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How much does the presence of a competitor modify the within-canopy distribution of ozone-induced senescence and visible injury?

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Summary

- Many natural vegetation species have been shown to be negatively affected by ozone. This study has investigated how the presence of competing species in a community affects two common responses to ozone: visible injury and senescence.
- Monocultures and mixtures of *Trifolium repens* and *Lolium perenne* grown in large containers were exposed in solardomes to either an episodic rural ozone profile (AOT40 of 12.86 ppm.h) or control conditions (AOT40 of 0.02 ppm.h) for 12 weeks.

- The proportion of ozone-injured or senesced leaves decreased in the order upper>edge>inner canopy for *T. repens* and *L. perenne*. The presence of *L. perenne* increased the proportion of ozone-injured leaves in *T. repens*, whilst the presence of *T. repens* decreased the proportion of senesced leaves in *L. perenne*. In *L. perenne*, the proportion of injured leaves at the edge and inner canopy decreased significantly when grown in competition, whilst for *T. repens* the reverse effect occurred in the inner canopy only.
- It is proposed that different mechanisms influence the interaction between response to ozone and competitors in these species: the response of *Lolium perenne* to ozone may have been related to nitrogen supply, whilst in *Trifolium repens* canopy structure was more important.

Key words

Ozone; visible injury; senescence; stomatal conductance; canopy; competition

Introduction

Ambient ozone concentrations in Europe have been shown to cause significant effects on a wide range of plant species. Although the effects vary between species, visible leaf injury and premature senescence are frequently reported from ozone exposure studies (e.g. Bergmann et al., 1999; Novak et al., 2003). In addition, approximately 80 species of semi-natural vegetation have been recorded with symptoms attributed to ozone in ambient air conditions (Hayes et al., 2007). There is a need to improve predications of the impacts of ambient ozone on natural vegetation communities, however, many studies investigate the effects of ozone using single species, and the presence of competing species in a community may affect the response to

47 ozone. Canopy structure and competition are two interlinked factors to be considered as
48 influences on the response to ozone in mixed vegetation communities. To our knowledge, no
49 other studies have investigated both of these factors together.
50
51 For some species, the magnitude of the response to ozone has been shown to be influenced by
52 competition, for example, the grass *Elymus glaucus* increased the impact of ozone exposure on
53 *Pinus ponderosa* (Anderson et al., 2001). Similarly *Poa pratensis* has been demonstrated to be
54 more sensitive to ozone (in terms of visible injury) when grown in competition with *Veronica*
55 *chamaedrys* compared to when grown as a monoculture but not when grown with other species
56 such as *Achillea millefolium* (Bender et al., 2005). In contrast, *Holcus lanatus, Lychnis flos-*
57 *cuculi, Molinia caerulea* and *Plantago lanceolata* showed no difference in response to ozone
58 when grown in monoculture compared to when grown in competition with *Agrostis capillaris*
59 (Tonneijck et al., 2004).
60
61 Presence within a canopy of vegetation may also influence the response of an individual
62 species to ozone. Few studies have investigated the changing profiles of ozone and light
63 through plant canopies, and these existing studies have tended to involve large canopies such
64 as forests (e.g. Utiyama et al., 2004). Lantinga et al. (1999) showed that PAR was dramatically
65 reduced inside a plant canopy, and in stands of a monoculture of cut-leaved coneflower
66 (*Rudbeckia laciniata* L.), ozone concentrations 20 cm above the ground were only half the
67 concentration of those just above the top of the canopy, which was approximately 1.5 – 2.0
68 metres high (Finkelstein et al., 2004). Within these large stands of cut-leaved coneflower, the
69 extent of ozone injury was lower on plants growing within the stand compared to those on the
70 edge (Chappelka et al., 2003). A similar pattern of ozone concentration within the canopy
71 occurred in the only study to investigate profiles of a grassland canopy, where leaves of low

