

# **QUANTIFYING THE IMPACTS OF OZONE POLLUTION ON THE SUSTAINABILITY OF PASTURE**

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## ABSTRACT

### QUANTIFYING THE IMPACTS OF OZONE POLLUTION ON THE SUSTAINABILITY OF PASTURE

Tropospheric ozone (O<sub>3</sub>) is a potent threat to food security. In recent years, increasing evidence suggests that ozone can have large effects on the growth and functioning of grassland species, although the responses of grassland to ozone are complex and difficult to predict. Given the global ecological and economic importance of grasslands, there is a continuing need to research the effects of ozone on grassland and pasture.

In this PhD project, the impacts of ozone on temperate managed pasture were investigated, primarily through a series of ozone-exposure experiments. Elevated ozone (seasonal mean concentration 30-67ppb) was found to have large impacts on pasture vegetation, including injury and premature senescence, reduced biomass production, declining production and yield, and impacts on forage quality. Ozone impacts on nodulation and nitrogen (N)-fixation in legumes have not extensively been investigated, but could have important consequences for pasture sustainability, with predicted reductions in the root nodule biomass of clovers (*Trifolium* spp.) of 12% or more in UK pasture. Ozone impacts on below ground biomass displayed a strong interaction with cutting, and intensive grazing could disproportionately increase the sensitivity of N-fixation in pasture to the effects of ozone. Flux-based ozone dose-response relationships are constructed for biomass, total injury rates, N-fixation and forage quality in high-sugar ryegrass (HSG) pasture mesocosms, which could assist in the development of new critical levels for the protection of pasture vegetation.

**DECLARATION**

I hereby declare that this work has been originally produced by me for the present Thesis and it has not previously been submitted for the award of a higher degree or professional qualification.

Daniel Kevin Leece Hewitt

April 2015

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# **1. INTRODUCTION – QUANTIFYING THE IMPACTS OF OZONE POLLUTION ON THE SUSTAINABILITY OF PASTURE**

## **1.1. Introduction**

Tropospheric ozone (O<sub>3</sub>) is considered the most important air pollutant in terms of its impact on the biosphere (Ashmore, 2005), and is a threat to food security (Mills et al. 2011a). Increasing evidence suggests grassland biomes and species can respond strongly to ozone. Although more attention has been paid to the response of grassland species to ozone in recent years, the impacts of ozone on intact grassland and pasture are complex with little information available (Fuhrer, 2009). Given the global ecological and economic importance of grasslands, there is a continuing need for research into the impacts of ozone on grassland species and biomes. In this PhD project, the impacts of ozone on the sustainability of temperate managed pasture were investigated.

## **1.2. Introduction to tropospheric ozone**

Ozone is a naturally-occurring chemical present in the Earth's atmosphere, present in both the stratosphere and troposphere. Ozone in the troposphere acts as a greenhouse gas (GHG), and is an air pollutant formed via the complex photochemical oxidation of nitrogen oxides (NO<sub>x</sub>), carbon monoxide (CO), methane (CH<sub>4</sub>) and other volatile organic compound (VOCs) emitted from natural and anthropogenic sources including wetlands, lightning, and industrial and vehicle emissions (Royal Society, 2008). Concentrations of tropospheric ozone are equilibrated by a number of complex non-linear photochemical reactions that also mediate ozone destruction via titration, with ozone concentration driven by inequalities in the NO<sub>x</sub>/VOC mixing ratio, and with

highest rates of ozone formation occurring in periods of high light intensity, warm temperatures and stable pressure (Sillman & He, 2002; Royal Society, 2008) (Figure 1.1). As well as chemical destruction, and via interactions between the troposphere and stratosphere (e.g. Beekman et al. 1997), ozone is removed from the atmosphere by dry deposition, where it reacts with land or ocean surfaces and vegetation (Royal Society, 2008).

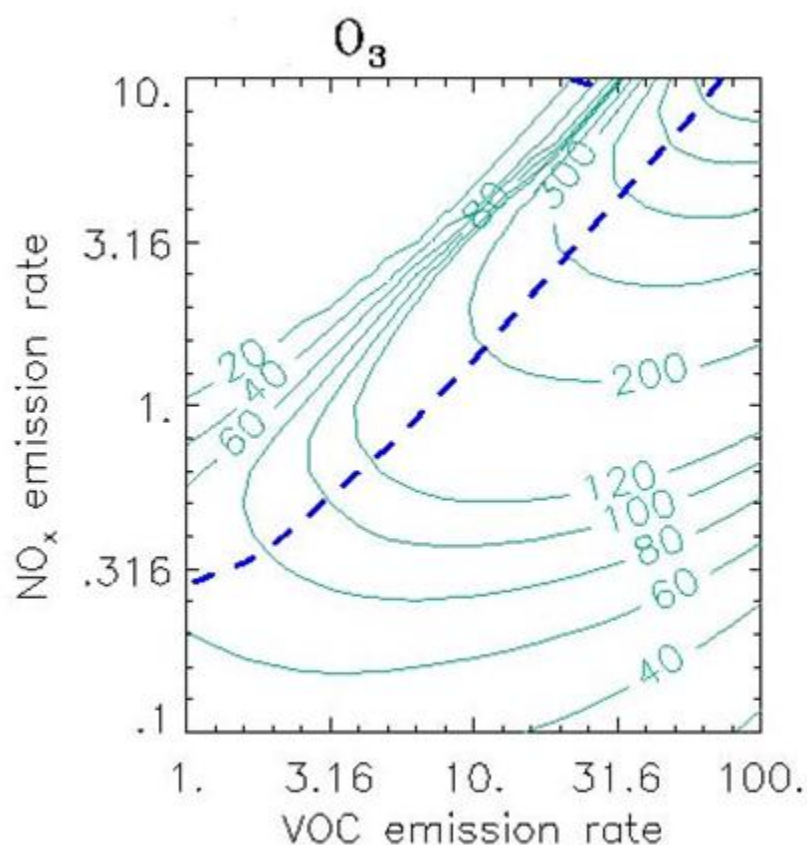


Figure 1.1: Zero-dimensional simulation of ozone formation isopleths (ppb) as a function of average diurnal NO<sub>x</sub> and VOC emission rate ( $10^{12}$  molecules  $\text{cm}^{-2}\text{s}^{-1}$ ). The blue dashed line represents the transition from VOC-sensitive to NO<sub>x</sub>-sensitive conditions (after Sillman & He, 2002).

Historical records of surface ozone are fragmented and poorly represented. However, it is clear that the pre-industrial levels of ozone were generally less than 20ppb (e.g. Bojkov, 1986; Sandroni & Anfossi, 1994). Observations indicate the mean background concentration of tropospheric ozone increased globally during the 20<sup>th</sup>



century, due to the increased anthropogenic emission of precursor compounds (Cooper et al. 2014). Maximum concentrations across the polluted Northern hemisphere now range ~55-80ppb (Figure 1.2), with a decadal increase of 0.5-2.0% in the last few decades and a global summertime mean of ~50ppb (Vingarzan, 2004; Royal Society, 2008; IPCC, 2013).

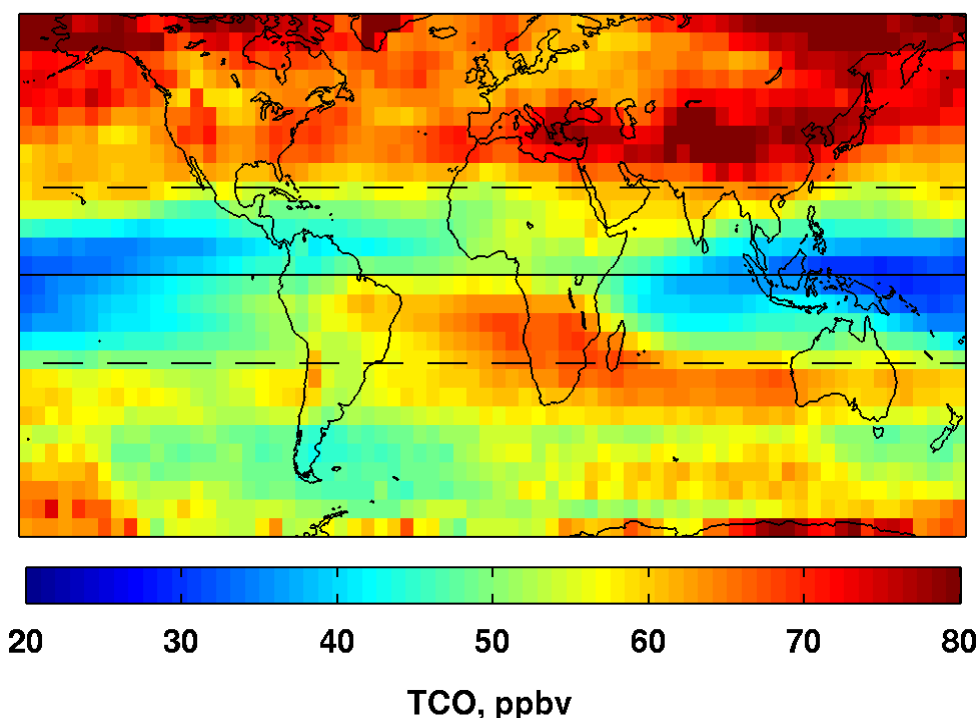


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Based on summarized trends, long-term (c.2100) annual mean surface concentrations of ozone are predicted to decrease globally, due to the continuing control of precursors, and increases in mean surface temperature and atmospheric water vapour content (IPCC, 2013). However, under most representative concentration pathways (RCPs), ozone concentrations will change little in the coming decades, with sustained increases in East Asia and polluted regions (IPCC, 2013), and with the tropospheric burden of ozone chiefly determined by the abundance of  $\text{CH}_4$  in the atmosphere (IPCC, 2013). Warming may increase the frequency and severity of acute short-term

ozone episodes (e.g. Solberg et al. 2005), and climate change may otherwise influence the regional potential for ozone formation due to changes in hemispheric transport of ozone precursors, and increases in VOC production (IPCC, 2013).

### **1.3. European grasslands and pasture**

Grasslands can be defined as any biome with a majority vegetative cover of true-grasses (*Poaceae*), and range from savannah, tropical, alpine and montane to temperate grasslands, covering approximately one fifth of global land surface. In Europe, the majority of grassland is anthropogenic in origin, and can be said to be part of the semi-natural landscape; maintained by grazing and without need for reseeded. The earliest anthropogenic grasslands arose during the Neolithic and Bronze Age (~6500-3000 years before present (BP)) on cleared calcareous soils, heathland and from natural riverine communities (Prins, 1998). The majority of hay meadows have their origin throughout the middle-ages and early modern era (~1500-200 BP), as a consequence of clearance on poorly-drained forest soils (Prins, 1998). Traditional low-intensity farming methods, including use of fallow land and draught animals, were ubiquitous in North-West Europe until the industrial era. However land-use change and agricultural improvement, in response to the demands of wartime production and a rising population, significantly decreased the land area of unimproved grassland in North-West Europe during the 20<sup>th</sup> century. In England and Wales, the area of unimproved grassland declined by 97% between 1930-1984, primarily, as a consequence of conversion to arable farmland and productive pasture (Fuller, 1987). A total area of  $1.2 \times 10^5$  km<sup>2</sup> of land area in the United Kingdom (UK) is now utilized as some form of pasture land, with rough grazing and temporary grasslands each accounting for  $1.1 \times 10^4$  km<sup>2</sup>, and with improved permanent grasslands occupying a further  $9.7 \times 10^4$  km<sup>2</sup> (DEFRA, 2013). Open pasture occupies ~35% of the

agriculturally utilized area in Europe, with the most productive grasslands occurring in Atlantic regions; including Great Britain, Ireland, France, Northern Germany and Southern Norway (Smit et al. 2008). Pastures in Western Europe may meet up to 75% of the dietary needs of grazing livestock (Mayne et al. 2000), and produce at least 75Mt whole cow milk, 3.5Mt cattle meat and 179kt sheep meat per annum, to a total value of €28 billion (FAO, 2015). In year 2013, UK pastures supported  $3.3 \times 10^6$  head of dairy and beef cattle and  $3.3 \times 10^7$  head of sheep (DEFRA, 2013), worth approximately £4 billion (DEFRA, 2013).

The transformation of unimproved grassland into productive pasture has been achieved by means of soil cultivation and irrigation, reseeding, use of inorganic fertilizer and herbicides, repeated silage cutting, and intensive grazing. The most productive pasture systems in Europe are typically comprised by high abundances of adaptable, palatable and high-yielding 'preferred' grasses such as ryegrass (*Lolium* spp.), meadow-grasses (*Poa* spp.) and fescues (*Festuca* spp.), as well as containing other common grass species such as cocksfoot (*Dactylis glomerata* L.) and timothy (*Phleum pratense* L.). Improved pastures may also contain smaller amounts of herbaceous forbs and nitrogen (N)-fixing legumes (*Fabaceae*).

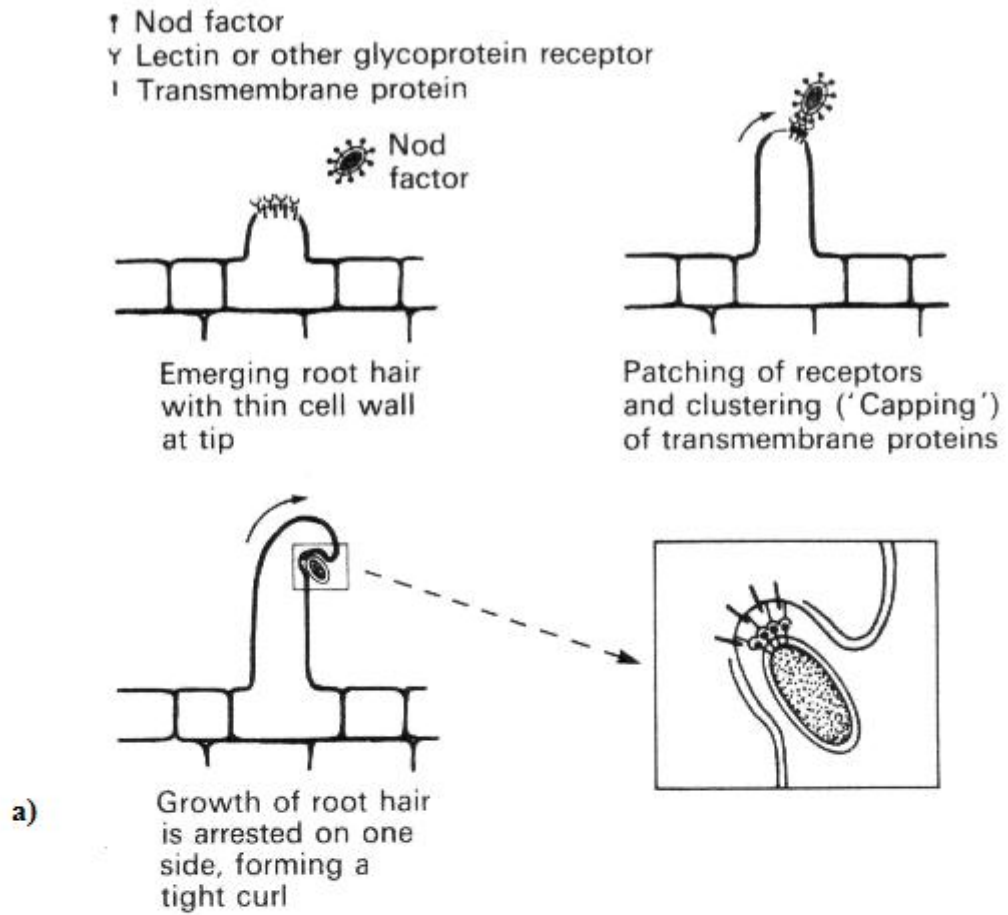
N-fixation in nodulating legumes, via symbioses with soil-dwelling N-fixing rhizobacteria, is a novel trait that is thought to have evolved during the early history of the legume family (Sprent, 2007). In exchange for carbohydrates, such as malate, legumes are able to obtain a source of fixed atmospheric N from rhizobia partners. The successful infection of root cortex cells by rhizobia and the formation of nodules (Figure 1.3a) is a complex process initiated by the secretion of flavonoid compounds from the roots (Hirsch, 1992), the recognition of bacterial nodulation (Nod) factors (Geurts & Bisseling, 2002) and the encapsulation of bacterial cells (Hirsch, 1992).

Although many of the mechanisms that regulate nodulation (Figure 1.3b) have yet to be elucidated (*sensu* Ryu et al. 2012; Aranjuelo et al. 2015), N-fixation is an energy-intensive process (Rees et al. 2005), and root nodule numbers are tightly controlled by plants through both local and long-distance signaling (Ferguson et al. 2010; Mortier et al. 2012; Ryu et al. 2012). Consequently, N-fixation rates or nodulation may be suppressed by excess soil N (e.g. Streeter and Wong, 1988), or disruptions in the supply of carbon assimilates, including by ozone stress (e.g. Pausch et al 1996b) or cutting (Avice et al. 1996).

Clovers (*Trifolium* spp.) are the most important N-fixer in intensive pasture systems, increasing the mineral and protein content, palatability, yield, and voluntary intake of pasture forage by grazing herbivores, and improving the available N content of soils via the rhizodeposition of root exudates and the build-up of weathered nutrients (e.g. Høgh-Jensen & Schjoerring, 1997; 2001). Clovers may also provide ancillary benefits to soil structure, improving nutrient retention and utilization (e.g. Høgh-Jensen & Schjoerring, 1997). Consequently, presence of clovers may also reduce inorganic fertilizer inputs and reduce nitrous oxide (N<sub>2</sub>O) emission from soils, making the use and maintenance of clover an important aspect for the economic sustainability and N-efficiency of organically-managed pasture systems (e.g. Parsons & Chapman, 2000; Ledgard et al. 2009). White clover (*T. repens* L.) represents the mainstay clover species for continuously grazed pasture across temperate Europe, with a stoloniferous habit increasing colonization and persistence as well as maintaining a high palatability by the continuous growth of new leaves (Parsons & Chapman, 2000). *T. repens*, on average, may input 100-200kg N ha<sup>-1</sup> y<sup>-1</sup> when included in low N- fertilizer sward mixtures (Parsons & Chapman, 2000). A 10% increase in the abundance of *T. repens* can increase the crude protein content of the

sward by 1% (Better Returns Programme, 2015), with a grass: clover ratio of 70:30 representing an optimum for forage yield, quality and productivity (Parsons & Chapman, 2000). Other clovers, such as red clover (*T. pratense*) and subterranean clover (*T. subterranean*) are also commonly used in pasture situations or as forage crops.

The widespread use, economic importance and sensitivity of *Trifolium* spp. also led to their use as ozone biomonitoring indicators throughout temperate regions (e.g. Mills et al. 2011b).



b)



Figure 1.3: (a) Nod-factor receptor model of initial root-hair infection by soil rhizobia (after Hirsch, 1991), (b) root nodules on the root system of white clover (*Trifolium repens* L.).

#### **1.4. Ozone as a stressor**

As a powerful oxidant, tropospheric ozone, in addition to being a hazard to human health (WHO, 2006), is a potent phytotoxin, inducing a state of oxidative stress in plants (Richards et al. 1959; Wilkinson et al. 2011). Upon uptake via the stomata, ozone diffuses through extracellular air spaces and dissolves in the apoplast to form reactive oxygen species (ROS), partially reduced oxygen intermediates (Laisk et al. 1989). Via ROS-signaling, ozone can induce the up or down-regulation of responsive genes, proteins and secondary metabolites, triggering functional and non-functional biochemical change throughout the cell, and causing an appropriation of normal cell processes and metabolism to repair and defense processes (Cho et al. 2011; Wilkinson et al. 2011). The downstream responses to ozone are also mediated by the local or long-distance action of phytohormones, including ethylene (ET), salicylic acid (SA), and abscisic acid (ABA) (Tamaoki, 2008). Thus, ozone appears to be an abiotic elicitor of plant defense responses, triggering a reaction similar to the hypersensitive response (HR) (e.g. Rao & Davies, 2001).

A major effect of ozone-induced oxidative stress is a reduction in net photosynthesis, accelerated cell death and leaf senescence (Figure 1.4), and through a loss of stomatal control (Cho et al. 2011; Wilkinson et al. 2011). Over time, these impacts lead to significant declines in the production of carbon (C) assimilates, and reduce vegetative and reproductive growth (Cho et al. 2011; Wilkinson et al. 2011). Amongst other effects, ozone can also cause alter the partitioning of assimilate, and other resources, in plant organs and structures, and lead to the blockage of phloem-sieve plates (e.g. Asensi-Fabado et al. 2010). Consequently, ozone can have large impacts on below-ground biomass (e.g. Cooley & Manning, 1987; Hewitt et al. 2014), including a reduced growth of storage organs (e.g. Vandermeiren et al. 2005), and

disruptions in mutualistic structures such as mycorrhiza (e.g. Andrew & Lilleskov, 2009) and root nodules (e.g. Letchworth & Blum, 1977; Hewitt et al. 2014). Reduced biomass production may impact the quality of soil, such as dissolved organic carbon content (DOC), (e.g. Jones et al. 2009), and could increase sensitivity of plants to water or nutrient stress (e.g. Grulke et al. 1998).

The sensitivity of plants to ozone can vary with a number of ecophysiological parameters, including - but not limited to - stomatal conductance ( $g_s$ ) (e.g. Pleijel et al. 2006; Danielsson et al. 2013), the antioxidant capacity and metabolism of leaves (Nali et al. 2005; Cho et al. 2011), canopy position (Hayes et al. 2009; 2010a) and water and nutrient availability (e.g. Bungener et al. 1999; Wagg et al. 2012; Wyness et al. 2013). Many staple agricultural crops are highly sensitive to ozone (Mills et al. 2011a). Global yield reductions due to ozone range 7-15% in wheat, 6-16% in soybean, 3-5% in maize and 3-4% in rice on an annual basis (Avnery et al. 2011a). Under the pessimistic RCP8.5 scenario (IPCC, 2013), the combined impact of ozone and climate change is estimated to reduce global crop production by 15% (Tai et al. 2014), with a concomitant 45% increase in undernourishment rates in developing countries by year 2050 (Tai et al. 2014). However, with the continued control of ozone precursors, reducing concentrations of ozone may partially offset some of the most severe impacts of climate change on global crop production (Tai et al. 2014).





Figure 1.4: Characteristic ozone-induced senescence on a leaf of white clover (*Trifolium repens* L.). Ozone has been recognized as a threat to plant health since at least the 1950s, when damage to grape and other crops was first reported in California (Richards et al. 1959).

### **1.5. Ozone metrics, and critical levels for grasslands**

Historically, ozone-effect relationships and protection efforts for natural and cultivated vegetation, have been focused on mean exposure (e.g. 7hr, 24hr or seasonal mean concentration) or accumulated exposure to ozone (e.g. accumulated exposure above a threshold of 40ppm (AOT40) or sum of hourly concentrations above 0.06ppm (SUM06)). Based on AOT40, a 3-month accumulated exposure of 3 ppm.h, or a 6-month exposure of 5ppm.h, are critical levels for the protection of grasslands and semi-natural vegetation in Europe (CLRTAP, 2011). However, the continuing rise in background ozone in the Northern Hemisphere in recent decades means concentration-based critical levels may now be constantly exceeded (CLRTAP, 2011; ROTAP, 2011). In addition, concentration-based indices provide greater statistical

weight to high concentrations of ozone (e.g. Samuelson & Kelly, 1996), and do not reflect actual ozone uptake or detoxification, leading to inaccurate assessments of ozone. The phytotoxic ozone dose above a threshold flux of  $\text{Ynmol m}^{-2} \text{s}^{-1}$  (PODY) is an exposure index based on the modelled flux of ozone into the plant via the stomatal pores. Stomatal conductance of ozone is estimated using the complex multiplicative Jarvis-type conductance model (Jarvis, 1976), which requires the parametisation of biotic and abiotic factors such as phenology, temperature and light (Emberson et al. 2000). Consequently, the ozone uptake-based POD model provides a better fit to effect-data, and stomatal flux-based critical levels of ozone have been developed for key arable crop and forest species under the auspices of the UN Convention of Long-Range Transboundary Air Pollution (CLRTAP) (e.g. CLRTAP, 2011; Mills et al. 2011b, c, d). For impacts on biomass production in pasture and grasslands of high conservation value, an accumulated 3-month  $\text{POD}_1$  of  $2\text{mmol m}^{-2}$  has been provisionally determined as the critical level, representing the ozone flux required to induce a 10% reduction in the shoot biomass yield of *T. repens* or other *Trifolium* species (CLRTAP, 2011; Mills et al. 2011b, d) (Figure 1.5).

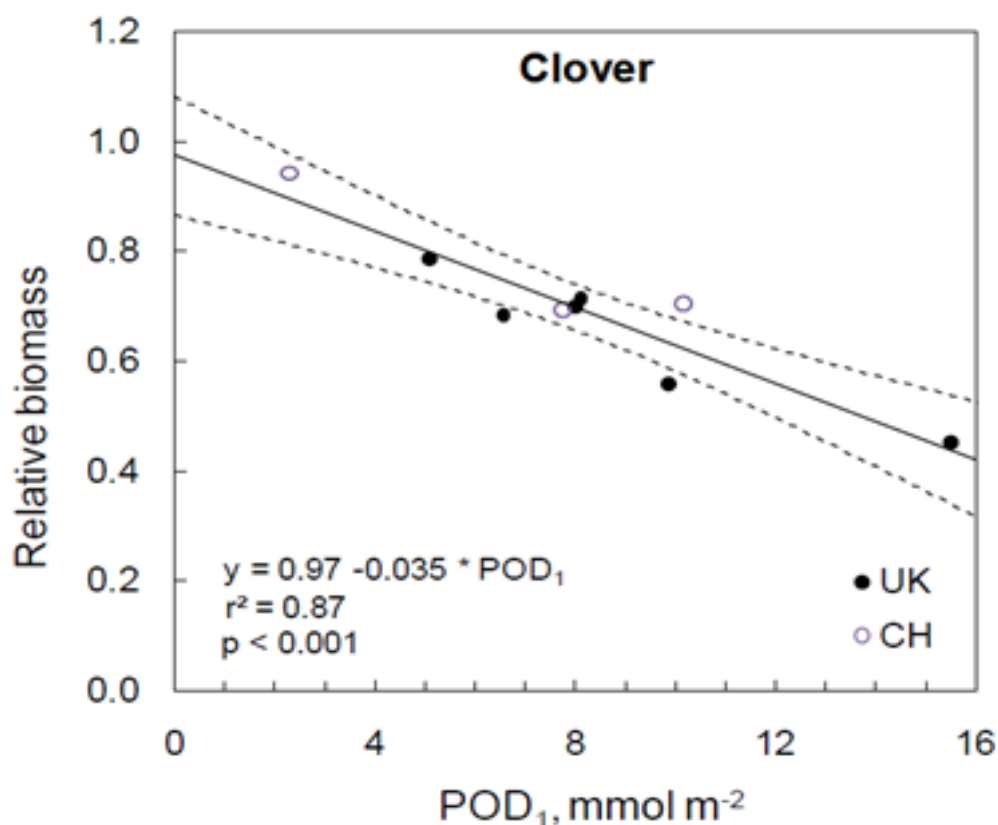


Figure 1.5: Flux-based ozone dose-response relationship for the yield of white clover (*Trifolium repens* L.), determined with data from clover grown at sites in the UK and Switzerland (after Mills et al. 2011b) (where POD=phytotoxic ozone dose; dashed lines are 95% confidence intervals).

### 1.6. Ozone deposition to European grasslands

There is generally a seasonal NW/SE gradient in the concentration of tropospheric ozone across Europe, reflecting region-specific inventories of NO<sub>x</sub> and VOC (Royal Society, 2008; van Goethem et al. 2013). Thus, the deposition and predicted flux of ozone to grassland biomes is highest in Southern and Central European regions providing there is adequate soil moisture (e.g. Ashmore et al. 2007; van Goethem et al. 2013), supported by observations of ozone-injury in *T. repens* (Mills et al. 2011c). However, surface concentrations frequently exceed concentration-based critical levels for the protection of grasslands throughout Europe (e.g. Fernàndez-Fernàndez et al. 2011; Mills et al. 2011c). Change in the regional and hemispheric production of ozone

precursors have also shifted the seasonal peak in North West Europe from early summer to spring (ROTAP, 2011; Cooper et al. 2014). In the UK, the Early-season (April-June) deposition of ozone to pasture based on the AOT40 index is high in a climate-typical year, with the highest exposure occurring in the South East, and lower ozone exposures in Western and Northern regions (Figure 1.6a). Approximately 70%, 26% and 22% of UK calcareous, acid and mesotrophic grasslands respectively exceed a 6-month critical level of 5 ppm.h (ROTAP, 2011). A full evaluation of flux-based critical levels for an assessment of impacts on UK grassland has not been performed to date. However, based on the predicted ozone flux to *T. repens* in E2 Mesic grassland habitat (EEA, 2012), the uptake of ozone is moderate across the UK, with the highest predicted fluxes in the South West (Figure 1.6b). Ozone concentrations are also typically higher in coastal and upland regions due to increased deposition by wind or decreased destruction (Royal Society, 2008; ROTAP, 2011; Cooper et al. 2014).

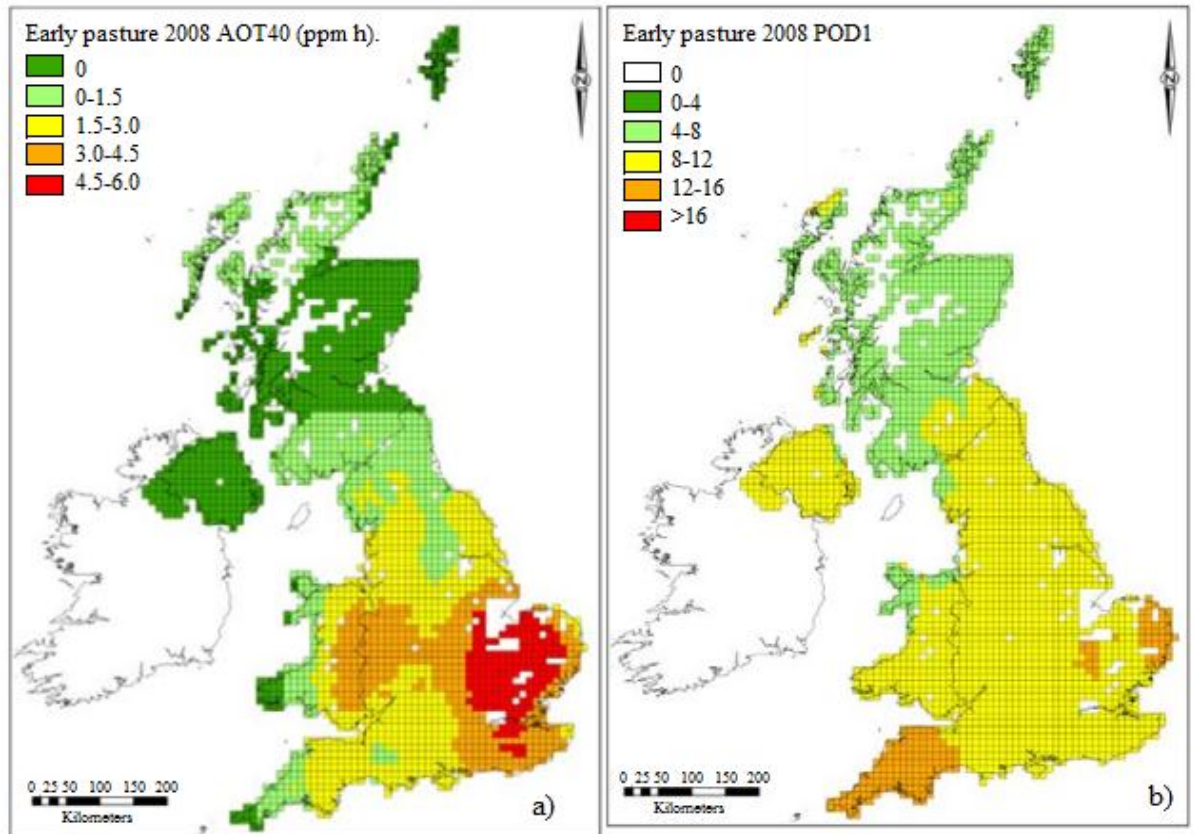


Figure 1.6: The spatial distribution of (a) accumulated ozone above a threshold of 40ppm (AOT40 ppm.h) (b) ozone flux to white clover (*Trifolium repens* L.) across E2 Mesic grassland in the early season (April-June) for a climate-typical year (year 2008) (where pasture >100 ha per 10 x 10 km grid square; POD=phytotoxic ozone dose) (Edited after Mills et al. 2011b).

