

Effects of tropospheric ozone and elevated nitrogen input on the temperate grassland forbs *Leontodon hispidus* and *Succisa pratensis*



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ABSTRACT

Atmospheric ozone (O₃) and nitrogen (N) pollution have increased since pre-industrial times and pose a threat to natural vegetation. The implications of these pollutants for the perennial temperate grassland species *Leontodon hispidus* (Rough Hawkbit) and *Succisa pratensis* (Devil's-bit Scabious) are largely unknown. Both species are important for pollinators and *Succisa pratensis* is the host plant for the threatened marsh fritillary butterfly (*Euphydryas aurinia*).

We examine growth and physiological responses (leaf cover, leaf litter, flowering, chlorophyll index [*Leontodon hispidus* and *Succisa pratensis*]; photosynthesis and stomatal conductance [*Succisa pratensis*]) using an outdoor Free Air Ozone Enrichment system. Plants were exposed to Low, Medium and High ozone treatments over three growing seasons (treatment means: 24, 40 and 57 ppb, respectively), with and without the addition of nitrogen (40 kg ha⁻¹ yr⁻¹) during the first year.

Decreases in leaf cover ($p < 0.001$) and chlorophyll index ($p < 0.01$) were observed with increased O₃ for *Leontodon hispidus*. The addition of N resulted in a higher chlorophyll index only at the uppermost O₃ level and also led to an overall increase in litter production of 6%. However, a stronger effect of both O₃ and N treatments was observed with *Succisa pratensis*. Litter production increased with increasing O₃ ($p < 0.001$) and an overall rise of 31% was recorded with added N ($p < 0.05$). However, O₃ had the biggest impact on *Succisa pratensis* foliage leading to more damaged leaves ($p < 0.05$). During summer resources were prioritised to new leaves, maintaining stomatal conductance and photosynthesis rates. However, this was not sustained during autumn and accelerated senescence occurred with higher ozone, and rates declined faster with added nitrogen ($p < 0.05$). Elevated O₃ also reduced *Succisa pratensis* flowering ($p < 0.01$).

These effects have implications for inter- and intra-specific competition, seed establishment, nutrient cycling, as well as the provision of general pollinator resources with specific issues for butterfly larvae. Results highlight the need for concerted action to reduce precursor ozone emissions to go alongside habitat management efforts to protect biodiversity.

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1. Introduction

At ground level, ozone (O₃) is a greenhouse gas and secondary air pollutant. It is formed from solar radiation-driven chemical reactions between pre-cursor gases, including carbon monoxide (CO), nitrogen oxides (NO_x), methane (CH₄) and non-methane volatile organic compounds (Monks et al., 2015; Royal Society, 2008; Simpson et al., 2014). Concentrations have increased since pre-industrial times by around 40% and O₃ pollution continues to be a threat globally to human health, natural vegetation and crops (Cooper et al., 2014; Fleming et al., 2018; Mills et al., 2018a, 2018b; Ronan et al., 2020). Whilst O₃ might affect plant community composition (Payne et al., 2011), little is known about its impact on biodiversity (Fuhrer et al., 2016). Atmospheric deposition of reactive nitrogen (N) is a major driver of biodiversity change, especially for nitrogen limited ecosystems where N deposition is a problem for many species (Sala et al., 2000; Bobbink et al., 2010). Dependent on climatic conditions, both O₃ and N can be transported distances away from urban and industrialised zones leading to pollution issues in rural areas (Lefohn et al., 2018).

In plants O₃ is predominantly taken up through leaf stomatal pores, where it reacts with the apoplastic fluid inside the leaf forming reactive oxygen species. In O₃ sensitive plants this can cause changes in cell structure and metabolism, and trigger cellular response defence mechanisms causing early senescence and the repression of growth and seed production (Ainsworth, 2017; Bergmann et al., 2017). Natural and semi-natural grasslands are a cause for conservation concern, being important globally for maintaining biodiversity and the provision of ecosystem services (Habel et al., 2013; Carbutt et al., 2017), and both O₃ and N sensitive taxa can be found as part of these ecosystems (Hayes et al., 2007; Stevens et al., 2010; Payne et al., 2013; Bergmann et al., 2017).

Recent (2013–2017) summer averages of O₃ concentrations across a range of European temperate zones have varied between 30 and 100 ppb (European Environment Agency (EEA), 2017). In grassland communities higher levels of O₃ have been found to result in visible leaf injury, increases and decreases in biomass (Bungener et al., 1999; Bergmann et al., 2017), changed timing of plant life-cycles (Hayes et al., 2011), and reduced pasture feed/forage quality (González-Fernández et al., 2008; Hayes et al., 2016), as well as impacting on species composition (Ashmore et al., 1995; Wedlich et al., 2012) and stomatal functioning (Mills et al., 2009; Wilkinson and Davies 2009).

Whilst increased N can result in increased growth and biomass in some species, it can also reduce plant coverage and be harmful to species adapted to low N conditions (Bobbink et al., 1998; Payne et al., 2013). This can lead to changes in grassland species composition and richness (Stevens et al., 2010, 2016).

However, less is known about the combined impact of O₃ and N, and contrasting results have been found. For grassland species and communities the interaction of O₃ and N has resulted in: no effect, damage limitation, and the increase of injury (Payne et al., 2011; Wyness et al., 2011; Bassin et al., 2013; Hayes et al., 2019). Responses are likely to vary depending on the species that make up the community and their sensitivity to O₃. For example, Hayes et al. (2019) found that dune grasslands with high levels of N deposition (over a number of sites along an N deposition gradient) had the least damage caused by elevated O₃, but that the plant community contained a high proportion of O₃ resistant forbs and grasses.

