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Contact CEH NORA team at
noraceh@ceh.ac.uk

1 **Ozone pollution affects flower numbers and timing in a simulated BAP Priority**
2 **calcareous grassland community**

3

4 Felicity Hayes^{a*}, Jennifer Williamson^a and Gina Mills^a

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6 ^aCentre For Ecology and Hydrology, Environment Centre Wales, Deiniol Road, Bangor,

7 Gwynedd, LL57 2UW, UK

8 *Corresponding author. Felicity Hayes

9 E-mail address: fhay@ceh.ac.uk

10 Tel.: +44-1248-374500

11 Fax: +44-1248-362133

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13 **Research highlights**

- 14 • An increase in ozone accelerated timing of maximum flowering in *Lotus corniculatus*
15 • Ozone reduced flower numbers in *Campanula rotundifolia* and *Scabiosa columbaria*
16 • Reduced water availability did not protect most species from the effects of ozone

17

18 **Abstract**

19 Mesocosms representing the BAP Priority habitat ‘Calcareous Grassland’ were exposed to
20 eight ozone profiles for twelve-weeks in two consecutive years. Half of the mesocosms
21 received a reduced watering regime during the exposure periods. Numbers and timing of
22 flowering in the second exposure period were related to ozone concentration and phytotoxic
23 ozone dose (accumulated stomatal flux). For *Lotus corniculatus*, ozone accelerated the
24 timing of the maximum number of flowers. An increase in mean ozone concentration from
25 30 ppb to 70 ppb corresponded with an advance in the timing of maximum flowering by six

26 days. A significant reduction in flower numbers with increasing ozone was found for
27 *Campanula rotundifolia* and *Scabiosa columbaria* and the relationship with ozone was
28 stronger for those that were well-watered than for those with reduced watering. These
29 changes in flowering timing and numbers could have large ecological impacts, affecting plant
30 pollination and the food supply of nectar feeding insects.

31

32 **Capsule**

33 Increased tropospheric ozone affected timing of flowering and maximum flower numbers in
34 calcareous grassland mesocosms.

35

36 **Keywords**

37 Ozone; accelerated flowering; stomatal flux; drought; phenology

38

39 **1. Introduction**

40 Concentrations of tropospheric ozone have been increasing steadily over the last 150 years as
41 a result of increasing emissions of precursor molecules such as oxides of nitrogen and VOCs
42 from anthropogenic sources (Solberg et al., 2005; Volz and Kley, 1988). Current mean ozone
43 concentrations are typically 35-40 ppb at Mace Head, Ireland (Derwent et al., 2007) and have
44 increased at a rate of 0.16 ppb per annum over the period 2000 – 2007 (Tripathi et al., 2010).
45 Tropospheric ozone concentrations across the northern hemisphere are predicted to increase
46 further as emissions of precursor molecules continue to rise (Vingarzan, 2004, Meehl et al.,
47 2007). Summer mean ozone concentrations across Europe are expected to reach 40 to 60 ppb
48 by 2030 (Royal Society, 2008) and some predictions are for concentrations to reach 60 ppb
49 across central and northwest Europe by 2100 (Andersson and Engardt, 2010). At current
50 ozone concentrations, visible effects have been observed on plants growing in natural

51 conditions across Europe (Mills et al., 2011a). Ozone exposure studies have demonstrated
52 that ozone pollution can affect species of (semi-) natural vegetation communities in many
53 ways including above-ground growth (Franzaring et al., 2000; Gimeno et al., 2004a; Hayes et
54 al., 2010), root growth (Franzaring et al., 2000; Batty and Ashmore 2003), biomass
55 partitioning (Cooley and Manning, 1987; Hayes et al., 2009), flowering (Rämö et al., 2007)
56 and seed output (Gimeno et al., 2004b; Black et al., 2000) with differential responses by
57 individual species.

58

59 Flowering is a critical stage in the life-cycle of a plant and alterations to this process could
60 influence species survival within a plant community and reduce the important ecosystem
61 services related to pollination and nectar sources. A recent meta-analysis of effects of ozone
62 on plant reproductive growth and development revealed that current ambient ozone
63 concentrations significantly reduced seed number, fruit number and fruit weight compared to
64 charcoal-filtered air conditions (Leisner and Ainsworth, in press). There have been several
65 studies that have shown changes in flower number or flower biomass in response to ozone,
66 although these studies have mainly used individual plants/monocultures, relatively few ozone
67 treatments (up to 4), and usually only occasional assessments of flower number even though
68 this is a very dynamic process. An exposure study of a sown species mixture in Finland
69 showed that the elevated ozone treatment corresponded with a significantly reduced number
70 of flowers in *Campanula rotundifolia* compared to the non-filtered air control (Rämö et al.,
71 2007). A reduction in flower biomass with increasing ozone exposure has been demonstrated
72 for *Trifolium cherleri*, *Trifolium subterraneum* and *Trifolium striatum* grown as individual
73 species (Gimeno et al., 2004b). Flower weights were also significantly reduced by ozone for
74 *Eupatorium cannabinum* and *Plantago lanceolata*, also grown as individual species
75 (Franzaring et al., 2000). There can be carry-over effects of ozone exposure which can

76 influence subsequent flowering. For example, following exposure to ozone of *Trifolium*
77 *striatum* when the plants were in a vegetative state there was reduced flowering that persisted
78 for one month following cessation of the ozone exposure (Sanz et al., 2007). In a separate
79 study *Carex echinata* exposed to elevated ozone showed a reduction in flower biomass of
80 approximately 30% in spring following exposure to ozone in the previous summer (Hayes et
81 al., 2006). In addition, for *Leontodon hispidus* there was acceleration in the progression from
82 flowers to seed-heads in the year following ozone exposure (Hayes et al., 2011).

83

84 Despite evidence of alterations in flower numbers/biomass following ozone exposure,
85 comparatively few studies have investigated the effect of ozone on the timing of flowering.
86 Comparing only two ozone treatments, *Campanula rotundifolia* and *Vicia cracca* showed
87 delayed flowering with increasing ozone exposure in the second year of exposure of
88 simulated meadow communities (Rämö et al., 2007). These two species also showed reduced
89 early season coverage in the high ozone treatment, suggesting that there was reduced resource
90 availability following the first year of exposure. In a single-season study *Spartina*
91 *alterniflora*, grown as individual plants, showed delayed flowering and a reduction in the
92 number of flower spikes produced in the elevated ozone treatment compared to control
93 (Taylor et al., 2002). These plants also showed reduced shoot and leaf number, again
94 suggesting reduced resource availability. In contrast, for some species there are suggestions
95 of earlier flowering with increasing ozone exposure. *Betula pendula* flowered earlier with
96 elevated ozone compared to the ambient air control, with a non-significant increase in female
97 flower formation (Darbah et al., 2007); the authors did not suggest a mechanism for this.
98 Similarly for *Rubus cuneifolius* an initial acceleration in flowering occurred in the second
99 year of ozone exposure for the highest ozone treatment compared to the lower treatments,
100 with increased flower numbers and an earlier time of peak production (Chappelka, 2002).

101 There was no overall relationship between timing of flowering and ozone concentrations in
102 this study as the flowering time of plants in charcoal-filtered air was intermediate to the 2x
103 ambient air and non-filtered air treatments.