72 growing *Trifolium repens* received approximately 30% less ozone than *Alopecurus pratensis,*
73 which itself was exposed to slightly lower ozone concentrations than those of the bulk air
74 above the canopy (Jäggi et al., 2006). Thus, there is the potential for differential effects of
75 ozone within mixed canopy grasslands.
76
77 Models of ozone fluxes to natural vegetation communities have been developed (e.g. Bassin et
78 al., 2004, Emberson et al., 2000, 2001, Simpson et al., 2003). These models currently include
79 the influence of environmental variables such as temperature on stomatal conductance and
80 therefore ozone fluxes. Use of a mechanistic model of canopy development of *Lolium perenne*
81 demonstrated the importance of simulation of canopy growth compared to a fixed seasonal
82 profile of leaf area index (Ashmore et al., 2007), however these models do not currently
83 account for differential ozone uptake within different portions of a plant canopy, or differential
84 uptake by different species or functional types.
85
86 In this study, responses of plants grown in monoculture were compared to the responses when
87 grown in mixture, using *Trifolium repens* and *Lolium perenne* as model species that respond to
88 ozone by the development of ozone injury and senescence. Detailed measurements of visible
89 injury and senescence were carried out at different positions in the canopy to investigate
90 whether the presence of a competitor modifies the extent and location of damage within the
91 canopy. Effects in *Trifolium repens* were related to within canopy variation in stomatal
92 conductance.
93

Materials and Methods

Plant material

Plant material was vegetatively propagated from *Lolium perenne* and *Trifolium repens* plants from turf samples of pasture managed for silage near Edinburgh, UK (Grid reference NT245642). Plants originating from different parents were randomised between different competition and ozone treatments. Individual plants were established for approximately eight weeks before monocultures and mixtures of plants were established for ozone exposure.

Experimental design

Large containers (35.5 cm x 45 cm x 25 cm deep), with holes for drainage, were lined with perforated plastic sheeting to prevent roots from growing out through the bottom and filled with multipurpose compost ('Gem' tub and planter).

In each pot twelve plants were planted in an evenly spaced arrangement, consisting of four central plants surrounded by eight additional plants. In each mixture, the four central plants were *Trifolium repens* and the eight surrounding plants were *Lolium perenne*. Three pots each of the *Lolium perenne* and *Trifolium repens* monocultures, and three pots of the *Lolium perenne* and *Trifolium repens* mixture were randomly allocated to each of four solardomes. Plants were exposed in the solardomes for twelve weeks, starting on 26th July 2002. The exposure period was divided into two harvest periods. Plants were cut back on 6th September, the intermediate harvest, and 16th October, when the final harvest occurred. Plants were kept well-watered throughout the experiment using a mist irrigation system, with additional watering by hand during periods of warm weather.

Ozone exposure

Four solardomes were used for exposure. Ozone was generated from oxygen using an ozone generator (Wallace and Tiernan). Ozone concentrations were measured every 30 minutes in each solardome using an ozone analyser (Dasibi 1003-AH) which sampled ozone for a minimum of 3.5 minutes from each solardome using a computer controlled sample selector. Two solardomes were used as controls, with ozone added to charcoal-filtered air using computer controlled (LabView version 6) mass flow controllers to give continuous ozone concentrations in each dome of 30 ppb (O_3(30)). An episodic rural ozone profile (O_3(30+peaks)) was given over the course of each week to the two other domes. The ozone exposure was programmed to reach a maximum concentration of 80 ppb on days 1 and 4, and a maximum concentration of 100 ppb on days two and three. Ozone concentrations increased from 30 ppb to the daily maximum over the course of 2 hours, remained at the daily maximum for 6 hours, then decreased back down to 30 ppb over the course of 2 hours. Ozone concentrations were programmed to remain at 30 ppb at all other times.

Visual assessments

Visual estimates of senescence and ozone-specific injury, apparent as white or pale yellow stipples on the leaf surface, were made for whole pots, because the individual plants had grown together and could not be separated. Leaves were classified as either senesced or injured if >25% of the leaf was senesced or injured respectively, otherwise they were classified as healthy. For *Lolium perenne* senescence of leaves started at the leaf tip and progressed along the leaf blade. The length of the senesced portion (in mm) of the leaf blade was measured on a sub-sample of twenty randomly chosen leaves in each pot.