Leontodon hispidus (Rough Hawkbit) and *Succisa pratensis* (Devil's-bit Scabious) are important summer flowering temperate grassland species for pollinators in particular (Hicks et al., 2016; Ceulemans et al., 2017; Cole et al., 2017). For example, *Succisa pratensis* is the host plant for the threatened marsh fritillary (*Euphydryas aurinia*) butterfly eggs and larvae (Johansson et al., 2019). Adapted to low nutrient conditions, *Succisa pratensis*, faces threats from land use change, eutrophication and acidification (from the addition of fertilizers and atmospheric deposition of N and sulphur), and is therefore the subject of conservation efforts aiming to restore or maintain suitable habitat conditions for the plant (Vergeer et al., 2003a; van der Meer et al., 2014; Brunbjerg et al., 2017; Johansson et al., 2019). Although defined as a stress tolerator by Grime et al. (1988), elevated O₃ has been found to induce premature senescence in this species (Franzaring et al., 2000). *Succisa pratensis* vitality can also be damaged by eutrophication (from N), leading to problems for long term population viability (Pauli et al., 2002; Vergeer et al., 2003b), but the combined effects of increased O₃ and N are not known.

Leontodon hispidus has previously been found to be moderately sensitive to O₃ in terms of plant biomass at O₃ concentrations of over 40 ppb (Hayes et al., 2007, 2011; Mills et al., 2009; Wilkinson and Davies 2009). In previous pot-based experiments *Leontodon hispidus* has displayed delayed senescence, reductions in root biomass (Hayes et al., 2011), and disruptions to stomatal control (Mills et al., 2009; Wilkinson and Davies 2009).

In this study we test growth and physiological responses (leaf cover, leaf litter, flowering, chlorophyll index, photosynthesis and stomatal conductance) of *Leontodon hispidus* and *Succisa pratensis* to ambient and elevated O₃ levels, with and without the addition of N.

2. Materials and methods

2.1. Plant growth method

Leontodon hispidus and *Succisa pratensis* plants were grown from plug plants (British Wildflower Plants, Norfolk, UK) and planted into the ground in April 2016 (soil classification: Orthic Podzol, pH 5, Wang et al., 2019) at Abergwyngregyn, North Wales, UK (Latitude 53.2389, Longitude -4.0185). The plants were arranged in 1 m separate blocks, alternating in a latin square design (see Supplementary Information (SI), Figure SI 1) within nine Free Air Ozone Enrichment (FAOE) rings.

Irrigation occurred naturally from precipitation, and no supplementary watering was required apart from that associated with the N addition.

2.2. Ozone and nitrogen treatments

During the first season (May to September 2016) the plants were subjected to three O₃ treatments (Low, Medium and High) targeted to replicate the range of concentrations found in temperate zones, each with and without the addition of N (applied weekly in 5 L of water, to the equivalent of 40 kg N ha⁻¹ yr⁻¹). The level of N addition was chosen to be at the top end of the range of European N deposition (Stevens et al., 2010). Five litres of water were also applied to the zero N addition plots at the same time. Each O₃ treatment was replicated using three FAOE rings of 4 m diameter with 10 m spacing between the ring centres (Fig. 1).

The control treatment was randomly assigned within each row, and was upwind (of the prevailing wind direction) to the highest O₃ treatments. O₃ was supplied at a height of 30 cm with delivery achieved using an O₃ generator (G11, Pacific Ozone Inc. California, USA) supplied with oxygen concentrated from ambient air (Integra 10, SeQual Technologies Co. Ltd, Taiwan) and distributed to the rings via computer-controlled (LabView version 2012) solenoid valves (using pulse width modulation). Fans (200 mm, Xpelair, Southampton, UK) drove the O₃ through the delivery pipe (65 mm diameter, with 3 mm holes every 20 cm) at a rate of 0.17 m³ s⁻¹. Wind speed (monitored using a WindSonic sensor, Gill Instruments Ltd, Lymington, UK) was used to instantaneously adjust solenoid operation, reducing O₃ release at wind speeds of less than 16 m s⁻¹ and stopping delivery at speeds below 2 m s⁻¹. The O₃ concentration within each ring (at a height of 30 cm) was sampled for ~3.5 min in every half-hour using an O₃ analyser (Thermo 49i, Thermo Fisher Scientific Inc. Massachusetts, USA). Air temperature (Skye Instruments Ltd, Llandrindod Wells, UK) on site was recorded with hourly averages calculated from 5 min interval raw data. Following the main plant trial from May 2016 to February 2017 the plants remained undisturbed and in-situ until the end of the 2018 season. No further N treatments were made although seasonal O₃ treatments continued (May to September 2016 and 2017; June to September 2018).

2.3. Plant measurements

2.3.1. Leaf cover and leaf litter

Leaf cover of both plant species was determined in October 2016 using grids (containing 100 squares) overlain onto photographs of the plant blocks, with total leaf area and the proportion of damaged leaves assessed. The occurrence of frost damage was surveyed in each plot in January 2017 using the same method. Leaf litter was collected monthly from July 2016 to February 2017 (excluding December 2016) and dry weights (after oven drying at 60 °C) were recorded.

