta T Devices, Cambridge, UK).

179

180 *Biomass harvest*

181 After 12 weeks of growth, the shoot, root and nodule mass of the plants from each  
182 cultivar was harvested. Shoot biomass was harvested for the entire pot in October. For  
183 rapidly-growing Merviot, a mid-season harvest of shoot biomass was also performed in  
184 late August after 7 weeks exposure by cutting back to 7cm. Below-ground biomass was  
185 determined from a representative quarter of each pot, due only to the extensiveness of  
186 the root system. Furthermore, below-ground biomass was determined in treatments 1, 4  
187 and 7 only, as harvest of the roots took almost 3 weeks; even with cold storage, it was  
188 considered inappropriate to store soil samples for longer than this due to the re-growth

189 or decomposition of root material. Nodules were excised from the root system, counted  
190 and weighed. Shoots and roots were dried for a minimum of 48 hours at 60°C or until  
191 constant mass was achieved. Nodule biomass was air dried and sized into two  
192 categories based on maximum length (<0.1-0.7mm; 0.7->1.5mm). Root biomass, nodule  
193 biomass and nodule numbers per pot were calculated as follows:

194

195  $\text{Root biomass pot}^{-1} = (\text{root biomass quarter}^{-1} / \text{soil mass quarter}^{-1}) * \text{soil mass pot}^{-1}$

196  $\text{Nodule biomass pot}^{-1} = \text{nodule biomass g root}^{-1} * \text{root biomass pot}^{-1}$

197  $\text{Nodules pot}^{-1} = \text{nodules g root}^{-1} * \text{root mass pot}^{-1}$

198

199 Mass-per-nodule, root:shoot, total biomass and root:total biomass were also determined.

200 To allow comparison with previously published data, and to facilitate analysis of ozone  
201 effects on a UK scale, biomass variables were expressed to accumulated exposures  
202 above a threshold of 40ppb during daylight hours at canopy height (AOT40, units  
203  $\text{ppmh}^{-1}$  (after Fuhrer (1994)).

204

205 *Acetylene reduction assays (ARA)*

206 Assessments of system nitrogenase activity were performed on Crusader in treatments 1  
207 and 7, using a method adapted from Lindstrom (1984). Two weeks prior to the assay,  
208 two sealable 400ml plastic bottles, with the bottom removed and fitted with a gas  
209 septum, were inserted to a depth of 2cm into the centre of each pot. For the assay, a  
210 10% acetylene atmosphere was generated inside one bottle by removing 10% of the air  
211 and immediately replacing it with acetylene gas (BOC, Guildford, UK). The second  
212 bottle acted as a control to determined baseline ethylene generated from the soil.

213 Acetylene was stored and transported to the solardome facility in inert gas bags

214 (SUPELCO, Bellefonte, US), which were vented to the atmosphere and flushed through

215 with N<sub>2</sub> after use. 15 ml gas samples were taken from the bottles at 0, 4 and 8 and 24  
216 hours, with a 1ml sub-sample analysed for ethylene content using a mass-selective  
217 detector (Model 6890, Agilent Technologies, Santa Clara, US). Ethylene peak area was  
218 determined using G1701DA analytical software (version D.00.00.38; Agilent  
219 Technologies, Santa Clara, US). Two assays were performed, in similar climate  
220 conditions, in the 10<sup>th</sup> and 11<sup>th</sup> weeks of exposure.

221

#### 222 *Statistical analyses*

223 The precise ozone control system used in the solardomes allowed small changes in  
224 ozone profile to be simulated, facilitating dose-response analyses. We note that the lack  
225 of treatment replication may raise concerns about pseudo-replication. However, we  
226 believe the benefit of using more treatments outweighs this limitation, as published  
227 previously by Mills *et al.* 2009, Hayes *et al.* 2012 and others. Air flow rates are matched  
228 between solardomes, and where recorded, climatic conditions did not vary significantly  
229 from solardome to solardome (e.g. leaf temperature, see supplementary information).  
230 For consistency with existing literature, injury and gs, variables were each analysed by  
231 general linear regression, with the 3 week (for injury data) or 12 week AOT40 value for  
232 each treatment applied as the predictor variable. For biomass and ARA variables,  
233 parameters were analysed via one-way analysis of variance (ANOVA) with 12 week  
234 AOT40 values in the former and 10 and 11 week AOT40 values in the latter applied as  
235 a factor. For nodule size, each size category was analysed separately against the 12  
236 week AOT40 value for each treatment. To exclude outliers due to very high or low  
237 PAR, a cohort of gs data for Crusader (n=133) and Merviot (n=104) was selected for  
238 analysis using the 25-75% quartile range of all recorded ambient PAR data for each  
239 cultivar respectively. Post hoc Tukey's honest significant difference tests were applied  
240 to assess pairwise differences between means where ANOVA revealed a significant

241 effect of ozone. Insufficient gs data was collected for the modelling of ozone flux-effect  
242 relationships. All analyses were conducted using R software version 2.15.2 (R Core  
243 Development Team, 2012).

244

## 245 **Results**

### 246 *Ozone concentrations and climate conditions*

247 During the course of the experiment, the seven ozone treatments generated seasonal 24  
248 hr means of 33, 36, 40, 45, 51, 54 & 66ppb and AOT40 values of 0.4, 1.0, 2.7, 5.2, 8.6,  
249 11.5 and 18.5ppm h<sup>-1</sup> (Figure 1; Table 1). Ozone concentrations increased in each  
250 treatment during the weekend reaching a maximum peak on Mondays, and a minimum  
251 on Thursdays (Figure 1). The ozone treatments successfully simulated decreasing peak  
252 and background concentrations, with greater reductions in peak than background ozone.  
253 Mean daylight (when PAR >50 μmol m<sup>-2</sup> s<sup>-1</sup>) air temperature and VPD were 21.3°C and  
254 0.84 kPa for the study period, with maxima of 24.6°C and 1.14 kPa. Mean daytime  
255 PAR was 521 μmol m<sup>-2</sup> s<sup>-1</sup>, with an average daily maximum of 814 μmol m<sup>-2</sup> s<sup>-1</sup>.