Harvests

All plants were cut back to a height of 7 cm on 6th September and 16th October, after exposure to the ozone regime for six weeks and 12 weeks respectively. The plants were harvested in separate layers: material growing outside the pot perimeter, material greater than 14 cm above soil level, and plant material between 7 cm and 14 cm above soil level. At the final harvest an additional layer with plant material 0 to 7 cm above soil level was also used. Fresh plant material from each layer was sorted into the component species at the time of harvest. Healthy and ozone-injured leaves of *Trifolium repens* were separated. *Lolium perenne* was sorted into healthy leaves and senesced leaves. Plant material was dried at 65°C for a minimum of 4 days before biomass was determined.

Stomatal conductance measurements

Measurements of stomatal conductance were made on *Trifolium repens* using a porometer (Delta-T AP4) on days of stable meteorological conditions after exposure to the ozone regime for 10/11 weeks. Measurements of stomatal conductance in the upper canopy (where leaves were in full sunlight) and the inner canopy (where leaves were more shaded) were taken, using six leaves (two per pot) for each canopy position in every solardome.

Chlorophyll content

Chlorophyll content (chlorophyll a + b) of leaves of *Trifolium repens* was measured using a SPAD meter (CCM-200, ADC Bioscientific Ltd., UK) after exposure to the ozone treatment for one week and ten weeks. 'Typical' leaves were used; therefore some ozone injury was present in some cases. The chlorophyll index, in relative units, given by the SPAD meter, were calibrated for *Trifolium repens* following determination of chlorophyll content by extraction with acetone and measurement of light absorption at wavelengths 470, 646 and 663 nm, according to the equations of Lichtenthaler and Wellburn (1983). The relationship between

chlorophyll index and measured chlorophyll (mg g^{-1} fresh weight) had an r^2 of 0.90 (data not presented) and was:

Chlorophyll content (mg g^{-1} FW) = (chlorophyll index * 30.448) + 417

Statistical analysis

For each parameter, values were averaged to provide a mean per solardome prior to subsequent analysis. Statistical analysis was based on these dome means. Visible injury and senescence data were arcsine transformed prior to analysis. Oneway ANOVA (Minitab version 14) was used for analysis of stomatal conductance data. Other comparisons were made in Genstat (version 8) using split-plot or split-split plot ANOVA. The main plot was ozone treatment and the sub-plots were monoculture/mixture. Sub-sub-plots of canopy position were used where appropriate.

Results

Ozone concentrations

The mean AOT40 for the two domes exposed to the O_3(30+peaks) episodic ozone regime was 9.98 ppm.h during the first harvest interval, and 11.89 ppm.h during the second harvest interval, giving a total of 21.86 ppm.h over the 12 week exposure period (Table 1). The difference in AOT40 between the two replicate O_3(30+peaks) solardomes was less than 2% for each harvest interval. In the two replicate O_3(30) solardomes, the mean AOT40 over the exposure period was less than 0.02 ppm.h. 24-hour mean, 12-hour mean and 12-hour mean of episode days also show small differences between the replicate solardomes (Table 1).

Influence of *Lolium perenne* on visible injury on clover

Visible injury caused by ozone on *Trifolium repens* was apparent first as small, yellow flecks on the leaves. As the severity increased, the extent of chlorosis increased until eventually the leaf was dry and curled. Visible injury symptoms caused by ozone were first observed on the clover plants after one week of exposure. Very little non-specific senescence (<1 % of leaves) was observed on *T. repens* leaves during the experiment; any senescence that corresponded with the presence of ozone injury symptoms was recorded as "visible injury".

At the intermediate harvest, a visual assessment of the O_3(30+peaks) treated *Trifolium repens* plants growing in monoculture showed that 69% of leaves per pot had visible injury symptoms compared to only 0.5% in the O_3(30) treatment ($p<0.001$). Similar proportions of injury were observed when *Trifolium repens* was grown in combination with *Lolium perenne* - 67% injured leaves in O_3(30+peaks) compared to 0% injured in O_3(30) ($p<0.001$). At the final harvest the proportion of injured *Trifolium repens* leaves per pot in the O_3(30+peaks) treatment was significantly higher when grown in the mixture compared to when grown in monoculture (77% compared to 67%, $p<0.01$). There was also an interaction between ozone treatment and whether the plants were grown in monoculture or in mixture ($p<0.01$), with a larger difference in the extent of visible injury between O_3(30) and O_3(30+peaks) if the plants were grown in mixture with *Lolium perenne*.