104

105 Future climate change scenarios predict changes in rainfall patterns (IPCC, 2007), with
106 reduced rainfall across many temperate regions and an increase in the frequency and severity
107 of summer droughts across much of Europe (Blenkinsop and Fowler, 2007; Lehner et al.,
108 2006), therefore any interaction between effects of ozone pollution and reduced rainfall on
109 plants is an important consideration when investigating effects of future ozone patterns.

110 Although drought itself has been shown to reduce growth on grassland species (e.g. Bahrani
111 et al., 2010), some studies have demonstrated that drought has a protective effect against
112 ozone, for example reduced visible injury caused by ozone exposure (Loew et al., 2006).

113 Effects of ozone pollution on vegetation have been shown to be more strongly related to flux
114 of ozone into the plant, rather than to ozone concentrations in the surrounding air and a
115 critical level approach using the modelled flux of ozone through the stomata has been
116 developed (LRTAP Convention, 2010, Mills et al., 2011b). It has been proposed that drought
117 can induce stomatal closure, therefore reducing ozone uptake and protecting against ozone
118 damage (e.g. for *Populus spp.*, Silim et al., 2009). However, some recent results have shown
119 that the response of the plant to ozone can interfere with the signalling process that induces
120 stomatal closure in response to drought, reducing the ability to tolerate drought conditions
121 (Mills et al., 2009; Wilkinson and Davies, 2010).

122

123 In this study we exposed mesocosms containing seven-species mixtures representing the
124 Biodiversity Action Plan (BAP) priority community 'Calcareous Grassland' to eight
125 simulated ozone regimes, with half of the mesocosms remaining well-watered and the other

126 half receiving a reduced water regime during the ozone exposure, but remaining well-watered
127 for the remainder of the experiment. The ozone regimes used were chosen to simulate
128 previous, current and projected future ozone concentrations in remote rural areas and were
129 applied in two successive growing seasons. Effects are reported for elevated ozone
130 treatments using both the 24h mean ozone concentration and species-specific stomatal ozone
131 flux (determined using the DO₃SE model, LRTAP Convention, 2010) as ozone metrics. We
132 focus here on the flowering response and report the results of detailed flower counts made
133 throughout the second exposure period. Thus, the overall aims of the study were to
134 investigate whether ozone influenced the timing and number of flowers in this ecologically
135 important community.

136

137 **2. Materials and Methods**

138

139 *Ozone system and treatments*

140 Plants were exposed to ozone in solardomes (hemispherical greenhouses 3m diameter, 2m
141 tall). Ozone was generated from oxygen concentrated from air (Workhorse 8, Dryden Aqua,
142 Edinburgh, UK) using an ozone generator (G11, Dryden Aqua, UK) and distributed to each
143 solardome via PTFE tubing. Ozone was delivered to each solardome using mass flow
144 controllers (Celerion, Dublin, Ireland) controlled by computer software (Labview version 7).
145 Ozone concentrations were continuously monitored in one solardome using a dedicated
146 ozone analyser (Thermo Electron, Waltham, MA, USA; Model 49C), allowing feedback to
147 compensate for small variations in ozone production. In all solardomes, the ozone
148 concentration was measured for 5 minutes in every 30 minutes using two additional ozone
149 analysers (Envirotech, St Albans, UK; Model API 400A) of matched calibration.

150

151 Eight ozone treatments were randomly allocated to the solardomes, with one solardome used
152 for each treatment. A weekly profile based on an ozone episode was used for each treatment.
153 The treatments used were increments above and below a simulated ambient profile of peaks
154 of +10 to +25 ppb on four days, followed by three days of low peaks (ca. 5 ppb)
155 superimposed on a background of ca. 45 ppb, mimicking an ambient episode at Keenley,
156 Northumberland, UK (20th-27th May 2008, Grid reference NY794562). The other seven
157 treatments increased or decreased concentrations by -30, -20, -10, +10, +20, +30 and +40
158 ppb. The target weekly ozone profile is shown in Figure 1.

159

160 ***Mesocosm set-up***

161 Mesocosms representing the BAP priority habitat ‘Calcareous Grassland’ were established in
162 spring 2009 in 14 litre pots (33.3 cm diameter x 24.0 cm deep), lined with perforated plastic
163 sheeting to deter roots from growing through the drainage holes in the base of the pot. Pots
164 were filled with a mixture of topsoil (Humax, UK), sand and grit in the ratio 50:3:3 by
165 volume. 200 g horticultural powdered lime (J. Arthur Bowers, UK) was added to each pot to
166 increase the soil pH. Mesocosms were established using plug plants (British Wildflower
167 Plants, UK) on 19th June 2009 and maintained in ventilated polytunnels until the experiment
168 started. Each mesocosm contained *Briza media* (2 ‘plugs’), *Festuca ovina* (2 ‘plugs’),
169 *Campanula rotundifolia*, *Sanguisorba minor*, *Scabiosa columbaria*, *Helianthemum*
170 *nummularium* and *Lotus corniculatus* (1 ‘plug’ of each), planted in an identical arrangement,
171 and watered as required.

172

173 Mesocosms (10 replicates per solardome) were moved into the solardomes (at 20 ppb ozone)
174 on 22nd July 2009, and the watering treatments were applied by hand from 17th July (before
175 the ozone exposure started) until the end of the ozone exposure on 14th October, with five

176 replicates of each watering regime per solardome. Soil moisture was continuously recorded
177 in two well-watered mesocosms (WW) and two reduced water (RW) mesocosms using theta
178 probes (ML2x, Delta-T, UK) attached to a datalogger (DL6, Delta-T, UK) and the mean soil
179 moisture content was 32% and 21% for the WW and RW mesocosms respectively. All of the
180 mesocosms were overwintered outdoors during 2009/10 and were exposed to a second 12-
181 week ozone exposure in 2010 from 21st April to 15th July, with hand watering to provide the
182 WW and RW regimes (mean soil moisture content 31% and 23% respectively).

183

184 *Assessments of flowering*

185 During the course of the ozone exposure in 2010 the numbers of flower buds and flowers
186 were counted weekly for all species in each mesocosm, with the exception of the grass
187 *Festuca ovina* (which had over 150 flowers per mesocosm and therefore could not be counted
188 due to time constraints).

189

190 *Stomatal conductance measurements and parameterisation of a flux-model*

191 Stomatal conductance measurements were made on *L. corniculatus* (216 measurements), *C.*
192 *rotundifolia* (205 measurements), *S. columbaria* (307 measurements), *B. media* (105
193 measurements) and *S. minor* (321 measurements) during the course of the two ozone
194 exposures using a porometer (AP4, Delta-T), with corresponding measurements of soil
195 moisture using a hand-held portable theta probe (ML2x probe attached to HH2 Moisture
196 meter, Delta-T, UK), and using climatic measurements within the solardomes made using an
197 on-site weather station. These measurements were made between 27th July and 8th October
198 2010, with some additional measurements made during June 2010, over a range of times and
199 weather conditions, and were used to parameterise a stomatal flux-model for each of these
200 species based on that described by Emberson et al., 2000 and LRTAP Convention (2010).