256

### 257 *Ozone injury and gs*

258 Both Crusader and Merviot displayed highly significant increases in visible leaf injury  
259 with increasing ozone concentrations ( $p<0.001$  and  $p=0.01$  respectively) (Figure 2a),  
260 with Crusader displaying a significantly higher injury rate with increasing ozone  
261 exposure ( $p<0.001$ ). Baseline injury and senescence were detectable in both cultivars in  
262 the lowest exposure treatment (24hr mean of 33 ppb, AOT40 of 0.4ppm h<sup>-1</sup>). There was  
263 no relationship between mean gs and increasing ozone in Crusader ( $r^2<0.10$ ;  $p=0.54$ ) or  
264 in Merviot ( $r^2=0.21$ ;  $p=0.09$ ) (Figure 2b). However, there was a pronounced cultivar  
265 effect, with Merviot displaying significantly higher mean gs rates than Crusader  
266 ( $p<0.001$ ).

267

268 *Biomass harvest*

269 Both cultivars had highly significant ozone-induced reductions in root biomass per pot,  
270 with a decrease of 61% in Crusader ( $p=0.01$ ) and 63% in Merviot ( $p=0.01$ ) in the  
271 highest ozone treatment 7 relative to the lowest treatment 1 (Figure 3a). End-of season  
272 shoot biomass for each cultivar, and shoot biomass of two individual harvests of  
273 Merviot, did not display any significant responses to ozone (Figure 3b). The reduction  
274 in root biomass also manifested as proportional declines in root:shoot and root:total  
275 biomass ratios for both cultivars (Figure 3c; Table 2). Each cultivar also had reductions  
276 in nodule number per pot, with a significant decrease of 36% in Crusader ( $p=0.02$ ) and  
277 reduction of 32% in Merviot ( $p=0.09$ ) (Figure 3d) in treatment 7 compared to treatment  
278 1. In Crusader, a decreased number of nodules per pot was accompanied by a 40%  
279 reduction in the proportion of larger nodules with a maximum length  $> 0.7\text{mm}$  ( $p=0.01$ )  
280 (Figure 4a). Consequently, Crusader pots had a 36% reduction in mass-per-nodule  
281 ( $p=0.04$ ) (Figure 3e) and a 60% reduction in nodule mass per pot ( $p=0.002$ ) (Figure 3f)  
282 relative to treatment 1. In contrast, nodule size, mass-per-nodule and nodule-mass-per-  
283 pot in Merviot were unaffected by increasing ozone (Figure 3e, f, Figure 4b). However,  
284 Merviot displayed increases of 128% in nodule number ( $p=0.01$ ) and 133% in nodule  
285 mass, per gramme of root material ( $p=0.02$ ), in the high ozone treatment 7 compared to  
286 treatment 1 (Table 2). Both Crusader and Merviot experienced a decline in total  
287 biomass, with a 13% reduction in the former ( $p=0.08$ ) and a significant 25% reduction  
288 in the latter ( $p=0.01$ ).

289

290 ARA

291 In both assays, a small amount of ethylene was detected after 0 hours, less than 1% of  
292 the amount present at the end of the incubation (not shown). In the week 10 assay, mean

293 ethylene evolution per cm<sup>2</sup> of soil surface showed a trend for a reduction in treatment 7  
294 after 4 hours incubation compared to treatment 1 ( $p=0.06$ , Figure 5a). In week 11,  
295 ethylene evolution per cm<sup>2</sup> was significantly reduced in treatment 7 after 8 hours  
296 ( $p=0.05$ , Figure 5b). No ethylene was detected in either assay after 24 hours.

297

## 298 **Discussion**

299 This study has updated existing knowledge of the effects of ozone on the growth and  
300 functioning of current clover cultivars in present and near-future ozone. We report  
301 increased foliar injury and decreased biomass of a white clover (Crusader) and red  
302 clover (Merviot) cultivar, with Crusader also displaying a consistent reduction in N-  
303 fixation in high ozone.. The implications of these effects are discussed below in relation  
304 to options for reduction in peak and background atmospheric ozone concentrations.

305 In the present study, Crusader and Merviot both displayed a partitioning of ozone  
306 effects, with systemic reductions in below-ground and total biomass, and an absence of  
307 ozone impacts on shoot biomass despite the occurrence of ozone-induced foliar injury  
308 and senescence. The maintenance of growth in the shoots at the expense of root biomass  
309 has been demonstrated previously in *Trifolium* spp. (*e.g.* Letchworth & Blum, 1977;  
310 Miller *et al.* 1997), and is otherwise extensively reported as a common response to  
311 ozone-induced oxidative stress. Foliar injury may similarly occur in chronic ozone  
312 exposures without an effect on above-ground biomass (*e.g.* in potato; Temmerman *et al.*  
313 2002). While foliar injury in *Trifolium* spp. may display closer correlations with ozone  
314 flux in pasture vegetation than when related to accumulated exposure indices (Mills *et*  
315 *al.* 2011b; 2011c), clear linear relationships were found with AOT40 values in the non-  
316 water limiting conditions of this study.

317 The overall reduction in nodules-per-pot observed in both cultivars may have arisen  
318 from a general reduction in the translocation of photoassimilates to the root system, but

319 more specifically due to an enhanced regulation of nodulation via downstream AON  
320 (Mortier *et al.* 2012). A reduction in nodule growth in Crusader, manifesting in a  
321 reduced mass-per-nodule and an increased proportion of small, likely non-fixing,  
322 pseudonodules (Figure 4), would also suggest a reduced availability of assimilate in the  
323 root system. This also explains consistent differences in nodule activity (measured by  
324 in-situ ARA) in Crusader between treatments 1 and 7. In Merviot, higher gs rates may  
325 hint at a greater capacity to supply root nodules with assimilates during ozone-induced  
326 oxidative stress (Figure 2), explaining why the growth of individual root nodules was  
327 unaffected (Figure 3e; 4b).