The proportion of injured leaves was also quantified by biomass. Separation of leaves into those that were healthy and those that were injured at the intermediate harvest showed that differences in the biomasses of both healthy leaves and ozone injured leaves were significantly affected by ozone in *Trifolium repens* growing both as a monoculture and as part of the mixture (Table 2). The biomass of injured leaves was approximately two thirds of the total leaf

biomass in O_3(30+peaks) treated plants, whereas the biomass of injured leaves was negligible in O_3(30) plants. At the final harvest the total leaf biomass and the biomass of both healthy and injured leaves were significantly affected by ozone in *Trifolium repens* growing both as a monoculture and as part of the mixture (Table 2). The proportion of injured leaves was negligible in O_3(30) treated plants and approximately 80% of the total leaf biomass in O_3(30+peaks) treated plants (Table 2). Due to the difference in the number of *Trifolium repens* plants per pot in the monoculture and mixture, statistical comparison was based on the proportion of injured leaves relative to healthy leaves, rather than the actual biomass. This showed that there was no significant interaction between ozone treatment and whether the plants were grown in monoculture or in mixture.

The proportion of injured leaves was different in the different regions of the canopy (Figure 1). At the intermediate harvest the highest proportion of injured leaves was in the plant material growing at the edge of the canopy – plant material growing outside the pot perimeter (75% of leaves were injured, $p<0.05$). The proportion of injured leaves was lower above 14cm – the upper canopy (67%) and lowest in the inner canopy (52%) – plant material between 7cm and 14cm. The pattern was similar in the monoculture, and there were no significant effects of whether the plants were grown in monoculture or in mixture, or any significant interaction between this and the ozone treatment.

At the final harvest the proportion of injured leaves in the monoculture was not significantly different in the different regions of the canopy. There was much less growth outside of the pot perimeter during the second harvest interval (data not presented). In addition, although there was reduced leaf biomass at the final harvest compared to the intermediate harvest (Table 2),

the canopy height was the same (data not presented) indicating that the canopy was much more open during the second harvest interval.

The proportion of injured leaves in the inner canopy (7 – 14cm) was higher in plants growing in mixture with *Lolium perenne* compared to those of the monoculture, where the proportions of injured leaves were 81% and 63% in the mixture and monoculture respectively at the final harvest (Figure 1, $p<0.01$). There was also an interaction between ozone exposure and whether the plants were grown in monoculture or in mixture for the proportion of injured leaves in the inner canopy ($p<0.05$), with ozone treatment corresponding with an increased proportion of injured leaves in the mixture. There were no significant differences and no interaction between ozone exposure and whether plants were grown in monoculture or in mixture for the proportion of injured leaves in the upper canopy or the canopy edge.

The influence of *Trifolium repens* on senescence of *Lolium perenne*

In contrast to *T.repens*, *L. perenne* responded to ozone by the development of non-specific senescence; no ozone-specific injury was observed during the course of the experiment.

The large difference in the extent of senescence of O_3(30+peaks) treated *Lolium perenne* compared to O_3(30) was significant at both harvests (Table 3, $p<0.05$ at each harvest). In the O_3(30+peaks) treatment at the intermediate harvest, the proportion of senesced leaves was approximately 50% for plants growing in the monoculture and in the mixture. At the final harvest, there was a further increase in senescence of plants in the O_3(30+peaks) treatment in the monoculture, to 68%, but a reduction in senescence for plants in mixture with *Trifolium repens* to 28%. There was also significantly less senescence of *Lolium perenne* when grown as a mixture compared to as a monoculture in the O_3(30) treatment (0% vs 28%, $p<0.001$).