2.3.2. Chlorophyll, photosynthesis and stomatal conductance

The following measurements were made on healthy leaves. The chlorophyll index (C.I.) was recorded weekly on both *Succisa pratensis* and *Leontodon hispidus* between 23 June and November 24, 2016 using a CCM200 m (Opti-Sciences, Hudson,

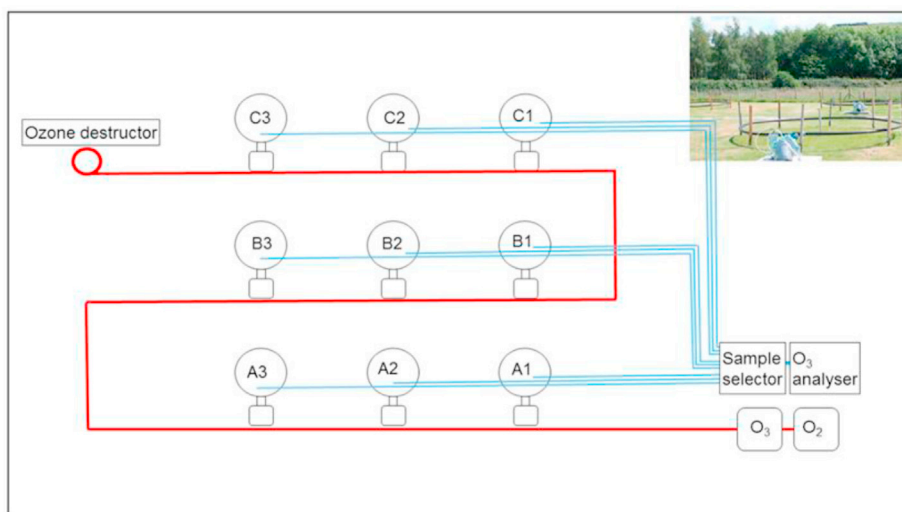


Fig. 1. Layout of the Free Air Ozone Enrichment (FAOE) rings. The rings were supplied with ozone (O₃) from an O₃ generator utilizing oxygen (O₂) concentrated from ambient air. O₃ concentrations within the rings were monitored with an O₃ analyser. O₃ treatments were assigned to the rings as follows: A1, B2, C3 Low; A3, B1, C2 Medium; A2, B3, C1 High. Inset: photograph of an O₃ delivery ring.

USA). In addition, light-saturated photosynthesis (Asat) and stomatal conductance (gs) measurements were also recorded on *Succisa pratensis* and were made every two weeks (between 13 July and October 5, 2016) using a LI-COR 6400XT (Nebraska, USA) portable gas analyser. Leaves completely filled the chamber, and mean chamber conditions were: air temperature 20 °C; incoming air CO₂ (carbon dioxide) concentration 400 μmol mol⁻¹; light 1500 μmol m⁻² s⁻¹ (6400 LED source). Outgoing air relative humidity was within the range 60–80% and leaf vapour pressure deficit between 0.8 and 1.2 kPa.

2.3.3. Flowering

A count of the number of *Leontodon hispidus* flowering stems was made in August 2016, and *Succisa pratensis* in September 2018 (*Succisa pratensis* did not flower in 2016).

2.4. Statistical analysis

All ± margins reported show the standard error of the mean. Tests for statistical significance were carried out with R (version 3.6.3 R Core Team, 2020). Generalized linear models ('glmer', package 'lme4', Bates et al., 2015) with poisson distribution were used for count data (i.e. cover determined from number of squares, and number of flower stems). Linear mixed models (package 'nlme', Pinheiro et al., 2017) were used for litter, C.I., Asat and gs data. Beta regression (package 'reghelper', Hughes 2020) was used for proportional data (percentage leaf damage). Leaf cover, leaf litter and chlorophyll data were tested against the fixed factors of date, O₃ treatment, N treatment and species (as well as their interactions) using the random effect of FAOE ring and maximum likelihood (ML) estimation. Response variables relating to Asat, gs, and flowering (where data was recorded on *Succisa pratensis* alone or in separate years for each species) the fixed factors of date, O₃ treatment and N treatment (and their interactions) were used. Model residuals and plots were checked for the appropriateness of each model (including testing for overdispersion with 'glmer'), and data were transformed where necessary (cube root with litter data and square root with purple coloured leaf damage data). Where data contained repeated measures, date was included either as a factor (litter, Asat and gs) or as a continuous variable (days after start of measurements (DAS) for chlorophyll content) depending on model fit. The suitability of the inclusion of an autoregressive (AR) correlation term was determined by plots and model fit. The best model fit was assessed using comparisons of Akaike's Information Criteria (AIC). With the exception of beta regression, model results were summarised using ANOVA 'type 3' (package 'car', Fox and Weisberg 2011) which performs a Wald chi-square test (χ^2). Tukey HSD post-hoc tests (package 'multcomp', Hothorn et al., 2008) and contrasts of means (package 'emmeans', Lenth, 2020) were carried out where appropriate.

3. Results

3.1. Ozone treatments and climatic conditions

The seasonal mean O₃ levels over the exposure periods were ~24 ppb for the Low treatment, ~40 ppb for the Medium treatment and ~57 ppb for the High treatment (Table 1). During the first season daily (24 h) mean levels reached 45 ppb, 66 ppb and 100 ppb in the Low, Medium and High treatments, respectively (Fig. 2a). Due to technical issues no O₃ was delivered to the Medium and High treatments between 7 and 9 August and 23–27 September 2016.

Climate conditions during the trial were within those generally expected for the site location in the western part of the UK (Fig. 2b and c). The maximum air temperature on-site was 32 °C recorded on July 19, 2016 (afternoon) and the lowest was 6 °C on May 24, 2016 (early morning).

3.2. Plant response to treatments

3.2.1. Leaf cover and damage: significant effects of ozone, but not nitrogen

Mean leaf cover (determined from count of number of squares) ranged from 3 (±6, High O₃, added N) to 12 (±6, Low O₃, no added N) for *Leontodon hispidus*, and from 61 (±6, High O₃, no added N) to 74 (±4 Medium O₃, no added N) for *Succisa pratensis*. No significant effect of N was found, however there was a significant interaction between O₃ and species (χ^2 (2) = 14.60, p < 0.001). *Leontodon hispidus* showed a trend of reducing leaf cover with increasing O₃ (p < 0.001, between Low and High O₃), whereas *Succisa pratensis* cover was slightly increased in the Medium O₃ treatment and slightly reduced in the High O₃ treatment this was not significant (Fig. 3).