328 The role of phytohormones in moderating above-ground stress responses to ozone is  
329 well established, (*e.g.* Rao & Davies, 2001; Wilkinson & Davies, 2009; Cho *et al.*  
330 2011), though the influence of ozone on their below-ground action and accumulation  
331 remains poorly characterised. In Merviot, the significant increase in nodule density per  
332 gramme of root biomass may suggest a decrease in ethylene sensitivity localised within  
333 the root vasculature to maintain plant growth (Lohar *et al.* 2009; Mortier *et al.* 2012;  
334 Chan *et al.* 2013). Ozone-induced stress ethylene is hypothesised as a general antagonist  
335 for ABA signalling (Wilkinson & Davies, 2009). We therefore speculate that an  
336 increase in nodule density may also have arisen due to a down-regulation in ABA  
337 synthesis and/or signalling, mediated by ozone-induced increases in below-ground  
338 ethylene. The results presented, here support the synthesis of published data by Hayes *et*  
339 *al.* 2007, which indicated a lower ozone sensitivity in *T. pratense* compared to *T.*  
340 *repens*, perhaps due to differences in photosynthetic capacity and resilience, and/or in  
341 the production and action of endogenous defence compounds (*e.g.* Francini *et al.* 2007).

342 In subterranean clover (*T. subterranean*), exposure to comparable mean  
343 concentrations of ozone affect forage quality in as little as 30 days (Sanz *et al.* 2005),  
344 with impacts in *T. repens* readily apparent after a 3 month period (González-Fernández

345 *et al.* 2008). However, ozone impacts on forage quality, and, in particular, the N and  
346 crude protein content of *Trifolium* forage, do vary, depending on exposure method and  
347 community composition, and it is unclear from the available literature how well these  
348 parameters lend to assessments of ozone sensitivity within and between *Trifolium* spp.  
349 (Letchworth & Blum, 1976; Blum *et al.* 1983; Montes *et al.* 1983, Fuhrer *et al.* 1994;  
350 Sanz *et al.* 2005). To some degree, ozone impacts on leguminous N-fixation can be  
351 compensated by an increased supply and uptake of soil N in short term exposures (*e.g.*  
352 Pausch *et al.* 1996; Cong *et al.* 2009). The effect of ozone on forage quality and the soil  
353 N pool, through leaf chemical composition and indirectly via reduced fixation, were not  
354 determined in this study, but are worthy of further study.

355 The most severe impacts of ozone on clover biomass, nodulation and N-fixing  
356 activity observed in this study occurred in a weekly repeated present-day ozone profile  
357 (treatment 7). Further investigations are needed to determine whether these effects  
358 presently occur on a landscape-scale basis, and indeed, whether such impacts translate  
359 to measureable declines in the productivity, and hence the profitability, of pasture.  
360 Nevertheless, on the basis of this study, average reductions in N-fixation, determined  
361 after an 8 hour ARA incubation, may potentially lead to an increased fertiliser usage in  
362 the highest ozone scenario with additional costs to producers, and potentially  
363 detrimental environmental impacts.

364

### 365 *Conclusions*

366 This study has provided for the first time some insight into beneficial effects of  
367 progressive controls on ozone precursors. On the evidence, controls leading to decreases  
368 in peak ozone concentrations by ~30ppb and baseline concentrations by ~10ppb may  
369 increase root nodule biomass of white clover by as much as 45%. Controls on the  
370 emission of ozone precursors have been included in recent multi-model predictions,

371 suggesting a globally reduced tropospheric ozone burden by year 2030 in most relative  
372 concentration pathways (RCPs) (Young *et al.* 2013), with regional concentrations  
373 displaying an increased sensitivity to climate change (Langner *et al.* 2013). The  
374 potential impacts of ozone on the biomass, nodulation and N-fixation of clover  
375 described in this study thus provide a continuing economic and environmental incentive  
376 for controls on the emission of trans-boundary ozone precursors.

377

### 378 **Acknowledgements**

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381

### 382 **References**

- 383 Ashmore, M.R., Ainsworth, N. 1995. The effects of ozone and cutting on the species  
384 composition of artificial grassland communities. *Functional Ecology*, **9**, 708-712.
- 385  
386 Avnery, S., Mauzerall, D.L., Liu, J., Horowitz, L.W. 2011. Global crop yield reductions  
387 due to surface ozone exposure: 1. Year 2000 crop production losses and economic  
388 damage. *Atmospheric Environment*, **45**, 2284-2296.
- 389  
390 Bassin, S., Volk, M., Fuhrer, J. 2007. Factors affecting the ozone sensitivity of  
391 temperate European grasslands: an overview. *Environmental Pollution*, **146**, 678-691.
- 392  
393 Blum, U., Heck, W.P. 1980. Effects of acute ozone exposures on snap bean at various  
394 stages of its life cycle. *Environmental and Experimental Botany*, **20**, 73-85.
- 395  
396 Blum, U., Tingey, D.T. 1977. A study of the potential ways in which ozone could  
397 reduce root growth and nodulation of soybean. *Atmospheric Environment*, **11**, 737-739.
- 398  
399 Blum, U., Heagle, A.S., Burns, J.C., Linthurst, R.A. 1983. The effects of ozone on  
400 fescue-clover forage: regrowth, yield and quality. *Environmental and Experimental*  
401 *Botany*, **23**, 121-132.
- 402  
403 British Grassland Society. 2013. *Recommended List of Grasses and Clover 2013*.  
404 Available at:[http://www.britishgrassland.com/document/recommended-grass-and-](http://www.britishgrassland.com/document/recommended-grass-and-clover-list-farmer-handbook)  
405 [clover-list-farmer-handbook](http://www.britishgrassland.com/document/recommended-grass-and-clover-list-farmer-handbook).
- 406  
407 Chan, P.K., Biswas, B., Gresshoff, P.M. 2013. Classical Ethylene Insensitive Mutants  
408 of the Arabidopsis EIN2 Orthologue Lack the Expected 'hypernodulation' response in  
409 *Lotus japonicas*. *Journal of Integrative Plant Biology*, **55**, 395-408.