260 However, there was no significant interaction between ozone treatment and whether the plants
261 were grown in monoculture or in mixture at either harvest.
262
263 In *Lolium perenne* plants, senescence started at the tip of the leaf blade and progressed back
264 towards the main plant. The extent of the senesced portion of leaf (in mm) was significantly
265 increased in O_3(30+peaks) treated plants compared to O_3(30) plants for both the monoculture
266 and the mixture at both harvests (Table 3, $p<0.05$). As with the proportion of senesced leaves,
267 the extent of senescence of both O_3(30+peaks) and O_3(30) treated plants was significantly less
268 in the mixture compared to the monoculture at both harvests ($p < 0.001$ in each case). Again,
269 there was no significant interaction between ozone treatment and whether the plants were
270 grown in monoculture or in mixture at either harvest.
271
272 The biomass of healthy leaves and senesced leaves were not affected by ozone at the
273 intermediate harvest (Table 4), and there was no significant difference in the proportion of
274 senesced leaves of plants grown in monoculture compared to those grown in mixture. The
275 senesced biomass was approximately four-times greater in the O_3(30+peaks) treatment in the
276 monoculture ($p<0.01$) and approximately two-times greater in the mixture ($p<0.1$, Table 4).
277 There was no significant interaction between ozone treatment and whether the plants were
278 grown in monoculture or in mixture.
279
280 At the final harvest there was a significant effect of ozone on the biomass of the senesced
281 leaves ($p<0.01$, Table 4). There was also a large reduction (80%) in the biomass of healthy
282 leaves in the O_3(30+peaks) treatment of the monoculture ($p<0.05$), whereas the biomass of
283 healthy leaves in the mixture was not significantly affected by ozone treatment (Table 4).
284

There was a significant effect of canopy position on the proportion of senesced leaves of *Lolium perenne* ($p<0.01$ at each harvest; Figure 2). The proportion of senesced leaves of *Lolium perenne* was much lower in the inner canopy than in the upper canopy or canopy edge for plants growing in both the monoculture and the mixture ($p<0.01$ in both cases). The proportion of senesced leaves of *Lolium perenne* was also much lower overall in the mixture than in the monoculture, although this difference was only statistically significant at the intermediate harvest ($p<0.01$). However there was no significant interaction between ozone treatment and whether the plants were grown in monoculture or in mixture.

Within-canopy variation in stomatal conductance

There were no significant differences in stomatal conductance of *Trifolium repens* in the monoculture compared to in mixture with *Lolium perenne* (data not presented). However, there was reduced stomatal conductance in the inner canopy compared to the upper canopy of *Trifolium repens* monocultures in both $O_3(30)$ ($p<0.05$) and $O_3(30+peaks)$ treatments ($p<0.05$, Table 5). There were also significant differences between the $O_3(30)$ and $O_3(30+peaks)$ treatments, with increased stomatal conductance in the inner canopy of $O_3(30+peaks)$ treated plants compared to $O_3(30)$ ($p<0.05$). There were no significant differences in stomatal conductance between treatments in the upper canopy.

Corresponding measurements of PAR, measured at the same time as stomatal conductance using a light sensor on the head of the leaf clip of the porometer, indicated that the PAR was different in the different regions of the canopy. PAR was reduced by 88% and 77% in the inner canopy compared to the upper canopy in the $O_3(30)$ and $O_3(30+peaks)$ treatments respectively (Table 5). The PAR in the inner canopy was significantly higher for canopies that received the $O_3(30+peaks)$ treatment compared to $O_3(30)$, $p<0.01$, however, there was no

309 difference in the relationship between PAR and stomatal conductance between the two ozone
310 treatments (data not presented).
311
312 Stomatal conductance was not related to leaf age. For *Trifolium repens* there was no difference
313 in stomatal conductance of different age leaves along a stolon (i.e. between Leaf 1 the newest
314 fully expanded leaf, Leaf 2 and Leaf 3) in either the O_3(30) or O_3(30+peaks) treatments (data
315 not presented).
316
317 There were no significant differences between ozone treatments in the stomatal conductance of
318 upper canopy leaves of *Lolium perenne* after exposure for 2, 4 or 10 weeks (data not
319 presented).

320 **Within-canopy variation in chlorophyll content**

321 Chlorophyll content of upper canopy leaves was reduced by approximately 12% in leaves of
322 *Trifolium repens* that had been exposed to O_3 (30+peaks) compared to the O_3(30) treatment
323 ($p<0.05$, Figure 3). However, there were no significant differences between ozone treatments
324 for leaves of the inner canopy.
325
326 There were no differences in the chlorophyll content of leaves of different ages in the O_3(30)
327 treatment, however, there was a significant decrease in the chlorophyll content with increasing
328 leaf age in the O_3(30+peaks) treatment (Figure 4), which corresponded with an increased
329 extent of ozone damage in older leaves. There were no significant differences in chlorophyll
330 content of plants grown in monoculture compared to plants grown in mixture (data not
331 presented).