201 For the parameterisations for the modification of stomatal conductance by light, temperature,
 202 VPD and soil water potential (f_{light} , f_{temp} , f_{VPD} and f_{SWP}) respectively, the x-axis was
 203 subdivided into segments and for each segment the 90th centile for relative stomatal
 204 conductance was calculated. A physiologically relevant curve, as described in Emberson et
 205 al., (2000) was then fitted to these datapoints. The values of the constants calculated for
 206 these parameterisations are indicated in Table 1. The phenology function f_{phen} was
 207 considered to be 1 throughout the growing season and f_{O_3} (the modification of stomatal
 208 conductance due to the ozone concentration) was not included in the model as there was
 209 insufficient data to show a clear effect of ozone on stomatal aperture. G_{max} (the species-
 210 specific maximum stomatal conductance) was calculated for each species as the 95th centile
 211 of the stomatal conductance measurements. G_{min} (the minimum stomatal conductance) was
 212 considered to be $0.1 * g_{\text{max}}$. These parameterisations were applied to the DO₃SE model
 213 (LRTAP Convention, 2010; Emberson et al., 2000) to calculate stomatal conductance (g_{sto}):

$$215 \quad g_{\text{sto}} = g_{\text{max}} * [\min (f_{\text{phen}}, f_{\text{O}_3})] * f_{\text{light}} * \max[f_{\text{min}}, (f_{\text{Temp}} * f_{\text{VPD}} * f_{\text{SWP}})] \quad [\text{Eq.1}]$$

216
 217 The stomatal flux of ozone (F_{stO_3}) was calculated according to the equation of Emberson et
 218 al. (2000), using a conversion factor of 0.663 to account for the ratio of the molecular
 219 diffusivity of ozone compared to that of water vapour (LRTAP Convention, 2010):

$$221 \quad F_{\text{stO}_3} = [\text{O}_3] * 0.663 * g_{\text{sto}} \quad [\text{Eq.2}]$$

222
 223 Calculations of stomatal fluxes were made using hourly averages of the variables needed for
 224 the model. It was assumed that for each species, light, VPD, and temperature were the same
 225 for each ozone and watering treatment. The hourly ozone fluxes were accumulated over a

226 threshold of 1 nmol for daylight hours (POD₁, the Phytotoxic Ozone Dose) and were summed
227 over the duration of both the first ozone exposure and the second exposure. This threshold
228 was used as it was selected by ‘expert judgement’ in the determination of flux-based critical
229 levels of ozone for trees and semi-natural vegetation within the LRTAP Convention, and
230 agreed at a LRTAP Convention workshop held in 2009 (Mills et al., 2011b), and represents
231 the detoxification capacity of the vegetation. POD₁ varied by 10-20% between the two
232 seasons (depending on the species), and effects are presented against the mean POD₁ for the
233 two exposure seasons.

234

235 *Data analysis and statistics*

236 Scatter plots of the number of flowers on each assessment date were used to determine the
237 Julian date of peak flowering. All datasets were analysed using the solardome (O₃ treatment)
238 mean values for each watering regime. Linear responses in the data were analysed using the
239 General Linear Model (Minitab, version 14), using 24h mean ozone concentration or POD₁
240 and watering regime as inputs to the model, or by linear regression.

241

242 **3. Results**

243 *Ozone exposure*

244 In 2009 the ozone exposure ranged from a seasonal 24h mean of 15.6 ppb to 73.0 ppb whilst
245 in 2010 the seasonal 24h mean ranged from 19.0 ppb to 73.3 ppb (Table 2). Mean
246 temperature within the solardomes during the ozone exposure was 20.6°C in 2009 and 20.4°C
247 in 2010, and mean humidity was 76.5% in 2009 and 68.6% in 2010.

248

249 *Lotus corniculatus*

250 Early season formation of flowers was accelerated with increasing ozone concentration for *L.*
251 *corniculatus* during the second ozone exposure period. Increasing ozone concentration
252 corresponded with a significantly earlier date on which 20% of the maximum number of
253 flowers (used as a surrogate for the start of flowering) was reached ($p=0.017$; Figure 2a). The
254 difference in the time taken to reach 20% of the maximum number of flowers varied across
255 the range of ozone exposures by nine days in the WW treatment and by seven days in the RW
256 treatment. In the early weeks of flowering for *L. corniculatus* this resulted in increased
257 numbers of flowers in the higher ozone treatments. For example, on 27th May, after exposure
258 to the ozone regime for five weeks in 2010, there was a linear increase in flower number with
259 increasing ozone exposure for the WW treatment ($r^2=0.67$, $p=0.013$) and a non-significant
260 increase for the RW treatment ($r^2=0.32$, $p=0.145$; Figure 2b). Despite the differences in
261 flower number between treatments in the early weeks of flowering, there were no differences
262 in the maximum number of flowers between ozone treatments for this species (Figure 4a).
263 However, as there were no significant differences in the time taken to increase from 20% to
264 either 50% or 90% of the maximum flower number with either the ozone or the watering
265 regime (data not presented), the date on which the maximum number of flowers occurred
266 during the second exposure season was increasingly earlier for *L. corniculatus* with
267 increasing ozone exposure ($p=0.009$; Figure 3a), with the total range for the date of
268 maximum flowering between treatments being 14 days. An increase in the mean ozone
269 concentration from 30 ppb to 70 ppb corresponded with maximum flowering occurring six
270 days earlier in both the WW and RW treatments. Flower numbers decreased to
271 approximately 50% of the maximum number by the final assessment, after exposure for 11
272 weeks (6th July, data not presented).

273

274 For all ozone treatments, flowering was slightly later in the RW treatment compared to the
275 WW treatment (Figure 3a), but this difference was not significant and there was no
276 significant interaction between ozone and watering regime. The relationship between the
277 date of maximum flowering and ozone concentration was linear for both watering regimes,
278 with the correlation coefficient having an r^2 of 0.46 for WW plants and 0.45 for RW plants
279 (Figure 3a). The difference in peak flowering date for plants in the WW compared to the RW
280 treatment can be explained by ozone flux as there was a linear relationship between ozone
281 flux and the date of peak flowering when the data were plotted together ($r^2=0.49$, $p=0.002$;
282 Figure 3b). Based on 95% confidence intervals for this relationship, the POD_1 needed to give
283 a significant change in flowering date was 2.5 mmol m^{-2} .

284

285 There was a decrease in the total number of flowers of approximately 50% in response in the
286 RW treatment compared to WW ($p=0.01$; Figure 4a). However, there was no relationship
287 between total flower number and ozone flux using either the actual flower number (data not
288 presented) or the relative flower number, normalised to account for the influence of watering
289 regime (Figure 4b).

290

291 *Campanula rotundifolia*

292 The maximum number of flowers of *C. rotundifolia* was significantly reduced with
293 increasing ozone concentration ($p=0.029$; Figure 5a). Although there was no significant
294 effect of watering regime, and no significant interaction between ozone and watering regime,
295 the relationship between ozone concentration and maximum flower number was much
296 stronger for plants of the WW treatment ($r^2=0.63$) compared to those of the RW treatment
297 ($r^2=0.20$), although due to the low numbers of flowers, these statistics should be interpreted
298 with caution. For the WW treatment, an increase in mean ozone concentration from 30 ppb

299 to 70 ppb corresponded to a 40% decline in flower number. Combining both watering
300 treatments, the decline in maximum flower number for *C. rotundifolia* showed a significant
301 linear relationship with POD_1 ($r^2=0.33$, $p=0.02$; Figure 5b). Based on 95% confidence
302 intervals for this relationship, the POD_1 needed to give a significant change in flower number
303 was 12.2 mmol m^{-2} .