410  
411 Cho, K., Tiwari, S., Agrawal, S.B., Torres, N.L., Agrawal, M., Sarkar, A., Shibato, J.,  
412 Agrawal, G.A., Kubo, A., Rakwal, R. 2011. Tropospheric Ozone and Plants:  
413 Absorption, Responses, and Consequences. *Reviews of Environmental Contamination*  
414 *and Toxicology*, **212**, 61-111.  
415  
416 Cong, T., Booker, F.L., Burkey, K.O., Shujjin, H. 2009. Elevated Atmospheric Carbon  
417 dioxide and O<sub>3</sub> differentially alter Nitrogen acquisition in Peanut. *Crop Science*, **49**,  
418 1827-1836. Ensing, J., Hofstra, G. 1982. Impact of the air pollutant ozone on acetylene  
419 reduction and shoot growth of red clover. *Canadian Journal of Plant Pathology*, **4**, 237-  
420 242.  
421  
422 Ensing, J., Hofstra, G., Roy, R.C. 1985. The impact of Ozone on Peanut exposed in the  
423 Laboratory and Field. *Phytopathology*, **75**, 429-432.  
424  
425 FAO. 2013. Food and Agriculture Organization of the United Nations database.  
426 Available at: <http://faostat.fao.org>  
427  
428 Francini, A., Nali, C., Pichhi, V., Lorenzini, G. 2007. Metabolic changes in white  
429 clover clones exposed to ozone. *Environmental and Experimental Botany*, **60**, 11-19.  
430  
431 Fuher, J. 1994. The critical level for ozone to protect agricultural crops-an assesment  
432 of data from European open-top chamber experiments, in; Fuhrer, J., Achermann, B  
433 (Eds), *Critical levels for ozone. A UN-ECE workshop report*. Schriftenreihe de FAC  
434 Liebfeld, **16**, 42-57.  
435  
436 Fuhrer, J. 2009. Ozone risks for crops and pastures and future climates.  
437 *Naturwissenschaften*, **96**, 173-194.  
438  
439 Fuhrer, J., Madari-Sharia, H., Perler, R., Tschannen, W., Grub, A. 1994. Effects of  
440 ozone on managed pasture: II. Yield, Species, Composition, Canopy Structure and  
441 Forage quality. *Environmental Pollution*, **86**, 307-314.  
442  
443 González-Fernández, I., Bass, D., Muntifering, R., Mills, G., Barnes, J. 2008. Impacts  
444 on ozone pollution on productivity and forage quality of grass clover swards.  
445 *Atmospheric Environment*, **42**, 8755-8759.  
446  
447 Hayes, F., Jones, M.L.M., Mills, G., Ashmore, M. 2007. Meta-analysis of the relative  
448 sensitivity of semi-natural vegetation species to ozone. *Environmental Pollution*, **146**,  
449 754-762.  
450  
451 Hayes, F., Mills, G., Ashmore, M. 2009. Effects of ozone on inter- and intra-species  
452 competition and photosynthesis in mesocosms of *Lolium perenne* and *Trifolium repens*.  
453 *Environmental Pollution*, **157**, 208-214.  
454  
455 Hayes, F., Wagg, S., Mills, G., Wilkinson, S., Davies, W. 2012. Ozone effects in a drier  
456 climate: implications for stomatal fluxes of reduced stomatal sensitivity to soil drying in  
457 a typical grassland species. *Global Change Biology*, **18**, 948-959.  
458

459 Heagle, A.S., Rebbeck, J., Shafer, S.R. Blum, U., Heck, W.W. 1989. Effects of long-  
460 term ozone exposure and soil moisture deficit on growth of a ladino clover-tall fescue  
461 pasture. *Phytopathology*, **79**, 128–136.

462  
463 Herridge, D.F., Peoples, M.B., Boddey, R.M. 2008. Global inputs of biological fixation  
464 in agricultural systems. *Plant Soil*, **311**, 1-18.

465  
466 Ito, S., Ohtake, N., Sueyoshi, K., Ohyama, T. 2007. Characteristics of initial growth of  
467 hypernodulation mutants, NOD1-3, NOD2-4 and NOD3-7, affected by inoculation of  
468 bradyrhizobia and nitrate supply. *Soil Science and Plant Nutrition*, **53**, 66-71.

469  
470 Jones, W., Mulchi, C.L., Kenworthy, W.J. 1985. Nodule Activity in Soybean Cultivars  
471 Exposed to Ozone and Sulfur Dioxide. *Journal of Environmental Quality*, **14**, 60-65.

472  
473 Langner, J., Engardt, M., Baklanov, A., Christensen, J.H., Gauss, M., Geels, C.,  
474 Hedegaard, G.B., Nuterman, R., Simpson, D., Soares, J., Sofiev, M., Wind, P., Zakey,  
475 A. 2012. A multi-model study of impacts of climate change on surface ozone in  
476 Europe. *Atmospheric Chemistry and Physics*, **12**, 10423-10440.

477  
478 Letchworth, M., Blum, U. 1976. Effects of acute ozone exposure on growth, nodulation  
479 and nitrogen content of ladino clover. *Environmental Pollution*, **14**, 303-312.

480  
481 Lindström, K. 1984. Analysis of factors affecting in situ nitrogenase (C<sub>2</sub>H<sub>2</sub>) activity of  
482 *Galega orientalis*, *Trifolium pratense* and *Medicago sativa* in temperate conditions.  
483 *Plant and Soil*, **79**, 329-341.

484  
485 Lohar, D., Stiller, J., Kam, J., Stacey, G., Gresshoff, P.M. 2009. Ethylene insensitivity  
486 conferred by a mutated *Arabidopsis* ethylene receptor gene alters nodulation in  
487 transgenic *Lotus japonicas*. *Annals of Botany*, **104**, 277-285.