### 1.7. The effects of ozone on grassland and pasture

Although grassland species are individually often sensitive to ozone (Hayes et al. 2007; Mills et al. 2007), the sensitivity of grassland vegetation to ozone is also influenced by mutualistic and competitive interactions (e.g. Nussbaum et al. 2000; Hayes et al. 2009), management factors (Wilbourn et al. 1995) and microclimatic conditions (e.g. Pleijel et al. 1995; Nussbaum et al. 2000), amongst other factors, and the response of grassland biomes to ozone are complex and difficult to predict (Fuhrer, 2009). Most research into ozone effects on grassland and pasture has come from Europe in the last few decades, and some of the main findings are summarized below.

Through its adverse effects on biomass accumulation, a primary effect of ozone is a change in floral composition. As determined from several ozone-exposure experiments, the composition of semi-natural grassland can respond to ozone with a decreased occurrence of dominant fine grass species, and a reduction in the cover of characteristic forbs and legumes (e.g. Ashmore et al. 1995; Hayes et al. 2006; 2010b; Samuelsson et al. 2006; Thwaites et al. 2006; Wedlich et al. 2012). In clovers, sensitivity to ozone may manifest in cumulative declines in photosynthesis rate, shoot and stolon biomass and density, and declines in stored energy reserves; ultimately exacerbating a reduction in the clover: grass biomass ratio of the sward (e.g. Rebbeck et al. 1988; Heagle et al. 1989; Fuhrer et al. 1994; Ashmore & Ainsworth, 1995; Nussbaum et al. 1995; Wilbourn et al. 1995; González-Fernández et al. 2008; Hayes et al. 2009, 2010b). Long-term change in the composition of grassland communities may also arise from ozone-impacts upon reproductive growth and fecundity (e.g. Whitfield et al. 1998; Gimeno et al. 2004; Schoene et al. 2004; Rämö et al. 2007; Martínez-Ghersa et al. 2008; Hayes et al. 2012). Declines in the biomass of ozone-sensitive species within grassland communities can lead to a reduction in total forage yield and productivity. For example, a long-term study on *in-situ* montane grassland found dry matter yields reduced by a quarter after a 5 year period of exposure to elevated ozone, primarily driven by declines in the legume fraction (Volk et al. 2006). Impacts on yield have also been reported in tall fescue-white clover pastures (e.g. Rebbeck et al. 1988; Heagle et al. 1989) and other grass/clover mixtures (e.g. Fuhrer et al. 1994; Wilbourn et al. 1995; Calvete-Sogo et al. 2014), and may also be driven by reduction in the biomass of forbs (e.g. Wedlich et al. 2012) and grasses (e.g. Nussbaum et al. 2000). An increased above-ground biomass of companion grasses, due to an ozone-induced exclusion of clover, can also compensate for losses in mixed communities,

such that impacts on total forage yield are reduced (Heagle et al. 1989; Fuhrer et al. 1994; Ashmore & Ainsworth, 1995; González-Fernández et al. 2008). Ozone may reduce the yearly gross primary production (GPP) of European vegetation by up to 22% (Anav et al. 2011), and global reductions in net primary production (NPP) for a range of functional groups, including C<sub>3</sub> and C<sub>4</sub> grasses, may range 5-30% (Ainsworth et al. 2012). Shortfalls in arable and grassland production, may necessitate up to a 3% increase in agricultural area in western Europe by year 2050 (Chuwah et al. 2015). However, mature and long-established perennial grasslands may also display a large degree of inertia to the effects of ozone on composition and yield than the sensitivity of component species would otherwise suggest (e.g. Stampfli & Fuhrer, 2010; Bassin et al. 2013; Volk et al. 2014).

Reduced forage quality in response to elevated ozone has also been reported in several important grassland species, including *P. pratensis* (Bender et al. 2006), *L. perenne* (González-Fernández et al. 2008), *T. repens* (Fuhrer et al. 1994; Muntifering et al. 2006; González-Fernández et al. 2008), *T. subterranean* (Sanz et al. 2005), *T. pratense* (Muntifering et al. 2006) and forage from mixed communities (e.g. Powell et al. 2003; Lewis et al. 2006; Gilliland et al. 2012; 2015). Ozone-induced reductions in forage quality arise from increases in senesced biomass, changes in the concentration of cell wall constituents such as neutral and acid detergent fibres, lignin and phenolics, and impacts on crude protein, N content, and micronutrient content per unit of dry matter. Impacts upon forage quality result in a reduced feed value and digestibility in ruminants and non-ruminants (e.g. González-Fernández et al. 2008; Frei et al. 2010; Gilliland et al. 2012) and lower milk and meat production (e.g. Krupa et al. 2004). By year 2020, predicted impacts on forage quality and yield across UK pasture could reduce the liveweight of lambs relative to year 2007 (Hayes et al., submitted).

Assuming livestock and dairy production respond linearly to pasture productivity, Holland et al. (2002), predicted a total ozone-induced cost to UK meat and milk production of £2.1 billion for year 2010. However, in a global analysis of ozone impacts, Reilly et al. (2007) suggest losses in arable grain, rather than grassland productivity, may be a more important determinant of livestock production over the course of this century.

Ozone may also affect a range of other grassland services. Ozone impacts on GPP could modify C sink strength of grassland biomes (e.g. Anav et al. 2011; Volk et al. 2011; Calvete-Sogo et al. 2014). For losses in the C storage capacity, biogeochemical models suggest rising concentrations of ozone may have reduced C storage potential of Chinese grasslands by 140 Tg C between 1961-2000 (Ren et al. 2007). However, impacts of ozone on net C balance in grassland biomes may also be absent (e.g. Volk et al. 2011), and in general, effects on C sequestration potential are still poorly understood (Harmens et al. 2012). Similarly, relatively little is also known about the impacts of ozone on nutrient cycling in grassland soils. Studies by Kanerva et al. (2006; 2007) and by Rämö et al. (2006) suggest that ozone-induced reductions impacts on biomass production in hay meadow communities were sufficient to reduce total community N pool, concentrations of ammonium ( $\text{NH}_4^+$ ) and mineral N; though total soil N, nitrate ( $\text{NO}_3^-$ ), decomposition rate and nitrification processes were not affected by elevated ozone. Increased litter production due to ozone can also increase the N pool in soil and microbial biomass in subalpine grassland (Bassin et al. 2015), and increased N availability may otherwise decrease, increase or have no effect on the sensitivity of grassland vegetation to elevated ozone (e.g. Bassin et al. 2009; Wyness et al. 2013). The effects of ozone on the hydrology of grassland communities are also largely unknown, though a loss of stomatal control in key species (e.g. Mills et al.



2009; Wagg et al. 2012) could reduce freshwater availability within grassland catchments and increase the sensitivity of vegetation to drought events (Mills et al. 2009; Wilkinson & Davies, 2010; Harmens et al. 2012).

## **1.8. Ozone impacts on leguminous N-fixation**

### **1.8.1. Introduction**

Ozone may also affect N-fixation in pasture legumes (e.g. Letchworth & Blum, 1977; Kochhar et al 1980; Montes et al. 1983; Hewitt et al. 2014). However, the impacts of ozone on N-fixation in legumes in general are poorly understood. The existing literature concerning the potential impacts of ozone on leguminous N-fixation is here summarized. The survey of literature identified a total of 26 studies related to ozone effects on N-fixation (Table 1.1). Most studies have been conducted on soybean (*Glycine max*) and clovers (*Trifolium spp.*), followed by beans and pulses (*Phaseolus spp.*; *Vicia spp.*; *Vigna spp.*), chickpea (*Cicer arietinum*) and peanut (*Arachis hypogaea*). There is wide variation in ozone exposure method and total duration, making a generalisation of effects difficult (Table 1.1). The majority of studies date from the 1970s and 1980s, with the earliest studies having utilised closed chamber controlled environment experiments, often with short exposures of acute concentrations of ozone (>300 ppb) (Table 1.1). In more recent years, experiments have been conducted in open-top chambers or field-based exposure facilities which more accurately reflect ambient, or likely near-future conditions, under naturally fluctuating climate conditions (Table 1.1).

Table 1.1: Summary of published literature concerning the effects of ozone on leguminous N-fixation, including reported effect-size relative to low or zero ozone controls, for impacts on Nodulation (nodule number, mass etc) and N-fixation parameters (e.g. nitrogenase activity, g N fixed, nodule activity). Key: CC=closed chambers; G=fumigated greenhouse; OTC=open top chambers; A=ambient; S=solardomes; minus and plus signs (-, +) indicate a negative or positive ozone effect respectively; n/a = not applicable; single asterisks (\*) denote a single experiment with multiple growth seasons; reported effects are significant at  $p \leq 0.05$  unless otherwise stated.

Reference	Year	Species	Exposure method	Effective ozone range/mean	Total exposure duration (per growth season)	Main ozone impacts	Effect size (Nodulation/N fixation).	Additional variables
Manning et al.	1971, 1972, 1973	Pinto bean	CC/G	~60-150 ppb	20-60 days	Nodulation (-)	34%	
Tingey & Blum	1973	Soybean	CC	750 ppb	1 hour	Nodulation (-)	10-14%	
Blum & Tingey	1977	Soybean	CC	500 ppb	4 hours	Nodulation (-)	60%	
Letchworth & Blum	1977	White clover	CC	300-600 ppb	4 hours	Nodulation (-), tissue N content (+)	34-36%	
Blum & Heck	1980	Common bean	CC	300-600 ppb	3 hours	Nodulation (-), tissue N content (+) total N content (-)	12-29%	
Reinhart & Weber	1980	Soybean	CC	250 ppb	~5 days	Nodulation (-)	33-72%	Sulphur dioxide
Kochhar et al.	1980	White clover	OTC/G	300 ppb	2 hours	Nodulation (-)	47%	Competition/inte raction, leachates
Ensing & Hofstra	1982	Red clover	OTC	A-200ppb	24-48 hours	N fixation (-)	24-50%	
Montes et al.	1983	White clover	OTC	~50-80 ppb	~4 months	N fixation (-), soil N inputs (-)	44-79%	Competition, N availability
Ensing et al.	1985	Peanut	A	A	~3 months	Nodulation (-), N fixation (-)	45-48%/57%	
Jones et al.	1985	Soybean	OTC/CC	~60 ppb	5 days	N fixation (-) ( $p < 0.10$ )	~8-16%	Sulphur dioxide
Flagler et al.	1987	Soybean	OTC	58-107 ppb	~6 months*	N fixation (-) ( $p < 0.10$ )	27%	Drought
Agrawal & Agrawal	1990	Chickpea, Faba bean	OTC	~100 ppb	30 days	Nodulation (-), N fixation (-), tissue N content (-); N metabolism (-)	12-34%/ 3-11%	
Smith et al.	1990	Soybean	CC	200 ppb	n/a	N fixation (-), N metabolism (-)	54-68%	N availability
Pausch et al.	1996a, b	Soybean	OTC	43-80 ppb	n/a	N fixation (-), total N content (-)	5-73%	
Nazzari	2002	Faba bean	CC	100 ppb	~3 days	Nodulation (-), N fixation (-)	34-40%/48-99%	Acid rain
Welfare et al.	2002	Chickpea	CC	85 ppb	~6 days	Nodulation (-)	18-21%	Salinity
Nasim et al.	2007	Mung bean	OTC/A	~40-80ppb	~4 months	Nodulation (-), N fixation (-)	21-70%/57-70%	Nitrous oxide
Umponstira et al.	2009	Cowpea	CC	40-70 ppb	7-74 days	Nodulation (-), N fixation (-), tissue N content (-), soil N inputs (-)	31-90%/30-33%	
Cong et al.	2009	Peanut	OTC	49-79 ppb	~4 months	N-fixation (-) (not directly determined), total N content (-)	n/a	Carbon dioxide
Cheng et al.	2011	Soybean	OTC	65 ppb	~6 months*	N-fixation/soil N inputs (-)	37-40%	Carbon dioxide
Zhao et al.	2012	Soybean	OTC	40-110 ppb	n/a	Nodulation (-)	Abstract only	
Hewitt et al.	2014	White clover/Red clover	S	45-66 ppb	3 months	Nodulation (-), N fixation (-)	33-60%/68-83%	

### 1.8.2. Evidence for effects of ozone on N-fixation

The most common effect of ozone in the available literature is on the number, size or mass of root nodules, with 17 studies reporting negative impacts on legume nodulation (Table 1.1). Where a range of ozone concentrations exists from multiple studies, the concentration of ozone reported as being effective for impacts on root nodules appears similar for agriculturally important legume species (Figure 1.7a), with effect size ranging 10-90% in available literature (Table 1.1). Acute concentrations of ozone  $\geq 500$ ppb may affect significant reductions of  $>10\%$  in soybean nodule number or mass after less than 5 hours exposure (e.g. Tingey & Blum, 1973; Blum & Tingey, 1977), though impacts of  $>20\%$  can also occur in legumes after the seasonal exposure to means  $>60$ ppb (e.g. Nasim et al. 2007; Hewitt et al. 2014). Several studies also report decreased N-fixation parameters, with the majority of concerned studies having focused on negative effects in clover and soybean (Table 1.1), although ozone-induced suppression of N-fixation rates have also been reported in peanut, chickpea, faba bean, mung bean and cowpea. As above, the effective range of ozone for a suppression of N-fixation rates does not appear to differ between legume species (Figure 1.7b), with reductions in N-fixation ranging from  $\sim 8$ -99% (Table 1.1). In peanut, seasonal exposure to treatments of 49-70 ppb is sufficient to reduce tissue N content and N-fixation rates compared to charcoal-filtered controls (Cong et al. 2009). Similarly, mean daytime exposures up to 107 ppb reduced soybean N-fixation rates by up to 58% (Flagler et al. 1987; Pausch et al. 1996b Cheng et al. 2011), with a significant decline in the number and dry mass of soybean root nodules also occurring with exposure to concentrations  $>100$  ppb (Zhao et al. 2012). N-fixation rates in white clover were reduced  $>50\%$  by seasonal means of 60-80 ppb (Montes et al. 1983, Hewitt et al. 2014). A seasonal mean of 66 ppb was also sufficient to reduce the

number, size and mass of clover root nodules, and increase the density of small, likely non-fixing pseudonodules (Hewitt et al. 2014). In general, seasonal ozone means of 40-60ppb or more, or short-term exposure to concentrations of >100ppb, appears sufficient to achieve a reduction in N-fixation rates or impact nodulation in the agricultural legumes assessed (Table 1.1; Figure 1.7). Although root nodule structure can differ substantially, ozone similarly affects legumes with determinate (e.g. soybean, common bean) or indeterminate root nodules (e.g. clover, chickpea) (Table 1.1). Ozone has also been shown to disrupt N-fixing symbioses in non-legumes, such as Alder (*Alnus* spp., Greitner and Winner, 1989).

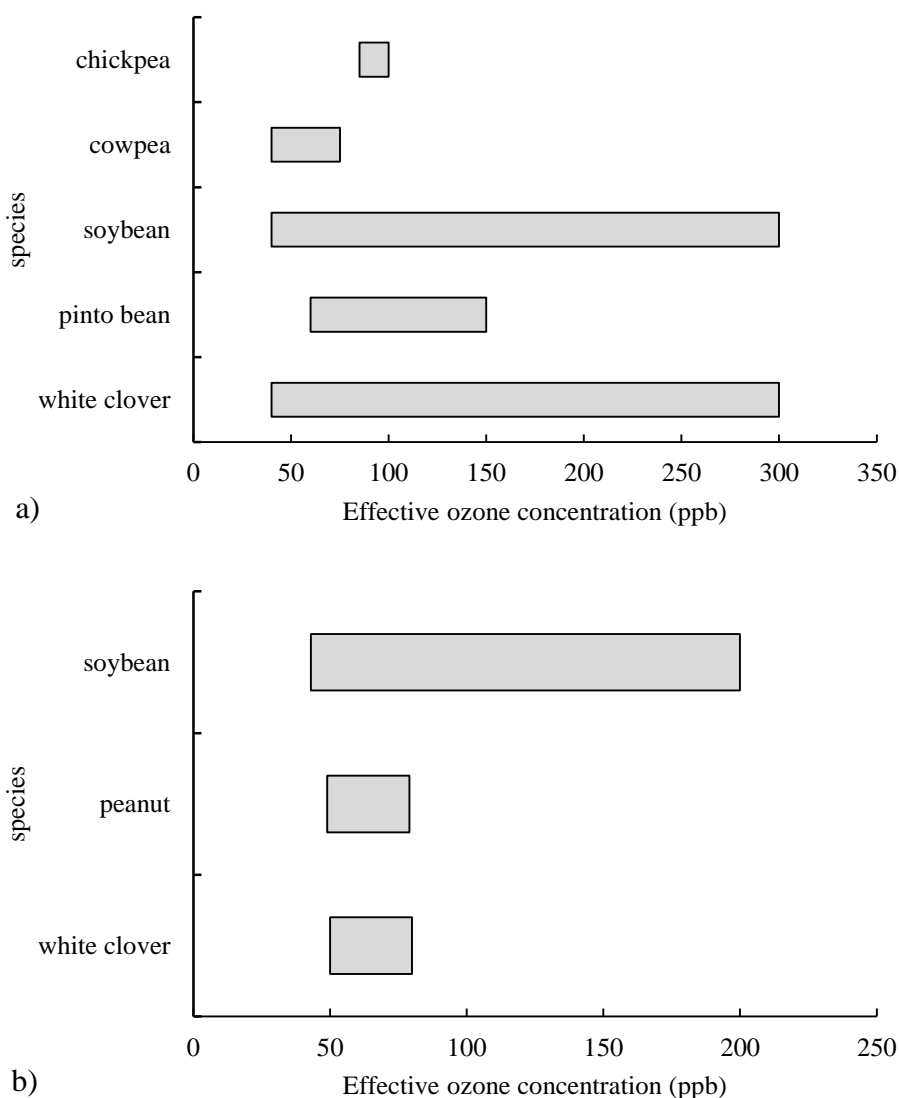


Figure 1.7: The approximate range of ozone concentrations reported as impacting on (a) the number, size or mass of root nodules, or (b) suppressing N-fixation rates, in agronomically-important legume species (concentrations above 300ppb are excluded).

### 1.8.3. The physiological basis of ozone impacts

Ozone enters the plant primarily through the stomata, and thus ozone uptake or flux is highly related to the hydrological status of the plant (Wilkinson and Davies, 2010). In addition to directly reducing photosynthetic rates and capacity, ozone exposure may cause the diversion of carbon and other resources to above-ground growth, defence and repair, including the synthesis of antioxidants and structural carbohydrates (Wilkinson et al. 2011). Callus tissue, built up in the phloem as a response to ozone

exposure, may also cause a mechanical hindrance to the flow of carbon and resources to the root system (Wilkinson et al. 2011). Thus, although the leaves are the main site of ozone exposure, the early limitation of growth often occurs in below ground organs (e.g. Hewitt et al. 2014). Reduced N-fixation rates in nodulating legumes could result from a decrease in root nodule size or number, or in the specific activity of the nitrogenases, the central group of oxygen-sensitive rhizobial enzymes responsible for the catalysis of atmospheric N into ammonia (Rees et al. 2005). Ozone does not directly affect root nodules as it does not penetrate through the soil surface (Blum and Tingey, 1977), though the leachates of ozonated plants may influence the growth and subsequent colonisation of soil rhizobia and root fungi (Manning et al. 1971; Kochhar et al. 1980; Umponistira et al. 2009). A reduced supply of detritus may lead to a suppression of N-fixation in soil microbial communities in legume agroecosystems (He et al. 2014). As determined from stable isotope studies, ozone-induced reductions in N-fixation rates are thought to ultimately arise from a reduced availability and translocation of carbon assimilates to the root system during growth (Pausch et al. 1996a, b, Cong et al. 2009), with a reduced sucrose flow to nodules the most likely cause (Udvardi & Poole, 2013). N-fixation rates may also recover from ozone episodes over time (e.g. Ensing & Hofstra, 1982; Flagler et al. 1987). In chronic ozone exposures, reduced N-fixation rates may be associated with reductions in root biomass, though they may occur independently of impacts on above-ground yield (e.g. Hewitt et al. 2014). In red clover (*Trifolium pratense*), the most severe impacts on root nodule number may be avoided due to higher stomatal conductance rate and possible ability to maintain a supply of assimilates to the root system (Hewitt et al. 2014).

As ozone impacts photosynthesis at a metabolic level (e.g. Sun et al. 2014), impacting root growth (Hewitt et al. 2014), disrupting flavonoid synthesis (Galant et

al. 2012) and reducing the leghaemoglobin content of root nodules (Blum and Heck, 1980), its proximate mechanisms are superficially similar to ultraviolet radiation (UVB) (e.g. Chouhan et al., 2008; Sharma and Guruprasad, 2012; Hectors et al. 2014). This is in contrast to below-ground stressors such as drought and salinity, which initially inhibit nodulation by directly disrupting the redox status of roots or nodules (e.g. Munoz et al. 2012; Esfahani et al. 2014). Although the molecular mechanism that regulates optimal root nodule number has not yet been elucidated (*sensu* Soyano et al. 2014), a role for all the main phytohormones has been implicated in nodule development (e.g. Ferguson et al. 2004; Mortier et al. 2012; Ryu et al. 2012). This may suggest some commonality with abiotic defence responses in the mesophyll, and hint at potential mechanisms by which ozone could cause reductions in nodule number, including the long-distance transport of ozone-induced stress hormones (Tamaoki, 2008), Mitogen-Activated Protein Kinase (MAPK) signalling cascades (Samuel et al. 2000), or a reduced flow of Auxin to the root system (Blomster et al. 2011). Ozone can decrease nitrate and nitrite reductase activity (Agrawal and Agrawal, 1990; Smith et al. 1990) and impair the mobilisation or partitioning of N (e.g. Pausch et al. 1996b; Zhang et al. 2014). Altered N or protein content of plant tissues may occur as a consequence of shortfalls in N-fixation (e.g. Agrawal and Agrawal, 1990). However no consistent effect is apparent in the available literature (Table 1.1), and ozone-induced impacts on N-fixation or N partitioning may increase (Letchworth and Blum, 1977; Blum & Heck, 1980), decrease (Blum and Heck, 1980; Flager et al. 1987; Agrawal and Agrawal, 1990; Cong et al. 2009; Umponistira, 2009) or have no effect (Montes et al. 1983; Pausch et al. 1996b) on the N content of tissues, organs or whole plants depending upon exposure method and duration, and ozone concentrations used (Table 1.1). Given the practical difficulty in observing *in vivo*

changes directly, below-ground responses to ozone in general remain poorly characterised and most studies concerned with this question have used destructive techniques (e.g. Letchworth & Blum, 1977). The acetylene reduction assay, the most commonly employed technique in the literature to assess N-fixation, can also be used non-destructively by measuring gas changes in a chamber placed over soil containing roots (Hewitt et al. 2014).

#### **1.8.4. Interactions between ozone and other stressors**

Eleven studies have considered the interactive or additive effects of ozone and other stressors or variables on N-fixation (Table 1.1), including interspecific competition (Kochhar et al. 1980; Montes et al. 1983), salinity (Welfare et al. 2002), sulphur dioxide and acid rain (Reinhart & Weber, 1980; Jones et al. 1985; Nazzar, 2002), nitrous oxide (Nasim et al. 2007) drought (Flager et al. 1987) and elevated concentrations of carbon dioxide (CO<sub>2</sub>) (Cong et al. 2009; Cheng et al. 2011). The effects of these additional environmental stressors are largely additive. Nodulation may be reduced in *T. repens* by leachates of ozone-exposed *Fescue* plants but not non-exposed plants (Kochhar et al. 1980), and competition with companion grasses may exacerbate ozone-induced reductions in N-fixation rate in clover-fescue pasture (Montes et al. 1983). Ozone-induced reductions in N-fixation and other effects may be ameliorated by the stimulatory effect of elevated CO<sub>2</sub> (Cong et al. 2009; Cheng et al. 2011).

Only two studies have considered the interactive effects of N-availability and ozone on N-fixation (Montes et al. 1983; Smith et al. 1990). As N-fixation is an energy-intensive process (Rees et al. 2005), excessive inputs of N to agricultural systems can suppress nodule development (e.g. Nanjareddy et al. 2014) or fixation



rates (Streeter and Wong, 1988), and lead to reduced growth or yield (e.g. McCurdy et al. 2014). In experiments on clover-fescue (*Festuca arundinacea*) pasture, no interaction between ozone and treatments of  $\text{NH}_4\text{NO}_3$  was apparent on fixation rate, though the factors may affect N-fixation singly (Montes et al. 1983). In soybean, interactions between ozone and  $\text{NO}_3^-$  treatment in nodule activity were unclear (Smith et al. 1990). In addition to artificial fertiliser, high levels of N deposition ( $>10\text{kg N ha}^{-1}\text{ yr}^{-1}$ ) co-occur across regions that have also seen large increases in tropospheric ozone (Dentener et al. 2006), making these two factors major drivers of change in polluted regions such as the Mediterranean (EEA, 2011). In Mediterranean pasture legumes, such as subterranean clover, (*T. subterraneum*), striated clover (*T. striatum*) and yellow serradella (*Ornithopus compressus*), N deposition and ozone may interact to drive reductions in nutritive quality (Sanz et al. 2005) and terpene emissions (Llusia et al. 2014), which may result in lower competitiveness (Llusia et al. 2014). However, N-deposition may also stimulate growth, reducing the sensitivity of legumes to impacts on above-ground biomass and ozone-induced foliar injury (Sanz et al. 2007; Calvete-Sogo et al. 2014). Thus, the N-enrichment of agricultural soils, via artificial fertiliser or N-deposition, has the potential to at least partially mitigate ozone impacts on N-fixation. This effect may be particularly important in low-growth rate grassland habitats, where species composition displays more sensitivity to levels of N than to long-term ozone pollution (e.g. Bassin et al. 2013). Adding extra N to compensate for ozone effects on N-fixation in agroecosystems may have important environmental consequences, including a decline in species diversity (Bassin et al. 2013) and increased GHG emissions (e.g. Flechard et al. 2007).

#### **1.8.5. Consequences for N availability in agroecosystems**

Few studies have reported decreased plant contributions to soil N as a consequence of reduced N-fixation rates (Table 1.1). Montes et al. (1983) report a linear decline in soil nitrate ( $\text{NO}_3^-$ ) concentrations in clover/fescue pasture after seasonal exposures to elevated ozone and reduced total N content of soil was found in short-term experiments with Cowpea (Umponstira et al. 2009). Reduced N inputs to soil were also found in 4 year-long experiments with soybean, although total N in the soil surface was unaffected (Cheng et al. 2011). Indeed, other than the studies by Cheng et al. (2011) and Flagler et al. (1987), wherein soybean N-fixation rates were annually assessed from low and high ozone plots for multiple growth seasons, there have been no long-term experiments to our knowledge that have investigated the consequences of ozone on N-fixation in any legume species (Table 1.1). It is therefore unknown whether decreased inputs of organic N due to ozone leads to lasting declines in soil fertility and an increased reliance on artificial fertiliser in agroecosystems.

### **1.9. Key uncertainties**

There are large uncertainties relating to the impacts of ozone on temperate grassland and pasture. The lack of quantification of the magnitude of effects of ozone, including impacts on species diversity, forage yield and quality, meat and dairy production, and impacts on other ecosystem services, is a long-standing issue, reflecting the high complexity of grassland systems and inherent difficulty in scaling observations from often short-term ozone-exposure experiments to an agroecosystem basis. A more complete understanding of the long-term effects of ozone on the ecological and economic sustainability of grasslands may develop over the coming years (Mills et al. 2011a).

On a more practical basis, there is little information available regarding the effects of ozone on modern varieties of clovers and companion grasses in managed pastures, though this need has been recognized (e.g. González-Fernández et al. 2008; Hewitt et al. 2014), as sensitivity to ozone is known to vary widely within cultivated species and could form an important basis for the future selection of cultivars (e.g. Pleijel et al. 2006; Danielsson et al. 2013). For example, although numerous studies have researched the impacts of ozone on the growth and functioning of white clover (*T. repens*), relatively few cultivars have been assessed, with an extensive focus since the early 1990s on physiological responses within the *T. repens* cv. Regal bioindicator system (e.g. Heagle et al. 1993;1995; Tang et al. 1999; Bermejo et al. 2002; Nali et al. 2005; Francini et al. 2007; González-Fernández et al. 2008; Berner & Krüger, 2009; Borowiak et al. 2011). In recent years, new varieties of clover have been developed for varied use in pasture and forage crops (British Grassland Society, 2015), stimulated by their potential to reduce agricultural GHG emissions (Smith et al. 2007), the high price of artificial fertilizers (Lynch, 2007) and by advances in the selection of legume germplasm for stress tolerance and improved performance (e.g. Abberton and Marshall, 2005). Environmental and economic concerns have led to the development of high-sugar ryegrass (HSG), designed to improve the uptake, digestion and N-utilization of grazing stock in managed pasture, and suggested as having the potential to increase production yields and reduce CH<sub>4</sub> and N emissions from livestock farming (Ellis et al. 2011; Staerfl et al. 2012).