304

305 *Scabiosa columbaria*

306 For *S. columbaria* the total numbers of buds were used for analysis as this species flowers
307 later and the end of the ozone exposure was before the maximum number of flowers was
308 reached. Overall, the maximum number of buds showed a large decline with increasing
309 ozone exposure ($p=0.043$; Figure 6a) in the WW treatment ($r^2=0.65$) but not in the RW
310 treatment ($r^2=0.04$), although the statistics should be treated with caution due to the low
311 numbers of buds per mesocosm. An increase in ozone concentration from 30 ppb to 70 ppb
312 corresponded to a 20% decline in flower number in the WW treatment only. There was a
313 reduction in maximum bud number in the RW compared to WW treatment that showed a
314 strong trend ($p=0.058$) but there was no significant interaction between ozone and watering
315 regime. When the number of buds was related to the calculated ozone flux there was a strong
316 trend for a reduction in maximum bud number with increasing POD_1 ($r^2= 0.19$, $p=0.096$;
317 Figure 6b), and no improvement to the relationship when the numbers of buds were
318 normalised to account for differences due to the influence of the watering regime (data not
319 presented). There were no significant effects of either ozone or watering regime on either the
320 onset of flowering (using the date when 20% of the maximum number of buds recorded was
321 reached) or the timing of peak bud number (data not presented).

322

323 *Briza media, Sanguisorba minor and Helianthemum nummularium*

324 The maximum number of flowers of *B. media* and *S. minor* showed no significant response to
325 either watering regime or ozone (data not presented). There was also no effect of either
326 ozone or drought on the timing of flowering for these species. *H. nummularium* flowered
327 sporadically throughout the exposure season, but the low numbers of flowers meant that it
328 was not possible to determine whether or not there were responses to ozone or watering
329 regime. A summary table showing F-values and significance for the relationships between
330 the maximum number of flowers for *L. corniculatus*, *C. rotundifolia*, *S. columbaria*, *S. minor*
331 and *B. media* in response to ozone, watering regime, the interaction between ozone and
332 watering regime, and the relationship with ozone flux (POD₁) is shown in Table 3.

333

334 **4. Discussion**

335 The detailed flowering assessment regime of this study has revealed effects that may have
336 been overlooked in previous studies where flower numbers have usually been counted on a
337 single occasion. For example, counts of *L. corniculatus* flowers early in the exposure period
338 indicated that there was a large effect of ozone on flower number, whereas subsequent counts
339 revealed that rather than affecting the maximum flower number, the effect of ozone was to
340 alter the timing of flowering in this species. Therefore, single assessments at different times
341 in the growing season would have indicated different results.

342

343 This study has revealed species-specific effects of both drought and ozone which could
344 potentially change the dynamics of calcareous grassland ecosystems. Of the six species that
345 had flowering assessed during this study, *L. corniculatus*, *C. rotundifolia* and *S. columbaria*
346 showed significant effects of increasing ozone on flower number or phenology. *H.*
347 *nummularium* did not have sufficient flowers to show any trends. Only *B. media* and *S.*
348 *minor* showed no effects of ozone. The high proportion of species from this community

349 responding to ozone is of concern for the viability of this habitat in future ozone conditions.
350 In addition, this study has shown that some species show an interaction between ozone and
351 watering regime, whereas others do not. The combination of reduced water and increased
352 ozone, as predicted in future ozone and climate scenarios, could therefore have a large effect
353 on the numbers, composition and timing of flowering of plant communities such as
354 calcareous grassland due to the species-specific responses. The linear relationships between
355 the timing of flowering and numbers of flowers in response to ozone shown in this study, and
356 evidence from a recent study indicating that in the UK 72% of lowland calcareous grassland
357 occurred in regions where the AOT40 was greater than 6.5 ppm h (averaged over 1999 –
358 2003; Morrissey *et al.*, 2007) implies that changes in flowering number and phenology of
359 species from native calcareous grassland habitats may already be occurring at current ambient
360 compared to pre-industrial ozone concentrations.

361

362 The consequence of earlier flowering of a species in a community as a result of ozone
363 exposure could be a lack of synchronicity with pollinating species. In a recent review of
364 plant and pollinator phenology in response to climate change, Hegland *et al.* (2009)
365 emphasised that, in many cases, both plant and insect phenology appear to be governed by
366 temperature, so that they remain synchronized. When synchronization is not maintained,
367 there can be severe consequences. For example, Kudo *et al.* (2004) found a mismatch
368 between early flowering plants in Japan, which advanced their flowering time in a warm
369 spring, and bumble bee emergence, which did not advance, resulting in a decreased seed-set
370 in bumble bee pollinated plants. It has also been shown that for some species the abundance
371 of other flowers before or during its own flowering can influence reproductive success due to
372 competition for pollination (Brown *et al.*, 2002). In addition to effects on the plant species,
373 when plants and pollinators do not move in parallel, then it is predicted that a large

374 proportion of pollinators may suffer population declines from a reduced diet breadth
375 (Memmott et al., 2007). Studies on interactions between pollinators and plant phenology in
376 response to ozone have not so far been carried out, but it is possible that these mismatches in
377 synchronicity normally associated with climate change may also occur, with possible
378 detrimental effects on both the plants and their associated pollinators as a consequence.

379

380 The current study has also shown large reductions in flower number in response to increased
381 ozone exposure for *C. rotundifolia* and *S. columbaria*. This is in agreement with other
382 studies that have shown reductions in flower numbers or flower biomass (e.g. Rämö et al.,
383 2007). In addition to the response to increased ozone concentrations, the current study has
384 also highlighted the differential response to drought of the component species of this
385 community. A reduction in watering of 30% corresponded with reductions in flower number
386 of 50% for *L. corniculatus* and 16% for *S. columbaria*. Reduced flower numbers would
387 result in reduced chances of pollination for these species and could therefore reduce the
388 reproductive success, thereby decreasing the long-term viability of these species within the
389 plant community. Although these reductions in flowering could be a result of reduced
390 resource availability, responses in reproductive structures do not always correspond to
391 reductions in growth, but may be a result of reduced resource allocation. This is in contrast
392 to the hypothesis of Saikkonen et al., 1998, who suggested that under stress conditions there
393 would be increased allocation to reproductive structures. These large effects of drought on
394 flower number also indicate that although it could be argued that reduced watering protects
395 some species from the effects of ozone, for others e.g. *L. corniculatus* and *S. columbaria* the
396 severe effect of the drought itself far outweighs any benefit of a reduction in ozone flux.