Discussion

By using two model species representing grasses and legumes, this study has revealed that the presence of a competitor modifies the extent and canopy distribution of two important responses to ozone: visible injury and senescence.

Overall, a higher proportion of leaves were injured by ozone when *T. repens* was grown in competition with *L. perenne* than when grown in monoculture, with this effect most significant in the inner canopy leaves. Increased sensitivity to ozone when grown in competition has previously been demonstrated on *Poa pratensis* (Bender et al., 2005), where *P. pratensis* developed more ozone injury when grown with competing species such as *Veronica chamaedrys* than when grown alone. In contrast, *L. perenne* was not affected as severely by ozone when growing in combination with *T. repens* compared to when growing in monoculture. Indeed, senescence was reduced in the mixture in both the O_3(30) and O_3(30+peaks) treatments, we speculate that in *L. perenne,* since nitrogen transfer from clover to grass in grass-clover swards has been demonstrated in several studies e.g. Sincik & Acikgoz (2007) and Goodman (1988) there is likely to have been an increased availability of nitrogen to *Lolium perenne* when it was grown with *Trifolium repens.* It has been shown that for some species, e.g. *Trifolium subterraneum*, increased nitrogen supply can partially counterbalance the effects of ozone exposure (Sanz et al., 2005). Some studies have shown that levels and activity of Rubisco were reduced following ozone exposure (Pell et al., 1997). Increased nitrogen availability may have increased turnover of the Rubisco enzyme in *L. perenne*, reducing leaf senescence.

The reduced chlorophyll content of *Trifolium repens*, which corresponds with increased visible injury, implies that there is a reduced capacity for photosynthesis following ozone exposure for

this species, which may have contributed to reduced plant growth (Hayes et al., in press). The proportion of leaves showing visible injury symptoms in *Trifolium repens* varied according to the position of the leaf in the plant canopy, with reduced injury in the inner canopy. This corresponded with reduced stomatal conductance in the inner canopy compared to the upper canopy. At the intermediate harvest, the proportion of leaves of *Trifolium repens* that had visible injury symptoms was lower in the inner canopy than in the upper canopy and the canopy edge. This pattern was not as pronounced at the final harvest, which may have been because there was less growth between the intermediate harvest and the final harvest, resulting in a more open canopy. This would allow increased light and ozone penetration into the inner canopy during the second harvest interval, reducing the differences in microclimate between the upper canopy/canopy edge compared to the inner canopy at the final harvest.

There was increased overall ozone leaf injury at the final harvest than at the intermediate harvest (using the proportion of injured leaves, quantified by biomass), even though the AOT40 value during the two harvest intervals was similar. This could have been due to the more open canopy, allowing greater penetration of ozone and light. However, this effect was also seen on the upper canopy and canopy edge leaves, so may have been due to a cumulative/carry-over effect of ozone on the plants. Cumulative effects caused by ozone on plant biomass have previously been shown for *Trifolium repens* (Fumagalli et al., 2003, Nussbaum et al., 1995). In these two studies, regrowth in subsequent growth periods was affected and the biomass differences were better related to the cumulative ozone than to the ozone dose from an individual growth period only. However, these cumulative effects have been shown only in biomass and not for visible injury on leaves produced in a subsequent growth period, as in this study.