488  
489 Ludidi, N.N., Pellny, T.K., Kiddle, G., Dutilleul, C., Van Heerden, P.D., Dutt, S.,  
490 Powers, S., Römer, P., Foyer, C.H. 2007. Genetic variation in pea (*Pisum sativum* L.)  
491 demonstrates the importance of root but not shoot C/N ratios in the control of plant  
492 morphology and reveals a unique relationship between shoot length and nodulation  
493 intensity. *Plant, Cell & Environment*, **30**, 1256-1268.

494  
495 Manning, W.J., Feder, W.A., Papia, P.M., Perkins, I. 1971. Influence of foliar ozone  
496 injury on root development and root surface fungi of pinto bean plants. *Environmental  
497 Pollution*, **1**, 305–312.

498  
499 Miller, J.E., Shafer, S.R., Schoeneberger, M.M., Pursley, W.A., Horton, S.J., Davey, C.  
500 1997. Influence of mycorrhizal fungus and/or Rhizobium on growth and biomass  
501 partitioning of subterranean clover exposed to ozone. *Water, Air and Soil Pollution*, **96**,  
502 233-248.

503  
504 Mills, G., Harmens, H., Vandermeiren, K., Bender, J. 2011a. Introduction in: Mills, G.,  
505 Harmens, H (Eds), *Ozone Pollution: A hidden threat to food security*. Programme  
506 Coordination Centre for the ICP Vegetation, Centre for Ecology and Hydrology,  
507 Bangor, UK.pp 13-18.Available at: <http://icpvegetation.ceh.ac.uk/>

508

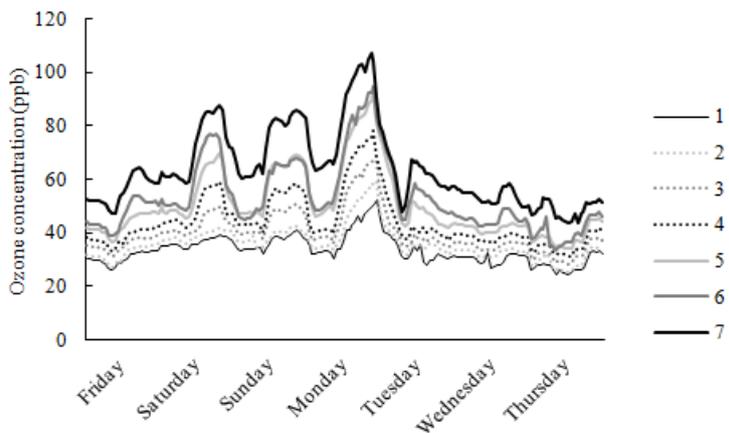
509 Mills, G., Hayes, F., Simpson, D., Emberson, L., Norris, D., Harmens, H., B ker, P.  
510 2011b. Evidence of widespread effects of ozone on crops and (semi-)natural vegetation  
511 in Europe (1990 – 2006) in relation to AOT40– and flux-based risk maps. *Global*  
512 *Change Biology*, **17**, 592-613.  
513  
514 Mills, G., Hayes, F., Norris, D., Hall, J., Coyle, M., Cambridge, H., Cinderby, S.,  
515 Abbott, J., Cooke, S., Murrells, T. 2011c. *Impacts of Ozone Pollution on Food Security*  
516 *in the UK: A Case Study for Two Contrasting years, 2006 and 2008*. Report for Defra  
517 contract AQ08610. Available at <http://icpvegetation.ceh.ac.uk/>  
518  
519 Mills, G., Hayes, F., Jones, M.L.M., Cinderby, S. 2007. Identifying ozone-sensitive  
520 communities of (semi-) natural vegetation suitable for mapping exceedance of critical  
521 levels. *Environmental Pollution*, **146**, 736-743.  
522  
523 Mills, G., Hayes, F., Wilkinson, S., Davies, W. 2009. Chronic exposure to increasing  
524 background ozone impairs stomatal functioning in grassland species. *Global Change*  
525 *Biology*, **15**, 1522-1533.  
526  
527 Mills, G., Soja, G., Volk, M. 2012. Potential for impacts on C sequestration in  
528 agricultural and grassland systems, in ; Harmens, H., Mills,G (Eds), *Ozone pollution:*  
529 *impacts on carbon sequestration in Europe*. Programme Coordination Centre for the  
530 ICP Vegetation, Centre for Ecology and Hydrology, Bangor, UK, pp 69-74. Available  
531 at <http://icpvegetation.ceh.ac.uk/>  
532  
533 Montes, R.A., Blum, U., Heagle, A.S., Volk, R.J.1983. The effects of ozone and  
534 nitrogen fertilizer on tall fescue, ladino clover and a fescue-clover mixture. II. Nitrogen  
535 content and nitrogen fixation. *Canadian Journal of Botany*, **61**, 2159-2168.  
536  
537 Mortier, V., Holsters, M., Goormachtig, S. 2012. Never too many? How legumes  
538 control nodule numbers. *Plant, Cell and Environment*, **35**, 245-258.  
539 Munifering, R.B., Chappelka, A.H., Lin, J.C., Karnosky, D.F., Somers, G.L . 2006.  
540 Chemical composition and digestibility of *Trifolium* exposed to elevated ozone and  
541 carbon dioxide in a free-air (FACE) fumigation system. *Functional Ecology*, **20**, 269-  
542 275.  
543  
544 Nussbaum, S., Geissmann, M., Fuhrer, J. 1995. Ozone exposure-response relationships  
545 for mixtures of perennial ryegrass and white clover depend on ozone exposure patterns.  
546 *Atmospheric Environment*, **29**, 989-995.  
547  
548 Pausch, R.C., Mulchi, C.L., Lee, E.H., Meisinger, J.J. 1996. Use of <sup>13</sup>C and <sup>15</sup>N isotopes  
549 to investigate O<sub>3</sub> effects on C and N metabolism in soybeans. Part II. Nitrogen uptake,  
550 fixation, and partitioning. *Agriculture, Ecosystems & Environment*, **60**, 61-69.  
551  
552 Parrish, D.D., Law, K.S., Staehelin, J., Derwent, R., Cooper, O.R., Tanimoto, H., Volz-  
553 Thomas, A., Gilge, S., Scheel, H.E., Steinbacher, M., Chan, E . 2012. Long-term  
554 changes in lower tropospheric baseline concentrations at northern mid-latitudes.  
555 *Atmospheric Chemistry and Physics*, **12**, 11485-11504  
556  
557 Parsons, A.J., Chapman, D.F. 1999. The principles of pasture growth and utilisation, in:  
558 *Grass: its production and utilisation* (Ed: Hopkins, A.), Blackwell Science, UK, pp31-  
559 79.