In addition, the critical level for productive grasslands is currently only based on UK and Swiss data (Mills et al. 2011d), and may overestimate the possible effect of ozone on *T. repens* yield in some circumstances (e.g. Mills et al. 2011b). Flux-based dose-response relationships suitable for derivation of critical levels for several other

ozone impacts, including forage quality, remain undeveloped. Consumable food value (CFV), for example, is an important index of forage quality, considering impacts to both yield and quality (e.g Krupa et al. 2004; González-Fernández et al. 2008) and making it particularly useful for the economic valuation of impacts of ozone in pasture (Mills et al. 2011a). However, flux-effect relationships for CFV, and other forage quality parameters in grassland vegetation, have not extensively been examined in the published literature (González-Fernández et al. 2008). Flux-effect relationships for impacts on the below-ground responses of key grassland species are also poorly understood, although reductions in root biomass, and nodulation and N-fixation in legumes, could have important ecological and economic consequences, and may be more responsive than effects on yield (e.g. Hewitt et al. 2014; Sanz et al. 2014).

Indeed, the effects of ozone on N-fixation in pasture (Letchworth & Blum, 1977; Kochhar et al 1980; Ensing & Hofstra, 1982; Montes et al. 1983; Hewitt et al. 2014), or in legumes in general, has been surprisingly neglected. Still fewer studies have explored the potential physiological mechanisms of ozone impacts on N-fixation, despite considerable overlap between abiotic defense (Rao & Davies, 2001; Tamaoki, 2008) and nodule regulation pathways (Ryu et al. 2012; Mortier et al. 2012). The impacts of ozone on N-fixation in pasture on an agroecosystem scale are also unknown.

### **2.0. Objectives**

This study aimed to investigate:

- 1) Effects of ozone on growth and functioning of modern cultivars of clover (*Trifolium* spp.) (Chapter 2).

- 2) Impacts of ozone on HSG pasture mesocosms, including the development of dose-response relationships for growth, N-fixation and forage quality (Chapter 3).
- 3) The consequences of ozone-impacts on clover nodulation for pasture sustainability (Chapter 4).
- 4) Interactions between cutting and ozone on injury and nodulation in white clover (*T. repens*) (Chapter 5).

Some of this work has been published by the author in peer-reviewed journals:

Hewitt, D.K.L., Mills, G., Hayes, F., Wilkinson, S., Davies, W. 2014. Highlighting the threat from current and near-future ozone pollution to clover in pasture. *Environmental Pollution*, **189**: 111-117.

Hewitt, D.K.L., Mills, G., Hayes, F., Norris, D, Coyle, M, Wilkinson, S., Davies, W. N-fixation in Legumes – an assessment of the potential threat posed by ozone pollution. *Environmental Pollution*, in press.

## 2. THE EFFECTS OF OZONE ON MODERN CULTIVARS OF CLOVER (*TRIFOLIUM* SPP.)

### 2.1. Introduction

Nitrogen (N) fixation by legumes (*Fabaceae*) is of vital agronomic importance. On a global scale, the legume-rhizobia symbiosis, contained within specialised organs called root nodules, is thought to add at least 30 Tg N annually to agricultural land (Herridge et al. 2008). At present, legume crops account for ~15% of utilised arable land area (FAO, 2013), constituting the primary source of dietary protein for a substantial proportion of the human population. Legumes are also an essential component of many pasture systems; improving the protein content, nutritional value and uptake of forage, as well as providing ancillary benefits to the structure and long-term fertility of soils (Parsons & Chapman, 2000). In temperate regions of Europe, Oceania and the Americas, clovers (*Trifolium* spp.) are the most important pasture legume. Surprisingly, given the agricultural importance of clover, little attention has been paid in recent decades to the fact that *Trifolium* spp. are amongst the most sensitive known to ground-level ozone pollution (e.g. Hayes et al. 2007). Worryingly, concentrations of tropospheric ozone have risen in that time over arguably all of the clover-growing regions of the world (Royal Society, 2008). The potential for losses in quantity and quality of pasture forage, with a concurrent need for increased usage of artificial fertiliser in current and near-future ozone regimes, formed the motivation for this study.

At present, background levels of tropospheric ozone are high enough to damage sensitive crops across the Northern Hemisphere (Mills et al. 2011a), with a mean concentration of 30-40ppb representing a doubling of the pre-industrial background

(Vingarzan, 2004). In respect of its threat to agricultural production and food security, tropospheric ozone is the most important air pollutant (Avnery et al. 2011; Mills et al. 2011a; Wilkinson et al. 2011). Ozone damage occurs in plants via the induction of oxidative stress, leading to foliar injury, impacts on gas exchange, photosynthesis, growth and eventual yield (Wilkinson et al. 2011).

Grassland systems and constituent species have been identified as particularly sensitive to ozone pollution (e.g. Hayes et al. 2007; Mills et al. 2007). Indeed, numerous studies have highlighted the complex response of managed grasslands to ozone (for reviews see Bassin et al. 2007 & Fuhrer, 2009), with pasture forage susceptible to reductions in quality and yield, as well as shifts in species composition, with uncertain effects upon the carbon (C) sink strength of grassland systems (see Harmens et al. 2012). Most previous experiments on ozone effects on clover were conducted in the 1970s and mid-1990s, usually with ozone profiles exhibiting high peaks and a low baseline concentration, no longer representative of current ambient conditions in Europe. Due to the improved control of precursor emissions, local peak concentrations of ozone have decreased in Europe in the last 20 years, whilst the baseline has steadily risen, in part due to the hemispheric transport of ozone precursors from other regions (Parrish et al. 2012). Furthermore, previous studies often used relatively high ozone concentrations, delivering unrealistically acute dosages (e.g. Letchworth & Blum, 1977; Blum et al. 1983). Results from studies with mixed-species swards are highly complex and range from a gradual reduction in yield of the *Trifolium* fraction to no overall effect on botanical composition (e.g. Blum et al. 1983; Rebbeck et al. 1988; Heagle et al. 1989; Fuhrer et al. 1994; Ashmore & Ainsworth, 1995; Pleijel et al. 1996; Nussbaum et al. 1995; Wilbourn et al. 1995; Gonzalez-Fernandez et al. 2008; Hayes et al. 2009). Differential sensitivity to ozone

induced foliar injury within *Trifolium* spp. lends utility for their use as ozone biomonitors (Mills et al. 2011c).

Nodulation in legumes is primarily controlled by long distance root and shoot-derived signalling (termed autoregulation of nodulation (AON)) (Mortier et al. 2012). A complete understanding regarding the molecular nature of AON signalling, and more generally, the role of C and N supply in the determination of nodule number, remains obscure (e.g. Ludidi et al. 2007; Mortier et al. 2012). N-fixation is an energy-intensive process, and nodules in legumes are a strong sink for assimilates, such that root and shoot growth may be suppressed in hypernodulating mutants (e.g. Ito et al. 2007; Yoshida et al. 2010). Superfluous nodulation is regulated by a shoot-derived inhibitor (SDI), with the long-distance transport and action of brassinosteroids and jasmonic acid (JA) suggested as likely candidates for the SDI signal (Mortier et al. 2012). Nodulation is also determined by local hormonal regulation, with JA, abscisic acid (ABA) and ethylene together acting as local negative regulators of nodule initiation (Mortier et al. 2012).

Ozone-impacts on nodulation or N-fixation have been shown in several legumes including soybean (Tingey & Blum, 1973; Reinhart & Weber, 1980; Jones et al. 1985; Pausch et al. 1996b), peanut (Ensing et al. 1985; Cong et al. 2009) and beans (Manning et al. 1971; Blum & Heck, 1980). Research by Blum & Tingey (1977) does not support a significant direct influence of ozone on legume root nodules, with reduced photosynthate translocation suggested by this, and other studies, as the cause for a reduction in nodule growth (e.g. Tingey & Blum, 1973; Reinhart & Weber, 1980). Stable isotope studies by Pausch et al. (1996b), and Cong et al. (2009), also attribute ozone impacts on N-fixation to a reduced availability of assimilate. However, relatively few studies have directly addressed the impacts of ozone on



clover nodulation; still less having explored the mechanistic basis of these effects, and the potential impacts on pasture sustainability caused by the current and near-future concentrations of ozone. Letchworth & Blum (1977) reported a reduction in nodule growth in *T. repens* in response to acute exposure in closed chamber studies, although nitrogenase activity per nodule, and per plant, was not significantly altered. In contrast, Ensing et al. (1982), and Montes et al. (1983), in open-top-chamber studies, reported ozone-induced reductions in N-fixation in *T. pratense* and *T. repens* respectively. Further, ozone-induced reductions in total N or % N in *T. repens* biomass are reported by Letchworth & Blum (1977), Blum et al. (1983) and Montes et al. (1983), with some studies reporting some effect upon the crude protein content (e.g. Blum et al. 1983; Fuhrer et al. 1994; Sanz et al. 2005) and digestibility (e.g. Fuhrer et al. 1994; Sanz et al. 2005; Muntifering et al. 2006; Gonzalez-Fernandez et al. 2008) of *Trifolium* forage. Ozone impacts may occur in earliest root tip development in *Trifolium* spp. (Vollsnes et al. 2010), whilst infection by rhizobia may afford some level of protection to ozone impacts on growth relative to non-inoculated controls (Miller et al. 1997).

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

## **2.2. Methods**

### **2.2.1. Clover cultivars**

*T. repens* cv. Crusader, a medium-leaved cultivar used for frequent cutting and grazing, and *T. pratense* cv. Merviot, used for cutting and finishing autumn stock, (hereafter referred to as Crusader and Merviot) were sown as seeds into cell trays in compost (John Innes No. 2; J. Arthur Bowers, Lincoln, UK) in late spring 2012. Seeds were obtained from a commercial seed supplier, and originated from the UK (Wynnstay Seeds; UK). Plants were propagated in plug-plant trays in an unheated glass-house, watered by hand as necessary and thinned when appropriate to one seedling per cell. After 3 weeks of growth, seedlings of each cultivar were transferred into 5L plant pots (22cm diameter x 19.1cm depth), filled with sterile topsoil (Gravelmaster, UK), with 4 seedlings arranged evenly in each pot. To introduce a soil microbe population, pots were inoculated with 200ml of a soil slurry mixture made from approximately 5kg of soil from agricultural grassland (Abergwyngregyn, North Wales, UK, 53°14'N, 4°01'W) and 14L water. Seedlings were grown for a further 4 weeks. On 06/07/2012, 42 pots per cultivar, selected for consistent size, were then transferred to a series of 7 'solardomes' (hemispherical glasshouses; 3m diameter, 2.1m high) at the CEH solardome facility near Bangor, North Wales, with 6 pots of each cultivar per solardome.

### **2.2.2. Ozone system and treatments**

Plants were then exposed to a range of ozone treatments based on an episodic profile recorded at a rural ozone monitoring site (Aston Hill, Wales, UK, 52°50'N, 3°03'W) with a unique treatment in each solardome. Treatments were designed to reflect future ozone scenarios, with peak concentrations reduced by more than the background

(Figure 2.1). Treatments were applied to the solardomes randomly. Plants were exposed to the ozone treatments for a three-month period, starting 11/07/2012 and finishing 03/10/2012.

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

### **2.2.3. Visual assessment**

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

### **2.2.4. Stomatal conductance (gs)**

Stomatal conductance (gs) of both cultivars was determined at intervals throughout the growth season across all ozone treatments in naturally fluctuating climatic

conditions. All measurements were made using a porometer (AP4, Delta T Devices, Cambridge, UK), between 10:00-16:00h, on the abaxial surface of leaves displaying <10% ozone injury and senescence. Solardomes were visited in random order, and measurements were made in the presence of ozone. Soil moisture content was determined after every measurement with a hand-held soil moisture probe and sensor (ML2x ThetaProbe, HH2 Moisture Meter; Delta T Devices, Cambridge, UK).

### **2.2.5. Biomass harvest**

After 12 weeks of growth, the shoot, root and nodule mass of the plants from each cultivar was harvested. Shoot biomass was harvested for the entire pot in October. For rapidly-growing Merviot, a mid-season harvest of shoot biomass was also performed in late August after 7 weeks exposure by cutting back to 7cm. Below-ground biomass was determined from a representative quarter of each pot, due only to the extensiveness of the root system. Furthermore, below-ground biomass was determined in treatments 1, 4 and 7 only, as harvest of the roots took almost 3 weeks; even with cold storage, it was considered inappropriate to store soil samples for longer than this due to the re-growth or decomposition of root material. Nodules were excised from the root system, counted and weighed. Shoots and roots were dried for a minimum of 48 hours at 60°C or until constant mass was achieved. Nodule biomass was air dried and sized into two categories based on maximum length (<0.1-0.7mm; 0.7->1.5mm). Root biomass, nodule biomass and nodule numbers per pot were calculated as follows:

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

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

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

Mass-per-nodule, root:shoot, total biomass and root:total biomass were also determined. To allow comparison with previously published data, and to facilitate analysis of ozone effects on a UK scale, biomass variables were expressed to accumulated exposures above a threshold of 40ppb during daylight hours at canopy height (AOT40, units ppm.h (after Fuhrer (1994))).

### **2.2.6. Acetylene reduction assays (ARA)**

Assessments of in-situ nitrogenase activity were performed on Crusader in treatments 1 and 7, using a method adapted from Lindström (1984). Two weeks prior to the assay, two sealable 400ml plastic bottles, with the bottom removed and fitted with a gas septum, were inserted to a depth of 2cm into the centre of each pot. For the assay, a 10% acetylene atmosphere was generated inside one bottle by removing 10% of the air and immediately replacing it with acetylene gas (BOC, Guildford, UK). The second bottle acted as a control to determined baseline ethylene generated from the soil. Acetylene was stored and transported to the solardome facility in inert gas bags (SUPELCO, Bellefonte, US), which were vented to the atmosphere and flushed through with N<sub>2</sub> after use. 15 ml gas samples were taken from the bottles at 0, 4 and 8 and 24 hours, with a 1ml sub-sample analysed for ethylene content using a mass-selective detector (Model 6890, Agilent Technologies, Santa Clara, US). Ethylene peak area was determined using G1701DA analytical software (version D.00.00.38; Agilent Technologies, Santa Clara, US). Two assays were performed, in similar climate conditions, in the 10<sup>th</sup> and 11<sup>th</sup> weeks of exposure.

### 2.2.7. Statistical analyses

The precise ozone control system used in the solardomes allowed small changes in ozone profile to be simulated, facilitating dose-response analyses. We note that the lack of treatment replication may raise concerns about pseudo-replication. However, we believe the benefit of using more treatments outweighs this limitation, as published previously by Mills et al. 2009, Hayes et al. 2012 and others. Air flow rates are matched between solardomes, and where recorded, climatic conditions did not vary significantly from solardome to solardome (e.g. leaf temperature, see Appendix Figure A1b). For consistency with existing literature, injury and gs, variables were each analysed by general linear regression, with the 3 week (for injury data) or 12 week AOT40 value for each treatment applied as the predictor variable. For biomass variables, parameters were analysed via two-way analysis of variance (ANOVA) with 12 week AOT40 values and cultivar applied as factors. For Merviot, shoot biomass was analysed individually for each harvest, and total shoot biomass for the season. For nodule size, each size category was analysed separately against the 12 week AOT40 values for the ozone treatments. For ARA data, one-way ANOVA was utilised to determine the effects of ozone, with ozone treatment applied as a factor. To exclude outliers due to very high or low PAR, a cohort of gs data for Crusader (n=133) and Merviot (n=104) was selected for analysis using the 25-75% quartile range of all recorded ambient PAR data for each cultivar respectively. Post hoc Tukey's honest significant difference tests were applied to assess pairwise differences between means where ANOVA revealed a significant effect of ozone. Insufficient gs data was collected for the modelling of ozone flux-effect relationships. All analyses were conducted using R software version 2.15.2 (R Core Development Team, 2012).

## 2.3. Results

### 2.3.1. Ozone concentrations and climate conditions

During the course of the experiment, the seven ozone treatments generated seasonal 24 hr means of 33, 36, 40, 45, 51, 54 & 66ppb and AOT40 values of 0.4, 1.0, 2.7, 5.2, 8.6, 11.5 and 18.5ppm.h (Figure 2.1; Table 2.1). Ozone concentrations increased in each treatment during the weekend reaching a maximum peak on Mondays, and a minimum on Thursdays (Figure 2.1). The ozone treatments successfully simulated decreasing peak and background concentrations, with greater reductions in peak than background ozone. Mean daylight (when PAR >50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) air temperature and VPD were 21.3°C and 0.84 kPa for the study period, with maxima of 24.6°C and 1.14 kPa. Mean daytime PAR was 521  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , with an average daily maximum of 814  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

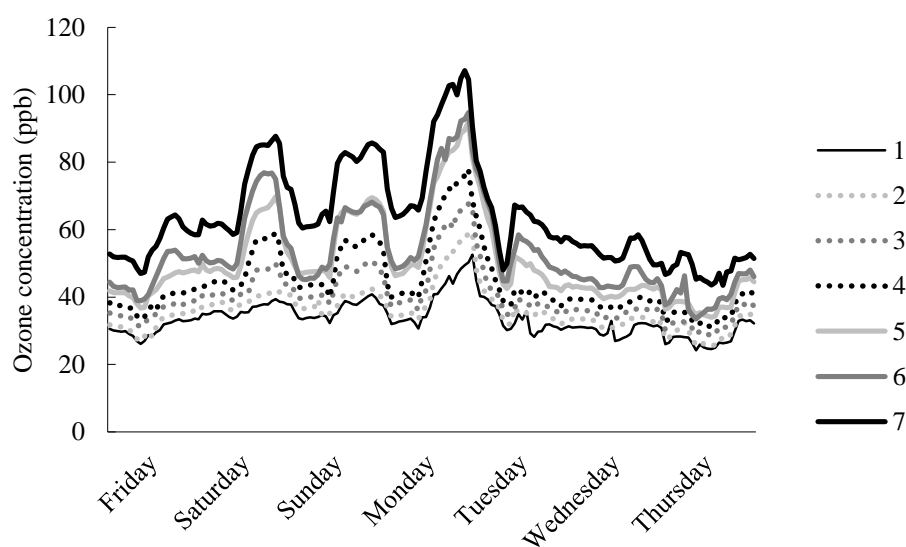


Figure 2.1: Average weekly ozone profile for the seven ozone treatments (see Table 2.1 for treatment details).

Table 2.1: Summary of ozone treatments, including minimum and maximum, and climate conditions for the duration of the experiment.

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

PAR, photosynthetically active radiation; VPD, vapour pressure deficit

Table 2.2: Summary of additional biomass data. Values are means and standard errors. Single and double asterisks (\*) denote a significant ozone effect at  $p < 0.05$  and  $p < 0.01$  respectively.

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



### 2.3.2. Visual assessment and stomatal conductance

Both Crusader and Merviot displayed highly significant increases in visible leaf injury with increasing ozone concentrations ( $p < 0.001$  and  $p = 0.01$  respectively) (Figure 2.2a), with Crusader displaying a significantly higher injury rate with increasing ozone exposure ( $p < 0.001$ ). Baseline injury and senescence were detectable in both cultivars in the lowest exposure treatment (24hr mean of 33 ppb, AOT40 of 0.4ppm.h). There was no relationship between mean  $g_s$  and increasing ozone in Crusader ( $r^2 < 0.10$ ;  $p = 0.54$ ) or in Merviot ( $r^2 = 0.21$ ;  $p = 0.09$ ) (Figure 2.2b). However, there was a pronounced cultivar effect, with Merviot displaying significantly higher mean  $g_s$  than Crusader ( $p < 0.001$ ).

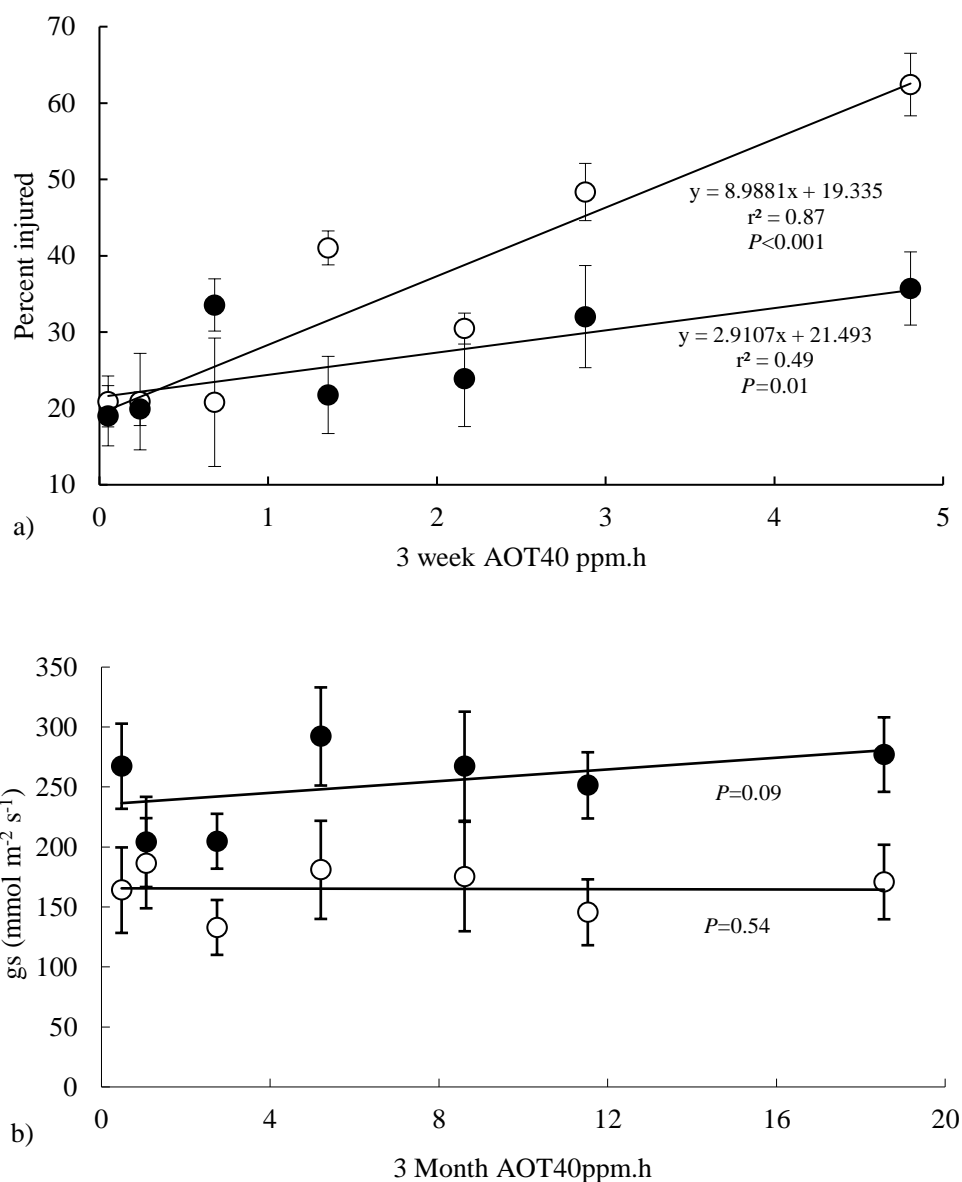


Figure 2.2: Effects of ozone exposure on (a) mean ozone-induced injury after 3 weeks exposure; (b) mean  $g_s$ , from measurements made in weeks 4, 5, 8 & 9 where PAR was 317-849  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (where white points = Crusader; filled points = Merviot; bars are standard errors).

### 2.3.3. Biomass harvest

Both cultivars had highly significant ozone-induced reductions in root biomass per pot (ozone =  $p=0.0006$ ), with a decrease of 61% in Crusader ( $p=0.01$ ) and 63% in Merviot ( $p=0.01$ ) in the highest ozone treatment 7 relative to the lowest treatment 1 (Figure 2.3a). No difference was apparent between the cultivars ( $p=0.22$ ). End-of

season shoot biomass for each cultivar (Figure 2.3b), and shoot biomass of two individual harvests of Merviot (data not shown), did not display any significant responses to ozone, although . The reduction in root biomass also manifested as proportional declines in root:shoot and root:total biomass ratios for both cultivars, with a significantly lower root:shoot in Merviot (Figure 2.3c; Table 2.2). Each cultivar also had reductions in nodule number per pot, with a significant decrease of 36% in Crusader ( $p=0.02$ ) and reduction of 32% in Merviot ( $p=0.09$ ) (Figure 2.3d) in treatment 7 compared to treatment 1, and with Merviot having a lower number of root nodules ( $p=0.01$ ). An interaction was also apparent, with ozone tending to have a lower effect on root nodule number in Merviot plants ( $p=0.06$ ; Figure 2.3d). In Crusader, a decreased number of nodules per pot was accompanied by a 40% reduction in the proportion of larger nodules with a maximum length  $> 0.7\text{mm}$  ( $p=0.01$ ) (Figure 2.4a). Consequently, Crusader pots had a 36% reduction in mass-per-nodule ( $p=0.08$ ) (Figure 2.3e) and a 60% reduction in nodule mass per pot ( $p=0.002$ ) (Figure 2.3f) in treatment 7 relative to treatment 1. In contrast, nodule size, mass-per-nodule and nodule-mass-per-pot in Merviot were unaffected by increasing ozone (Figure 2.3e, f; Figure 2.4b), but these parameters tended to be lower than in Crusader ( $p<0.10$ ), with a reduced effect of ozone nodule-mass-per-pot in Merviot ( $p=0.01$ ) (Figure 2.3e,f). Merviot also had increases of 128% in nodule number ( $p=0.01$ ) and 133% in nodule mass, per gramme of root material ( $p=0.02$ ), in the high ozone treatment 7 compared to treatment 1 (Table 2.2). Both Crusader and Merviot experienced a decline in total biomass, with a 13% reduction in the former ( $p=0.08$ ) and a significant 25% reduction in the latter ( $p=0.01$ ) (Table 2.2).

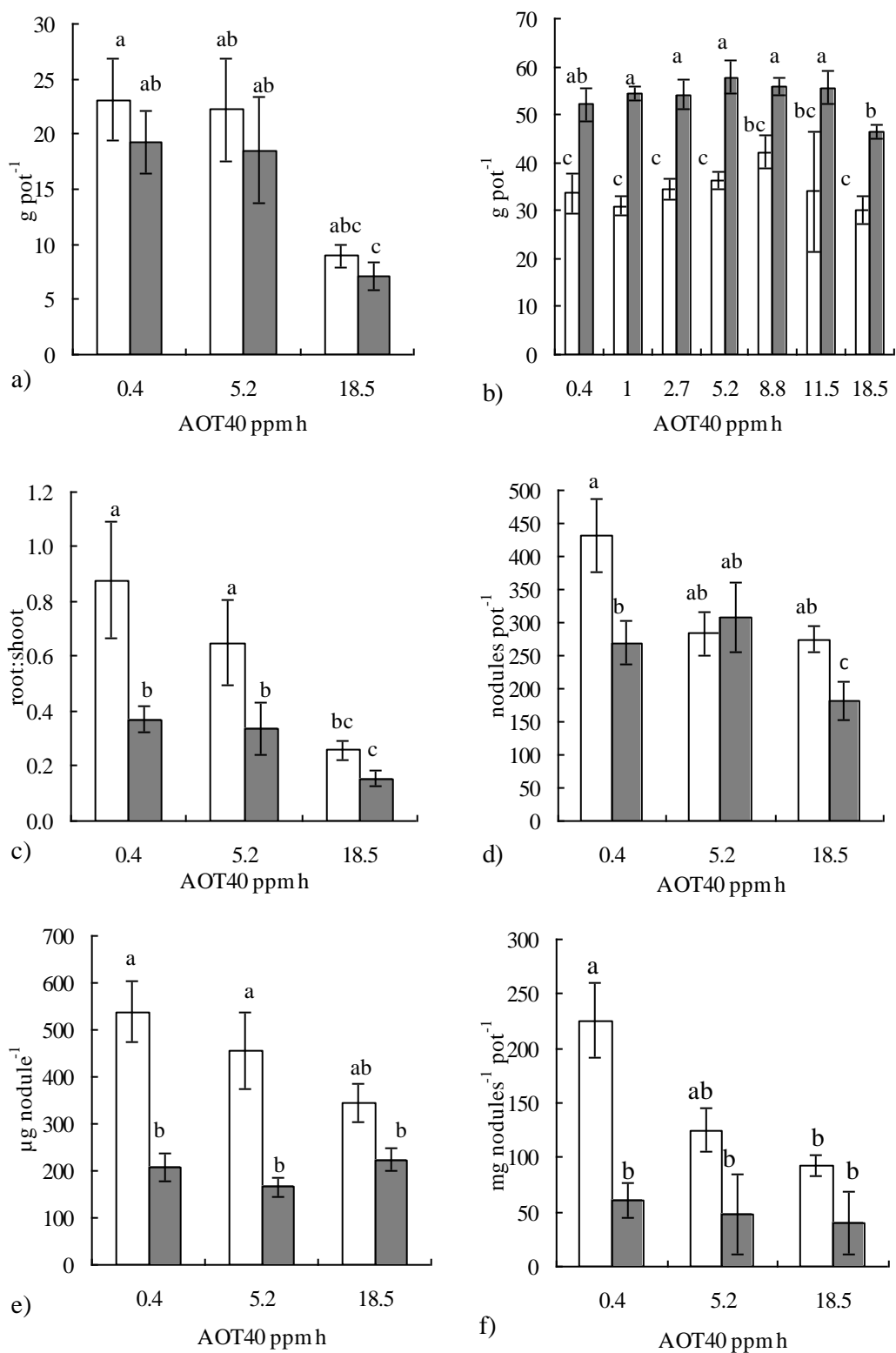
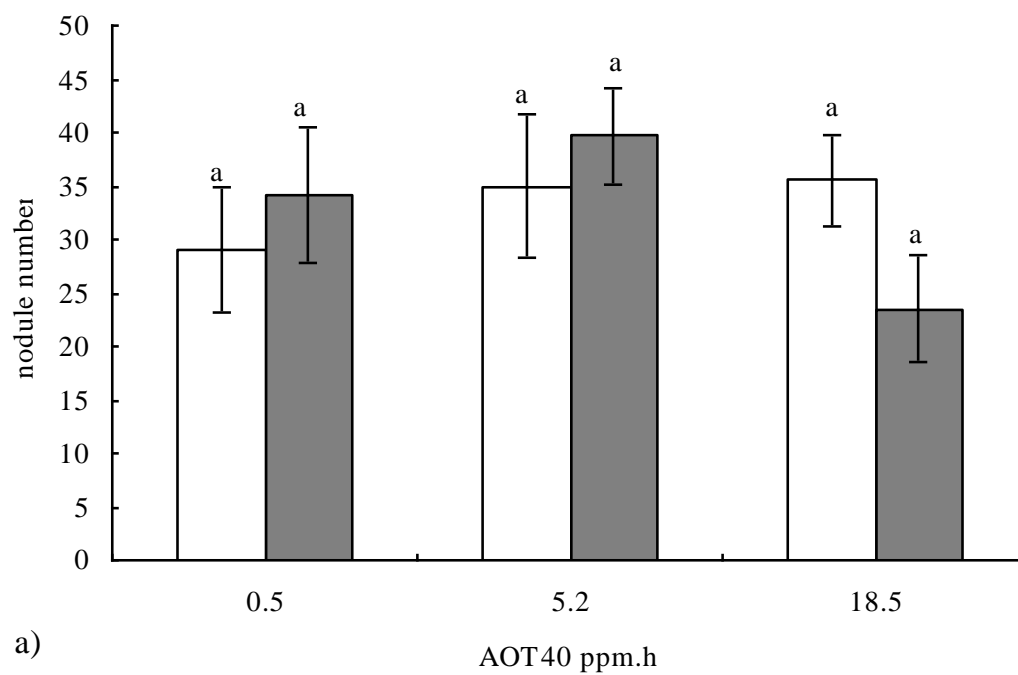
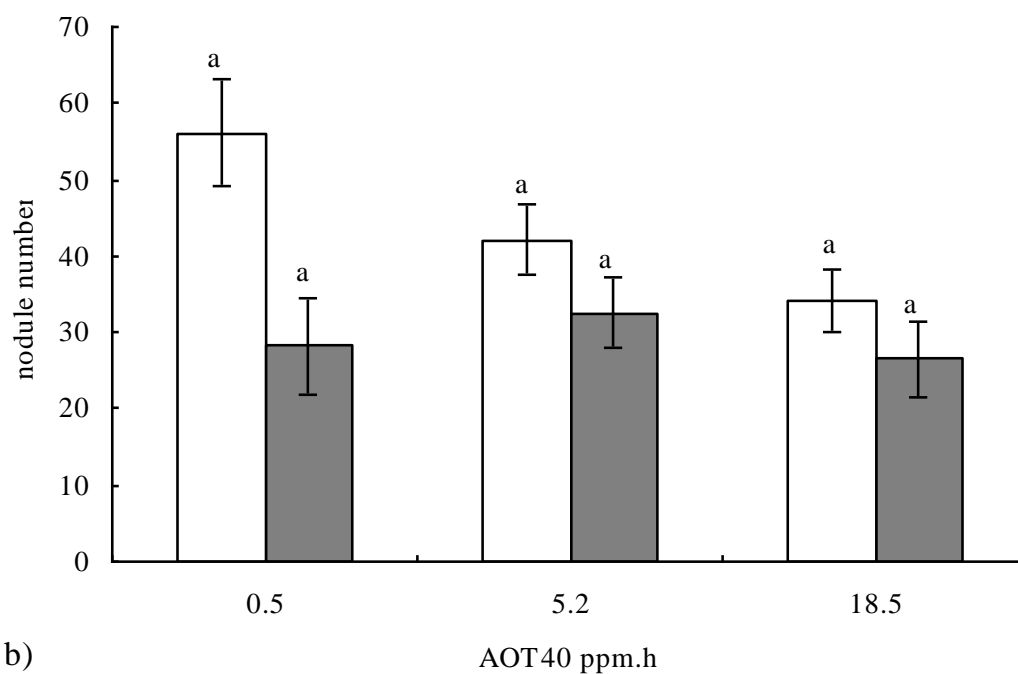


Figure 2.3: Change in (a) root biomass  $\text{pot}^{-1}$  (b) seasonal shoot biomass  $\text{pot}^{-1}$  (c) root:shoot (d) nodules  $\text{pot}^{-1}$  (e) mass nodule  $\text{pot}^{-1}$  & (f) nodule mass  $\text{pot}^{-1}$  in relation to 3 month AOT40. For shoot biomass  $\text{pot}^{-1}$ , pairwise differences are excluded for clarity (where white bars = Crusader; grey bars= Merviot; different letters denote a difference at the  $p=0.05$  level after post-hoc Tukey tests).



a)



b)

Figure 2.4: Effects of ozone on nodule size (a) nodules between 0.1mm-0.7mm maximum length (b) nodules >0.7mm long (where white bars = Crusader; Grey bars = Merviot. Different letters denote a difference at the  $p=0.05$  level after post-hoc Tukey tests).