Table 1
Seasonal ozone levels (ppb) across each treatment (Low, Medium and High).

	Low	Medium	High
2016 (May–Sep)	22 (±0.5)	41 (±3.7)	59 (±7.1)
2017 (May–Sep)	24 (±0.8)	41 (±2.1)	51 (±5.0)
2018 (Jun–Sep)	25 (±2.6)	38 (±4.8)	60 (±5.1)

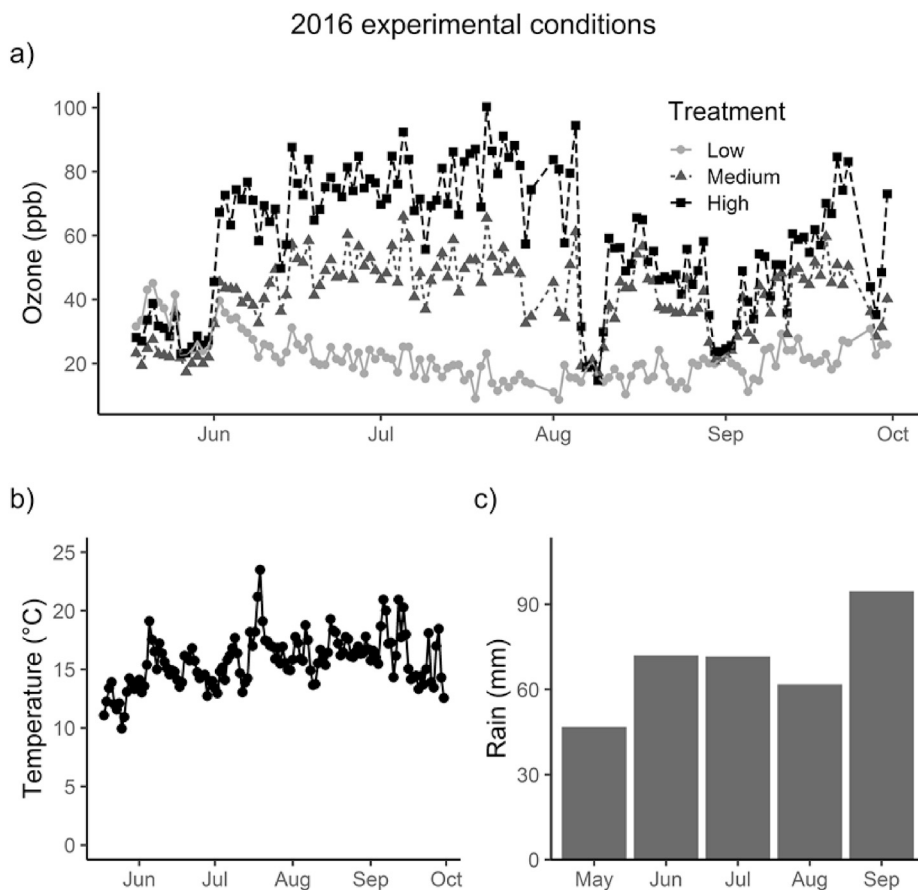


Fig. 2. **a)** Mean daily (24 h) ozone (ppb) for the three treatments, over the exposure period 18 May – September 30, 2016; **b)** On-site mean daily (24 h) air temperature (°C) for the experimental period 18 May – September 30, 2016; **c)** Monthly total rainfall (May–September 2016) recorded at the nearest Met Office recording station at Valley, Anglesey (Met Office, n.d.).

No leaf damage was noted for *Leontodon hispidus*. However, for *Succisa pratensis* the cover of damaged leaves (purple coloured, as a proportion of total cover) in the autumn increased with O_3 level ($\beta = -0.93$, $t(6) = -4.47$, $p = 0.004$, Fig. 4a), although there was no significant effect of N or its interaction with O_3 . Whilst greater O_3 concentrations increased the proportional cover of frost damaged leaves for *Succisa pratensis* ($\beta = -0.70$, $t(6) = -2.84$, $p = 0.03$, Fig. 4b), the addition of N did not significantly affect the level of damage (compared to those without added N).

3.2.2. Leaf litter: significant ozone and nitrogen effects

No significant effect of O_3 level on litter production was recorded for *Leontodon hispidus*. However, *Succisa pratensis* autumn litter mass was greater with increased O_3 , and at the highest O_3 treatment level the timing of the peak litter production was also affected ($\chi^2(12) = 37.63$, $p < 0.001$, for month $\times O_3$ * species, Fig. 5). The increase in litter was only related significantly to plant cover at the Low O_3 level (Figure SI 2).

Higher litter production occurred earlier with *Leontodon hispidus* than with *Succisa pratensis*. Peak litter production was in August for *Leontodon hispidus* where the mean weight of litter was 7.0 g (± 1.0), 7.1 g (± 1.5) and 7.2 g (± 1.9) for the High, Medium and Low O_3 treatments (respectively). The peak mean weight of litter for *Succisa pratensis* was 8.4 g (± 1.1) in November for the High O_3 treatment and 9.8 g (± 1.2) and 5.5 g (± 1.7) in January for the Medium and Low O_3 treatments (respectively).

Overall litter production was increased with added N ($\chi^2(1) = 4.36$, $p = 0.037$), but there were no interactions with the other model terms. The total weight of litter over the measurement period was increased by 6% by the addition of N for *Leontodon hispidus* and by 31% for *Succisa pratensis*.