397

398 For *L. corniculatus*, *C. rotundifolia* and *S. columbaria* there were significant (or nearly
399 significant) relationships between POD_1 and the timing or number of flowers ($p=0.002$, 0.010
400 and 0.096 respectively), with the calculated fluxes incorporating the reduction in stomatal
401 conductance due to drought. As there were also no significant interactions between ozone
402 concentration and drought for any of the response parameters, the differences in flower
403 numbers and timing reported can be attributed to ozone uptake in these species. Flowering of
404 plants is controlled by complex and highly regulated signalling pathways. It is thought that
405 for one of the pathways abscisic acid (ABA) affects hormone signalling processes in plants
406 including the transition from the vegetative to reproductive phase (see review by Barth et al.,
407 2006). Recent studies have shown that ozone reduces the responsiveness of plants to ABA
408 (Mills et al., 2009; Wilkinson and Davies 2009, 2010), and this could potentially be
409 happening in the flowering response. Effects of ozone crosstalk with the flowering signalling
410 pathways are thus worthy of further investigation.

411

412 In this study the acceleration of flowering in *L. corniculatus* with an increase in mean ozone
413 concentration from 30 to 70 ppb was six days. This suggests that the increases in ozone
414 concentration expected over the next few decades may accelerate flowering in this, and
415 possibly other species. In comparison, in Europe a comprehensive analysis of a large
416 systematic phenological dataset has shown that the phenological response to climate change,
417 based on temperature, shows an advance in spring/summer of 2.5 days per decade (Menzel et
418 al., 2006). Although slightly smaller than the changes associated with predicted increases in
419 temperature, the potential acceleration in timing of flowering in response to increases in
420 ozone concentration could result in significant ecological impacts on plant communities, and
421 should be studied further.

422

423 **5. Conclusions**

424 Increased ozone concentrations affected flower numbers and timing in calcareous grassland
425 species. Decreased flower numbers for *C. rotundifolia* and *S. columbaria* may have resulted
426 from decreased resource availability; however, an observed acceleration in the timing of
427 maximum flowering for *L. corniculatus* may have been a consequence of crosstalk to one of
428 the flowering signalling pathways. These effects on flowering were observed in the second
429 consecutive ozone exposure, demonstrating the importance of longer-term studies to
430 investigate responses. The results found suggest that increases in tropospheric ozone
431 concentrations could have indirect effects on plant pollinators, although further studies would
432 be needed to confirm this.

433

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438

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605 Wilkinson S, Davies WJ (2010) Drought, ozone, ABA and ethylene: new insights from cell
606 to plant to community. Plant Cell and Environment 33(4): 510-525

607 Table 1: Values of the constants used for parameterisation of the stomatal flux model for *L.*
 608 *corniculatus*, *C. rotundifolia*, *S. columbaria*, *B. media* and *S. minor*.

Parameter	Units	<i>L.</i>	<i>C.</i>	<i>S.</i>	<i>B. media</i>	<i>S. minor</i>
		<i>corniculatus</i>	<i>rotundifolia</i>	<i>columbaria</i>		
g_{\max}	$\text{mmol m}^{-2} (\text{H}_2\text{O})$	246	550	660	210	900
T_{\min}	$^{\circ}\text{C}$	12	15	11	1	11
T_{opt}	$^{\circ}\text{C}$	22	26	26	23	26
T_{\max}^1	$^{\circ}\text{C}$	32	35	35	30	35
VPD_{\max}	kPa	2.1	2.1	2.1	2.1	2.1
VPD_{\min}	kPa	3.5	3.5	3.5	3.5	3.5
L	Constant	-0.007	-0.007	-0.007	-0.007	-0.007
SWP_{\max}	MPa	0	0	0	0	0
SWP_{\min}	MPa	-0.03	-0.60	-0.45	-0.25	-0.45

609 ¹Outside the range of temperature measurements made and interpolated from available data.

610 Table 2: Season 24h mean ozone concentrations for each ozone treatment in 2009 and 2010,

Ozone treatment	2009 Season 24h mean ozone, ppb	2010 Season 24h mean ozone, ppb
AA-30	15.6	19.0
AA-20	23.2	25.5
AA-10	31.7	34.8
AA	40.3	40.8
AA+10	50.1	51.2
AA+20	57.4	60.3
AA+30	68.8	66.2
AA+40	73.0	73.3

611

612

613 Table 3: F-values for the maximum number of flowers of selected species, using General
 614 Linear Model, in response to ozone, watering regime and the interaction between ozone and
 615 watering regime, and relationship between maximum number of flowers and time integrated
 616 ozone flux (POD₁) using regression analysis ** , * and (*) indicate significant differences at
 617 p<0.01, p<0.05 and p<0.1 respectively.

Species	Ozone	Watering regime	Interaction between ozone and watering	Ozone flux, POD ₁
<i>L. corniculatus</i>	0.15	10.19 **	0	2.93
<i>C. rotundifolia</i>	6.17 *	0.27	0.08	6.89 *
<i>S. columbaria</i>	5.12 *	4.38 (*)	2.10	3.19 (*)
<i>S. minor</i>	0.12	1.95	0.02	0
<i>B. media</i>	2.18	0.20	0.53	2.40

618

619 Figure 1: The target weekly ozone regime used in the solardomes in 2009 and 2010.

620

621 Figure 2: (a) The Julian date when flower number reached 20% of maximum in *L.*

622 *corniculatus* in response to ozone concentration in both the WW and RW treatments and (b)

623 Mean number of flowers per mesocosm on 27th May (after 5 weeks of exposure in 2010).

624

625 Figure 3: Julian date of maximum flower number for *L. corniculatus* in response to (a) ozone

626 concentration and (b) ozone flux in both the WW and RW treatments.

627

628 Figure 4: Maximum flower number in the WW and RW treatments for *L. corniculatus* in

629 response to (a) ozone concentration and (b) in relation to ozone flux, normalised for the effect

630 of watering regime.

631

632 Figure 5: Maximum flower number for *C. rotundifolia* in the WW and RW treatments in

633 relation to (a) ozone concentration and (b) ozone flux.

634

635 Figure 6: Maximum flower number in the WW and RW treatments for *S. columbaria* in

636 relation to (a) ozone concentration and (b) ozone flux.