### 2.3.4. ARA

In both assays, a small amount of ethylene was detected after 0 hours, less than 1% of the amount present at the end of the incubation (not shown). In the week 10 assay, mean ethylene evolution per  $\text{cm}^2$  of soil surface showed a trend for a reduction in treatment 7 after 4 hours incubation compared to treatment 1 ( $p=0.06$ , Figure 2.5a). In week 11, ethylene evolution per  $\text{cm}^2$  was significantly reduced in treatment 7 after 8 hours ( $p=0.05$ , Figure 2.5b). No ethylene was detected in either assay after 24 hours.

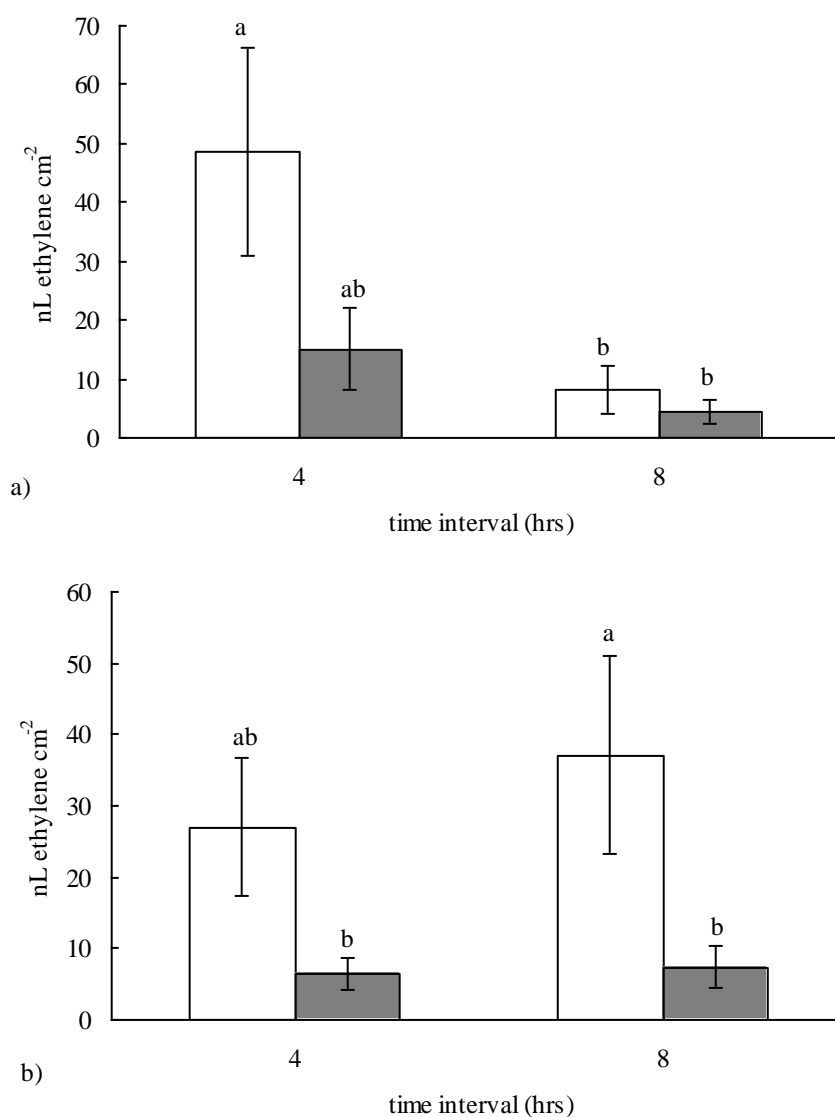


Figure 2.5: Ethylene evolution in (a) week 10 and (b) week 11 ARAs (where white bars = low ozone treatment 1; grey bars = high ozone treatment 7; different letters denote a difference at the  $p=0.05$  level after post-hoc Tukey tests).

## 2.4. Discussion

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

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

The overall reduction in nodules-per-pot observed in both cultivars may have arisen from a general reduction in the translocation of photoassimilates to the root system, but more specifically due to an enhanced regulation of nodulation via downstream AON (Mortier et al. 2012). A reduction in nodule growth in Crusader, manifesting in a reduced mass-per-nodule and an increased proportion of small, likely

non-fixing, pseudonodules (Figure 2.4), would also suggest a reduced availability of assimilate in the root system. This also explains consistent differences in nodule activity (measured by in-situ ARA) in Crusader between treatments 1 and 7. In Merviot, higher gs rates may hint at a greater capacity to supply root nodules with assimilates during ozone-induced oxidative stress (Figure 2.2), explaining why the growth of individual root nodules was unaffected (Figures 2.3e; 2.4b).

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

In subterranean clover (*T. subterranean*), exposure to comparable mean concentrations of ozone affect forage quality in as little as 30 days (Sanz et al. 2005), with impacts in *T. repens* readily apparent after a 3 month period (González-Fernández et al. 2008). However, ozone impacts on forage quality, and, in particular,



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

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

### **2.5. Conclusions**

This study has provided for the first time some insight into beneficial effects of progressive controls on ozone precursors. On the evidence, controls leading to decreases in peak ozone concentrations by ~30ppb and baseline concentrations by ~10ppb may increase root nodule biomass of white clover by as much as 45%.

Controls on the emission of ozone precursors have been included in recent multi-model predictions, suggesting a globally reduced tropospheric ozone burden by year 2030 in most representative concentration pathways (RCPs) (Young et al. 2013), with regional concentrations displaying an increased sensitivity to climate change (Langner et al. 2013). The potential impacts of ozone on the biomass, nodulation and N-fixation of clover described in this study thus provide a continuing economic and environmental incentive for controls on the emission of trans-boundary ozone precursors.

### **3. DEVELOPING OZONE-DOSE RELATIONSHIPS FOR IMPACTS ON THE FUNCTIONING AND QUALITY OF TEMPERATE PASTURE**

#### **3.1. Introduction**

Concentrations of tropospheric ozone have increased worldwide since the start of the industrial era (IPCC, 2013) and ground-level ozone is presently thought to be the most important air pollutant that directly affects vegetation (Ashmore, 2005). The largest increases in background ozone have occurred over the industrialised northern hemisphere, which reports an annual average of 30-40ppb over the mid-latitudes (Royal Society, 2008) with additional increases likely in the coming decades (IPCC, 2013). In Europe, ambient surface concentrations frequently exceed critical thresholds for plant protection (EEA, 2011, Mills et al. 2011a). Despite sustained efforts to control the emission of ozone precursor compounds, decreased cloud cover and increasing temperature are expected to increase the potential for ozone formation in the European region during this century (Katrakou et al. 2011; IPCC, 2013).

Historically, most studies concerning the effects of ozone have focused on the impacts on arable crops and forests, though in recent years more attention has been paid to the responses of natural and semi-natural grassland communities (e.g. Råmo et al. 2006; Hayes et al. 2007, 2010b; Wagg et al. 2013; Wyness et al. 2011). In Europe, open-pasture grasslands constitute ~35% of the agriculturally utilised area (Smit et al. 2008), and are of high value in terms of biodiversity and food production. As previously stated by Ashmore et al. 2007, the importance of grassland as a large continental sink for the deposition of ozone, coupled with evidence of significant ozone effects on grassland species and communities, highlights the need to quantify

the risks of rising ozone. Amongst other effects, ozone induces premature senescence (e.g. Hayes et al. 2010) and decreases the forage quality (González-Fernández et al. 2008; Gilliland et al. 2012), gross primary production (GPP) (Calvete-Sogo et al. 2014) and yield of grassland vegetation (e.g. Danielsson et al. 2013). Grasslands may contain species both tolerant and sensitive to ozone (Hayes et al. 2007), and grassland legumes are thought to be particularly sensitive (e.g. Hayes et al. 2007). However, intact mature and long-established grassland may display a large degree of inertia to ozone stress (e.g. Stampfli & Fuhrer, 2010; Bassin et al. 2013; Volk et al. 2014), and, in general, the response of temperate grasslands to rising ozone is uncertain.

Perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) are globally-important components of natural, semi-natural and annually seeded grasslands. Several previous studies have considered the effects of ozone on the functioning of *Lolium/Trifolium* pasture, with most having focused on the response of above-ground biomass (e.g. Fuhrer et al. 1994; Nussbaum et al. 1995; Wilbourn et al. 1995). The effects of ozone may vary substantially in key agricultural species, such that more modern cultivars, bred for higher productivity, may display increased sensitivity to ozone (e.g. in wheat, Velissariou et al. 1991; Pleijel et al. 2006). The effects of ozone on modern cultivars of both *L. perenne* and *T. repens* are virtually unknown (González-Fernández et al. 2008; Hewitt et al. 2014). In recent years, environmental and economic concerns have led to the development of high-sugar ryegrass (HSG), designed to improve the uptake, digestion and nitrogen (N)-utilisation of grazing stock in managed pasture, and suggested as having the potential to increase production yields and reduce methane (CH<sub>4</sub>) and N emissions from livestock farming (Ellis et al. 2011; Staerfl et al. 2012). In parallel, rising fertiliser costs, the low N-use efficiency of traditional fertilisers in pasture, and climate change, have also spurred

the development of new clover varieties for varied functions in pasture and fodder (British Grassland Society, 2014).

The phytotoxic ozone dose above a threshold of  $\text{Ynmol m}^{-2} \text{ s}^{-1}$  ( $\text{POD}_Y$ ) is an exposure index based on the modelled flux of ozone into the plant via the stomatal pores. Stomatal conductance of ozone is estimated using the complex multiplicative Jarvis-type conductance model (Jarvis, 1976), which requires the parametisation of biotic and abiotic factors such as phenology, temperature and light (Emberson et al. 2000). Consequently, the ozone uptake-based POD model provides a better fit to effect-data than concentration-based exposure-based indices, and flux-based critical levels of ozone have been developed for key arable crop and forest species (*e.g.* Mills et al. 2011; Danielsson et al. 2013). For productive grasslands, and grasslands of high conservation value, an accumulated 3-month  $\text{POD}_1$  of  $2\text{mmol m}^{-2}$  has been provisionally determined as the critical level, representing the ozone flux required to induce a 10% reduction in the shoot biomass yield of *T. repens* (Mills et al. 2011b). This critical level is currently only based on UK and Swiss data, and dose-response relationships suitable for derivation of critical level for several other aspects of managed pasture, including N-fixation and forage quality, remain undeveloped.

In this study, we investigate the changing effects of ozone on pasture mesocosms containing a variety of HSG (*L.perenne* cv. AberMagic) and a modern cultivar of white clover (*T. repens* cv. Crusader). Flux-based dose-response relationships are constructed for above and below-ground biomass, nodulation, injury, N-fixation and forage quality parameters.

## **3.2. Methods**

### **3.2.1. Pasture mesocosms**

In early April 2013, *Lolium perenne* cv. AberMagic, recommended for long-term pasture (British Grassland Society, 2014), was sown as seed at a density of 0.28g directly into 10 L pots (27.5 cm diameter x 22cm height) filled with compost (John Innes No. 2; J. Arthur Bowers, Lincoln, UK). *T. repens* cv. Crusader plants, recommended for general use in grassland leys (British Grassland Society, 2014), were also propagated from seed, grown in the same compost in plug-plant trays in an unheated glasshouse. Seeds were obtained from a commercial seed supplier (Wynnstay Seeds; UK). After 4 weeks of growth, 3 clover plants were transferred to each pot, with one clover plant in each third of the pot. A plastic collar (6cm diameter, 5cm depth) was inserted to a depth of 4cm within the centre of each pot for subsequent acetylene reduction assays. To introduce a soil microbe population, pots were inoculated with 400ml of a soil slurry mixture made from 5kg of soil from agricultural grassland (Abergwyngregyn, North Wales, UK, 53°14'N, 4°01'W) and 14L water. Mesocosms were grown for a further 4 weeks in ventilated greenhouses under optimum watering conditions. On 07/06/2013, 24 pots, of equal size and distribution of clover and grass, were transferred to each of 6 'solardomes' (hemispherical glasshouses; 3m diameter, 2.1m high) at the CEH solardome facility near Bangor, North Wales.

### **3.2.2. Ozone system and treatment**

Ozone exposure was based on an episodic profile recorded at a rural ozone monitoring site (Aston Hill, Wales, UK, 52°50'N, 3°03'W) with a unique, weekly repeating treatment in each solardome as described previously (Hewitt et al. 2014). Treatments were applied to the solardomes randomly. In one solardome, ambient air temperature, soil moisture content, photosynthetically active radiation (PAR) and relative humidity

were continuously monitored by an automatic weather station and soil moisture probes (Theta Probe, Skye Instruments Ltd, Llandridod Wells, UK; Delta-T Devices Ltd, Cambridge, UK). Plants were rotated within each dome weekly and watered twice-weekly, with additional watering when necessary to maintain soil moisture content at or near field capacity. Plants were exposed to the ozone treatments for a 16 week period, starting 11/06/2013 and finishing 01/10/2013 (Table 3.1). Although ozone treatments were not replicated, numerous previous studies have established the statistical validity of experiments conducting using the solardome facility with 6-8 ozone treatments (e.g. Hayes et al. 2009; 2010; Hewitt et al. 2014; Wagg et al. 2013).

Table 3.1: Start, end and harvest dates for the experiment.

Date	Activity
11/06/2013	Experiment start
09/07/2013	1 <sup>st</sup> harvest
06/08/2013	2 <sup>nd</sup> harvest
03/09/2013	3 <sup>rd</sup> harvest
01/10/2013	4 <sup>th</sup> harvest; experiment end.

### 3.2.3. Biomass assessments

Assessments of above-ground biomass were performed every 4 weeks (Table 3.1).

Within each solardome, the shoot biomass in 6 randomly-selected pots was cut to soil level and sorted by species, and the pots were subsequently removed from the experiment. For the remaining pots, a maintenance cut was performed by cutting back to 4cm. After 16 weeks, the root biomass of each cultivar was determined on a representative quarter of the 6 remaining pots in each ozone treatment. In Crusader, root nodules were also carefully excised from the root system. Shoots and roots were dried for a minimum of 48 hours at 60°C or until constant mass was achieved. Nodule biomass was air dried, sized and weighed. Root biomass, nodule mass and nodule numbers per pot were calculated as described previously (Hewitt et al. 2014).

#### **3.2.4. Visual assessment**

Prior to the harvest of shoot biomass, a representative quarter of each pot was selected and the total amount of injured or senesced *T. repens* leaves scored. As previously described (Hayes et al. 2010a; Hewitt et al. 2014), leaves were classified as injured or senesced if >25% of the adaxial leaf surface was affected by injury or senescence respectively. To account for differences in shoot biomass between treatments, injury and senescence was expressed on a percentage basis. Injury could not easily be scored on *L. perenne* after 4 weeks.

#### **3.2.5. Nitrogen fixation**

Assessments of clover nitrogenase activity were performed every four weeks, prior to cutting, using an acetylene reduction assay (ARA) method adapted from Lindström (1984) and Hewitt et al. (2014). A sealable 400ml plastic bottle, with the bottom removed and fitted with a gas septum, was inserted to a depth of 2cm into the soil within the collar previously placed in the centre each pot. For the assay, a 10% acetylene atmosphere was generated inside the bottle by removing 10% of the air and immediately replacing it with acetylene gas (BOC, Guildford, UK). Acetylene was stored and transported to the solardome facility in inert gas bags (SUPELCO, Bellefonte, US), which were vented to the atmosphere and flushed through with N<sub>2</sub> after use. 15 ml gas samples were taken from the bottles at 0 and 3hrs incubation, with a 1ml sub-sample analysed for ethylene content using a mass-selective detector (Model 6890, Agilent Technologies, Santa Clara, US). Ethylene peak area was determined using G1701DA analytical software (version D.00.00.38; Agilent Technologies, Santa Clara, US). ARA were performed prior to the maintenance cut-backs at 4, 8, 12 and 16 weeks.



### 3.2.6. Forage quality

At each 4-week harvest, the dried shoot biomass, including the total forage of both AberMagic and Crusader, was pooled in each ozone treatment. A range of nutritive quality characteristics were determined for each ozone treatment by near-infrared reflectance (NIR) using 20-30g of the mixed shoot biomass per parameter (AUNIR Ltd, England). The relative feed value (RFV) of the forage was calculated based on a standard control mixture containing 28% neutral detergent fibre (NDF) and 22% acid detergent fibre (ADF). The consumable food value (CFV) was calculated as follows,

$$\% \text{ CFV} = 100 (\text{RY} * \text{RFV})$$

with RY representing dry biomass relative to the lowest-ozone treatment, treatment 1 (Table 3.5).

### 3.2.7. Ozone dose-response relationships

Ozone flux was calculated according to the Convention on Long-range transporting Air Pollution Modelling and Mapping manual (CLRTAP, 2010), using the DO<sub>3</sub>SE model (Deposition of Ozone for Stomatal Exchange, available at <http://sei-international.org/do3se>). For Crusader, the DO<sub>3</sub>SE model parameterisation was determined from monitored environmental variables and accumulated abaxial *g<sub>s</sub>* measurements (n=607), adapted from a default parameterisation available with the DO<sub>3</sub>SE model (Table 3.2). Due to insufficient *g<sub>s</sub>* data, a default parameterisation for *Lolium perenne* was used for AberMagic (Table 3.2) (Ashmore et al. 2007).

Accumulated stomatal flux was calculated for each cultivar based on ozone flux in the upper leaves, with accumulation thresholds ('Y' in POD<sub>Y</sub>) of 6 nmol m<sup>-2</sup> s<sup>-1</sup> (POD<sub>6</sub>) and 1 nmol m<sup>-2</sup> s<sup>-1</sup> (POD<sub>1</sub>) for AberMagic and Crusader respectively. Although a good fit (*r*<sup>2</sup>) to biomass data was also found with other Y values after testing (Figure 3.1),

these values were chosen due to their closeness to recommended selection criteria (within 3% of an intercept of 1). The selected Y-values also have their decent spread along the X-axis, and consistency with existing literature (Ashmore et al. 2007; Mills et al. 2011d). In addition to calculating the flux above the selected Y values,  $POD_0$  values, assuming no threshold for effects (Sanz et al. 2014), were also determined for each species. Leaf area index was determined from dried biomass samples and ranged 0.37-4.17  $m^2/m^2$  for AberMagic and 0.95-5.15  $m^2/m^2$  for Crusader. For both cultivars, the phenology function ( $f_{phen}$ ) was assumed to be fixed at 1 throughout the season due to the frequent cuts. For Crusader, leaf dimension, canopy height and root depth were set at 0.07m, 0.30m and 0.15m respectfully. Total accumulated flux values were determined in each ozone treatment at 4, 8, 12 and 16 weeks. In addition to total flux accumulated at each harvest point, the flux accumulated in the preceding 4 weeks before each harvest was also determined (“growth cycle flux”).

Table 3.2: Parameterisations for stomatal flux algorithms for *T. repens* cv. Crusader and *L. perenne* cv. AberMagic (where  $G_{max}$  = maximum stomatal conductance,  $G_{light}$  =  $f_{light}$ ,  $T_{max}$  = maximum temperature,  $T_{min}$  = minimum temperature,  $T_{opt}$  = optimum temperature,  $VPD_{max}$  = maximum vapour pressure deficit,  $VPD_{min}$  = minimum vapour pressure deficit,  $SWP_{min}$  = minimum soil moisture potential,  $SWP_{max}$  = maximum soil moisture potential;  $f_{phen}$  = phenology function).

	AberMagic	Crusader
$G_{max}$	295	580
$G_{light}$	0.009	0.007
$T_{max}$	40 °C	30.9 °C
$T_{min}$	10 °C	16 °C
$T_{opt}$	25 °C	24.5 °C
$VPD_{max}$	2.0 kPa	2.1 kPa
$VPD_{min}$	4.0 kPa	3.5 kPa
$SWP_{min}$	-1.5 MPa	-0.04 MPa
$SWP_{max}$	-0.49 MPa	-0.01 MPa
$f_{phen}$	1	1

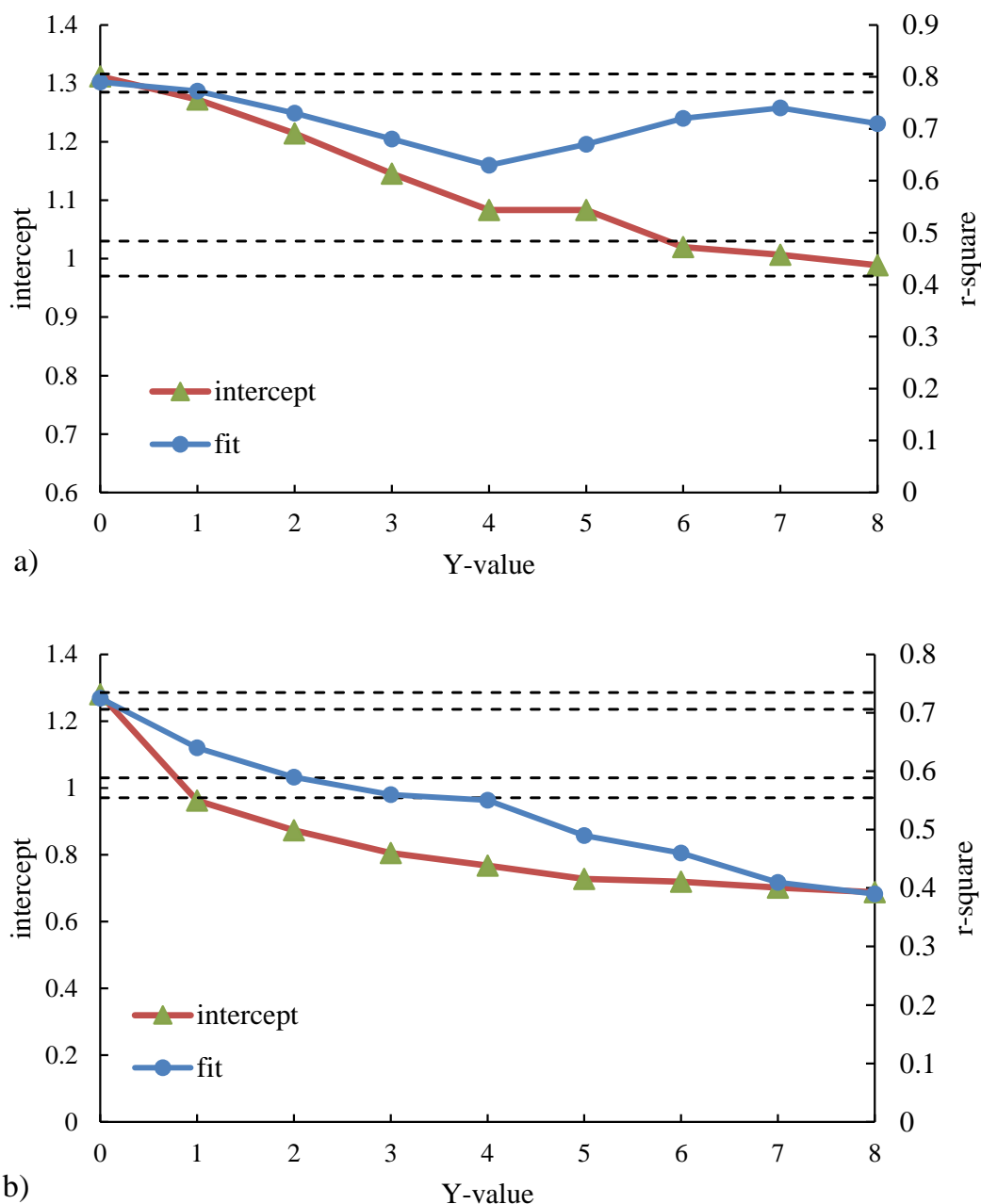


Figure 3.1: Intercept and fit ( $r^2$ ) of modelled Y-values against root biomass in AberMagic (a) and Crusader (b). Dashed lines indicate selection criteria range for Y-values, based on an intercept within 3% of 100 and an  $r^2$  value within 2% of the maximum.

### 3.2.8. Statistical analysis

For consistency with existing literature, mean biomass variables were converted to relative values based on zero PODy. Injury data was arcsine transformed prior to analysis. Shoot biomass, injury, N-fixation and forage quality parameters were

analysed by linear regression against growth cycle flux. For shoot biomass and forage quality parameters, analysis was also conducted on a seasonal basis using total accumulated fluxes. For below-ground biomass variables, determined only at the end of the experiment, linear regression was performed against the 16 week accumulated flux values. In the case of forage quality parameters, variables could not be attributed to an individual cultivar, and regression analysis was instead conducted for seasonal data against ozone flux for the mixed canopy. This was estimated as the weighted average of  $POD_0$  for the two species, determined using the percentage of each in the upper canopy (>4cm) at the time of harvest (see Table 3.5). All analyses were conducted in R software (Version 3.1.1).

### 3.3. Results

#### 3.3.1. Ozone concentrations and climate conditions

During the experiment, the six ozone treatments generated seasonal 24 hr means of 35, 41, 47, 51, 59 and 67ppb and accumulated daylight exposures above a threshold of 40ppb (AOT40) of 1.7, 5.2, 8.9, 12.0, 18.2 and 25.0 ppm.h respectively (Figure 3.2; Table 3.3). Mean daylight (when  $PAR > 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) air temperature and VPD were 18.4°C and 1.25 kPa for the study period, with maxima of 34.3°C and 3.05 kPa (Table 3.4). Mean daytime PAR was  $542 \mu\text{mol m}^{-2} \text{s}^{-1}$ , with an average daily maximum of  $1322 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Table 3.4).

Table 3.3: Seasonal mean, and accumulated ozone exposure (accumulated exposure over a threshold of 40ppb (AOT40)) for ozone treatments.

Treatment	Mean ozone (ppb)	AOT40 (ppm.h)
1	35	1.7
2	41	5.2
3	47	8.9
4	51	12.0
5	59	18.2
6	67	25.0

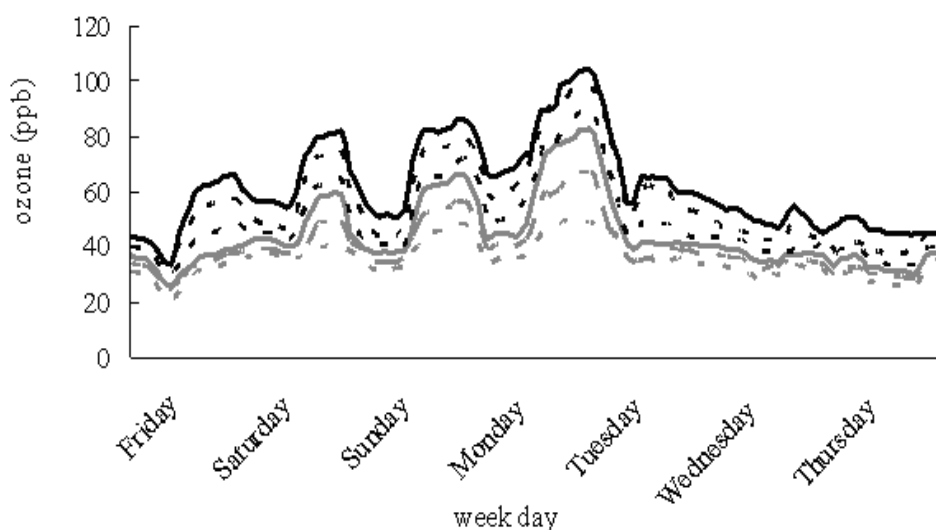


Figure 3.2: Average weekly ozone profile for the seven ozone treatments (see Table 3.4 for treatment details).