3.2.3. Chlorophyll index (C.I.): significant ozone and nitrogen interactions

The chlorophyll content varied with species and date. *Leontodon hispidus* C.I. remained between 30 and 40 for the measurement period, however C.I. for *Succisa pratensis* steadily increased from 30 to 50 from July to November (Fig. 6). C.I. for both species declined after a November peak. The mean maximum index values in November with and without the addition of N

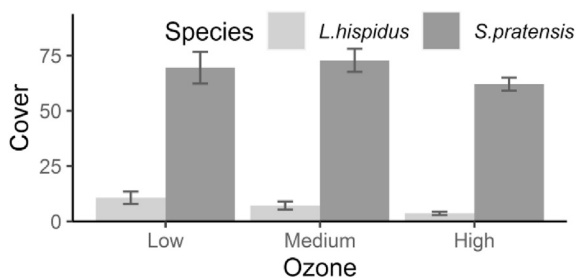


Fig. 3. Leaf cover for both species derived from a count of the number of squares covered by leaves for each ozone treatment (Low, Medium and High), with and without the addition of nitrogen. Bars show the standard error. A significant interaction was found between O_3 and species ($p < 0.001$).

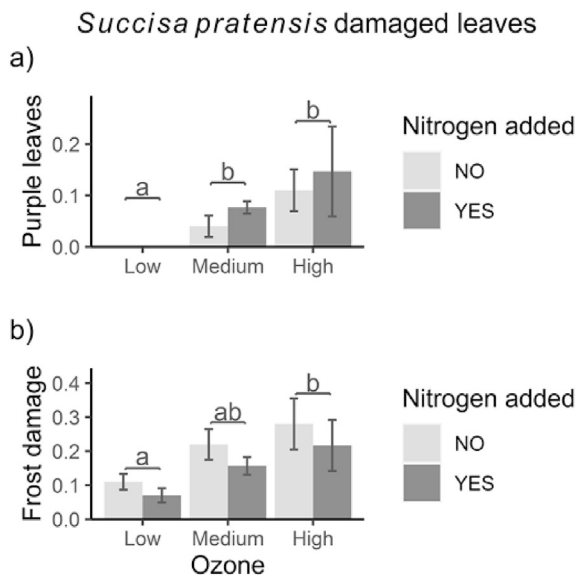


Fig. 4. O_3 and N impacts on **a)** Proportional cover of damaged leaves (derived from the total count of number of squares covered by leaves) as indicated by their purple colouring (recorded in October 2016). The significance level is $p < 0.001$ obtained using square root transformed data; **b)** Cover of frost damaged leaves as a proportion of the total leaf cover, derived from the count of number of squares covered by the frost affected leaves (recorded in January 2017). The significance level is $p < 0.05$. Treatments shown are ozone concentrations (Low, Medium, and High), with and without the addition of nitrogen. Different letters denote significance between the three ozone treatments and bars are standard error.

(respectively) were: $37 (\pm 0.9)$ and $36 (\pm 0.9)$ in the Low treatment, $36 (\pm 1.0)$ and $37 (\pm 1.3)$ in the Medium treatment, and $39 (\pm 0.9)$ and $36 (\pm 1.0)$ in the High treatment for *Leontodon hispidus*; and $48 (\pm 1.1)$ and $46 (\pm 1.0)$ in the Low treatment, $51 (\pm 0.9)$ and $47 (\pm 0.9)$ in the Medium treatment, and $49 (\pm 1.2)$ and $48 (\pm 0.8)$ in the High treatment for *Succisa pratensis*.

A significant interaction was found between date, species, O_3 , and N ($\chi^2 (2) = 9.38$, $p < 0.01$).

Leontodon hispidus C.I. was reduced in the Medium and High O_3 treatments compared to the Low treatment during October to November, with the addition of N resulting in an increase in C.I. in the highest O_3 treatment compared to the Medium and Low levels (Fig. 6a). *Succisa pratensis* C.I. was increased with increasing O_3 concentrations during August and September, and this was further increased by the addition of N (Fig. 6b).

3.2.4. Photosynthesis (Asat) and stomatal conductance (gs): significant ozone and nitrogen effects (Asat); significant ozone and nitrogen interaction (gs)

Light-saturated photosynthesis (Asat) and stomatal conductance (gs) were measured on *Succisa pratensis* only. Asat declined in the autumn compared to summer rates. The decline with time was enhanced at the High O_3 level ($\chi^2 (10) = 25.79$, $p = 0.004$) both with and without additional N (Fig. 7a). In the added N treatment Asat also reduced at a faster rate with time ($\chi^2 (5) = 12.77$, $p = 0.03$).

Gs followed the same trend as Asat by declining in the autumn compared to values during the summer. This decline was accelerated in the High O_3 treatment when there was no additional N. When N was added, gs was maintained across the different O_3 treatments (with a slight increase at High O_3 in October), although the seasonal decline was still present ($\chi^2 (10) = 21.38$, $p = 0.02$ (for date $\times O_3 \times N$), Fig. 7b).