381 The structure of the canopy is also important in influencing the impact of the ozone exposure.
382 O_3(30+peaks) treated *Trifolium repens* had a more open canopy due to reduced leaf biomass
383 and the leaves curling due to ozone injury. Similarly, reduced leaf-area index of a soybean
384 (*Glycine max)* canopy has been demonstrated due to increased senescence following ozone
385 exposure (Dermody et al., 2006). Differences in leaf-area index have been related to
386 differences in penetration of PAR through plant canopies (Shulski et al., 2004). In the current
387 study the microclimate of the canopy was altered following ozone exposure and light levels of
388 the inner canopy were higher than those from the O_3(30) treatment. Other factors such as
389 temperature and windspeed may also have been affected, but were not measured. In this study,
390 the difference in stomatal conductance between the upper and inner canopy of *Trifolium repens*
391 was reduced in the O_3(30+peaks) treatment compared to O_3(30) and this corresponded to less
392 dense leaf growth giving a more open canopy in the O_3(30+peaks) treatment. This would
393 reduce the differences in microclimatic conditions between the upper and inner canopy,
394 particularly for light. Models of stomatal conductance in response to climatic conditions have
395 shown a strong influence of light (e.g. Emberson et al., 2000), and in the current study the
396 differences in stomatal conductance between the upper and inner canopy were attributed to
397 differences in light conditions rather than alterations in the relationship between stomatal
398 conductance and light. It is also possible that chronic exposure to ozone increased the
399 sluggishness of stomata of the inner canopy leaves as found in other studies (Mills et al., in
400 press; Paoletti, 2005).
401
402 Stomatal conductance of *Trifolium repens* was similar to that of *Lolium perenne,* indicating
403 that differences in sensitivity to ozone of the two species are not linked to stomatal
404 conductance. There was no evidence that the stomata of *Trifolium repens* in comparable upper
405 canopy leaves were being closed by ozone treatment, in contrast to the assumptions made by

406 Sitch et al. (2007), where models predicted further increases in atmospheric carbon dioxide
407 concentrations due to ozone induced stomatal closure. However, in this study measurements of
408 stomatal conductance were only carried out on 'non-episode days', when the ozone
409 concentration was the same (approximately 30 ppb) in the two treatments. It is possible that
410 plants may respond to high ozone concentrations by closing their stomata during the period of
411 exposure only.

412

413 This study has shown that interspecific interactions can modify the response to ozone of both
414 *T. repens* and *L. perenne*, with the direction of the interaction dependant on the species. In
415 addition, within-canopy variations in the response to ozone occur, with inner canopy leaves
416 having less response to ozone. The influence of neighbouring species and the effects these
417 species have on the canopy and microclimate should be considered in future studies. There is a
418 need for studies on more complex plant communities to further investigate whether species are
419 as sensitive to ozone as predicted from experiments on monocultures and binary mixtures, and
420 to further investigate the role of microclimate in influencing the response to ozone.
421

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424

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532 Table 1: Ozone exposure characteristics for the O_3(30) and O_3(30+peaks) treatments. Standard

533 errors are shown in brackets

		First harvest interval	Second harvest interval
AOT40 (ppm.h)	O_3(30)	0.02 (0.02)	0 (0)
	O_3(30+peaks)	9.98 (0.10)	11.89 (0.08)
24 hour mean (ppb)	O_3(30)	28.0 (1.4)	27.1 (1.5)
	O_3(30+peaks)	41.8 (0.9)	46.2 (1.0)
12 hour mean (episode days, ppb)	O_3(30)	27.8 (1.4)	28.9 (1.4)
	O_3(30+peaks)	65.1 (0.0)	61.4 (0.0)

534

535

Table 2: Biomass of injured and healthy leaves of *Trifolium repens* at the intermediate and final harvests from the O_3(30) and O_3(30+peaks) treatments of plants growing in monoculture and in mixture. Standard errors are shown in brackets. ***/**/* indicates significant differences at $p<0.001$, $p<0.01$ and $p<0.05$ respectively.

		Intermediate harvest		Final harvest	
		Healthy (g)	Injured (g)	Healthy (g)	Injured (g)
Monoculture	O_3(30)	70.2 (7.0)	0.04 (0.0)	48.6 (1.9)	0 (0)
	O_3(30+peaks)	13.2 (1.8)	31.4 (4.1)	5.7 (0.4)	23.4 (1.6)
Mixture	O_3(30)	45.1 (2.0)	1.2 (0.7)	43.1 (2.7)	0 (0)
	O_3(30+peaks)	10.3 (0.9)	20.3 (1.7)	2.8 (1.1)	15.4 (0.1)
	Significance of ozone treatment	***	***	***	**
	[1]Significance of mixture vs monoculture		ns		*
	[1, 2]Significance of interaction		ns		ns

[1] Using the proportion of injured to healthy leaves.

[2] Significance of the interaction between whether plants are grown in monoculture or mixture and ozone treatment.