Table 3.4: 24hr mean, and mean, maximum and minimum daytime value for meteorological parameters during the course of the experiment (where PAR = photosynthetically active radiation; VPD = vapour pressure deficit).

Climate averages	24hr mean	daytime min.	daytime max.	daytime mean
PAR ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )			1322	542
Air temperature ( $^{\circ}\text{C}$ )	20.2	15.6	34.3	18.4
VPD (kPa)	0.76	0.13	3.05	1.25

### 3.3.2. Biomass assessments

For raw biomass data see Table 3.5. On a seasonal basis, above ground biomass of *L. perenne* was unaffected by total accumulated ozone (Figure 3.3a), and was not significantly correlated with  $\text{POD}_6$  at any individual harvest (Figure 3.3c; Table 3.6).

In contrast, the relative yield of *T. repens* had a significant ( $p=0.02$ ) negative relationship with accumulated  $\text{POD}_1$  over the course of the season (Figure 3.3b).

Analysis of individual harvest data also revealed strong negative responses in *T. repens* yield to  $\text{POD}_1$  between weeks 4 and 8, and between 8 and 12, with the largest

increase in  $\text{POD}_1$  occurring in the latter period (Figure 3.3b; Table 3.6). Negative responses were found in the relative root biomass of both species, with a ~8% reduction in *L. perenne* ( $p=0.03$ ) and a 9% reduction in *T. repens* ( $p=0.05$ ) at a  $\text{POD}$  of  $2\text{mmol m}^{-2}$  (Figure 3.4a, b). Strong negative responses were also revealed in *T. repens* nodule biomass per pot ( $p=0.01$ ) and mass-per-nodule ( $p=0.007$ ), with >50% reduction in each parameter at an accumulated  $\text{POD}_1$  of  $11\text{mmol m}^{-2}$  (Figure 3.5a, b). The relative density of nodules on clover roots displayed a trend towards a linear increase in response to ozone uptake ( $p=0.08$ ) (Figure 3.5c). Root nodule number per pot was unaffected by ozone (data not shown).

Table 3.5: Biomass variables including mean (n=6) and standard error (SE), and intercept after regression against accumulated POD<sub>6</sub> or POD<sub>1</sub> values, to derive the means as relative values.

cultivar	variable	mean	SE	ozone treatment	harvest week	intercept	% of upper canopy (>4cm)	
AberMagic	shoot biomass (g pot <sup>-1</sup> )	21.6	1.9	1	4	25.96	81	
		29.7	3.2	2		25.96	88	
		27.9	3.7	3		25.96	89	
		20.7	2.3	4		25.96	89	
		31.4	2.4	5		25.96	89	
		24.3	1.8	6		25.96	87	
			13.4	1.2	1	8	11.99	38
			12.5	0.8	2		11.99	32
			11.0	1.0	3		11.99	27
			13.0	1.7	4		11.99	41
			14.6	1.3	5		11.99	43
			14.7	1.6	6		11.99	51
			12.4	2.7	1	12	11.48	15
			9.4	2.2	2		11.48	15
			8.5	1.7	3		11.48	20
			9.4	2.9	4		11.48	17
			10.1	0.9	5		11.48	12
			9.9	2.4	6		11.48	23
			11.5	2.8	1	16	10.11	15
			9.2	0.6	2		10.11	9
			9.0	0.8	3		10.11	10
			10.3	0.8	4		10.11	15
			8.6	0.8	5		10.11	11
			9.2	0.7	6		10.11	12
	root biomass (g pot <sup>-1</sup> )	320.3	101.8	1	16	284.16		
		231.2	71.2	2		284.16		
		291.1	53.1	3		284.16		
		210.6	50.4	4		284.16		
		223.2	23.8	5		284.16		
		146.4	31.9	6		284.16		
Crusader	shoot biomass (g pot <sup>-1</sup> )	5.3	1.1	1	4	5.18	19	
		5.2	1.0	2		5.18	12	
		4.1	0.7	3		5.18	11	
		2.6	0.6	4		5.18	11	
		3.6	0.8	5		5.18	11	
		4.4	0.6	6		5.18	13	
			11.3	1.1	1	8	16.55	62
			14.3	1.3	2		16.55	68
			13.4	1.3	3		16.55	73
			7.6	0.7	4		16.55	59
			8.5	1.4	5		16.55	57
			6.7	0.3	6		16.55	49
			27.8	2.4	1	12	33.01	85
			25.2	3.3	2		33.01	85
			26.5	2.4	3		33.01	80
			23.7	3.2	4		33.01	83
			19.1	1.5	5		33.01	88
			20.6	2.4	6		33.01	77
			20.0	2.7	1	16	24.165	85
			20.5	1.5	2		24.165	91
			19.0	1.8	3		24.165	90
			15.3	1.9	4		24.165	85
			16.2	1.4	5		24.165	89
			15.0	2.1	6		24.165	88
	root biomass (g pot <sup>-1</sup> )	27.0	1.1	1	16	27.63		
		19.6	4.9	2		27.63		
		15.5	4.2	3		27.63		
		13.6	4.1	4		27.63		
		15.0	5.8	5		27.63		
		12.7	1.8	6		27.63		
	nodule biomass (mg pot <sup>-1</sup> )	164.7	26.4	1	16	219		
		187.0	39.3	2		219		
		109.8	22.0	3		219		
		105.8	51.2	4		219		
		90.1	13.2	5		219		
		74.3	18.4	6		219		
	mass nodule <sup>-1</sup> (µg)	137.1	22.6	1	16	157		
		114.5	22.0	2		157		
		91.0	19.8	3		157		
		73.4	25.4	4		157		
		73.7	9.9	5		157		
		59.6	10.8	6		157		
	nodule number gramme <sup>-1</sup>	4.6	0.9	1	16	4.75		
		8.4	1.1	2		4.75		
		8.7	2.2	3		4.75		
		9.3	2.8	4		4.75		
		8.9	1.5	5		4.75		
		10.3	1.6	6		4.75		

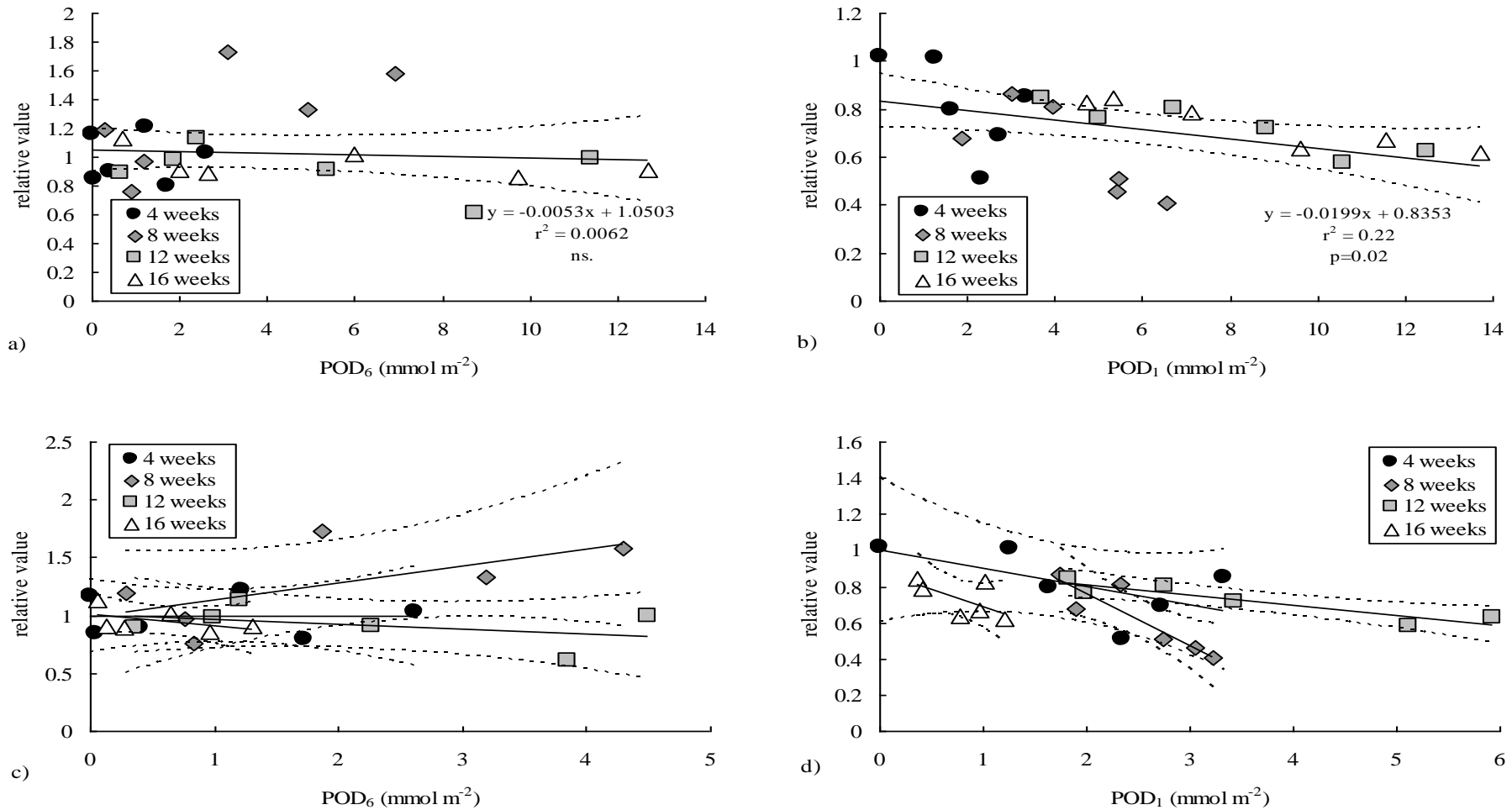


Figure 3.3: Ozone dose-response relationships for seasonal above-ground biomass production in (a) AberMagic and (b) Crusader and biomass production against growth cycle flux in (c) AberMagic (d) Crusader (where black circles = week 4; dark grey diamonds = week 8; light grey squares = week 12; white triangles = week 16;  $n=6$ ; dashed lines indicate 95% confidence intervals). See Table 3.6 for response function details of individual harvests.



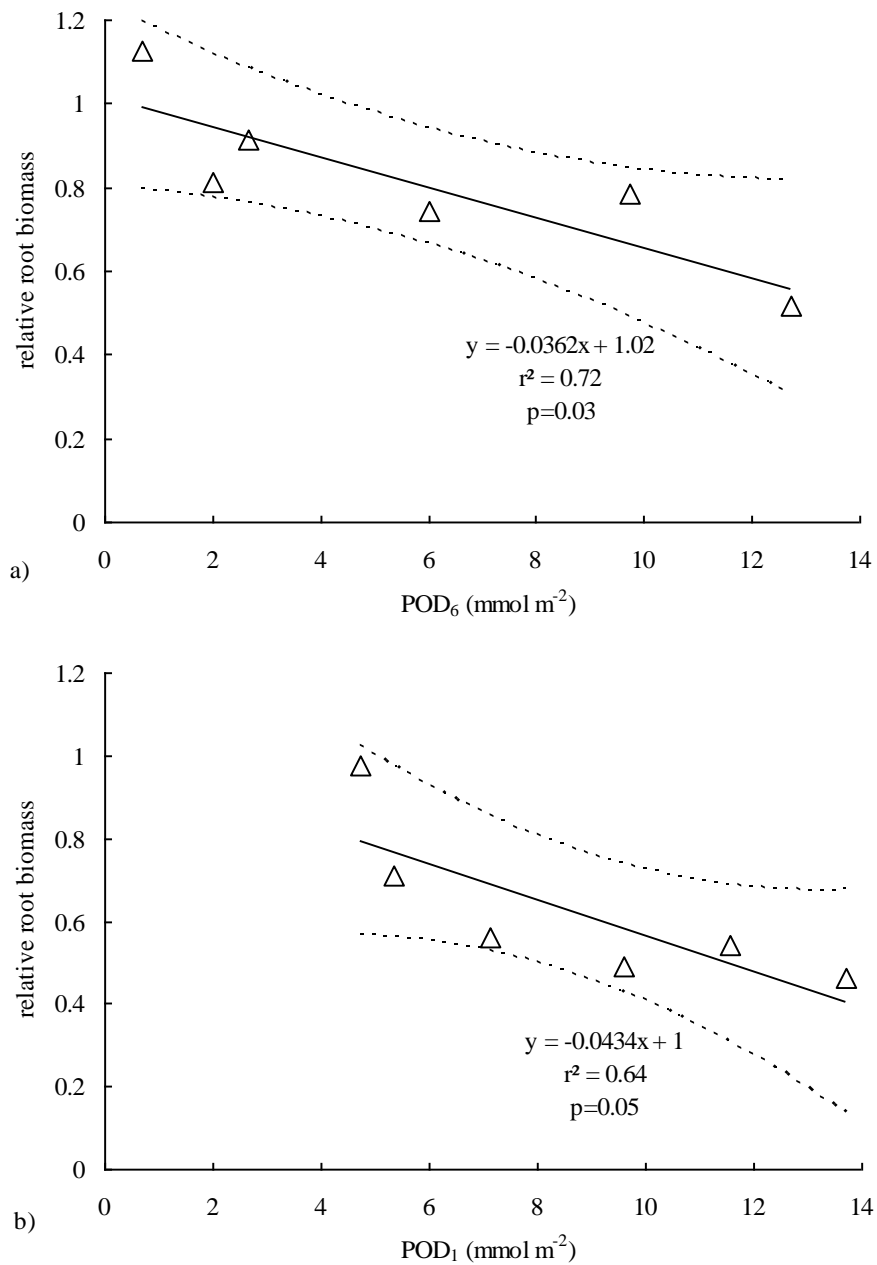


Figure 3.4: Ozone dose-response relationships for root biomass in (a) AberMagic and (b) Crusader against 16 week accumulated  $POD_6$  or  $POD_1$  values respectively.

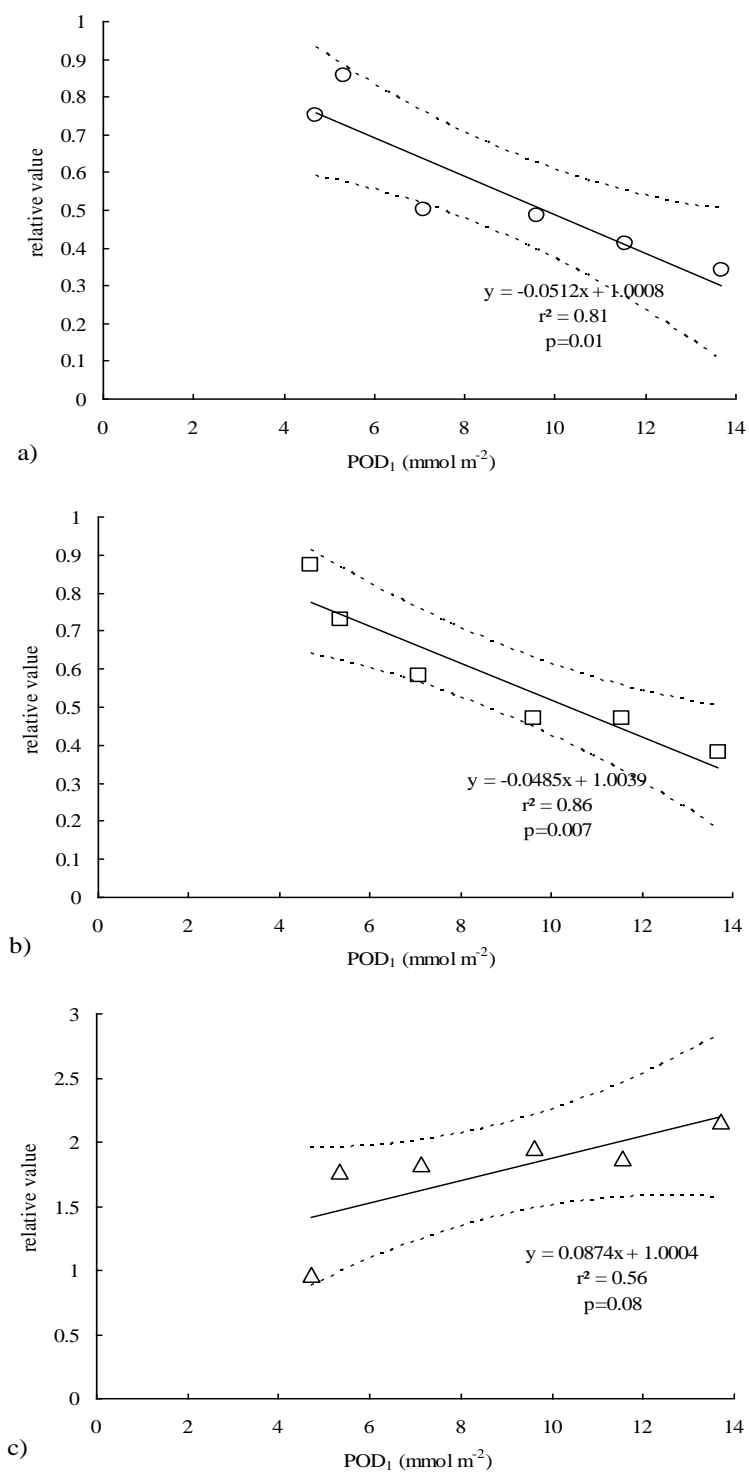


Figure 3.5: Dose-response relationships for mean nodule biomass per pot (a) mass-per-nodule (b) and nodule density (c) of crusader plants against 16 week accumulated  $POD_1$  (n=6; dashed lines indicate 95% confidence intervals).

### 3.3.3. Visual assessments

Analysis revealed strong positive responses in total ozone injury in *T. repens* to growth cycle flux assessed at weeks 4, 8 and 12 (Figure 3.6; Table 3.6), with a decreasing occurrence of injury over time. Between weeks 12 and 16, injury was not significantly related to  $POD_1$ . Although natural senescence was detectable during the latter half of the experiment, senescence without the presence of ozone-injury was not counted.

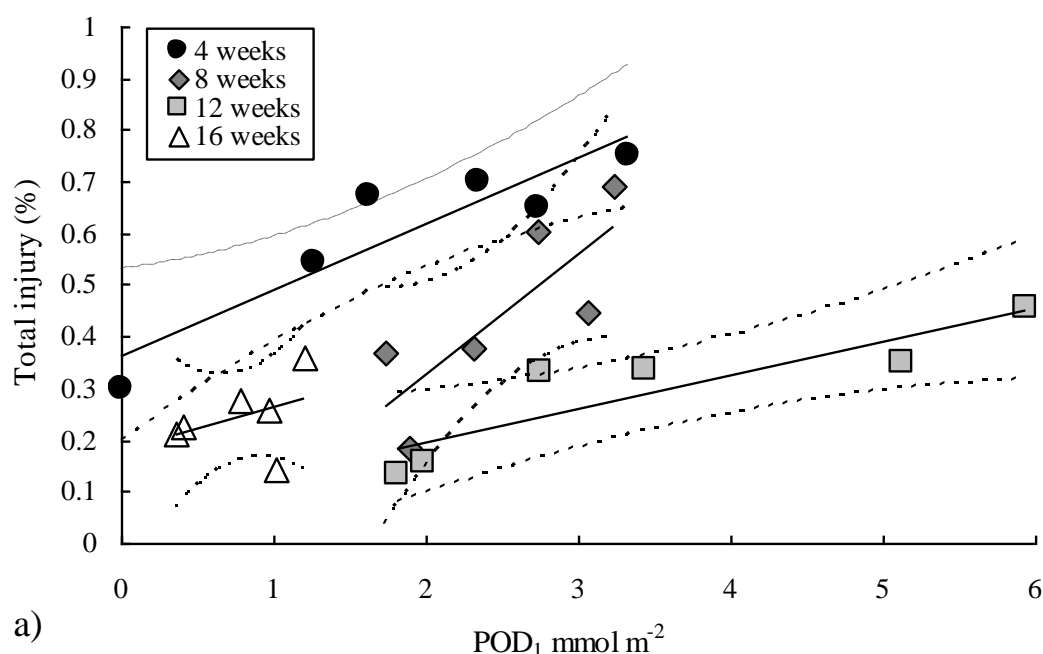


Figure 3.6: Dose-response relationships for mean ozone injury in *T. repens* cv. Crusader (injury + senescence) against growth cycle flux (where black circles = week 4; dark grey diamonds = week 8; light grey squares = week 12; white triangles = week 16;  $n=6$ ; dashed lines indicate 95% confidence intervals). See Table 3.6 for response function details.

### 3.3.4. Nitrogen fixation

No ethylene was detected at 0 hours incubation in any of the ARAs. After 3 hours incubation, measured nitrogen fixation rates had a strong linear decline against  $POD_1$  in week 4 ( $r^2=0.67$ ;  $p=0.04$ ) (Figure 3.7; Table 3.6) and between weeks 4 and 8 ( $r^2=0.82$ ;  $p=0.01$ ) (Figure 3.7; Table 3.6). AR activity showed no response to ozone between weeks 8 and 12, and between weeks 12 and 16 (Figure 3.7; Table 3.6).

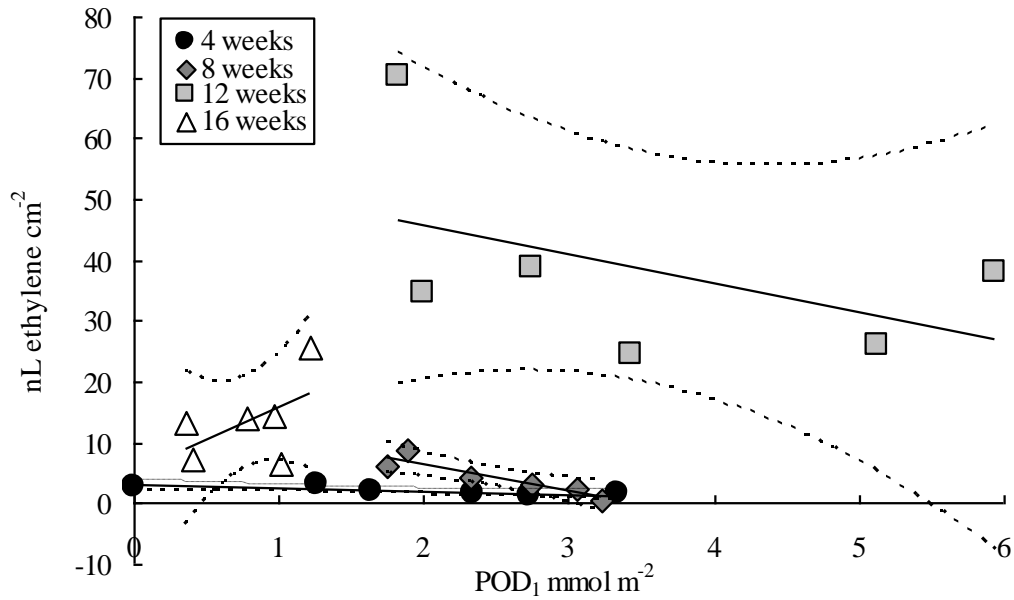


Figure 3.7: Dose-response relationships for mean nitrogen fixation rates in pasture mesocosms, determined after 3 hours ARA incubation prior to harvest and analysed against growth cycle flux (where black circles = week 4; dark grey diamonds = week 8; light grey squares = week 12; white triangles = week 16;  $n=6$ ; dashed lines indicate 95% confidence intervals). See Table 3.6 for response function details.

Table 3.6: Response functions for ozone effects on shoot biomass production in AberMagic/Crusader pasture mesocosms, and for total ozone-injury and ARAs determined against growth cycle fluxes. Asterisks denote a significant ozone effect at  $p \leq 0.05$ .

Relative yield				
cultivar	harvest week	response function	$r^2$	p-value
AberMagic	4	$-0.0005 \times \text{POD}_6 + 0.99$	<10	0.95
	8	$0.145 \times \text{POD}_6 + 0.98$	0.39	0.18
	12	$-0.0424 \times \text{POD}_6 + 1.01$	0.16	0.42
	16	$-0.0953 \times \text{POD}_6 + 1.00$	0.21	0.35
Crusader	4	$-0.1001 \times \text{POD}_1 + 1.00$	0.36	0.20
	8	$-0.2809 \times \text{POD}_1 + 1.32$	0.80	0.01*
	12	$-0.0561 \times \text{POD}_1 + 0.91$	0.83	0.01*
	16	$-0.0263 \times \text{POD}_1 + 0.95$	0.37	0.19
Total ozone injury				
Week	response function	$r^2$	p-value	
4	$0.1274 \times \text{POD}_1 + 0.36$	0.79	0.01*	
8	$0.236 \times \text{POD}_1 - 0.1467$	0.65	0.05*	
12	$0.0651 \times \text{POD}_1 + 0.06$	0.79	0.01*	
16	$0.0838 \times \text{POD}_1 + 0.18$	0.16	0.43	
nL ethylene $\text{cm}^{-2}$				
Week	response function	$r^2$	p-value	
4	$-0.4923 \times \text{POD}_1 + 2.99$	0.67	0.04*	
8	$-4.2801 \times \text{POD}_1 + 14.896$	0.82	0.01*	
12	$-4.759 \times \text{POD}_1 + 55.57$	0.23	0.33	
16	$10.571 \times \text{POD}_1 + 5.15$	0.27	0.28	

### 3.3.5. Forage quality

The acid detergent fibre fraction (ADF) was unaffected by ozone (data not shown).

The acid detergent lignin fraction of forage (ADL) gradually increased during the experiment in the lowest ozone treatment, and was further increased with accumulated pasture  $POD_0$ , resulting in a strong positive relationship ( $r^2=0.76$ ,  $p<0.001$ ; Figure 3.8a).

In contrast, the neutral detergent fibre (NDF) fraction was unaffected by flux accumulated through the season (Figure 3.8b). Total sugar content declined overall during the experiment, driven by a steep decline at the 4 week harvest (Table 3.7).

This produced a polynomial relationship with  $POD_0$ , reaching a minimum after an accumulated  $POD_0$  of  $22 \text{ mmol m}^{-2}$  ( $r^2=0.74$ ;  $p=0.01$ ) (Figure 3.8c). In addition, the crude protein content, relative food value (RFV) and consumable food value (CFV) of the mixed forage all had weak negative associations with accumulated  $POD_0$  during the course of the season ( $r^2=0.42$ ,  $p<0.001$ ;  $r^2=0.15$ ,  $p=0.05$  and  $r^2=0.30$ ,  $p=0.005$  respectively) (Figure 3.8d, e, f). Forage quality parameters were not significantly affected by ozone flux accumulated at individual harvests, except for total sugar content and CFV, which were negatively affected at weeks 4 and 12 ( $r^2=0.70$ ,  $p=0.03$  and  $r^2=0.78$ ,  $p=0.01$  respectively) (Table 3.7). This coincides with the largest increases in  $POD_0$  (Figure 3.8e) or maximum productivity (Table 3.5)

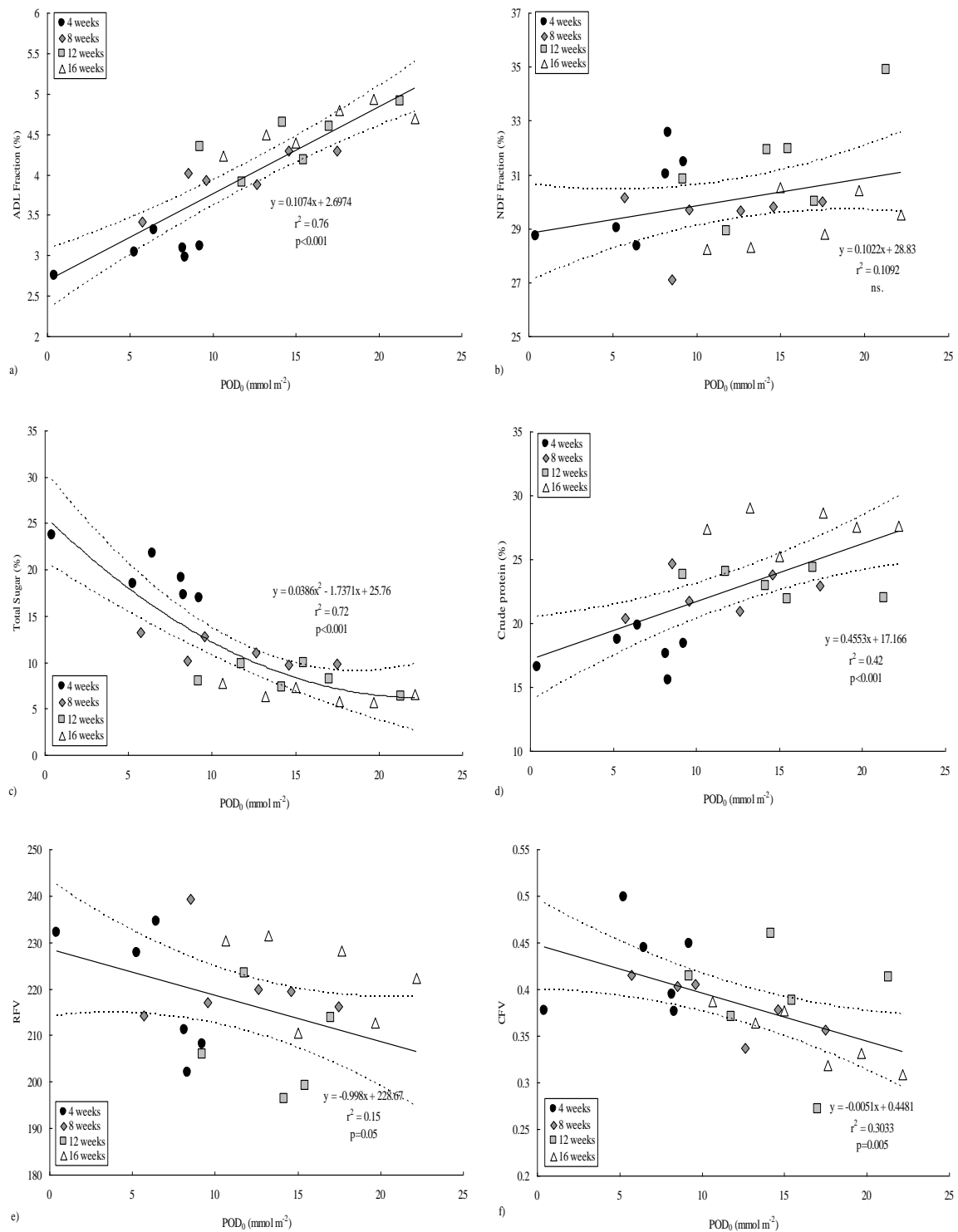


Figure 3.8: Effect of accumulated pasture  $POD_0$  against seasonal (a) acid detergent lignin (ADL) fraction, (b) neutral detergent fibre fraction (NDF), (c) total sugar content (d) total protein content (e) relative food value (RFV) (f) consumable food value (CFV) of AberMagic/Crusader forage (where black circles = week 4; dark grey diamonds = week 8; light grey squares = week 12; white triangles = week 16; dashed lines indicate 95% confidence intervals). Each value represents the mean of 6 pooled and mixed samples in each ozone treatment.

