

Article (refereed) - postprint

Hewitt, D.K.L.; Mills, G.; Hayes, F.; Wilkinson, S.; Davies, W. 2014.
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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9 **Full research paper**

10

11 **Abstract**

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

22

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

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

25 **Introduction**

26 Nitrogen (N) fixation by legumes (Fabaceae) is of vital agronomic importance. On a
27 global scale, the legume-rhizobia symbiosis, contained within specialised organs called
28 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 30-40 ppb 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 biomonitoring (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 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 determine 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 N₂ 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 10th and 11th 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⁻¹ (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⁻² s⁻¹) 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⁻²s⁻¹, with an average daily maximum of 814 µmol m⁻² s⁻¹.

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⁻¹). 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 cm² 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 cm² 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. subterranea*), 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**

379 Special thanks to Aled Williams, Steve Hughes and Dave Norris for their advice and
380 assistance and to NERC for funding this PhD Studentship (project code NEC04456).

381

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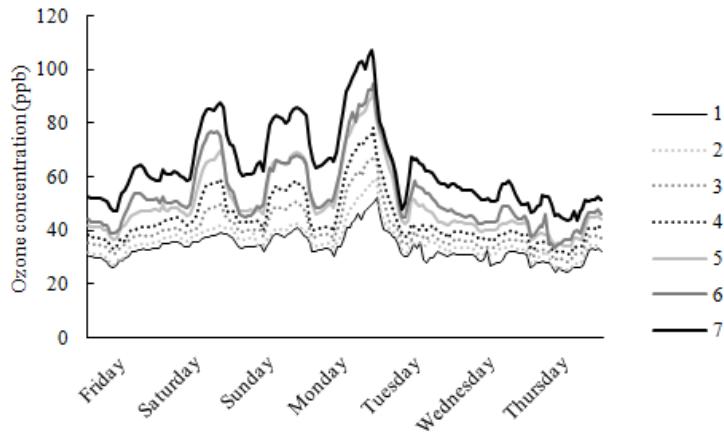
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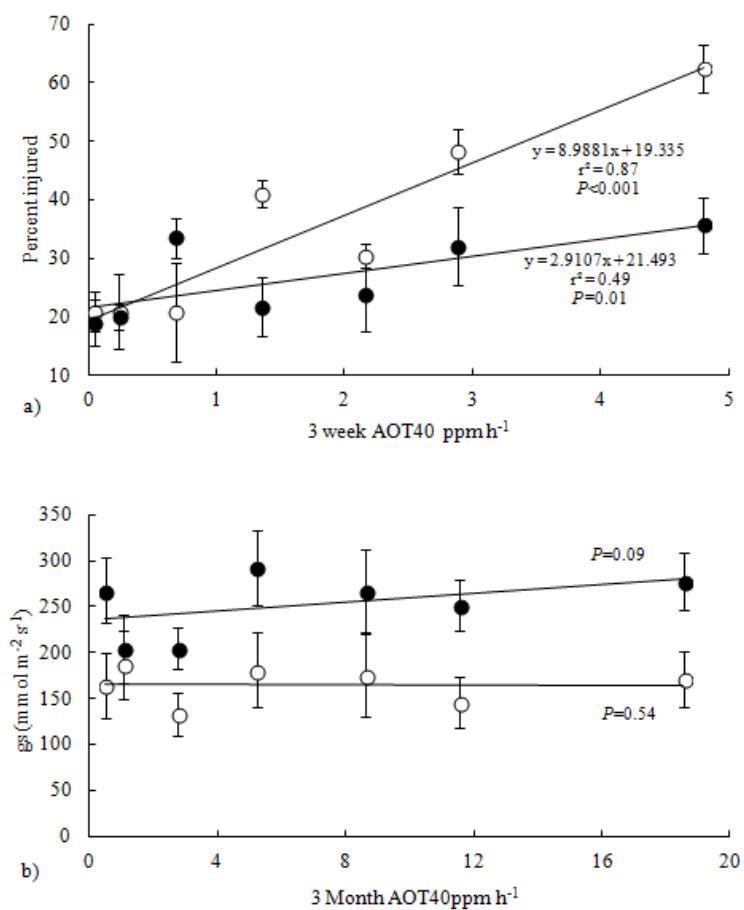
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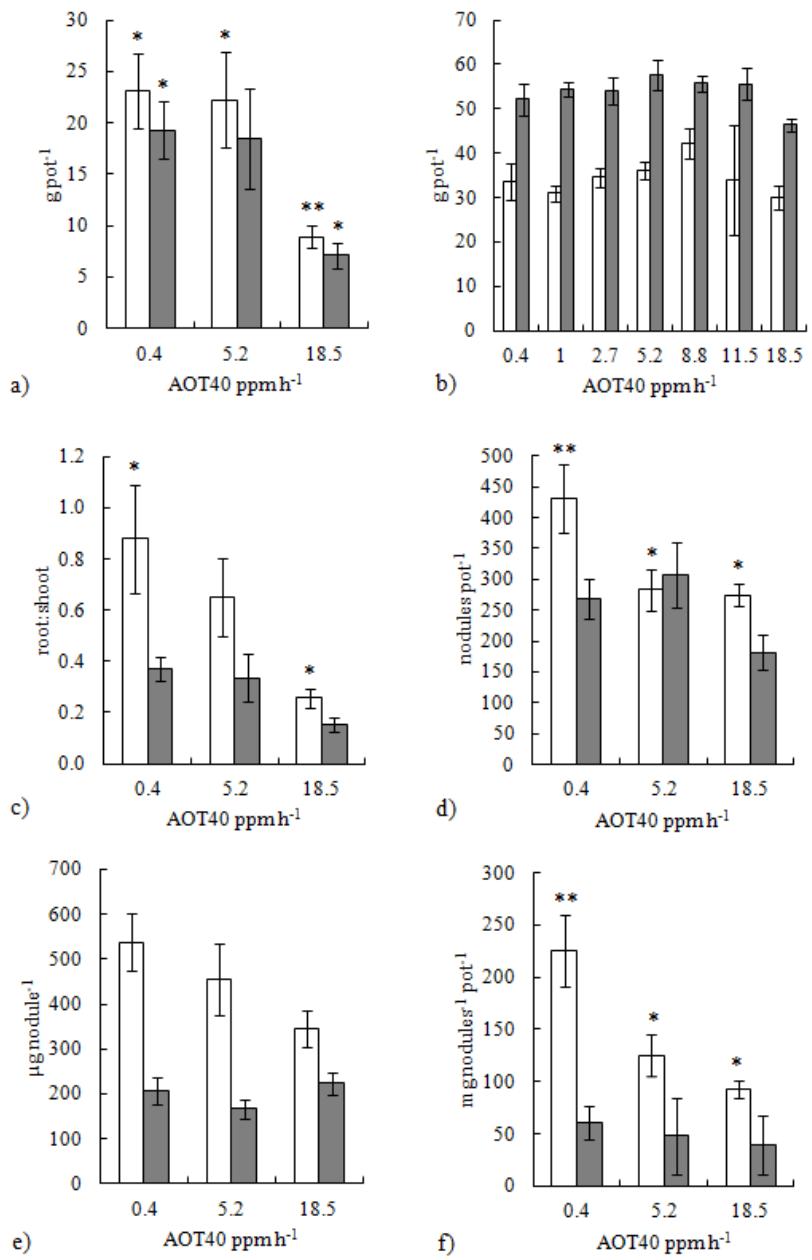
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630 Figure 1: Average weekly ozone profile for the seven ozone treatments (see Table 1 for
631 treatment details).