Table 3: Senescence of *Lolium perenne* at the intermediate and final harvests from the O_3(30) and O_3(30+peaks) treatments of plants growing in monoculture and in mixture. Standard errors are shown in brackets. ***, * and (*) indicate differences at $p<0.001$, $p<0.05$ and $p<0.1$ respectively.

		Intermediate Harvest		Final Harvest	
		Senescence (%)	Senescence (mm from tip)	Senescence (%)	Senescence (mm from tip)
Monoculture	O_3(30)	9 (8.9)	25.8 (20.9)	28 (10.0)	28 (6.7)
	O_3(30+peaks)	52 (5.0)	96.7 (16.7)	68 (5.0)	74 (0.8)
Mixture	O_3(30)	4 (2.8)	14.0 (8.4)	0 (0)	0.5 (0.3)
	O_3(30+peaks)	49 (4.1)	61.0 (19.2)	28 (3.3)	45 (2.5)
	Significance of ozone treatment	*	*	*	*
	Significance of mixture vs monoculture	ns	***	*	***
	Significance of interaction[1]	ns	ns	ns	ns

[1] Significance of the interaction between whether plants are grown in monoculture or mixture and ozone treatment.

551 Table 4: Biomass of senesced and healthy leaves of *Lolium perenne* at the intermediate and
552 final harvests from the O_3(30) and O_3(30+peaks) treatments of plants growing in monoculture
553 and in mixture. Standard errors are shown in brackets. **/* indicates significant differences at
554 $p<0.01$ and 0.05 respectively.

		Intermediate harvest		Final harvest	
		Healthy (g)	Senesced (g)	Healthy (g)	Senesced (g)
Monoculture	O_3(30)	16.3 (4.8)	1.4 (0.3)	5.8 (4.1)	1.5 (1.1)
	O_3(30+peaks)	10.0 (2.9)	5.8 (1.4)	1.2 (0.3)	2.0 (0.2)
Mixture	O_3(30)	12.6 (2.1)	2.5 (0.4)	4.6 (4.1)	0 (0)
	O_3(30+peaks)	11.5 (3.1)	4.8 (0.9)	3.7 (1.7)	1.8 (0.2)
	Significance of ozone treatment	ns	ns	ns	**
	Significance of mixture vs monoculture	ns	*	ns	ns
	Significance of interaction[1]	ns	ns	ns	ns

555 [1] Significance of the interaction between whether plants are grown in monoculture or mixture
556 and ozone treatment.
557

Table 5: Stomatal conductance and PAR of *Trifolium repens* (monoculture) leaves from the inner and upper canopy. Standard errors are shown in brackets. ** and * indicate significant differences between ozone treatments at $p<0.01$ and $p<0.05$ respectively.

	Inner Canopy		Upper Canopy	
	O_3(30)	O_3(30+peaks)	O_3(30)	O_3(30+peaks)
Stomatal Conductance (mmol m^{-2} s^{-1})	66 (7)	119 (7) *	338 (44)	291 (9)
PAR (μmol m^{-2} s^{-1})	94 (13)	220 (0) **	814 (176)	951 (13)

563 Figure 1: Percentage of injured leaves (determined by biomass) of *Trifolium repens* in
564 different regions of the canopy at the intermediate harvest (A) and final harvest (B) from the
565 O_3(30+peaks) treatment of plants growing in monoculture and in mixture. Bars are standard
566 errors. ** indicates a significant difference at $p<0.01$.
567
568 Figure 2: Percentage of senesced leaves (determined by biomass) of *Lolium perenne* in
569 different regions of the canopy at the intermediate harvest (A) and final harvest (B) from the
570 O_3(30+peaks) treatment of plants growing in monoculture and in mixture. Bars are standard
571 errors. * indicates a significant difference at $p<0.05$.
572
573 Figure 3: Chlorophyll content of leaves from the inner and upper canopy of *Trifolium repens*
574 exposed to O_3(30) or O_3(30+peaks). Bars are standard errors. * indicates significant
575 differences at $p<0.05$.
576
577 Figure 4: Chlorophyll content of leaves of *Trifolium repens* exposed to O_3(30) or
578 O_3(30+peaks). Leaves were numbered from Leaf 1 (newest fully expanded leaf) to Leaf 3 (3^{rd}
579 newest fully expanded leaf). Bars are standard errors. * indicates significant differences at
580 $p<0.05$.