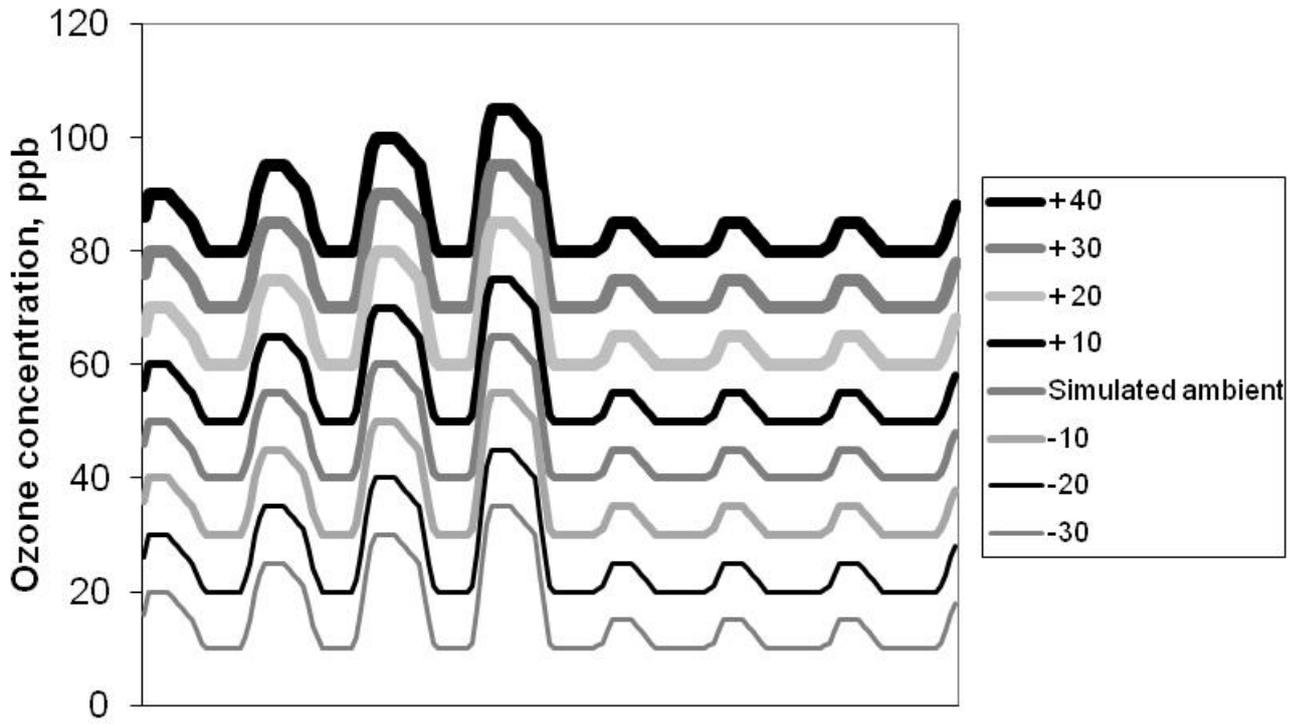


Figure 1: The target weekly ozone regime used in the solardomes in 2009 and 2010.

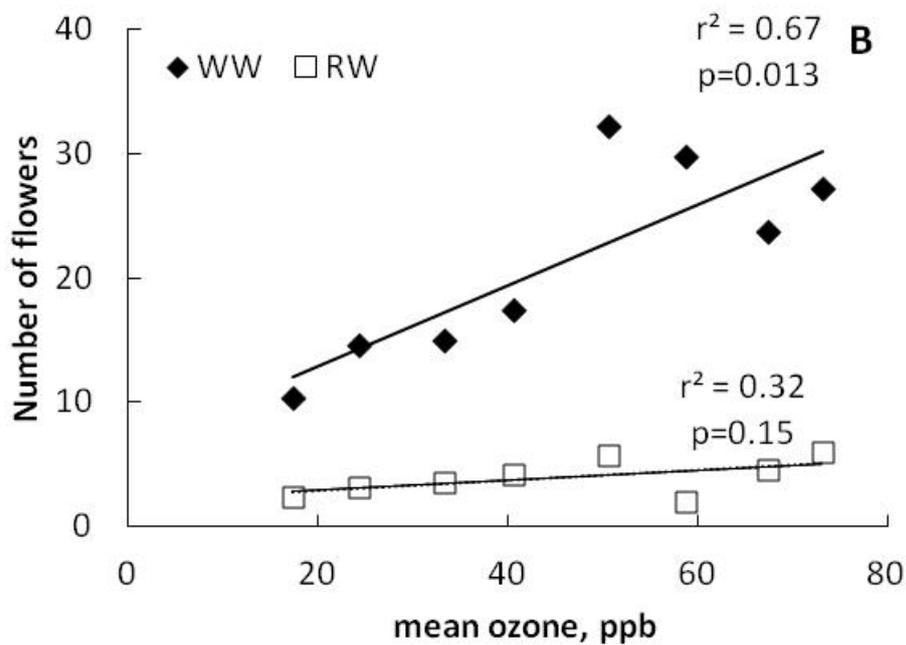
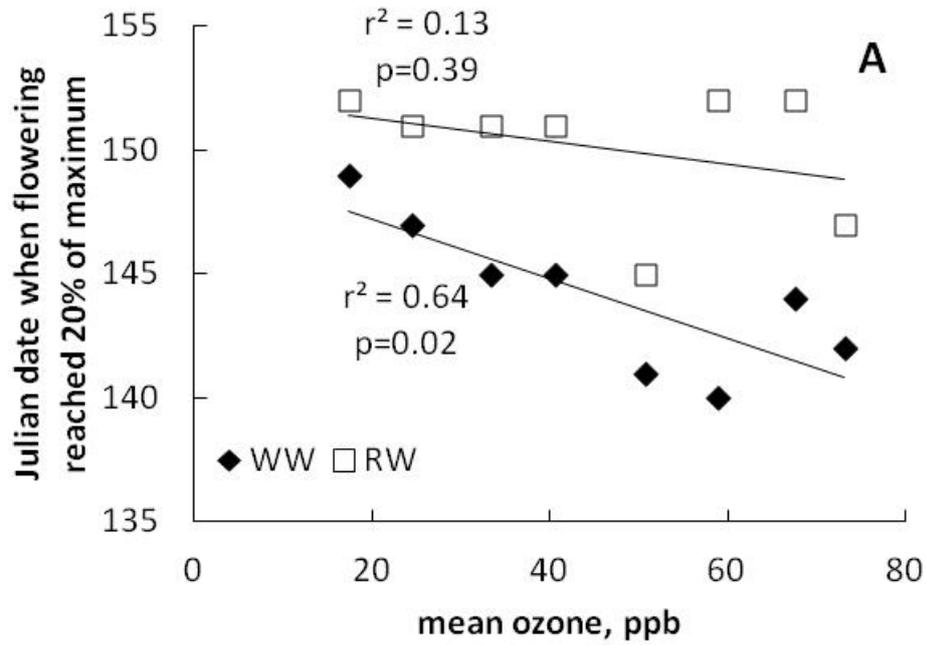
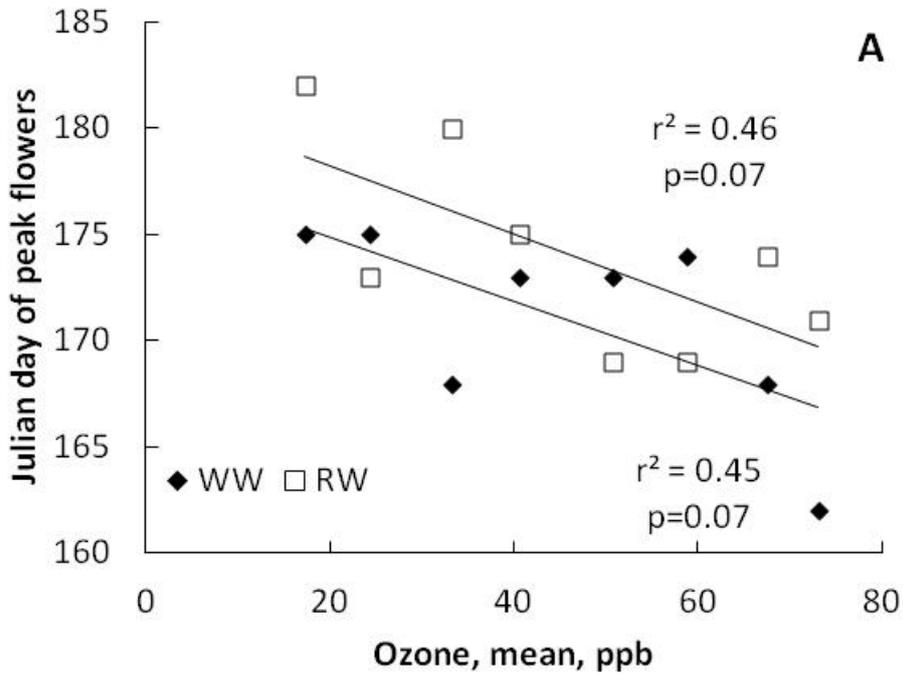
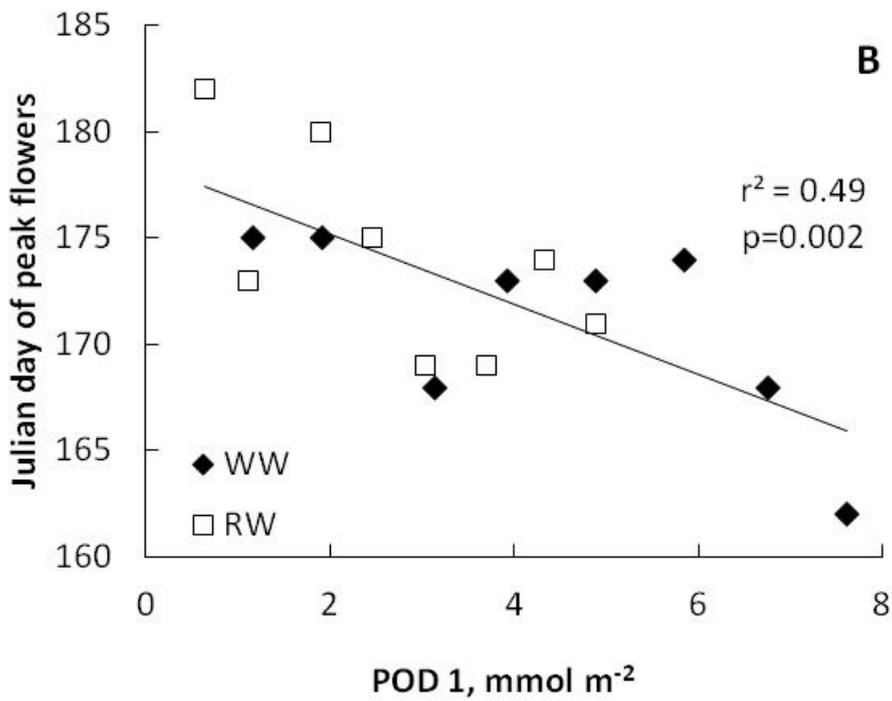