Table 3.7: Response functions for ozone effects on forage quality parameters in AberMagic/Crusader pasture mesocosms, determined against growth cycle fluxes. Asterisks denote a significant ozone effect at  $p \leq 0.05$  (where RFV= relative food value, CFV=consumable food value).

variable	harvest week	response function	$r^2$	p-value
ADL fraction (%)	4	$0.00372 \times \text{POD}_0 + 2.81$	0.41	0.16
	8	$-0.1118 \times \text{POD}_0 + 4.36$	0.35	0.38
	12	$0.0066 \times \text{POD}_0 + 4.30$	<0.10	0.92
	16	$0.2373 \times \text{POD}_0 + 4.22$	0.42	0.15
NDF fraction (%)	4	$0.377 \times \text{POD}_0 + 27.81$	0.49	0.11
	8	$0.3085 \times \text{POD}_0 + 27.83$	0.28	0.27
	12	$1.2034 \times \text{POD}_0 + 27.33$	0.19	0.37
	16	$0.045 \times \text{POD}_0 + 29.22$	<0.10	0.95
Total Sugar (%)	4	$-0.7028 \times \text{POD}_0 + 28.98$	0.70	0.03*
	8	$-0.3423 \times \text{POD}_0 + 12.90$	0.19	0.37
	12	$-1.068 \times \text{POD}_0 + 11.89$	0.32	0.23
	16	$-0.796 \times \text{POD}_0 + 7.83$	0.50	0.11
Crude Protein (%)	4	$0.0639 \times \text{POD}_0 + 17.36$	<0.10	0.80
	8	$0.0686 \times \text{POD}_0 + 22.05$	<0.10	0.87
	12	$-0.4549 \times \text{POD}_0 + 24.73$	0.10	0.53
	16	$0.8168 \times \text{POD}_0 + 26.29$	0.20	0.36
RFV	4	$-3.194 \times \text{POD}_0 + 239.44$	0.55	0.09
	8	$-2.220 \times \text{POD}_0 + 237.37$	0.23	0.35
	12	$-10.076 \times \text{POD}_0 + 237.33$	0.25	0.30
	16	$-0.1421 \times \text{POD}_0 + 222.85$	<0.10	0.98
CFV	4	$0.0042 \times \text{POD}_0 + 0.40$	<0.10	0.76
	8	$-0.0065 \times \text{POD}_0 + 0.41$	0.17	0.41
	12	$0.0733 \times \text{POD}_0 + 0.13$	0.78	0.01*
	16	$-0.014 \times \text{POD}_0 + 0.37$	<0.10	0.54

### 3.4. Discussion

As with previous assessments of the ozone sensitivity of *L. perenne* and *T. repens* (e.g. Hayes et al. 2007; Nussbaum et al. 1995), the above-ground biomass of *L. perenne* appeared unresponsive to ozone throughout the season while that of *T. repens* was strongly affected. The changing slope of Crusader shoot biomass to ozone flux at each growth cycle (-0.1001, -0.2809, -0.0561, -0.0263), could suggest a decrease in sensitivity to ozone after 8 weeks exposure, though a good fit ( $r^2$ ; 0.36, 0.80, 0.83, 0.37) also indicates a strengthening effect of ozone up to 12 weeks. Decreasing sensitivity to ozone is also supported by lower injury in Crusader foliage during the



course of the experiment. Although leaf biochemistry was not determined, an acclimation to ozone could reflect ozone-induced increases in antioxidant metabolism or capacity (Gillespie et al. 2012; Dumont et al. 2014), or be a more passive effect related to the repeated wounding of plants and the pre-activation of abiotic defence pathways (Koch et al. 1998; Tamaoki, 2008). Compared to the critical level (10% reduction in clover biomass) for productive grasslands, equating to a 3 month  $POD_1$  of  $2 \text{ mmol m}^{-2}$  ( $RY = -0.035 \times POD_1 + 0.97$ ; Mills et al. 2011d), the newer cultivar, Crusader, had a smaller response to ozone over the course of the experiment, ( $RY = -0.0199 \times POD_1 + 1$ ) with a 10% reduction occurring at a  $POD_1$  of  $4 \text{ mmol m}^{-2}$ . This suggests the critical level would overestimate the effect of ozone on *T. repens* yield in the present study. The mixed canopy of the present study may have reduced the effective dose to *T. repens* (Hayes et al. 2010a). In general, the low fit of accumulated  $POD$  to seasonal biomass production in the cultivars highlights the importance of other modifying factors, including interspecific competition and growth habit (Hayes et al. 2009; 2010a) and grazing pressure (Wilbourn et al. 1995). The absence of ozone effects on AberMagic yield also supports the basis for the critical level for above-ground production in pasture to be based on the response of *T. repens*, which would indirectly protect that of *L. perenne* (Mills et al. 2011). In contrast to relative above ground biomass, both cultivars had strong ( $r^2 > 0.60$ ) linear reductions in root biomass, with significant effects occurring at an accumulated  $POD_6$  of  $\sim 7 \text{ mmol m}^{-2}$  and  $POD_1$  of  $\sim 10 \text{ mmol m}^{-2}$  for AberMagic and Crusader respectively. Numerous previous studies have shown a reduced root: shoot in plants due to chronic ozone exposure (e.g. Landolt et al. 2000; Hewitt et al. 2014), with a preferential partitioning of C and other resources to above-ground growth, repair or defence (e.g. Cooley & Manning, 1988; Cho et al. 2011). Depressed root growth may also increase the sensitivity of pasture to

drought and nutrient stress (e.g. Vollnes et al. 2010). Thus a focus on above-ground productivity alone may risk understating the sensitivity of *L. perenne* to ozone. As previously stated by Hayes et al. (2009), the initial sensitivity of *T. repens* to ozone may lead to a competitive disadvantage in grassland communities. However, the negative effect seen here on the root biomass of *L. perenne*, and the changing sensitivity of *T. repens* forage, suggests the effects of ozone on the competitive ability of pasture species are highly complex. Indeed, although repeated harvesting may exacerbate ozone-induced reductions in clover/grass shoot biomass ratio (Nussbaum et al. 1995; Heagle et al. 1989; González-Fernández et al. 2008), *T. repens* here increased in abundance throughout the experiment.

For the first time, this study has also provided flux-based dose-response relationships for leguminous nodulation and N-fixation. There were strong negative responses in root nodule-mass-per-pot and mass-per-nodule to accumulated  $\text{POD}_1$ . Ozone flux resulted in ~10% loss in both parameters at a threshold of  $2\text{mmol m}^{-2}$ , and ~50% loss at an accumulated  $\text{POD}_1$  of  $11\text{mmol}^{-2}$ , with significant effects occurring after a minimum ozone flux of  $\sim 8\text{mmol m}^{-2}$ . Ozone impacts on the nodulation of *T. repens* have previously been reported in monoculture (e.g. Hewitt et al. 2014), and this study now confirms that impacts on the nodulation of *Trifolium* spp. also occur in mixed communities. Ozone flux to the upper leaves explained a large degree of the variation in nodule biomass ( $r^2 > 0.80$ ). In comparison to the slope for Crusader root biomass (0.043), the steeper slope of nodule-mass-per-pot (0.051) and mass-per-nodule (0.048) implies prioritisation in *T. repens* to the diversion of photosynthates away from the energetically-expensive process of N-fixation and nodule growth when under ozone stress (Rees et al. 2005). As root nodule number itself was unaffected (data not shown), this may represent the most severe ozone effect on the development

of root nodules in legumes, which is more likely with acute ozone concentrations (e.g. Letchworth & Blum, 1977), or where the effective dose is higher (Hayes et al. 2010; Hewitt et al. 2014). The N-fixing capacity of *T. repens* is associated with the mass of nodules (Crush & Caradus, 1996; Selge & Higuchi, 2000), suggesting sustained ozone concentrations would have the potential to limit N-fixation rates in mixed pastures over time. The flux-based dose-response relationships for root and nodule biomass constructed here did not pass through the intercept at or near the lowest ozone treatment used, indicating that deleterious effects may still be seen at even lower accumulated  $\text{POD}_1$ . The existing 3-month critical level of a  $\text{POD}_1$  of  $2 \text{ mmol m}^{-2}$  may therefore additionally serve to protect below-ground growth in pasture, including the architecture of N-fixation in *T. repens*.

When plotted against the  $\text{POD}_1$  accumulated by Crusader between harvests, a surprising range of effects were apparent in measured AR rates, with significant negative responses in ethylene concentration up to week 8. The large increase in AR rates in week 12 coincided with the seasonal peak biomass of *T. repens*, suggesting our ARA technique did accurately reflect the above-ground status of the plants. However, as ethylene concentration provides only a single point measurement, the ARA procedure is thought to have less accuracy than other methods of assessing N-fixation rates such as the  $\text{N}_{15}$  difference method (Hardarson & Danso, 1993). Thus, although individual ARA data had a good fit to accumulated  $\text{POD}_1$ , the variation in direction of effects seen here indicates that this response is unsuitable for the development of a specific flux-based critical level. Instead, optimum N-fixation rates may be best maintained by protecting against the less transient change occurring in root and nodule growth. One limitation of this study is the N content of individual tissues could not be determined due to small sample weights. In general, the close fit

of root biomass, nodule mass and N-fixation to ozone flux found in this study would support the development of additional critical thresholds for grasslands based on below ground responses (Mills et al. 2011a).

Consistent with previous findings, the chronic exposure to ozone led to increases in the ADL fraction, and had no effect on the NDF fraction of mixed forage (Gonzalez-Fernandez et al. 2008). The effects of ozone on N-fixation rate did not reduce the crude protein content of the pasture forage, which may be closely related to the increasing abundance of clover in the pasture during the course of the experiment (Better returns programme, 2014). As with crude protein content, the total sugar content of forage is a complex parameter and may be closely linked to the relative abundance of the cultivars during the experiment, although the present analysis suggests sensitivity to ozone may be retained up to an accumulated  $POD_0$  of  $\sim 22 \text{ mmol m}^{-2}$ . As in previous studies (Bender et al. 2006; Gonzalez-Fernandez et al. 2008), ozone flux also decreased the RFV and CFV of forage. As the majority of adverse effects of ozone on total sugar content and RFV occurred in the first 4 weeks of the experiment, the early season, occurring from April to June, appears to be the most sensitive time for impacts on forage quality, coinciding with naturally-high productivity in *L. perenne* and *T. repens* (Parsons & Chapman, 1999), and with peak concentrations of ozone in North-West Europe (Mills et al. 2011b). The comparatively low  $POD_0$  values necessary for adverse effects suggests pasture quality may retain sensitivity to acute episodes of ozone despite a reduction in injury and impacts on shoot biomass (Hayes et al. 2010b). A reduced sugar content may detract from the N-efficiency of HSG (Staerfl et al. 2012), and, coupled with deleterious effects on the ADL fraction and seasonal CFV, would have important implications for the diet of grazing ruminants (González-Fernández et al. 2008). However caution is required in

these interpretations, as the scarcity of ozone effects at individual harvests throughout the experiment suggests the passive effect of change in canopy composition and growth was overall a more important determinant of pasture quality than ozone directly. For example, the large increases in clover seen in the experiment may have increased the relative proportion of indigestible grass stem (Søegaard, 1993), partially explaining the increase in the ADL fraction (Søegaard, 1993), and gradual decreases in RFV and CFV (González-Fernández et al. 2008). Thus, in their present form, the dose-response relationships constructed here may overestimate the impacts of ozone pollution on pasture quality, and ozone may have a larger effect by affecting negative change in species abundance (e.g. Wilbourn et al. 1995). Although the dose-response relationships for forage quality parameters are specific to this study, they provide a novel indication of the effects ozone flux may have on the seasonal productivity of HSG pasture under a range of current and near-future ozone scenarios, and assist towards development of critical levels for forage quality. A close fit may also be found in forage quality parameters with PODYs with higher Y values e.g.  $POD_8$  (González-Fernández et al. 2008).

### **3.5. Conclusion**

The growth dynamics of HSG pasture may display considerable variation in response to ozone exposure over the course of a season. The current flux-based critical level may overestimate predicted ozone impacts on *T. repens* biomass in some circumstances, and a focus on above-ground productivity alone may understate the sensitivity of companion grasses such as *L. perenne*, which responds strongly to ozone flux with a reduction in root biomass. Negative dose-response relationships may also be found in *T. repens* root nodule biomass, although, at present, a critical level for N-

fixation itself cannot be developed due varying effects and insufficient data. Ozone flux to the canopy may adversely impact the quality of HSG forage, including reduced total sugar content, and a decline in relative food and consumable food values. A reduction in root growth, reduced sugar content of forage, and a potential shortfall in N-fixation capacity and rates, may increase the N-demand of managed pasture, and thereby detract from some of the suggested benefits of HSG in future ozone-rich climates.

## **4. THE POTENTIAL THREAT POSED BY OZONE POLLUTION TO N-FIXATION IN PASTURE**

### **4.1. Introduction**

Concentrations of tropospheric ozone ( $O_3$ ), a short-lived greenhouse gas produced via the complex photochemical reaction of volatile organic compounds (VOCs), carbon monoxide (CO) and nitrogen oxides ( $NO_x$ ), have increased around the world since the start of the industrial era (IPCC, 2013). The polluted Northern Hemisphere has the highest surface concentrations, with annual mean values of 30-40 ppb and increases of 0.5-2.0% occurring on an annual basis in the mid-latitudes (IPCC, 2013). The regional production of ozone is highest during periods of warm temperature, high radiation levels and stable pressure. Spring and summertime mean concentrations exceed 40 ppb across Central and Northern Europe, and 50 ppb across the Mediterranean basin, central Asia and the sub-tropics (Royal Society, 2008). Enhanced ozone destruction, due to increases in temperature and atmospheric water vapour, is expected to partially decrease surface ozone concentrations across much of the world in this century, though continuing increases in concentration are likely in the most polluted regions due to the increasing production of primary precursors (IPCC, 2013). Warming may also increase the regional or seasonal potential for ozone formation due to changes in the hemispheric transport of ozone precursors and increases in VOC production (IPCC, 2013).

Nitrogen (N) is an essential macronutrient for plants, and it is estimated that legumes contribute up to 70 Tg N annually to agricultural systems (Herridge et al. 2008). N-fixation in nodulating legumes, via symbioses with soil-dwelling N-fixing rhizobacteria, is a novel trait that is thought to have evolved during the early history of

the legume family (*Fabaceae*) (Sprent, 2007). In exchange for carbohydrates such as malate, legumes are able to obtain a source of fixed atmospheric N from rhizobia partners; an interplay that requires specified chemical signal exchanges and structures (Geurts and Bisseling, 2002; Rees et al. 2005) and developmental plasticity (Ferguson et al. 2010). Recently, legume crops and fodder have occupied >15% of all agricultural utilised area (FAO, 2014) as a component of intensive, organic, low input, and subsistence agriculture, and are a primary source of dietary protein. The increasing expense of artificial fertilisers, resulting from rising energy costs, has highlighted the importance of legume-based agriculture as a source of crop N, particularly for smallholder producers in developing regions (Lynch, 2007; De Schutter, 2013).

As a powerful oxidant, tropospheric ozone is considered the most damaging air pollutant to food production (Ashmore, 2005). Like other plant groups, a typical response of legumes to chronic ozone exposure is reduced productivity, which translates to measureable declines in yield and harvest index (e.g. Betzelberger et al. 2012). Global estimates of yield losses are unavailable for most legume crops, though it is estimated, based on concentration-based exposure indices, that losses due to ozone exceed 10% of soybean yield on an annual basis, equating to some \$2.9-4.9 billion (Avnery et al. 2011). In addition to declining yield, ozone impacts on nutritive quality have also been reported in several arable and forage legumes (Morgan et al. 2003; Gonzalez-Fernandez et al. 2008; Iriti et al. 2009). In grassland situations, ozone pollution may also contribute towards a reduction in the legume fraction of the community (e.g. Wilbourn et al. 1995; Volk et al. 2006), with implications for the ecological and economic sustainability of grasslands (Mills et al. 2011b).



Numerous previous studies have highlighted the often high sensitivity of agronomically-important legumes, including soybean, clover, beans, and pulses to ground level ozone (e.g. Hayes et al. 2007; Mills et al. 2007). However, the impacts of ozone on the N-fixing capacity of legumes remain surprisingly neglected, especially at current and near-future concentrations of ozone. In this study, experimentally-derived response functions, the effects of ozone that may occur on an agroecosystem scale by predicting reductions in clover (*Trifolium* spp.) root nodule biomass in United Kingdom (UK) pasture based on ozone concentration data for a “high” (2006) and “average” ozone year (2008).

## **4.2. Methods**

### **4.2.1. Ozone exposure experiments**

In our earlier study (Hewitt et al. 2014), a cultivar of white clover (*Trifolium repens* cv. Crusader) and red clover (*Trifolium pratense* cv. Merviot) recommended for general use in grazed pasture (British grassland society, 2014), were exposed to a range of current and near-future ozone scenarios (treatment means: 33, 35, 40, 45, 51, 54, 66ppb) in year 2012 in solardomes (hemispherical glasshouses) at the CEH Solardome facility near Bangor, North Wales. After extraction of root biomass material in selected treatments, the cultivars showed ozone-induced reductions in nodule development, and *in situ* measurements on the white clover revealed that reduced nodule mass was associated with a significantly reduced N-fixation rate determined after 12 weeks ozone exposure by acetylene reduction assay (ARA) (Hewitt et al. 2014). A second, previously unpublished ozone-exposure experiment, using the same experimental set up and treatments, was conducted in year 2013 on clover/ryegrass pasture mesocosms using *T.repens* cv. Crusader and *Lolium perenne*

cv. Abermagic. *L. perenne* cv. AberMagic was sown at a rate of 0.28g per pot directly into 10 L pots (27.5 cm diameter x 22cm height) filled with compost (John Innes No. 2; J. Arthur Bowers, Lincoln, UK). The total nitrogen content of soils ranged 1-3% (data not shown). *T. repens* cv. Crusader plants were also propagated from seed, grown in the same compost in plug-plant trays in an unheated glasshouse. Seeds were obtained from a commercial seed supplier, and originated from the UK (Wynnstay Seeds; UK). After 4 weeks of growth, 3 clover plants were transferred to each pot containing growing *L. perenne*, with one clover plant in each third of the pot. To introduce a soil microbe population, pots were inoculated with 400ml of a soil slurry mixture made from 5kg of soil from agricultural grassland (Abergwyngregyn, North Wales, UK, 53°14'N, 4°01'W) and 14L water. Mesocosms were grown for a further 4 weeks in ventilated greenhouses under optimum watering conditions. On 07/06/2013, 24 pots, of equal size and distribution of clover and grass, were transferred to each of 6 'solardomes' and exposed to the ozone treatments for 16 weeks. N-fixation was determined on pots every 4 weeks by ARA, and was reduced by ozone up to 8 weeks exposure (data not shown). As part of a final destructive harvest, clover root and nodule biomass was extracted from 6 pots in each ozone treatment as described previously (Hewitt et al. 2014). For more details regarding experimental procedure, including details of the climate conditions and ozone control system, see Hewitt et al. (2014). In both years, root nodule biomass per pot (n=6 per treatment), was analysed by linear regression, with either ozone exposure (accumulated ozone above a threshold of 40 ppb, AOT40 (ppm.h)) or seasonal mean O<sub>3</sub> concentration (ppb) at the time of harvest applied as the predictor variable.

#### **4.2.2. Predicting spatial patterns in nodule biomass loss in the UK**

The root nodule biomass per pot for *T. repens* and *T. pratense* in year 2012, and *T. repens* in year 2013, were expressed as relative values determined by regression against the AOT40 for each treatment and dividing by the value of the intercept, indicating zero ozone exposure (0 AOT40 ppm.h) or 0ppb seasonal mean O<sub>3</sub> concentration. Although *T. repens* and *T. pratense* differed in their response in root nodule biomass in the 2012 study, the slopes for relative root nodule biomass were not significantly different for red and white clover (linear regression;  $p=0.99$ ), nor was there a significant difference between the responses of *T. repens* cv. Crusader to ozone in 2012 and 2013 ( $p=0.88$ ), when interactions between species or year were considered. All data were combined, and linear regression was performed to provide one exposure-response function for effects of ozone on the root nodule biomass of white and red clover. To aid with a comparison to existing studies, the response of nodule biomass to seasonal mean ozone was also presented (Figure 4.1b).

The exposure-response function was then used to predict percentage reductions in UK pasture in year 2006, a hot and dry year with relatively high ozone concentrations, and 2008, a lower ozone year, and more typical of current climate conditions. The ozone concentration data, for the early season (April-June) and late season (July-Sept), were obtained from the UK air quality monitoring stations (<http://uk-air.defra.gov.uk/>) and used to estimate AOT40 values calculated across a 1km x 1km grid, based on the Ordnance Survey grid (Coyle et al. 2002). As described previously (Mills et al. 2011b), the 1km<sup>2</sup> grid data was aggregated to 10km x 10km squares for comparison with land-cover data obtained from the Convention on Long-Range Transboundary Air Pollution (CLRTAP) harmonised land cover map (Cinderby et al. 2007). Pasture was identified according to the distribution of the European Nature Information System (EUNIS) E2 Mesic grassland habitat (EEA, 2012). Grid squares

with <1% (100ha) pasture land-cover were excluded from the analysis. Maps were constructed using ArcGIS (version 10.1).

All statistical analyses were conducted in R software (version 3.0.2).

### **4.3. Results and discussion**

#### **4.3.1. Ozone exposure-response relationships for root nodule biomass**

When the data for white and red clover and 2012 and 2013 experiments were combined, the response function for relative root nodule biomass displayed a strong negative relationship with accumulated ozone exposure ( $r^2=0.72$ ;  $p=0.004$ , Figure 4.1a), with a 10% reduction at an AOT40 of 5 ppm.h, and >50% reduction in relative nodule biomass occurring at AOT40s of > 20 ppm.h (Figure 4.1a). These results are comparable with the accumulated ozone exposure necessary to achieve a 10% reduction in the shoot biomass of *T. repens*, requiring a 12 week AOT40 of 4.7 ppm.h (Mortensen and Bastrup-Birk, 1996). A significant effect of ozone on relative root nodule biomass did not occur below an AOT40 of 6 ppm.h (Figure 4.1a). This is above a critical level of 3 ppm.h for the protection of European grasslands (CLRTAP, 2011), although surface concentrations of ozone across Europe frequently exceed this exposure level (Fernández-Fernández et al. 2011; Mills et al. 2011b). For easy comparison with the other studies discussed, a strong negative relationship for relative nodule biomass was also found against seasonal mean ozone concentration ( $r^2=0.60$ ;  $p=0.002$ ; Figure 4.1b). However, the response against accumulated ozone exposure is preferred due to the higher fit ( $r^2=0.72$ ) and passage through the intercept at the concentrations assessed (Figure 4.1a).

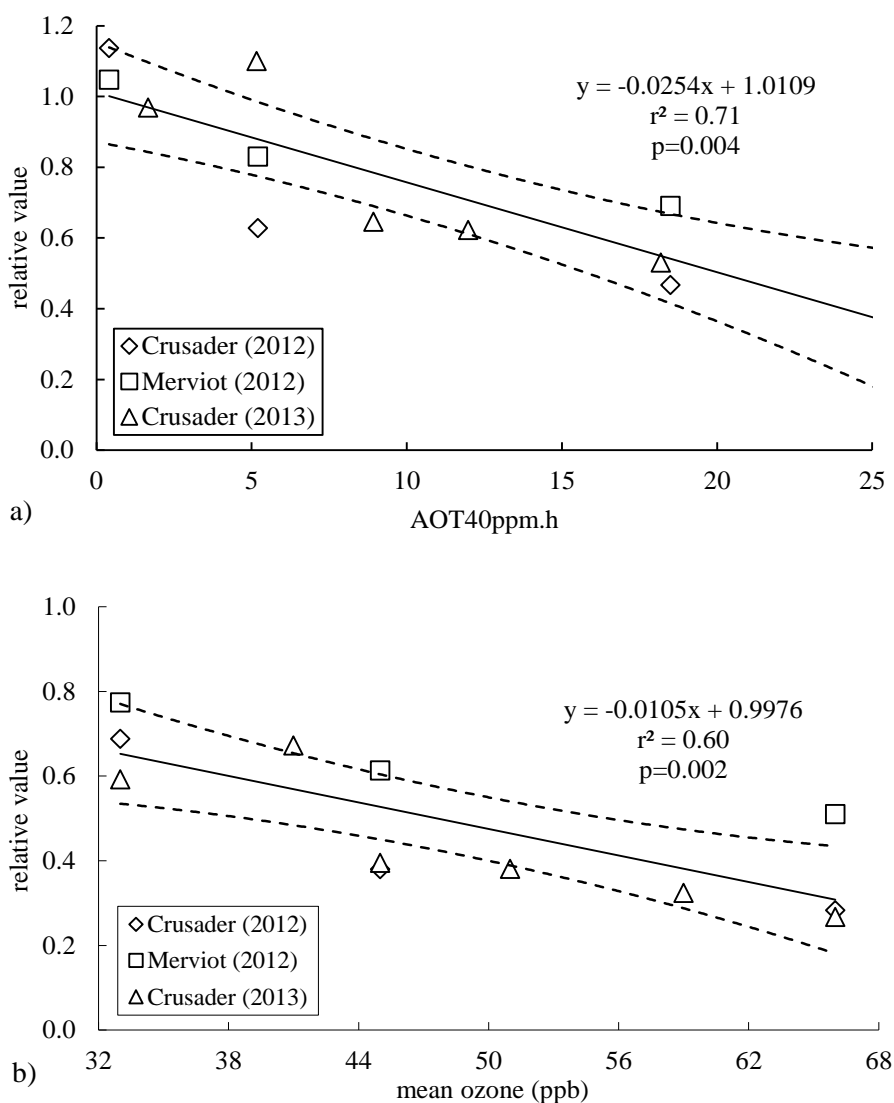


Figure 4.1: Response relationships for relative root nodule biomass in *Trifolium* spp. combined from experiments with white clover (*T. repens* cv. Crusader) and Red clover (*T. pratense* cv. Merviot) in years 2012 and 2013 with (a) accumulated ozone exposure (AOT40ppm.h) ( $y = -0.0254x + 1.0109$ ;  $r^2 = 0.72$ ;  $p = 0.004$ ;  $n = 6$ ) (b) seasonal mean ozone concentration (ppb) ( $y = -0.0105x + 0.9976$ ;  $r^2 = 0.60$ ;  $p = 0.002$ ;  $n = 6$ ).

#### 4.3.2. Spatial patterns of nodule biomass loss in 2006/2008

A total of 1124 10 x 10km grid-squares across the UK contained >1% pasture cover.

Predicted reductions in relative nodule biomass were most widespread in the spring of year 2008, with >8% reductions predicted across most of Wales, western areas of England and southern Scotland (Figure 4.2). In the spring of 2006 and 2008, ozone effects were predicted to be greatest for pasture in coastal or upland regions, with

pronounced effects in pasture-rich areas of central Wales and the South-West in both years (Figure 4.2). The mean percentage reductions were similar for both spring periods, but the inter-quartile range for year 2006 was larger at 6.2-13% compared to 9.2-12.6% in the spring of 2008 (Figure 4.2). In the late season (July-Sept) of both years, it was predicted that ozone impacts on relative root nodule biomass were lower than in the spring (Figures 4.2 and 4.3). This effect was most pronounced in the cooler, relatively wet late-season of 2008, with a mean reduction in relative nodule mass of 1.4% and an inter-quartile range of 0.9-1.6% (Figure 4.3). Thus, in a hot, dry, year such as 2006, with prolonged periods of high ozone during the spring and summer, sustained impacts on clover nodule biomass may potentially occur across UK pastures, though some impacts may be also be apparent in the spring of cooler, wetter years. The largest predicted losses in root nodule biomass, occurring in the early season (April-June) in Wales and western regions of the UK in both years, are consistent with a broader trend of current and near-future ozone impacts on agriculture in North-West Europe (Mills et al. 2011a).

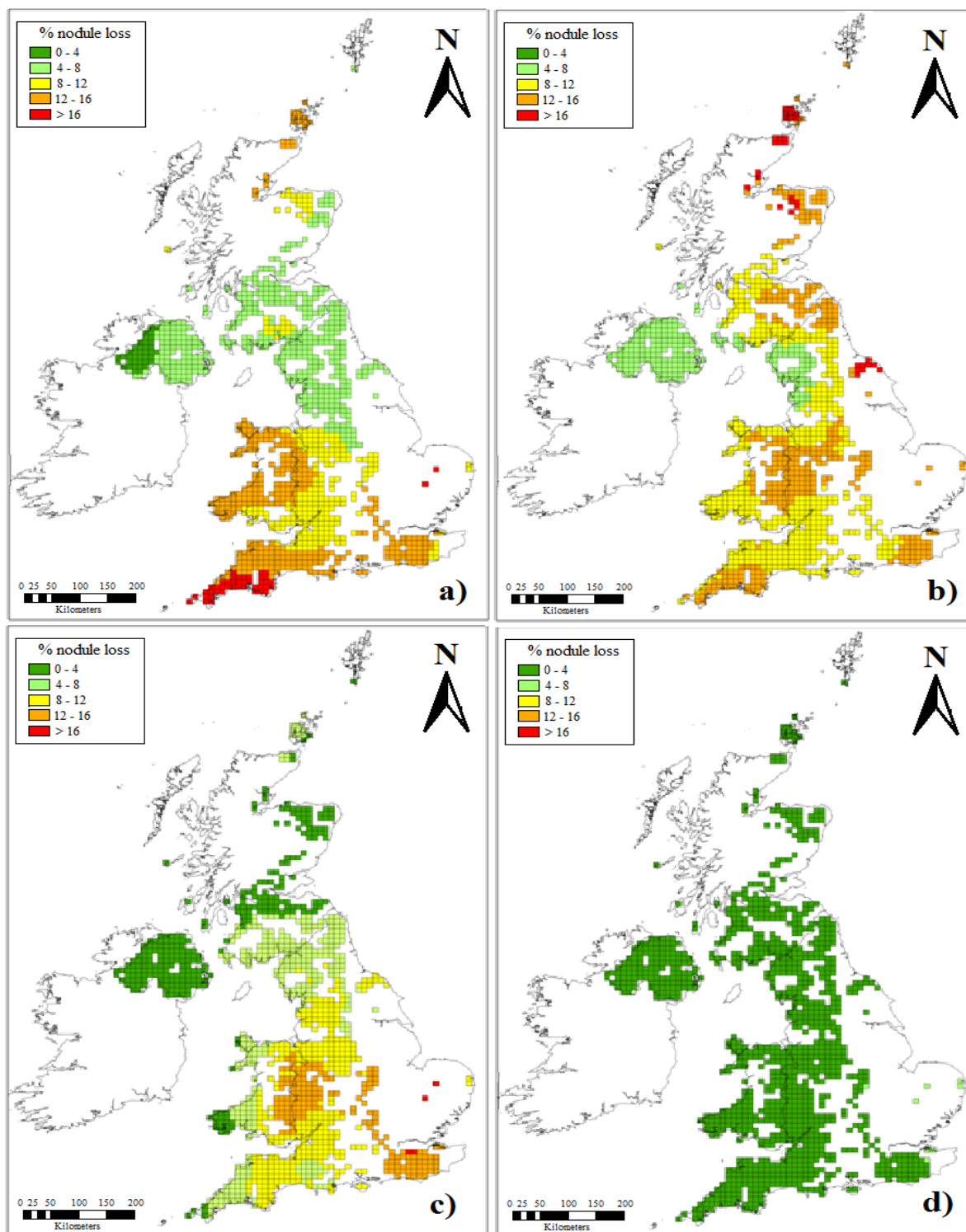


Figure 4.2: AOT40-based assessments of predicted percent reductions in clover root nodule biomass in the UK scaled for 10 x 10km grid squares, where pasture is present in >1% of the grid-square, and based on the combined response function of two ozone-exposure experiments in Figure 4.1: (a) Early-season (April-June) in year 2006; (b) Early-season in year 2008; (c) Late-season (July-Sept) in year 2006; (d) Late-season in year 2008.