560  
561 Pleijel, H., Karlsson, G.P., Sild, E., Danielsson, H., Skärby, L., Selldén, G. 1996.  
562 Exposure of a grass-clover mixture to ozone in open-top chambers effects on yield,  
563 quality and botanical composition. *Agriculture, Ecosystems and Environment*, **59**, 55-  
564 62.  
565  
566 Rao, M.V., Davies, K.R. 2001. The physiology of ozone induced cell death. *Planta*,  
567 **213**, 682-690.  
568  
569 Rebbeck, J., Blum, U., Heagle, A.S. 1988. Effects of ozone on the regrowth and energy  
570 reserves of a ladino clover-tall fescue pasture. *Journal of Applied Ecology*, **25**, 659-681.  
571  
572 Reinhart, R.A., Weber, D.E. 1980. Ozone and Sulfur Dioxide-Induced Changes in  
573 Soybean Growth. *Phytopathology*, **70**, 914-916.  
574  
575 Sanz, J., Muntifering, R.B., Bermejo, V., Gimeno, B.S., Elvira, S. 2005. Ozone and  
576 increased nitrogen supply effects on the yield and nutritive quality of *Trifolium*  
577 *subterraneum*. *Atmospheric Environment*, **39**, 5899-5907.  
578  
579 Temmerman, L.D., Karlsson, G.P., Donnelly, A., Ojanperä, K., Jäger, H.J., Finnan, J.,  
580 Ball, G. 2002. Factors influencing visible ozone injury on potato including the  
581 interaction with carbon dioxide. *European Journal of Agronomy*, **17**, 291-302.  
582  
583 The Royal Society. 2008. Ground-level ozone in the 21st century: future trends, impacts  
584 and policy implications. Policy document, 15/08, RS1276. Available at:  
585 [http://royalsociety.org/Report\\_WF.aspx?pageid=7924&terms=ground-level+ozone](http://royalsociety.org/Report_WF.aspx?pageid=7924&terms=ground-level+ozone).  
586  
587 Tingey, D.T., Blum, U. 1973. Effects of ozone on soybean nodules. *Journal of*  
588 *Environmental Quality*, **2**, 341-342.  
589  
590 Vingarzan, R. 2004. A review of surface ozone background levels and trends.  
591 *Atmospheric Environment*, **38**, 3431-3442  
592  
593 Vollnes, A.V., Kruse, O.M.O., Eriksen, A.B., Oxaal, U., Futsaether, C.M. 2010. In vivo  
594 root growth dynamics of ozone exposed *Trifolium subterraneum*. *Environmental and*  
595 *Experimental Botany*, **69**, 183-188.  
596  
597 Wilbourn, S., Davison, A.W., Ollerenshaw, J.H. 1995. The use of an unenclosed field  
598 fumigation system to determine the effects of elevated ozone on a grass-clover mixture.  
599 *New Phytologist*, **129**, 23-32.  
600  
601 Wilkinson, S., Davies, W.J. 2009. Ozone suppressed soil drying and abscisic acid  
602 (ABA)-induced stomatal closure via an ethylene-dependant mechanism. *Plant, Cell &*  
603 *Environment*, **32**, 949-959.  
604  
605 Wilkinson, S., Mills, G., Illidge, R., Davies, W.J. 2011. How is ozone pollution  
606 reducing our food supply? *Journal of Experimental Botany*, **63**, 527-536.  
607  
608 Yoshida, C., Funayama-Noguchi, S., Kawaguchi, M. 2010. *plenty*, a Novel  
609 Hypernodulation Mutant in *Lotus japonicas*. *Plant Cell Physiology*, **51**, 1425-1435.  
610

611 Young, P.J., Archibald, A.T., Bowman, K.W., Lamarque, J.F., Naik, V., Stevenson,  
612 D.S., Tilmes, S., Voulgarakis, A., Wild, O., Bergmann, D., Cameron-Smith, P., Cionni,  
613 I., Collins, W.J., Dalsøren, S.B., Doherty, R.M., Eyring, V., Faluvegi, G., Horowitz,  
614 L.W., Josse, B., Lee, Y.H., MacKenzie, I.A., Nagashima, T., Plummer, D.A., Righi, M.,  
615 Rumhold, S.T., Skeie, R.B. 2013. Pre-industrial to end 21st century projections of  
616 tropospheric ozone from the Atmospheric Chemistry and Climate Model  
617 Intercomparison Project (ACCMIP). *Atmospheric Chemistry and Physics*, **13**, 2063-  
618 2090.