Figure 2: (a) The Julian date when flower number reached 20% of maximum in *L. corniculatus* in response to ozone concentration in both the WW and RW treatments and (b) Mean number of flowers per mesocosm on 27th May (after 5 weeks of exposure in 2010).



1



2

3 Figure 3: Julian date of maximum flower number for *L. corniculatus* in response to (a) ozone
 4 concentration and (b) ozone flux in both the WW and RW treatments.

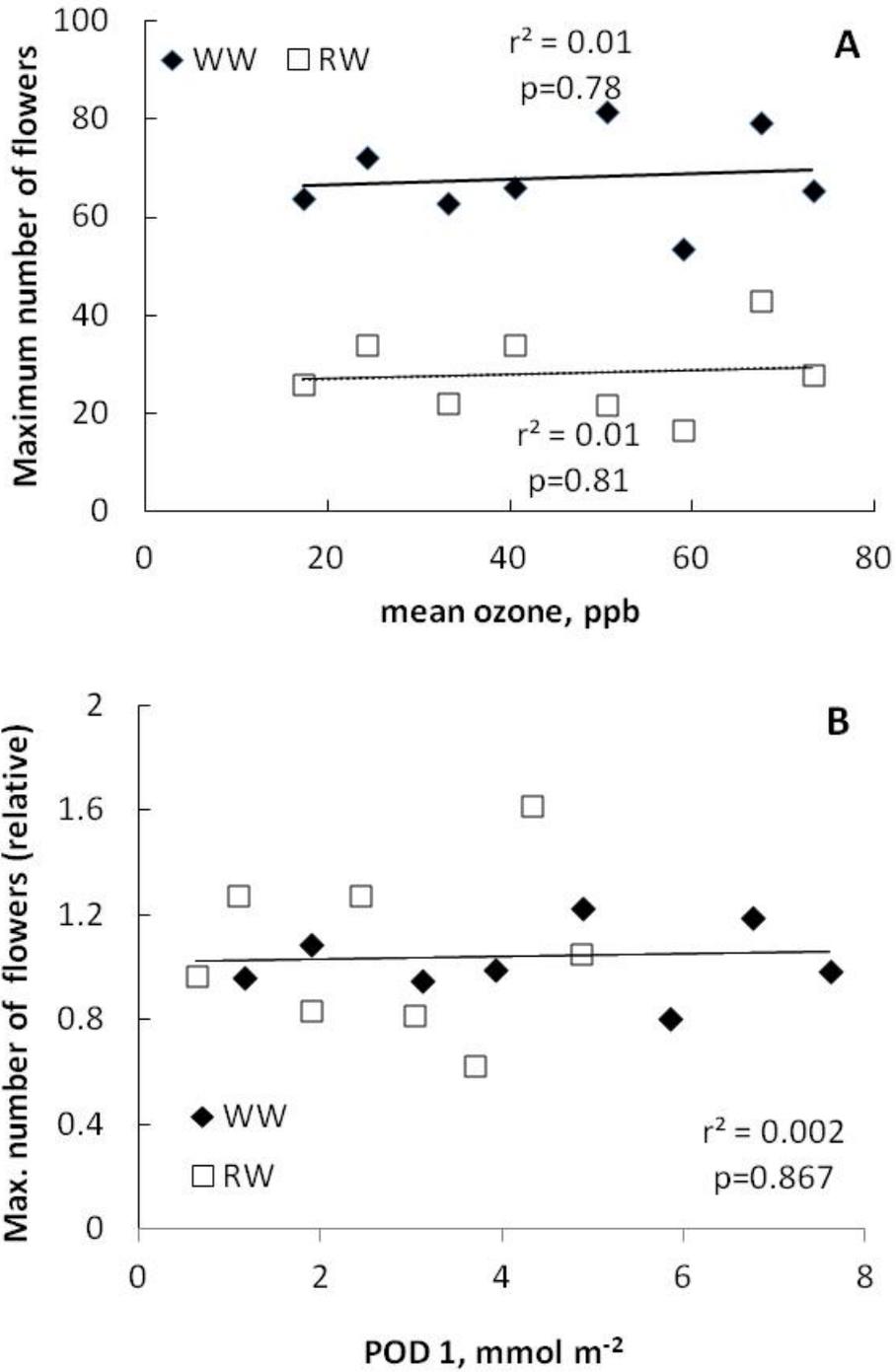


Figure 4: Maximum flower number in the WW and RW treatments for *L. corniculatus* in response to ozone concentration and (b) in relation to ozone flux, normalised for the effect of watering regime.