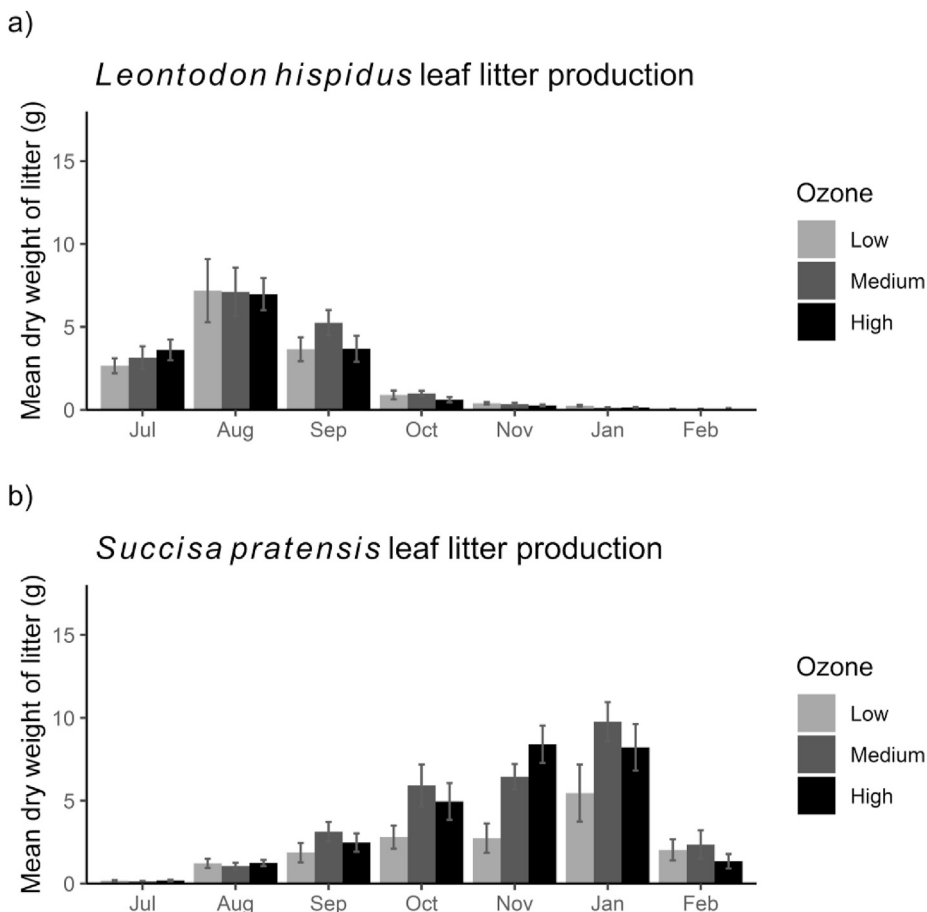


Fig. 5. Mean weight of litter (bars show the standard error) for each ozone treatment (Low, Medium and High) for species **a)** *Leontodon hispidus* and **b)** *Succisa pratensis*. A significant interaction was found for month \times O₃*species ($p < 0.001$).

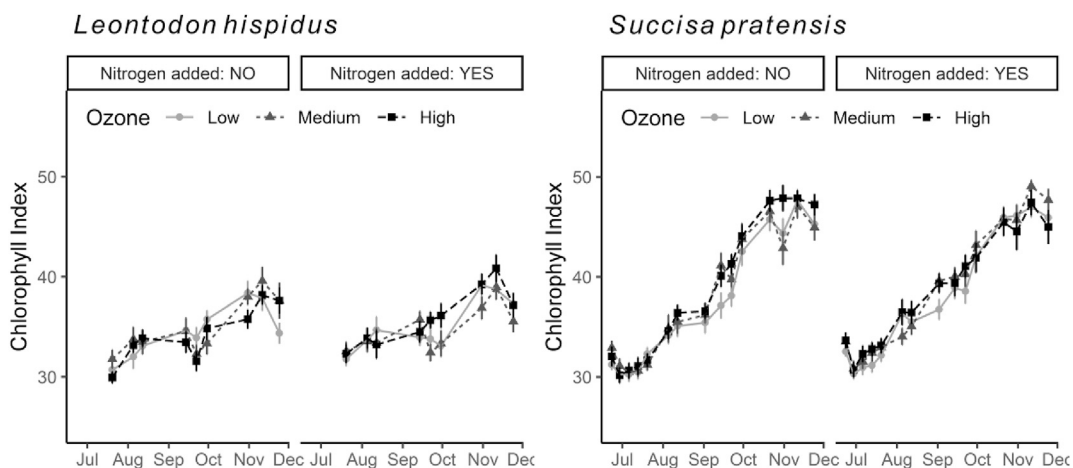


Fig. 6. Mean chlorophyll index for ozone concentrations (Low, Medium, and High), with and without the addition of nitrogen (bars are standard error) for species **a)** *Leontodon hispidus* and **b)** *Succisa pratensis*. A significant interaction was found between date, species, O₃, and N ($p < 0.01$).

3.2.5. Flowering: significant effect of ozone, but not nitrogen

The number of *Leontodon hispidus* flowering stems (recorded in 2016) was similar across the treatments with no effect of O₃ or N treatments (Fig. 8a). *Succisa pratensis* did not flower in 2016 and the number of flowering stems two years later were

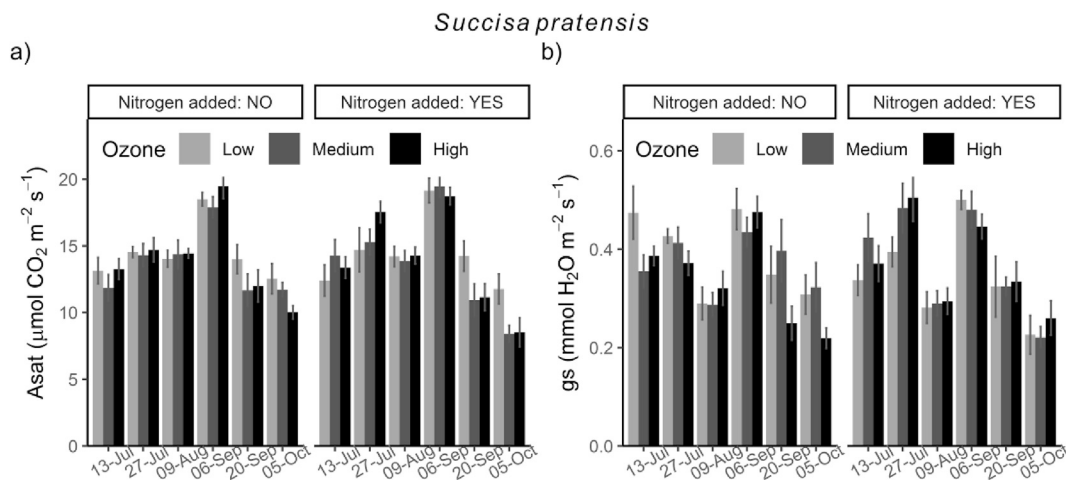


Fig. 7. Treatments shown are ozone concentrations (Low, Medium, and High), with and without the addition of nitrogen. Bars are standard error. **a)** *Succisa pratensis* mean light-saturated photosynthesis (Asat) for each treatment. The interactions between date and O_3 , and between date and N are significant at $p < 0.05$; **b)** *Succisa pratensis* mean stomatal conductance (gs) for each treatment. The interaction between date, O_3 and N is significant at $p < 0.05$.