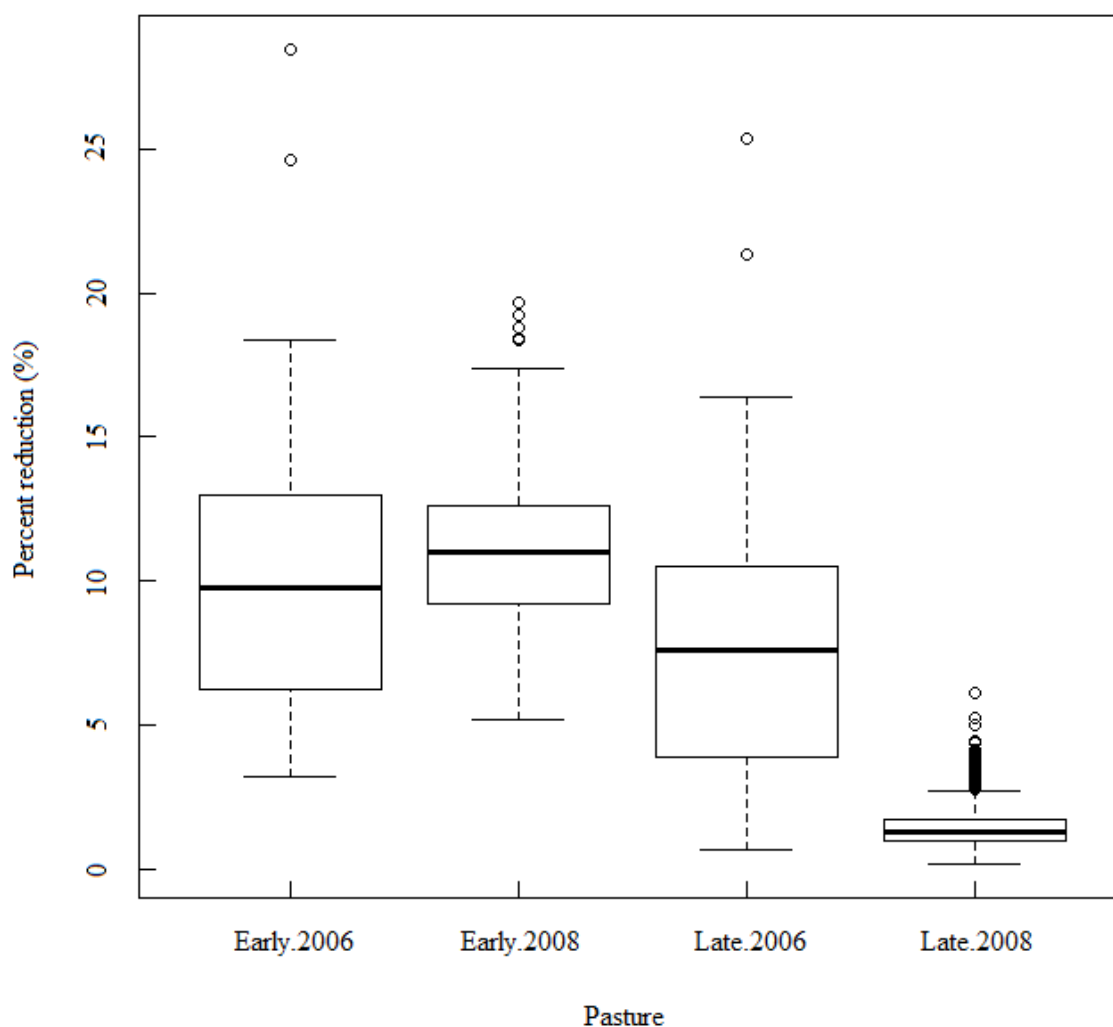


Figure 4.3: Quantification of ozone-induced reductions in clover root nodule mass for early season (April-June) and late season (July-Sept) pasture in years 2006 and 2008 using the grid-square values in Figure 4.2. Boxes display minimum, median, lower and upper quartiles and maximum values for grid squares ( $n=1124$ ). Open circles indicate potential outlier values.

We appreciate that our predicted impacts on root nodule biomass assume non-limited nodulation, and do not consider other important factors such as N-availability (Chmelíková and Hejcman, 2014), soil quality (Manier et al. 2009), hydrological status (Wilkinson and Davies, 2010), or the antioxidant capacity of plants (Scabba et al. 2003). In addition, our predicted impacts are based on modern commercial cultivars of clover, which may differ in sensitivity to established clover in M2 grassland habitat, although clover from this habitat does respond strongly to ozone



(e.g. Hayes et al. 2010). Similarly, the abundance of clover within pasture grid-squares, which could not be controlled for, would substantially influence N-fixation rates (Better returns programme, 2015), and could itself be negatively influenced by ozone pollution (e.g. Wilbourn et al. 1995). Ozone-effect data also has a closer fit to stomatal-flux based indices (Mills et al. 2011, a, b), leading exposure-based indices to overestimate losses. In general, mixed and established grassland communities may display a large degree of inertia to ozone stress, and the effects of rising ozone on European grasslands are uncertain (e.g. Stampfli and Fuhrer, 2010; Bassin et al. 2013; Volk et al. 2014). These limitations are key sources of uncertainty to this assessment, and the potential for exaggerated ozone-impacts on clover nodulation are recognised by the authors. Nevertheless, the modelling exercise presented here provides the first indication of the potential effects ozone pollution may have on legume nodulation on an agroecosystem scale, based on data from realistic experiments. It is unknown how well direct reductions in root nodule biomass would translate to N-fixation rates in pasture, though clover N-fixation rates are often associated with nodule weight or size (Crush and Caradus, 1996; Selge and Higuchi, 2000; Hewitt et al. 2014). Using averaged N-fixation data from the 2012 and 2013 experiments, root nodule biomass explained 50% of the variation in N-fixation rate ( $r^2=0.50$ ;  $p=0.04$ ; Appendix Figure A2). However, as N-fixation rates were determined from these studies by ARA (units: nL ethylene cm<sup>2</sup> soil surface), scaling this chemical data to a landscape scale would require a number of additional measurements and assumptions at this stage.

#### **4.4. Conclusion**

For the first time, this study has considered the potential impacts of ozone on leguminous N-fixation in a systematic way. The paucity of studies concerned with

ozone effects on N-fixation is noteworthy considering the vast number that have highlighted the impacts of ozone on legume growth and functioning. As determined from several ozone-exposure experiments, seasonal exposure to mean ozone concentrations of 40-60 ppb, or to short-term acute concentrations of >100ppb, is sufficient to reduce N-fixation rates or impact nodulation in important agricultural legumes. Effects of ozone on N-fixation and nodulation ultimately result from a reduced availability of C assimilates. However, the possible proximate effects of ozone, such as the action of long-distance stress hormones, and disruptions in the flow of auxin to developing nodules, have not been investigated and are worthy of further study. Based on ozone exposure (AOT40), annual impacts on clover root nodule biomass may occur in UK and temperate pastures, with sustained impacts occurring in years with higher ozone concentrations. Potential short-falls in N-fixation, and economic and environmental consequences, have not yet been considered on an agroecosystem basis and also present an important subject for further investigation. From the few interactive studies conducted, an increasing global burden of CO<sub>2</sub>, the use of artificial fertiliser, and reactive N pollution, may mitigate some of the impacts of ozone on leguminous N-fixation.

The use of legumes for agronomic purposes will likely increase in the future, stimulated by their potential to reduce agricultural greenhouse gas emissions (Smith et al. 2007), the high price of artificial fertilisers (Lynch, 2007) and by advances in the selection of legume germplasm for stress tolerance and improved performance (e.g. Abberton and Marshall, 2005). Multiple interacting environment stressors, such as ozone, drought, elevated CO<sub>2</sub> and N deposition will be increasingly important in determining the sustainability of legume-based agriculture and food production,

particularly in parts of the world where severe issues with food security already exist (e.g. De Schutter, 2013).

## 5. EXPLORING THE EFFECTS OF CUTTING ON OZONE-INJURY AND BIOMASS PRODUCTION OF WHITE CLOVER

### 5.1. Introduction

Tropospheric ozone (O<sub>3</sub>) is a powerful oxidant that can induce a state of oxidative stress in plants (Wilkinson et al. 2012). Current concentrations of ozone are causing damage to cultivated and natural vegetation, and ozone is considered a threat to food security (Wilkinson et al. 2012). The negative effects of ozone on the biosphere will persist as further increases are likely in the level of ozone over the coming century due to the continued emission of volatile precursor compounds (IPCC, 2013). Sensitivity to ozone is known to vary widely between (e.g. Hayes et al. 2007) and within species (e.g. Khan et al. 2013; Danielsson et al. 2013). Grassland and pasture legumes (Fabaceae), such as clover (*Trifolium* spp.), are valued for nitrogen (N) fixation and high palatability in pasture, are often considered amongst the most sensitive species to ozone pollution (Hayes et al. 2007), which has important implications for the economic and ecological sustainability of grasslands (Mills et al. 2011a).

Ozone primarily enters plants via the stomata, dissolving in the apoplast and causing a burst of reactive oxygen species (ROS) that mimics the hypersensitive response (HR) (Rao & Davies, 2001). Consequently, ozone can rapidly react with plants at a metabolic level, inducing foliar injury and premature senescence, and driving reductions in photosynthesis rates, growth, and yield (Wilkinson et al. 2012). Species or cultivar-specific sensitivity to ozone has been linked to a number of ecophysiological parameters such as ozone uptake rate via the stomata (Danielsson et al. 2013), growth habit (Hayes et al. 2010) and leaf ultrastructure (e.g. Postiglione et al. 2000). Antioxidant status also influences the response to ozone (Cho et al. 2011).

In addition to transient increases in antioxidant capacity or metabolism of leaves due to ozone-stress (e.g. Betzelberger et al. 2010; Wang et al. 2014), the activation of defence genes and change in the antioxidant status of leaves may be induced by mechanical wounding or herbivory (e.g. Kang et al. 2002; Suza et al. 2010). Thus, the pre-activation of the wounding response prior to ozone exposure may reduce or prevent ozone injury (e.g. Örvar et al. 1997; Koch et al. 1998) and chronic ozone exposure, in turn, can reduce the value of forage to herbivores (e.g. Gilliland et al. 2012; Khaling et al. 2015).

In addition to the shared pathways and cross-talk between wounding and oxidative stress pathways (e.g. Rao & Davies, 2001; Sasaki-Sekimoto et al. 2005), many of the signalling molecules that mediate defence responses, including ethylene (ET), and abscisic (ABA), salicylic (SA) and Jasmonic (JA) acids, are also involved in the positive or negative regulation of nodulation, locally or via autoregulation of nodulation (AON) (For reviews see: Susuki et al. 2004; Ryu et al. 2012; Mortier et al. 2012). Legumes, including clovers, can respond to ozone exposure with impacts on nodulation and N-fixation rate (e.g. Pausch et al. 1996; Hewitt et al. 2014). The impacts of ozone on legume nodulation and N-fixation are thought to result from reduced flow of carbon (C) assimilates to the root system (e.g. Letchworth & Blum, 1977, Pausch et al. 1996; Hewitt et al. 2014; Hewitt et al., submitted). However, the possible proximate influence of ozone-induced stress hormones on legume nodulation or N-fixation has not been investigated to date.

In this study, we test the hypothesis that wounding by cutting reduces ozone injury and senescence rates in a cultivar of white clover (*T. repens*) in a short-term ozone-exposure experiment. We compare the effects of cutting on injury and biomass production to uncut control plants, and with uncut plants treated with methyl

jasmonate (MeJa), a methyl ester of JA, and a potent inducer of defence genes and suppressor of ozone-induced ET (Koch et al. 1998). Lastly, we investigate the association of clover root nodule biomass to change in above and below-ground biomass and ozone injury parameters in cut and MeJa-treated plants.

## **5.2. Methods**

### **5.2.1. Plant material and treatments**

Seedlings of *T. repens* cv. Crusader, a medium-leaved cultivar used for frequent cutting and grazing (British Grassland Society, 2015), were propagated in plug-plant trays in early spring 2014. Seeds were obtained from a commercial seed supplier (Wynnstay Seeds; UK). After 3 weeks of growth, seedlings were transferred into 7.5L plant pots (27.5cm diameter x 21.5cm depth), and filled with compost (John Innes No. 2; J. Arthur Bowers, Lincoln, UK), with 3 seedlings per pot. To introduce a soil microbe population, pots were inoculated with 200ml of a soil slurry mixture made with 5kg of soil from agricultural grassland (Abergwyngregyn, North Wales, UK, 53°14'N, 4°01'W) and 15L of water. Pots were then divided between 4 treatments in a factorial experiment: cut+ MeJa- (with a single cut to 4cm height immediately prior to ozone exposure), cut- MeJa+ (with a weekly application of MeJa solution during the experiment), cut+ MeJa+ (with a pre-experiment cut and a weekly application of MeJa) and cut- MeJa- (with neither a pre-experiment cut or weekly MeJa application). A fresh MeJa stock solution (8 mM) was prepared in alcohol prior to spraying. The stock solution was then diluted with distilled water to a 500 µM solution, adjusted to a final volume with 0.1% ethanol (see appendix, Figure A3, for concentration-effect data). The canopy in treatments cut+ MeJa+ and cut- MeJa+ was sprayed once weekly in the late afternoon until run-off. To ensure there were no unaccounted for effects of

spraying, cut+ MeJa- and cut- MeJa- were sprayed with 0.1% alcohol solution.

Immediately after spraying, pots were enclosed overnight in transparent plastic bags to prevent cross-contamination by MeJa vapour.

Seedlings were grown for a further 4 weeks. On 23/05/2014, 15 pots, selected for consistent size, were then transferred to each of 6 'solardomes' (hemispherical glasshouses; 3m diameter, 2.1m high) at the CEH solardome facility near Bangor, North Wales, with 5 replicate pots per treatment.

### **5.2.2. Ozone exposure**

As described previously (Hewitt et al. 2014, Hewitt et al. in prep), ozone profiles (Table 5.1) were applied to the solardomes randomly. In one solardome, ambient air temperature, soil moisture content, photosynthetically active radiation (PAR) and relative humidity were continuously monitored by an automatic weather station and soil moisture probes (Theta Probe, Skye Instruments Ltd, Llandridod Wells, UK; Delta-T Devices Ltd, Cambridge, UK). Plants were rotated within each dome weekly and watered twice-weekly, with additional watering when necessary. Plants were exposed to the ozone profiles for a 4 week period, with the experiment starting on 28/05/2014 and finishing on 24/06/2014. Although individual ozone treatments were not replicated, numerous previous studies have established the statistical validity of experiments conducted using the solardome facility with 6-8 ozone treatments (*e.g.* Hayes et al. 2010; Hewitt et al. 2014; Wagg et al. 2013).

### **5.2.3. Visual assessment**

A representative quarter of each pot was selected and recordings of healthy, injured or senesced leaves made on in weeks 2, 3 and 4. As previously described (Hayes et al.

2010; Hewitt et al. 2014), leaves were classified as injured or senesced if >25% of the adaxial leaf surface was affected by injury or senescence respectively. Due to differences in shoot biomass between treatments, injury and senescence was expressed on a percentage basis.

#### **5.2.4. Biomass assessment**

The harvest of above and below-ground biomass was performed after 4 weeks on each pot. Shoot biomass, and root biomass from a representative quarter of the pot, were dried for a minimum of 48 hours at 60°C. Root nodule biomass was also excised from the root system, and was air dried, sized and weighed. Root biomass, nodule mass and nodule numbers per pot were calculated as described previously (Hewitt et al. 2014). Leaf area index (LAI) of undamaged leaves was determined from dried forage.

#### **5.2.5. Statistical analysis**

In order to broadly consider the effect of ozone background, ozone profiles were divided into low (profiles 1, 2), medium (3, 4) and high treatments (5, 6) and pooled. Mean injury and senescence data across the recordings was determined for each pot, and was arcsine transformed prior to analysis. Injury and senescence data, and selected biomass data, were then analysed by 3-way analysis of variance (ANOVA), with ozone background, cutting (+,-) or MeJa treatment (+,-) applied as factors, and solardome included as an error term. Post-hoc Tukey's honest significant difference tests were used to determine the significance of factors where appropriate. Relationships between total root nodule biomass and biomass, and raw injury parameters for each treatment were also investigated via Pearson's correlation analysis. All analyses were conducted in R Software (version 3.1.2).



### 5.3. Results

#### 5.3.1. Ozone concentrations and climate conditions

During the experiment, the six ozone profiles generated seasonal 24 hr means of 15, 29, 43, 49, 66, and 69ppb and accumulated daylight exposures above a threshold of 40ppb (AOT40) of 0, 5, 14, 18, 27 and 29ppm.h respectively (Table 5.1). This generated seasonal means of 21, 45 and 67ppb for low, medium and high ozone-backgrounds treatments respectively. Mean daylight (when PAR >50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) air temperature and VPD were 17°C and 0.48 kPa for the study period, with maxima of 29°C and 1.00 kPa respectively (Table 5.2). Mean daytime PAR was 639  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , with a maximum of 1550  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Table 5.2).

Table 5.1: Seasonal mean, and accumulated ozone exposure (accumulated exposure over a threshold of 40ppb (AOT40)) for ozone treatments.

Treatment	Mean ozone (ppb)	AOT40ppm.h	Ozone range
1	15	0	Low
2	29	5	Low
3	43	14	Medium
4	49	18	Medium
5	66	27	High
6	69	29	High

Table 5.2: 24hr mean, and mean, maximum and minimum daytime values for meteorological parameters during the course of the experiment (where PAR = photosynthetically active radiation; VPD = vapour pressure deficit).

Climate averages	24hr mean	daytime min.	daytime max.	daytime mean
PAR ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )			1550	639
Air temperature (°C)	16	11	29	17
VPD (kPa)	0.23	0.06	1.00	0.48

#### 5.3.2. Injury and senescence rates

As described previously (Hayes et al. 2010), visible injury caused by ozone on *T. repens* appears as small, yellow flecks on the leaves. Senescence that corresponded with the presence of ozone injury was recorded, and the presence of non-specific senescence was also low in the present study (<1%) (Hayes et al. 2010).

Mean injury and senescence rates were significantly increased by ozone background ( $p < 0.05$ , in each case), with an average increase of 154% and 100% between low and high ozone backgrounds for injury and senescence respectively (Figure 5.1a, b) (Table 5.3). On average, cutting significantly increased injury rates by 12% ( $p = 0.002$ ) and reduced senescence by 44% ( $p < 0.001$ ). Injury rates were additionally affected by MeJa treatment, both singly ( $p = 0.04$ ), and in an interaction with ozone ( $p = 0.02$ ) (Table 5.3), with MeJa tending to reduce injury in a high ozone background (Figure 5.1a, b) (Table 5.3).

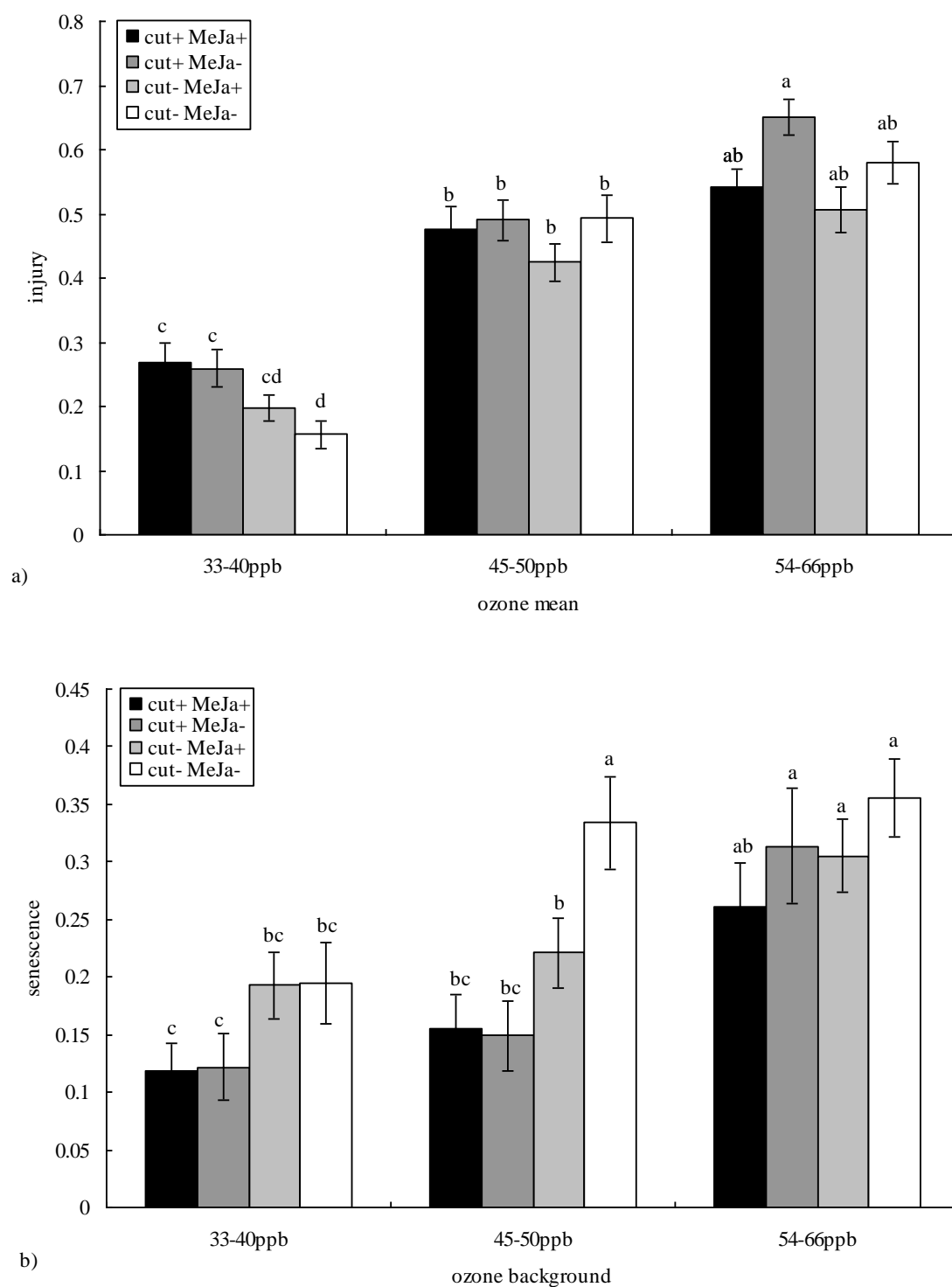


Figure 5.1: Mean percentage of injury (a) and senescence (b) across cut+ MeJa+, cut+ MeJa-, cut- MeJa+ and cut- MeJa- across low (33-40ppb), medium (45-50ppb) and high ozone (54-66ppb) backgrounds during the course of the experiment (different letters denote a significant difference at  $p < 0.05$ ; bars are standard error;  $n=30$ ).

### 5.3.3. Biomass

Shoot biomass, and resultant LAI, were negatively affected by ozone background and cutting ( $p < 0.001$  in each case) (Figure 5.2a, b) (Table 5.3), and shoot biomass was additionally impacted by MeJa treatment ( $p < 0.001$ ). On average, shoot biomass was reduced by 12% between the low (33-40ppb) and medium (45-50ppb) ozone backgrounds, with a 28% decrease between the low and high (54-66ppb) ozone backgrounds (Figure 5.2a). Pre-experiment cutting and treatment with MeJa on average caused additional reductions of 36% and 11% in shoot biomass respectively, though neither factor had an interaction with ozone background ( $p > 0.05$ ) (Figure 5.2) (Table 5.3). Root biomass was also strongly affected by ozone ( $p < 0.001$ ) with an average reduction of 14% between low and medium ozone backgrounds and 29% between low and high backgrounds (Figure 5.3a). As with above-ground biomass, cutting also negatively affected root biomass ( $p < 0.001$ ), causing an average reduction of 19% although no interaction was apparent with ozone (Figure 5.3a) ( $p > 0.05$ ) (Table 5.3). MeJa treatment had no effect on root biomass ( $p > 0.05$ ) (Figure 5.3a) (Table 5.3). Root nodule biomass per pot, nodule number per pot and mass-per-nodule were not significantly affected by ozone or MeJa treatment (Figures 5.3b, c, d) ( $p > 0.05$ , in each case) (Table 5.3). However, nodule number and nodule biomass per pot were both reduced in cut treatments, with a 18% reduction in the former and a concomitant decrease in the latter 28% (Figure 5.3b, c) ( $p < 0.05$ , in each case) (Table 5.3). In addition, nodule biomass of cut pots displayed a clear positive interaction with increasing ozone ( $p = 0.07$ ), with an average reduction of 55% in a high ozone background compared to uncut pots (Figure 5.3c) (Table 5.3).

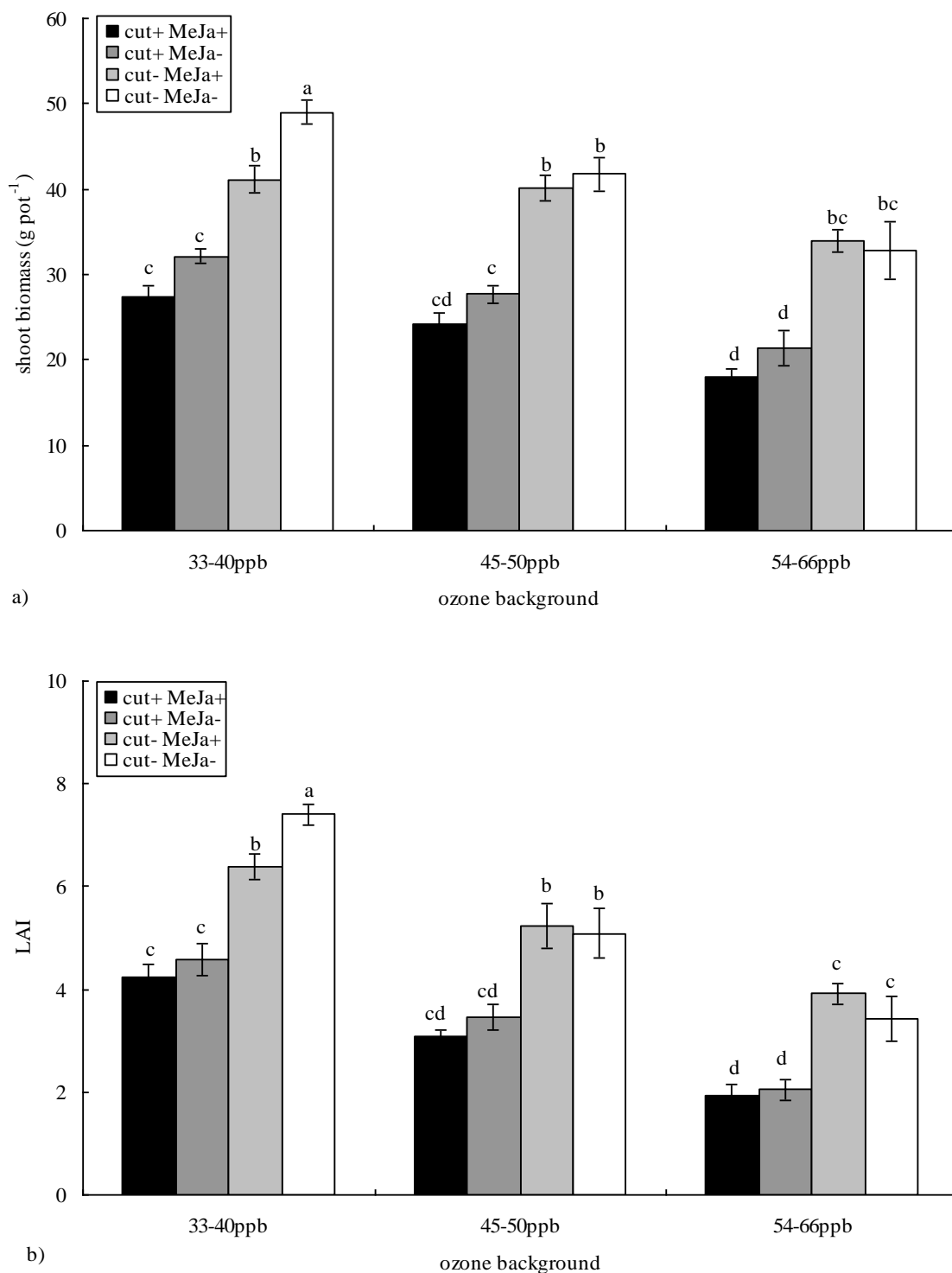


Figure 5.2: Effect of ozone background and cutting/MeJa treatment on mean (a) shoot biomass & (b) LAI after 4 weeks of growth (where LAI= leaf area index; different letters denote a significant difference at  $p < 0.05$ ; bars are standard error;  $n=10$ ).