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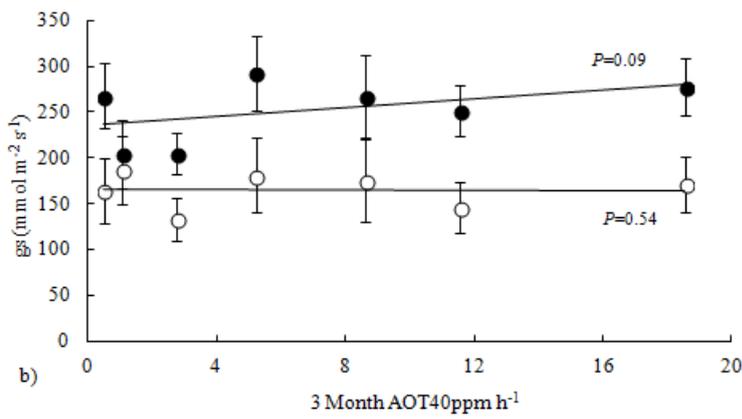
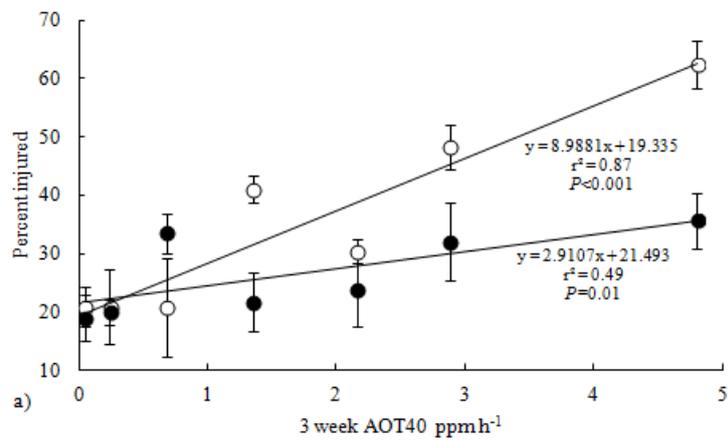
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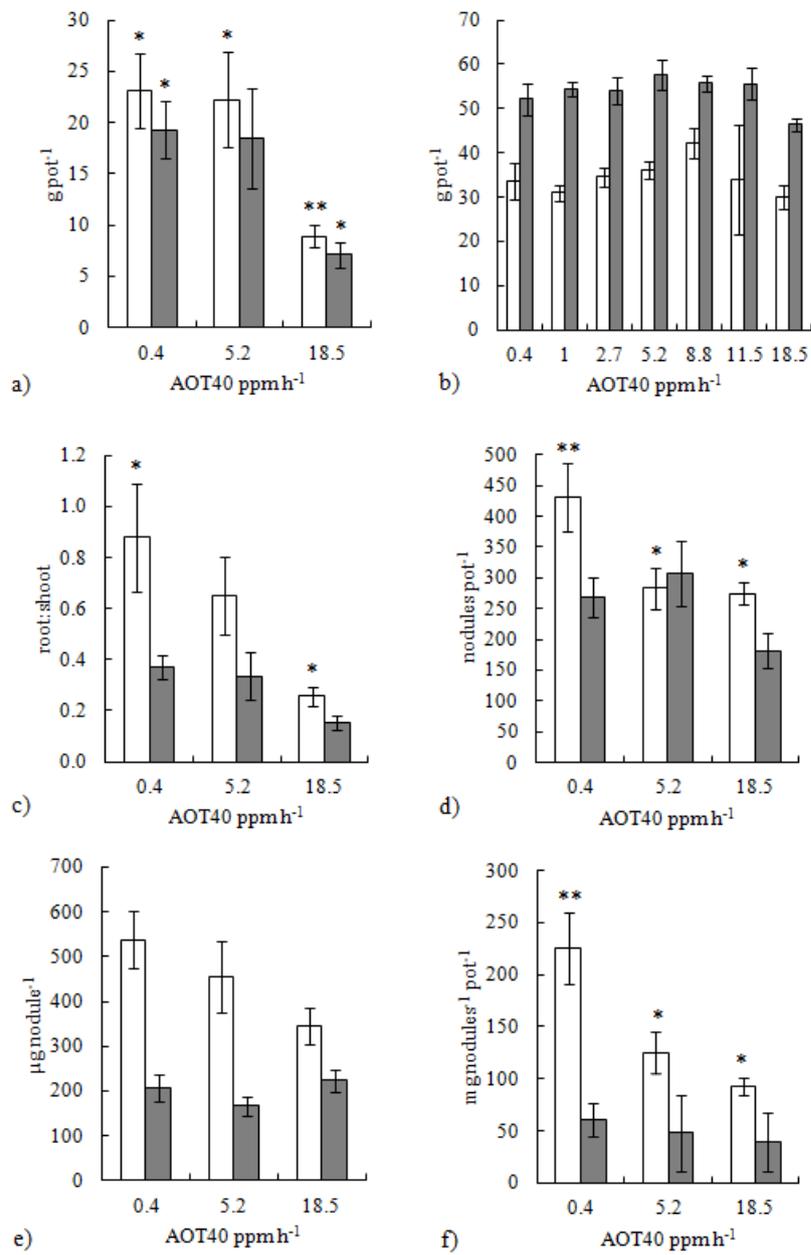
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630 Figure 1: Average weekly ozone profile for the seven ozone treatments (see Table 1 for  
631 treatment details).



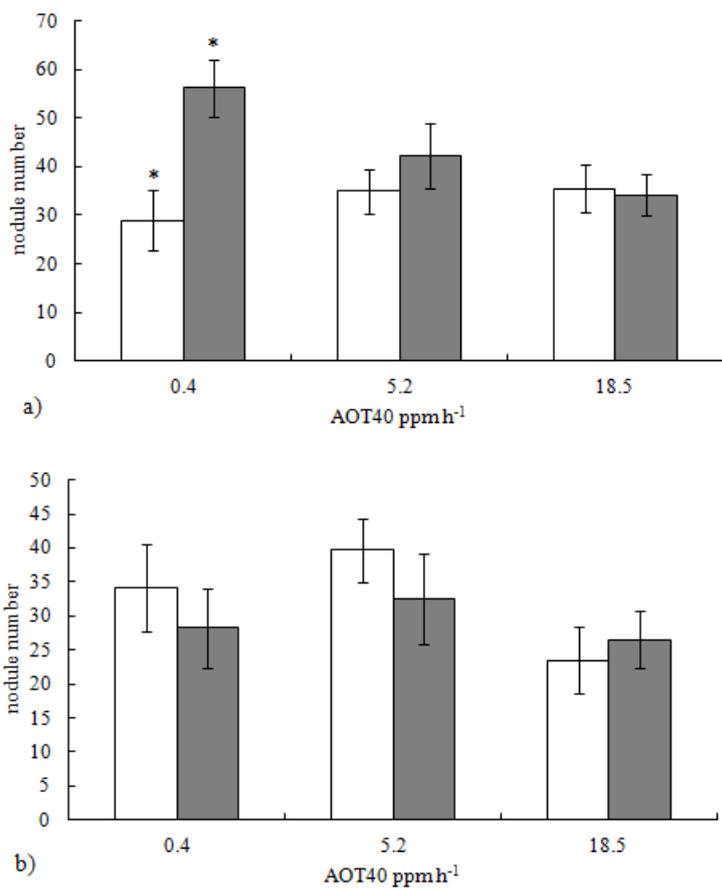
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634 Figure 2: Effects of ozone exposure on (a) mean ozone-induced injury after 3 weeks  
635 exposure; (b) mean  $g_s$ , from measurements made in weeks 4, 5, 8 & 9 where PAR was  
636 317-849  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (where white points = Crusader; filled points = Merviot; bars are  
637 standard errors).