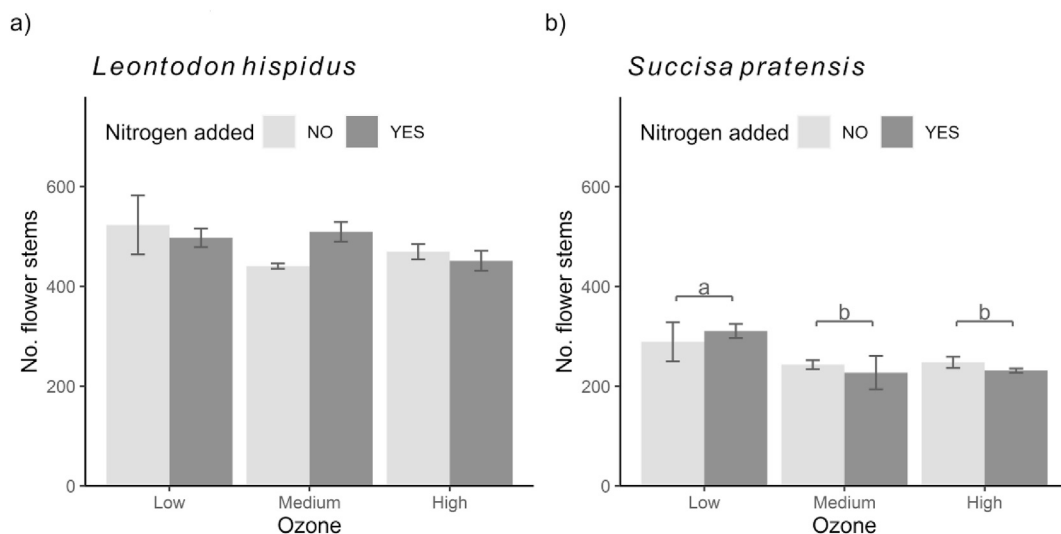


Fig. 8. Mean number of flowering stems for ozone concentrations (Low, Medium, and High), with and without the addition of nitrogen (Bars are standard error) for species **a)** *Leontodon hispidus* (2016) and **b)** *Succisa pratensis* (2018). Different letters denote significance between the three O_3 treatments at $p < 0.01$.

reduced in the elevated O_3 treatments ($\chi^2(2) = 13.41$, $p = 0.001$, Fig. 8b). There was no significant effect of the different N levels, or the interaction between O_3 and N treatments.

4. Discussion

This study has shown that the important grassland species are negatively affected by elevated O_3 even at a moderate level (seasonal mean 40 ppb), with few impacts exacerbated by elevated N.

Small decreases in leaf cover and chlorophyll index were observed with increased ozone for *Leontodon hispidus*, with the addition of N resulting in an increase in chlorophyll index only at the highest O_3 level and resulting in an overall increase in litter production. However, no effect of O_3 or N on flowering were found during the first season. Other studies into the effects of elevated O_3 with *Leontodon hispidus* have reported delayed senescence, root biomass reductions (Hayes et al., 2011), and alterations in stomatal control (Mills et al., 2009; Wilkinson and Davies 2009). It is, therefore, possible that the species was affected in aspects not measured (e.g. photosynthesis or below ground biomass).

A stronger effect of both O_3 and N treatments was seen with *Succisa pratensis*. After only three years of exposure to O_3 pollution, at concentrations similar to recent levels recorded across some European temperate grassland areas (European

Environment Agency (EEA, 2017), flowering of *Succisa pratensis* was reduced suggesting O₃ is already causing a reduction in pollinator resources (although we acknowledge that there may have been other interacting factors not taken into account, e.g. climate or pest species). Even though species like *Succisa pratensis*, with low specific leaf areas and relative growth rates, have been identified as more stress tolerant (Grime et al., 1988), our findings agree with the results of an earlier study into the effects of O₃ on the plant. In a pot based, open top chamber experiment Franzaring et al. (2000) observed that elevated O₃ (seasonal maximum of 77 ppb) did not influence growth (biomass) but that premature senescence was recorded with leaf litter significantly affected. In this current trial, whilst leaf cover was not impacted to a great extent, there was a strong effect of O₃ on leaf litter production and leaf damage caused by frost after only one season's exposure.

The increasing chlorophyll content with elevated O₃ implies that more resources were allocated to the newest leaves as the older ones died back prematurely and fell as litter (measurements were made on replacement healthy leaves). During the summer months resources were prioritised to new leaves so that stomatal conductance and photosynthesis could be maintained, but during the autumn this was not sustained and stomatal conductance declined with increasing O₃ concentrations. However, the decline in stomatal conductance observed at high O₃ was mitigated by adding N. Slow or impaired stomatal response due to elevated O₃ has been found at times for other plants including grassland species (Mills et al., 2009, 2013). The rate of photosynthesis is normally higher during the summer/flowering months and this was true for all of the treatments, but it should be noted that this also corresponds with the period when ambient O₃ levels are typically higher. *Succisa pratensis* is therefore susceptible to uptake of O₃ through stomatal fluxes during high ozone episodes. Although senescence occurred in October for all treatments, we noted a steeper decline in the rate of photosynthesis in the higher O₃ treatments during September and October as winter die-back was accelerated. This occurred both with and without additional N.