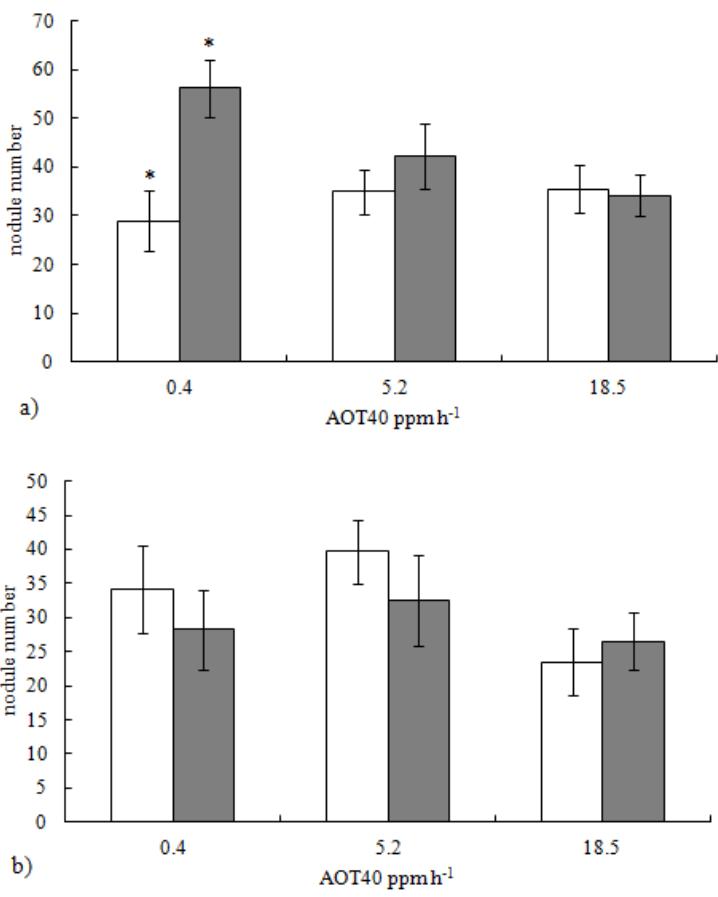


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Figure 2: Effects of ozone exposure on (a) mean ozone-induced injury after 3 weeks exposure; (b) mean g_s , from measurements made in weeks 4, 5, 8 & 9 where PAR was $317-849 \mu\text{mol m}^{-2} \text{s}^{-1}$ (where white points = Crusader; filled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

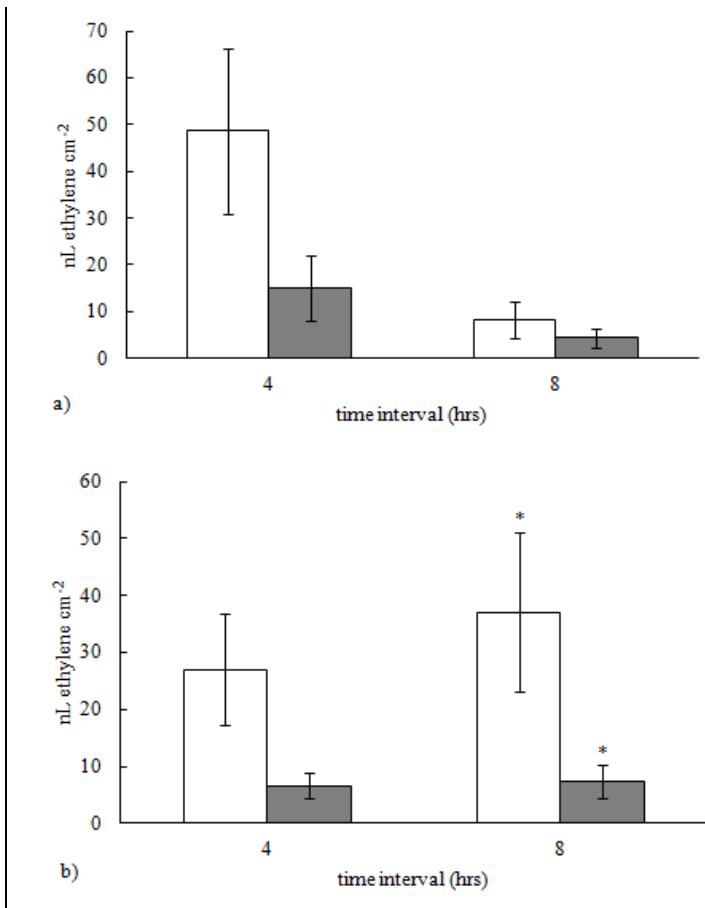


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640 Figure 3: Change in (a) root biomass pot⁻¹ (b) shoot biomass pot⁻¹ (c) root:shoot (d)
641 nodule mass pot⁻¹ & (e) nodule mass pot⁻¹ in relation to 3 month AOT40
642 (where white bars = Crusader; grey bars= Merviot; asterixes (*) denote a difference at
643 the p=0.05 level after post-hoc Tukey tests).
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Figure 4: Effects of ozone on nodule size in (a) Crusader; (b) Merviot (where white bars = number of nodules between 0.1mm-0.7mm maximum length; grey bars = number of nodules $>0.7\text{mm}$ long; asterixes (*) denote a difference at the $p=0.05$ level after post-hoc Tukey tests).



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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 ⁻¹	22	23	27	30	34	36	44
AOT40 ppmh ⁻¹	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 ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	
	24hr mean	Daylight mean	Mean Max.	24hr mean	Daylight mean	Mean Max.	Daylight Mean Max.
	19	21.3	24.6	0.54	0.84	1.14	521 814

PAR, photosynthetically active radiation; VPD, vapour pressure deficit

657

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

659

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

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