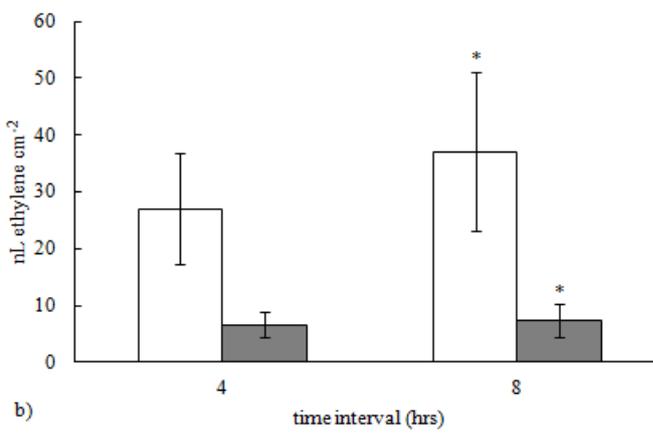
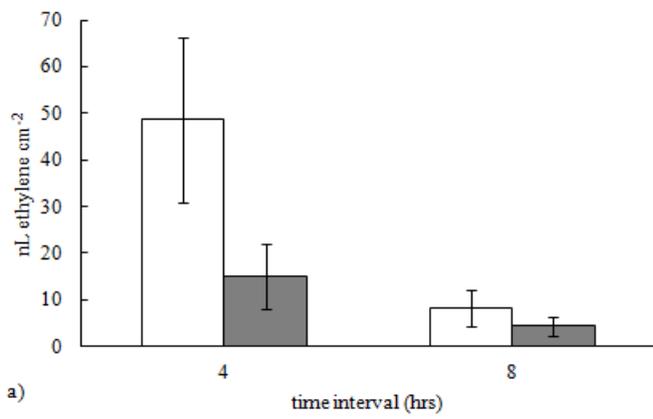


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Figure 3: Change in (a) root biomass pot<sup>-1</sup> (b) shoot biomass pot<sup>-1</sup> (c) root:shoot (d) nodules pot<sup>-1</sup> (e) mass nodule<sup>-1</sup> & (f) nodule mass pot<sup>-1</sup> in relation to 3 month AOT40 (where white bars = Crusader; grey bars= Merviot; asterixes (\*) denote a difference at the  $p=0.05$  level after post-hoc Tukey tests).



645  
 646 Figure 4: Effects of ozone on nodule size in (a) Crusader; (b) Merviot (where white  
 647 bars = number of nodules between 0.1mm-0.7mm maximum length; grey bars = number  
 648 of nodules >0.7mm long; asterixes (\*) denote a difference at the  $p=0.05$  level after post-  
 649 hoc Tukey tests).



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 651  
 652 Figure 5: Ethylene evolution in (a) week 10 and (b) week 11 ARAs (where white bars =  
 653 low ozone treatment 1; grey bars =high ozone treatment 7; asterixes (\*) denote a  
 654 difference at the  $p=0.05$  level after post-hoc Tukey tests).  
 655

656 Table 1: Summary of ozone treatments, including minimum and maximum, and climate conditions for the duration of the experiment.

Treatment	1	2	3	4	5	6	7	
24hr mean (ppb)	33	35	40	45	51	54	66	
AOT0 ppmh <sup>-1</sup>	22	23	27	30	34	36	44	
AOT40 ppmh <sup>-1</sup>	0.48	1.05	2.74	5.19	8.60	11.53	18.55	
Season min. conc. (ppb)	25	26	29	32	34	33	44	
Season max. conc. (ppb)	53	59	68	78	92	95	107	
Climate (seasonal mean)	air temperature (°C)			VPD (kPa)			PAR (μmol m <sup>-2</sup> s <sup>-1</sup> )	
	24hr	Daylight	Mean	24hr	Daylight	Mean	Daylight	Mean
	mean	mean	Max.	mean	mean	Max.	Mean	Max.
	19	21.3	24.6	0.54	0.84	1.14	521	814

PAR, photosynthetically active radiation; VPD, vapour pressure deficit

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Table 2: Summary of additional biomass data. Values are means and standard errors.. Significant p values are highlighted in bold.

Treatment	Crusader				Merviot			
	1	4	7	<i>p</i>	1	4	7	<i>p</i>
Nodule number (g <sup>-1</sup> root biomass <sup>-1</sup> )	23±6.5	14±3.0	33±4.0	0.11	14±1.5	19±2.5	32±8.5	<b>0.01</b>
Nodule biomass (mg g <sup>-1</sup> root biomass <sup>-1</sup> )	11±2.0	6±1.0	12±2.5	0.56	3.0±0.5	3.0±0.5	7.0±2.0	<b>0.02</b>
Root: total biomass	0.43±0.02	0.37±0.05	0.20±0.009	<b>0.001</b>	0.26±0.02	0.23±0.04	0.13±0.02	<b>0.008</b>
Total biomass (g pot <sup>-1</sup> )	51±1.0	57±2.0	44±0.5	0.08	71±5.0	76±4.0	53±2.0	<b>0.01</b>

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