These stress responses are likely to cause a decline, or change in allocation of, assimilated photosynthates with detrimental effects for plant health in subsequent years (Cooley and Manning 1987; Mills et al., 2013). This could in turn have implications for species that depend on the plant's health, density, and food quality (i.e. nectar, pollen and green leaves) (Tjørnløv et al., 2015; Brunbjerg et al., 2017; Ceulemans et al., 2017; Ghidotti et al., 2018). Detrimental impacts on plant health and ability to cope with environmental stresses were evident in the increased leaf damage and reduced flowering for plants that were exposed to elevated O₃. This has further consequences for the long-term viability of populations where reducing numbers of *Succisa pratensis* can reduce genetic diversity (Vergeer et al., 2003a). Furthermore, elevated O₃ increased the weight of litter in the Medium and High treatments (regardless of the amount of plant cover) from mid-August onwards. A lower number of green leaves during late summer will impact food resources of the larvae of the marsh fritillary (*Euphydryas aurinia*) butterfly that, almost exclusively, use the plant as host for food and form silken protective and hibernation webs amongst the leaf litter (Meister et al., 2015; Tjørnløv et al., 2015; Johansson et al., 2019). The larvae have limited mobility and whilst they are able to move to neighbouring plants this exposes them to predation and adverse weather conditions. Anthes et al. (2003) noted that the few larval groups (of *Euphydryas aurinia*) observed to move to another plant were those that had completely defoliated their host plant.

In contrast to *Leontodon hispidus*, the weight of *Succisa pratensis* litter remained higher in the elevated O₃ treatments during autumn and into winter, even though O₃ treatments stopped at the end of September. This prolonged reduction in plant health and early senescence may also influence the plants ability to compete with other grassland species, thereby reducing habitat quality and biodiversity (Vergeer et al., 2003a). This change in microhabitat structure may result in less suitable locations for egg placement by *Euphydryas aurinia* females who prefer sites with less dense vegetation that allows light to penetrate to lower layers (Pielech et al., 2017). The balance of leaf cover between the two species tested was not influenced by elevated O₃ or added N suggesting that (at the levels added) these treatments did not affect direct competition between them.

Increased litter also has the potential to impact on seed germination and establishment, as well as the microhabitats. Hovstad and Ohlson (2008) found that the addition of 900 g litter m⁻² produced a negative response in *Succisa pratensis* seedling emergence, and the amount of litter from competitor species also negatively affected *Succisa pratensis* in another grassland study (Hurst and John 1999). Extra carbon (C) and N released from greater amounts of litter may favour competition from other plant species and, in the potential change to soil C and N ratios, temperature, and moisture status, alter microbial activity (Steinbeiss et al., 2008; Kuzyakov 2010; Lange et al., 2015).

To our knowledge this is the first published study including the interaction of O₃ and N for a forb grassland species adapted to low nutrient conditions. Eutrophication has previously been shown to damage viable populations of *Succisa pratensis* by either reducing biomass (Pauli et al., 2002), or conversely by increasing biomass and seed production but reducing reproductive fitness and germination success (Vergeer et al., 2003b). In this study, the addition of N had no visible effect on either *Leontodon hispidus* or *Succisa pratensis* plant cover or flowering (although *Succisa pratensis* flowering was recorded two years after the N treatment was applied when it is less likely to have had an impact). However, N addition accelerated the developmental cycle of *Succisa pratensis* and produced a higher litter quantity for both species. The N treatment applied (40 kg N ha⁻¹ yr⁻¹) was twice the amount suggested as the point at which N deposition has been shown to reduce growth (20 kg N ha⁻¹ yr⁻¹, Payne et al., 2013), but was within the European range of between 2 and 44 kg N ha⁻¹ yr⁻¹ (Stevens et al., 2010).

Climate change-induced earlier vegetation growth in spring is exacerbated by N deposition, and can cause a cooling of the microclimate in the immediate zone of spring developing larvae with negative implications for butterfly populations (Wallis

de Vries and Van Swaay, 2006). It is not known how the increase of litter into the immediate area of the hibernating larval web would interact with this cooling issue, but alterations in humidity and temperature are likely.

Whilst some negative impacts have been documented as part of this study there may be other detrimental effects that will have implications for the grassland ecosystem. For example, in a study of grass and buttercup species the interaction of O₃ and N caused negative effects on root biomass (Wyness et al., 2011), a possible outcome not measured as part of this trial. O₃ pollution has also been shown to disrupt plant to plant, plant to herbivore, and plant to pollinator signalling for a number of species through reducing the lifetime of biogenic Volatile Organic Compounds (BVOCs) or by causing the emission of stress-induced BVOCs (Llusià et al., 2002; Pinto et al., 2010; Owen and Peñuelas, 2013; Farré-Armengol et al., 2013). It is known that caterpillars feeding on *Succisa pratensis* induce emissions of herbivory BVOCs that influence the behaviour of nearby plants as well as herbivores and their predators (Peñuelas et al., 2005). Indications are that herbivory BVOCs are degraded by O₃ pollution (Pinto et al., 2007; Fuentes et al., 2013), but it is not known if O₃ affects *Leontodon hispidus* or *Succisa pratensis* signalling in this way or whether other floral signalling mechanisms are affected. O₃ air pollution continues to be a problem for natural vegetation and crops (Yan et al., 2019; Ronan et al., 2020) and this study showing the impact on a high value plant for pollinators further highlights the need for concerted action at the global scale to reduce pre-cursor O₃ emissions alongside habitat management efforts.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gecco.2020.e01345>.

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