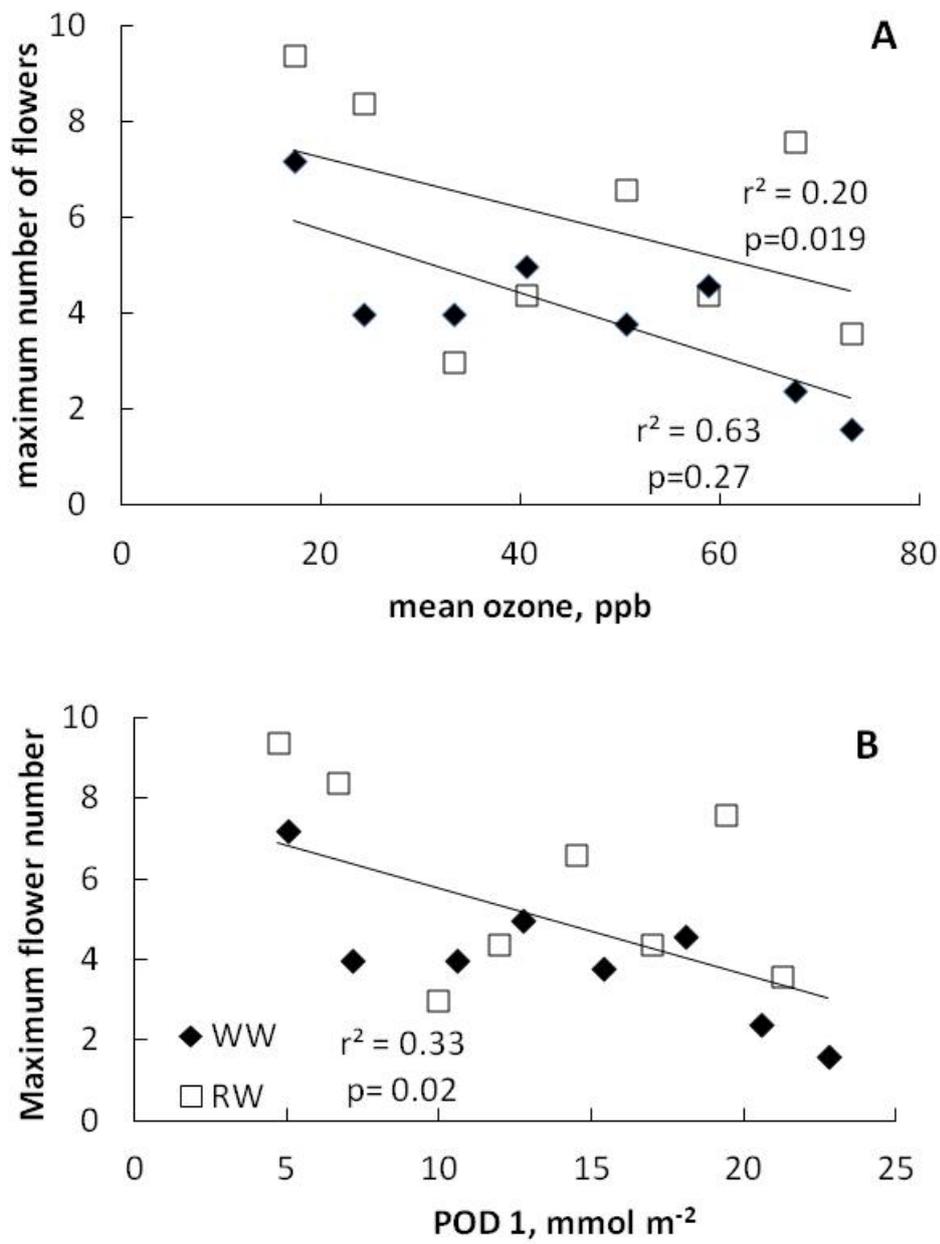


Figure 5: Maximum flower number for *C. rotundifolia* in the WW and RW treatments in relation to (a) ozone concentration and (b) ozone flux.

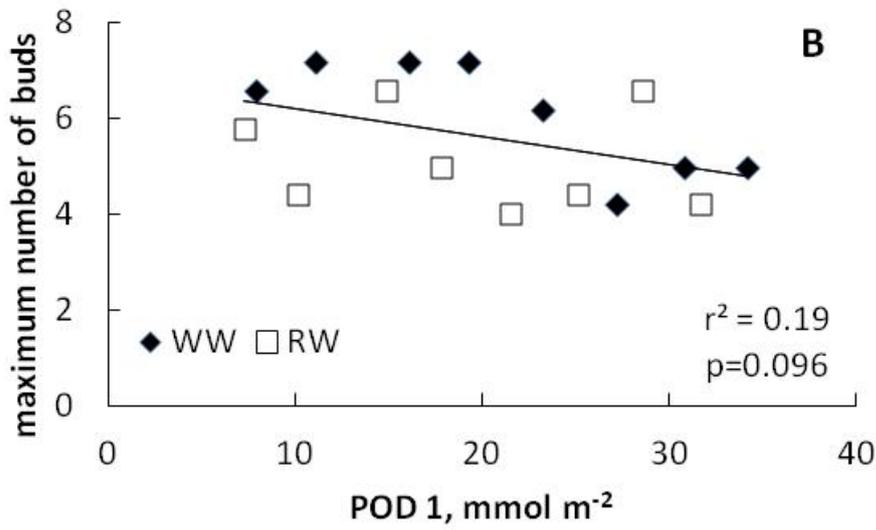
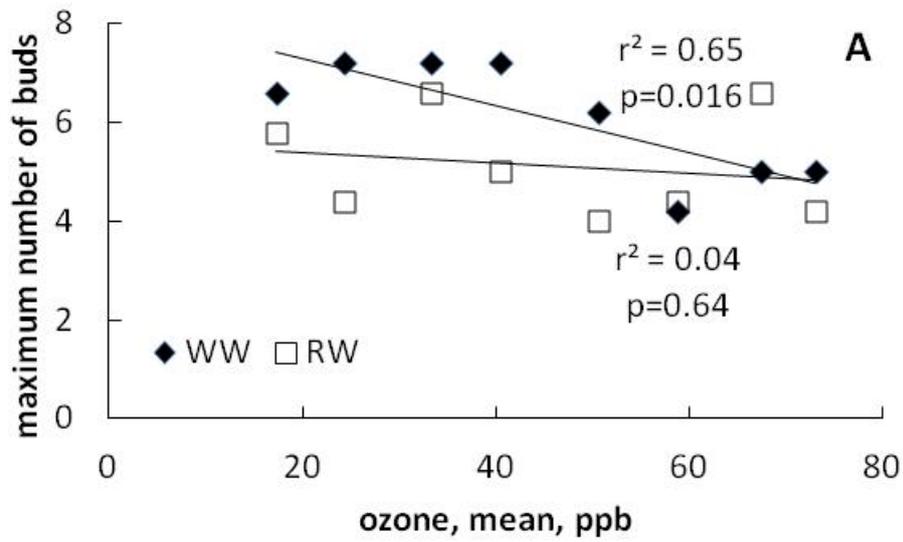


Figure 6: Maximum flower number in the WW and RW treatments for *S. columbaria* in relation to (a) ozone concentration and (b) ozone flux.