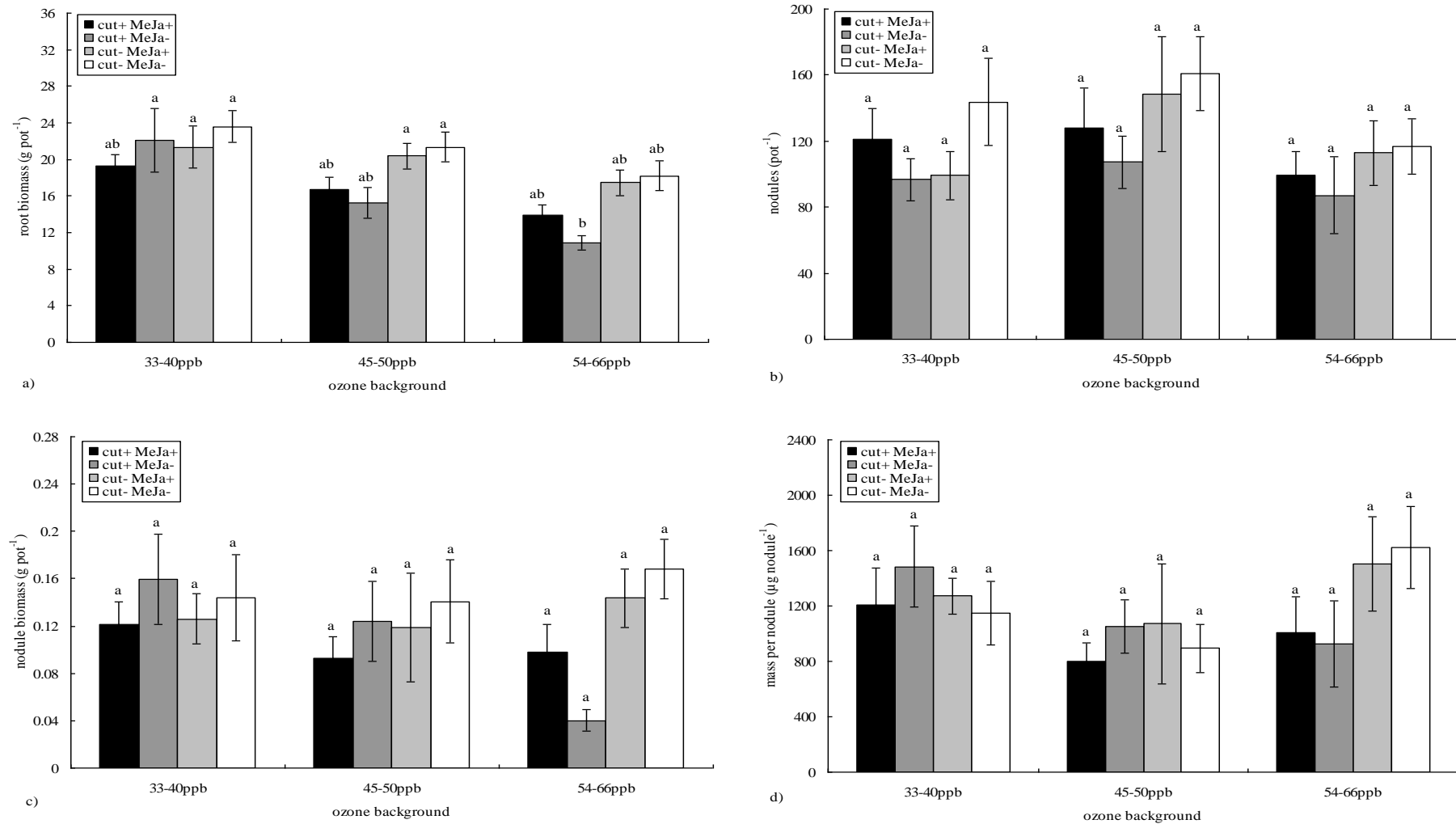


Figure 5.3: Effect of ozone background and cutting/MeJa treatment on mean (a) root biomass pot<sup>-1</sup> (b) nodule number pot<sup>-1</sup> (c) nodule biomass pot<sup>-1</sup> & (d) mass per nodule (different letters denote a significant difference at p<0.05; bars are standard error; n=10).

#### **5.3.4. Correlation analysis**

Root nodule biomass in cut+ MeJa+, cut- MeJa- and cut- MeJa+ pots was not significantly correlated with assessed biomass or injury parameters (Tables 5.3, 5.4). However, analysis revealed significant correlations for cut+ MeJa-, with a positive association with root biomass ( $r=0.84$ ;  $p < 0.001$ ) and LAI ( $r=0.48$ ;  $p=0.006$ ) (Table 5.3). Nodule biomass in cut+ MeJa- was also negatively associated with ozone injury rates in week 2 ( $r=-0.38$ ;  $p=0.03$ ) and 3 ( $r=-0.35$ ;  $p=0.05$ ) and senescence in week 3 ( $r=-0.37$ ;  $p=0.04$ ) (Table 5.4).

Table 5.3: Effects of ozone, methyl jasmonate and cutting treatment on injury, senescence and biomass parameters. Values are F-values (where df= degrees of freedom, ns=not significant, o=ozone, j=methyl jasmonate, c=cutting. Single, double or triple asterisks (\*) denote a significant effect at  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$  respectively after F-tests).

Variable	o (df=2)	j (df=1)	c (df=1)	o:j (df=2)	o:c (df=2)	j:c (df=1)	o:j:c (df=2)
Injury (%)	138.78***	4.18*	9.66**	3.70*	1.06 <sup>ns</sup>	0.01 <sup>ns</sup>	0.64 <sup>ns</sup>
Senescence (%)	19.97***	3.30 <sup>ns</sup>	16.69***	0.71 <sup>ns</sup>	1.47 <sup>ns</sup>	0.94 <sup>ns</sup>	1.03 <sup>ns</sup>
Shoot biomass (g pot <sup>-1</sup> )	42.61***	11.54***	226.31***	2.49 <sup>ns</sup>	0.26 <sup>ns</sup>	0.29 <sup>ns</sup>	1.37 <sup>ns</sup>
Leaf area index	89.50***	1.38 <sup>ns</sup>	137.36***	2.17 <sup>ns</sup>	1.86 <sup>ns</sup>	0.16 <sup>ns</sup>	1.48 <sup>ns</sup>
Root biomass (g pot <sup>-1</sup> )	13.33***	0.13 <sup>ns</sup>	15.43***	1.15 <sup>ns</sup>	1.24 <sup>ns</sup>	0.85 <sup>ns</sup>	0.38 <sup>ns</sup>
Nodules (pot <sup>-1</sup> )	2.35 <sup>ns</sup>	0.00 <sup>ns</sup>	3.72 <sup>ns</sup>	0.15 <sup>ns</sup>	0.34 <sup>ns</sup>	2.60 <sup>ns</sup>	0.39 <sup>ns</sup>
Nodule biomass (g pot <sup>-1</sup> )	0.71 <sup>ns</sup>	0.54 <sup>ns</sup>	4.05*	0.74 <sup>ns</sup>	2.59 <sup>ns</sup>	0.27 <sup>ns</sup>	0.92 <sup>ns</sup>
Mass ( $\mu\text{g nodule}^{-1}$ )	1.84 <sup>ns</sup>	0.08 <sup>ns</sup>	1.23 <sup>ns</sup>	0.81 <sup>ns</sup>	1.99 <sup>ns</sup>	0.46 <sup>ns</sup>	0.44 <sup>ns</sup>

Table 5.4: Correlation analysis of total root nodule biomass data, pooled from low, medium and high ozone backgrounds, with biomass parameters. Data are Pearson's correlation coefficients (where LAI = leaf area index; \*\*=significant at  $p \leq 0.01$ ; \*\*\*=significant at  $p \leq 0.001$ ).

Treatment	Root biomass (g pot <sup>-1</sup> )	Shoot biomass (g pot <sup>-1</sup> )	LAI
cut+ MeJa+	0.03	0.07	0.11
cut+ MeJa-	0.86***	0.26	0.48**
cut- MeJa+	0.19	-0.01	-0.04
cut- MeJa-	0.16	0.23	0.06

Table 5.5: Correlation analysis of total root nodule biomass data, pooled from low, medium and high ozone background treatments, with raw percentage injury and senescence data. Data are Pearson's correlation coefficients (\*=significant at  $p \leq 0.05$ ).

Treatment	Injury (week 2)	Senescence (week 2)	Injury (week 3)	Senescence (week 3)	Injury (week 4)	Senescence (week 4)
cut+ MeJa+	-0.13	-0.08	-0.17	-0.13	-0.13	-0.05
cut+ MeJa-	-0.38*	-0.13	-0.35*	-0.37*	-0.25	-0.09
cut- MeJa+	-0.10	-0.24	-0.21	-0.04	-0.20	-0.07
Cut- MeJa-	0.27	0.03	0.06	0.17	0.05	0.12



#### 5.4. Discussion

Contrary to the hypothesis, cutting tended to increase mean ozone injury rates, and cutting did not consistently affect lower senescence rates relative to control in the short term. Thus the decrease in total ozone injury rates in *T. repens* over time (e.g. Hayes et al. 2010; Hewitt et al., in prep) could indicate a passive whole-plant response to ozone, similar to SA-mediated systemic acquired resistance (e.g. Sharma et al. 1996; Tamaoki, 2008; Xu & Brosché, 2014). The relatively high levels of injury in cut+ MeJa- throughout the experiment suggest an enhanced sensitivity of the younger canopy, perhaps due to elevated ET production during leaf development (Hunter et al. 1999). In the present study, MeJa treatment reduced ozone injury only in the highest ozone background, and MeJa may induce additional ET emissions in ozone enriched environments causing a higher baseline injury (e.g. Grant & Vu, 2012). Regrettably, it is a limitation of the study that, owing to the short duration of the experiment, sufficient stomatal conductance ( $g_s$ ) data for each treatment could not be collected to investigate any possible effect of treatment on  $g_s$ , and thus ozone flux to the canopy (Mills et al. 2011). The exogenous application of MeJa can induce stomatal closure at as little as 10  $\mu\text{M}$  (e.g. Hossain et al. 2011), though applications of 100 $\mu\text{M}$  had no effect on  $g_s$  in ozonated tobacco (Örvar et al. 1997). Although  $g_s$  was not determined, MeJa treatment had a significant effect on shoot biomass, suggesting normal gas exchange may have been suppressed. In *Trifolium* spp., older leaves are thought to be more sensitive to ozone due to a higher conductance rate (e.g. Karlsson et al. 1995).

In addition to increasing ozone-injury rate, cutting clearly increased the sensitivity of *T. repens* to ozone-impacts on below ground biomass, after only 4 weeks ozone exposure and a single cut, including the architecture of N-fixation. Cut plants had a lower number of nodules in low and high ozone, and root nodule biomass in cut+

MeJa- displayed a strong ( $r=0.86$ ) positive relationship with root biomass, reflecting the primary importance of roots in providing carbon assimilates, nutrients, shoot-derived signalling and structure necessary for normal root nodule growth and development (e.g. Hirsch, 1992; Kinkema et al. 2006; Ferguson et al. 2010; Deinum et al. 2012). Ozone-induced reductions in root biomass reflect a preferential partitioning of C and other resources to above-ground growth, repair or defence (e.g. Cooley & Manning, 1988; Cho et al. 2011) and blockages in the phloem (e.g. Asensi-Fabado et al. 2010). Depressed root growth may increase the sensitivity of *Trifolium* spp. to drought and nutrient stress (*sensu* Vollnes et al. 2010) and could detract from the benefits of *T. repens* to soil structure in pasture (Mytton et al. 1993). Although root nodule biomass was independent of shoot biomass in cut+ MeJa-, it was associated with LAI ( $r=0.48$ ), and to lesser degree, with the percentages of injured or even senesced - and dead – leaves in the canopy. Consequently, nodulation in *T. repens* can reflect both the longer-term effects of ozone on whole-plant biomass, and changing short-term effects of ozone on the standing leaf area. This concurs with previous assessments of ozone sensitivity in *Trifolium*, which found differential sensitivity was related to leaf area and other foliar parameters (Postiglione et al. 2000; Francini et al. 2007; Vollnes et al. 2009). Increasing LAI enhances photosynthesis and plant productivity by providing the potential for light interception. Associations with LAI and leaf-injury therefore indicate the ultimate dependency of root nodules on leaves as a carbohydrate source and for AON (Ferguson et al. 2010). Although AON, and its interactions with cutting, are still poorly understood in legumes (*sensu* Aranjuelo et al. 2015), cutting can rapidly decrease N-fixation rates (Vance et al. 1979), induce the mobilisation of C and N reserves from roots to the shoots (Avicé et al. 1996), cause oxidative stress in nodules (Aranjuelo et al. 2015) and reduce nodule activity during

regrowth (Ta et al. 1990). Coupled to a reduced abundance of clover in pasture due to ozone (e.g. Heagle, 1989), the additive effects of cutting or grazing pressure could therefore rapidly, and disproportionately, increase the sensitivity of N-fixation in pasture to ozone (e.g. Montes et al. 1983; Hewitt et al. 2014). LAI and ozone-injury rate may initially be suitable proxies to gauge impacts on root nodule biomass, although this suitability may be lost as the pasture matures and develops a resistance to ozone (Hewitt et al., in prep). Interestingly, in frequently-cut plants, ozone impacts on the nodulation of *T. repens* can persist through an entire season despite some apparent recovery in above-ground effects (Hewitt et al., in prep). For the first time, this study has considered the possible influence of ozone-induced stress hormones on legume nodulation. An absence of ozone-impacts on nodule parameters in cut- MeJa+ and cut+ MeJa+ suggest foliar jasmonates may serve some protective effect on nodulation, although further work is needed to identify any possible role of JA in ozone-impacts on nodulation. The actions of other long-distance signalling phytohormones, such as ABA and auxin, are known to be affected by ozone-induced ROS signalling (Blomster et al. 2011; Wilkinson et al. 2010) and are worthy of further investigation.

## 5.5. Conclusion

In the present study, cutting did not significantly reduce ozone-injury rates in *T. repens* compared to uncut controls, and observations of reducing injury in *Trifolium* over time suggest a passively-acquired resistance to ozone irrespective of treatment. Cutting significantly increased the sensitivity of below ground biomass to the effects of ozone, with impacts on nodule number and root biomass, and additive interactions with ozone occurring in root nodule parameters. It is hypothesised that ozone-induced

impacts on root biomass and the architecture of N-fixation do not directly occur via JA dependant pathways, and root nodule biomass may display significant correlations with root biomass, LAI and ozone-injury parameters in cut plants.

## 6. GENERAL DISCUSSION

### 6.1. Overview of results

The main findings of this PhD project were:

- 1) Modern, previously untested varieties of clover (*Trifolium* spp.), recommended for use in pasture, can respond strongly to ozone, developing symptoms of injury (Figure 2.2a) and with impacts on biomass production, including reduced nodulation (Figures 2.3, 2.4). In white clover, *T. repens* cv. Crusader, ozone can also reduce nitrogen (N)-fixation rates as determined by the acetylene reduction assay (Figure 2.5).
- 2) Ozone can decrease the yield, production, N-fixation and quality of high sugar grass (HSG) pasture mesocosms. Effects on the yield of companion grasses (*Lolium perenne* cv. AberMagic) may be marginal, but they may respond strongly with a reduced root biomass (Figure 3.4). Impacts on the nodulation and N-fixation of white clover (*T. repens*) can occur within mixed canopies (Figures 3.5, 3.7), although symptoms of ozone on injury rates and yield can reduce over time, such that the current critical level for productive grasslands (Mills et al. 2011d) may over-estimate impacts on yield. Ozone may also impact a range of forage quality parameters, including relative (RFV) and consumable food values (CFV), and total sugar content, which may detract from the suggested benefits of HSG pasture (Figure 3.8). A good fit ( $r^2$ ) may be found with below-ground parameters and ozone flux (Figures 3.4, 3.5), though it may be hard to derive a critical level for impacts on forage quality in pasture where constituent species have a different threshold for effects (PODY), but where those impacts cannot be separated.

- 3) Ozone impacts on N-fixation have been reported in several important agronomic legume species, including clovers, but have not extensively been investigated (Table 1.1). Ozone-impacts on N-fixation are thought to arise from shortfalls in carbon (C) assimilation or translocation, but could also reflect change in autoregulation of nodulation processes, including the action of phytohormones. Based on ozone data for a high (year 2006) and average ozone year (2008), and an experimentally-derived exposure-response function (Figure 4.1), predicted reductions of up to 12% or more can occur in the root nodule biomass of clover across UK pasture (Figure 4.2). Further work is required to improve the understanding of the impacts of ozone pollution on N-fixation in legumes, but reactive N pollution, and increasing concentrations of carbon dioxide (CO<sub>2</sub>), may mitigate some of the most severe impacts.
- 4) Impacts on root and nodule biomass in white clover (*T. repens*) display a strong positive interaction with cutting (Figure 5.3), supporting a reduced flow of C to the root system as a cause for reduced N-fixation, and possibly hinting at a persistent oxidative stress in root nodules during ozone exposure due to reduced nodule respiration (Aranjuelo et al. 2015). Impacts on nodulation did not result from the application of exogenous jasmonates, and reduced injury over time likely reflects a systemic acclimation to ozone. Grazing pressure may increase the sensitivity of N-fixation in pasture to the effects of ozone, with root nodule biomass displaying negative correlations with injury and senescence in the canopy (Table 5.4).

## 6.2. Discussion

In the present study, the higher tolerance of red clover (*Trifolium pratense* cv. Merviot) manifested in reduced ozone injury rates and impacts on biomass production

(Figure 2.2a, 2.3) compared to white clover (*T. repens* cv. Crusader), which could reflect a significantly different stomatal conductance (Figure 2.2b), and possible capacity of the plant to maintain normal photosynthesis rates. Amongst other factors, differential sensitivity within *Trifolium* may also be expressed in Chlorophyll a fluorescence (Scebba et al. 2003), pigment content and antioxidant status (Scebba et al. 2003; Chaudhary & Agrawal, 2013), protein content (Kollner et al. 2002), and leaf temperature (Futsaether et al. 2015), although these parameters were not assessed. As for many cultivated species (e.g. Pleijel et al. 2006; Singh & Agrawal, 2011; Inada et al. 2012), evidence of the differential sensitivity of modern clover cultivars is therefore an important basis for the future selection of resistant varieties due to little change in the level of near-future ozone (IPCC, 2013). Compared to the *T. repens* cv. Regal bioindicator system, the new cultivar used throughout this study, *T. repens* cv. Crusader, seems sensitive to ozone, with symptoms of ozone damage occurring at comparable exposures to those of ozone-sensitive biotype (e.g. Postiglione et al. 2000). This study also supports a difference in sensitivity between *T. repens* and *T. pratense* (e.g. Hayes et al. 2007).

Although the effects of ozone on yield may be absent, an effect common to *Trifolium* in short-term exposures are large ozone-induced impacts on below ground biomass production (Chapter 2; Chapter 5) (Vollsnes et al 2010; Sanz et al. 2014). Reductions in root biomass (Chapters 2, 3 and 5) could increase the sensitivity of clover in pasture to the effects of drought and nutrient stress (*sensu* Vollsnes et al. 2010), and ultimately contribute towards an ozone-induced reduction in the abundance of clover in pasture over time (e.g. Hayes et al. 2009). The effects of ozone on nitrogen (N)-fixation in legumes remains poorly understood, this study having considered this effect on a systematic basis for the first time. Impacts on N-fixation

capacity or measured activity have been determined in a number of agriculturally-important legume species with variable effects on a whole-plant scale (Table 1.1). Relatively few experimental studies have investigated this question, often with wide variation in methodology. In general, seasonal exposure to mean ozone concentrations of 40-60ppb or short-term acute exposures to concentrations >100ppb, appear sufficient to reduce N-fixation rates or impact nodulation in a number of important legumes, including soybean, peanut and white clover (Table 1.1). Using ozone data for a year with high ozone concentrations (2006) and more average year (2008), reductions in the root nodule biomass of *Trifolium* spp. by  $\geq 16\%$  may be found in some UK pasture systems (Figure 4.2). The predicted losses in root nodule biomass assumes ideal nodulation, were not based on flux-based measures, and do not take into account a range of other important ecophysiological parameters, which are key sources of uncertainty in this assessment. Using averaged N-fixation data from the 2012 (Chapter 2) and 2013 (Chapter 3) experiments, root nodule biomass explained 50% of the variation in N-fixation rate ( $r^2=0.50$ ;  $p=0.04$ ; Appendix, Figure A2). However, as N-fixation rates were determined from these studies by acetylene reduction assay (ARA) (units: nL ethylene cm<sup>2</sup> soil surface), scaling this chemical data to a landscape scale would require a number of additional measurements and assumptions.

Impacts on the nodulation and N-fixation rate of *T. repens* may occur in monoculture (Chapters 2, 5) or in mixed communities (Chapter 3). The most severe effects of ozone on legume nodulation, such a reduced nodule number, occur in acute ozone exposures, or when the likely dose to ozone to plants is higher (e.g. Hayes et al. 2010b). A good fit ( $r^2$ ) can be found between below ground biomass, including root nodule biomass (Figure 3.5) and N-fixation (Figure 3.7), and ozone flux to *T. repens*



via the upper leaves, supporting the development of additional flux-based critical thresholds for below-ground responses (Mills et al. 2011d). However, the variation in effects on N-fixation data found in Chapter 4 (Figure 3.7) indicates that this response may be unsuitable for the development of a specific flux-based critical level. Instead, optimum N-fixation rates may be best maintained by protecting against the less transient change occurring in root and nodule growth (Chapters 2, 3 and 5). Interestingly, although impacts on nodulation may be found in uncut plants after 3 months exposure (Figure 2.3), impacts of chronic ozone do not occur in uncut plants in the short term (Chapter 5; Figure 5.3). This supports the hypothesis that impacts on nodulation or N-fixation quickly result from disruption in the flow of C assimilates (Pausch et al. 1996a, b; Cong et al. 2009; Cheng et al. 2011), and could also indicate a persistent oxidative stress in root nodules due to a cessation of nodule respiration (e.g. Aranjuelo et al. 2015). In cut plants, the root nodule biomass of *T. repens* may display significant correlations with root biomass, leaf area index and with raw percent injury and senescence data (Chapter 5). With cumulative cuttings, these impacts can persist throughout an entire season, despite a large reduction in above-ground ozone effects over time (Chapter 3). This effect may be particularly important in established grasslands where the apparent impacts of ozone are absent, but where legumes are an important and characteristic component (e.g. Stampfli & Fuhrer, 2010; Bassin et al. 2013; Volk et al. 2014). Although Jasmonic acid (JA) is mutually involved in defence responses to ozone and in the negative regulation of legume nodulation (e.g. Tuominen et al. 2004; Susuki et al. 2011), the absence of effects of methyl-jasmonate (MeJa) treatment were found here (Chapter 5). Intensive grazing pressure could substantially increase the sensitivity of N-fixation in pasture to the effects of ozone.

*T. repens* in managed pasture may acclimate to ozone over time, with a reduced effect of ozone on above-ground biomass and total ozone-induced injury and senescence (Chapter 4). In the short-term, this change in sensitivity to ozone is not attributable to defence pathways induced by cutting, against the initial hypothesis (Chapter 5). Instead, the acclimation to ozone could reflect a passive whole-plant response akin to systemic acquired resistance (e.g. Sharma et al. 1996; Tamaoki, 2008; Xu & Brosché, 2014), which may be reflected in increases in antioxidant capacity or metabolism of plants (Wang et al. 2014).

Ozone may also impact production and forage quality of managed pasture. Compared to the current critical level for impacts on biomass in productive pasture (Mills et al. 2011d), a lower effect was seen on the clover in this study (Chapter 3). The critical level may be unrepresentative of pasture communities in some circumstances, as a reduced effect of ozone on clover yield may occur over time (Chapter 3; Figure 3.3), and the dose of ozone to clover may be lower in mixed canopies (Hayes et al. 2010b). The impacts of ozone on the yield of *L. perenne* cv. AberMagic were marginal (Figure 3.3). Although this would support a difference in sensitivity between white clover and ryegrass in pasture, *L. perenne* did respond strongly with a reduction in root biomass (Chapter 3; Figure 3.4), which may indicate a subtler, longer-term effect of ozone. This study has also developed new dose-response relationships for acid detergent lignin (ADL) and neutral detergent fibre (NDF) fractions, total sugar and crude protein content, and relative (RFV) and consumable food values (CFV) of mixed high-sugar ryegrass (HSG) pasture (Chapter 3; Figure 3.8). Together, these could assist in the development of a critical level for impacts of ozone on the quality of pasture (Mills et al 2011a). However, caution is needed in this interpretation in order not to exaggerate the effects of ozone. For

example, these relationships are based on an ozone threshold of  $0\text{nmol m}^{-2}\text{ s}^{-1}$  ( $\text{POD}_0$ ) - assuming no threshold for effects. Although this is not without precedent (Sanz et al. 2014), thresholds for the effects of ozone are well established (CLRTAP, 2011), as some ozone can be detoxified without effect (Cho et al. 2011). In addition, the analysis of individual harvests against flux accumulated in the preceding 4 weeks (“growth cycle flux”) revealed only two occasions in the experiment in which ozone had a significant ( $p \leq 0.05$ ) effect on pasture quality parameters (Chapter 3, Table 3.8). Without a consistent impact of ozone, this suggests the seasonal effects of  $\text{POD}_0$  accumulation on forage quality parameters could also be largely attributed to passive change in the abundance of the two cultivars, and specifically, the increasing abundance of clover in the pasture mix (Chapter 3, Table 3.5). Given these considerations, these dose-response functions would therefore over-estimate the effects of ozone if applied to an agroecosystem scale, although they highlight the practical difficulty of deriving a critical level for impacts in pasture communities, where constituent species have different thresholds for effects, and where ozone effects are in practice often hard to distinguish against a background of natural variability (Chapter 3) (e.g. Stampfli & Fuhrer, 2010). Impacts upon forage quality result in a reduced feed value and digestibility in ruminants and non-ruminants (Gonzalez-Fernandez et al. 2008). Reduced sugar content of HSG pasture forage could detract from the suggested benefits of HSG grass varieties (Ellis et al. 2011; Staerfl et al. 2012), and coupled with potential shortfalls in N-fixation, detract from the N-use efficiency of pasture.

In addition to the limitations previously mentioned in this study, a further consideration is that, in the experimental chapters (Chapter 2, 3 and 5), pasture plants or mesocosms were grown in black plastic pots. Although soil temperature was not

determined, it is possible that increased soil temperature due to UV absorption could have affected root or nodule respiration and N-fixation activity. Plant pots similarly could have also increased root deformation and reduced production (e.g. Amoroso et al. 2011). Acetylene reduction assays, the N-fixation assay used throughout the study, would also be sensitive to ethylene originating from the decomposition of organics in soils. In contrast, other methods of N-fixation determination (e.g.  $N^{15}$ -difference method; total N content) would provide an assessment of N-fixation activity less susceptible to contamination and temporal variation. Although the exetainers used for the collection of gas samples were new, and samples were quickly processed, no testing of gas-tightness or loss rate was performed, which could have affected the absolute ethylene concentration measured. Irrespective, these limitations would have occurred across all ozone treatments, and do not detract from the main ozone-effects investigated in this the study. Unfortunately, in the experiment detailed in Chapter 3, low sample weights prevented the determination of total N, owing from the need to have sufficient material for forage quality analysis. Soil N levels, determined from samples in the experiment detailed in Chapter 2, showed no effect of ozone treatment (data not shown) despite the impacts on clover N-fixation, and a detectable effect of ozone on N availability was perhaps unlikely given the short-duration of the studies. Further testing and validation of the constructed dose-models (Chapter 3, Table 3.2) is also required; for example, a statistical testing of the goodness-of-fit between predicted stomatal conductance and observable measurements, as well as direct determination of ozone concentrations in the leaf-intracellular airspace and canopy boundary layer, and a quantification of cuticular and stomatal resistances. Lastly, in the experiment detailed in Chapter 5, there was insufficient time to discern a potential impact of MeJa treatment on gas

exchange, which may have caused an observable decrease in shoot biomass in MeJa-treated plants (Chapter 5, Figure 5.2).

### **6.3. Future work**

There are several avenues for future work. Additional research should be conducted on new clover and grass cultivars to improve knowledge of ozone impacts and help identify varieties resistant to ozone, both within, and between, species. Although it is clear ozone can impact N-fixation and nodulation in important agronomic legumes, including clovers in pasture, the extent of this effect is unknown and is based on relatively few studies. In addition to using a flux-based response-function for root nodule biomass (Chapter 3), predicted losses in clover nodulation across UK pasture (Chapter 4) could be further improved by correlating spatial data to known silage or pasture production data, or fertiliser usage data for the same period, which would help identify regions sensitive to impacts on N-fixation, and the magnitude of effects. The potential mechanisms of ozone-impacts on N-fixation are also an important avenue of future work. Although no evidence was found for an impact of JA on nodulation in the present study, further work is needed to rule out a role of JA, and the downstream effects of ozone could potentially disrupt the action of a range of other phytohormones, including auxin (Blomster et al. 2011), abscisic acid (Wilkinson & Davies, 2010) and gibberellins (Li et al. 2011), which all have an important role in legume nodulation (Maekawa et al. 2009; Ferguson et al. 2010; Mortier et al. 2012; Ryu et al. 2012). The large interactive effect of cutting and ozone on nodulation is noteworthy (Chapter 5), and additional work with selective cutting experiments could help identify the pathways shared between abiotic stressors (e.g. Aranjuelo et al. 2015). Flux-based dose-response relationships for biomass and forage quality remain

underdeveloped and simple, and more data is needed to better replicate the complexity and changing dynamics of pasture. In particular, it is a difficult, but important, question to quantify the impacts of ozone on pasture where constituent species have different thresholds for effects. In general, this study would support the development of dose-response relationships based on below-ground responses, which can be more responsive (Chapters 2 and 5), and have greater permanency (Chapter 3) than above-ground effects, which may be absent in established pastures (e.g. Stampfli & Fuhrer, 2010; Bassin et al. 2013; Volk et al. 2014). Lastly, although large impacts were found in this study, long-term ozone-exposure experiments are needed in all cases to determine the magnitude of effects of ozone on pasture yield and production, composition, quality and N-fixation.

#### **6.4. Conclusion**

In this PhD project, the impacts of ozone on the sustainability of pasture were quantified by investigating:

- 1) Effects of ozone on growth and functioning of modern cultivars of clover (*Trifolium* spp.).
- 2) Impacts of ozone on HSG pasture mesocosms, including the development of dose-response relationships for growth, N-fixation and forage quality.
- 3) The potential effect of ozone on clover nodulation in pasture and consequences for pasture sustainability.
- 4) Interactions between cutting and ozone on injury and nodulation in white clover (*T. repens*).

Chronic exposure to ozone pollution (seasonal mean concentrations (30-67ppb)) can affect a variety of impacts in the short-term on modern pasture vegetation, including; reduced biomass production in clovers and companion grasses, impacts on nodulation and nitrogen fixation, reduced pasture yield and productivity, and impacts on forage quality. Pasture vegetation can also display a large variation or resilience to effects, with transient impacts on biomass production and yield, forage quality parameters and N-fixation rates. Ozone impacts on N-fixation in legumes have not extensively been studied but are thought to ultimately arise from a reduced supply of C assimilates to the root system, though a strong interaction with cutting might hint at other potential mechanisms, including the persistence of oxidative stress in nodules. Based on an experimentally-derived ozone-exposure response function, large reductions in root nodule biomass ( $\geq 16\%$ ) may occur across UK pasture. The current flux-based critical level for impacts of biomass in productive pasture may be unrepresentative of managed pasture in some circumstances, and a focus on above-ground productivity alone may under-estimate the sensitivity of grass in pasture to ozone. The development of a critical level for impacts on forage quality requires an improved understanding of modelling impacts for mixed pasture. A good fit ( $r^2$ ) may be found with ozone flux in below ground variables in pasture vegetation, which, in general, often respond strongly to the effects of ozone.

## APPENDIX

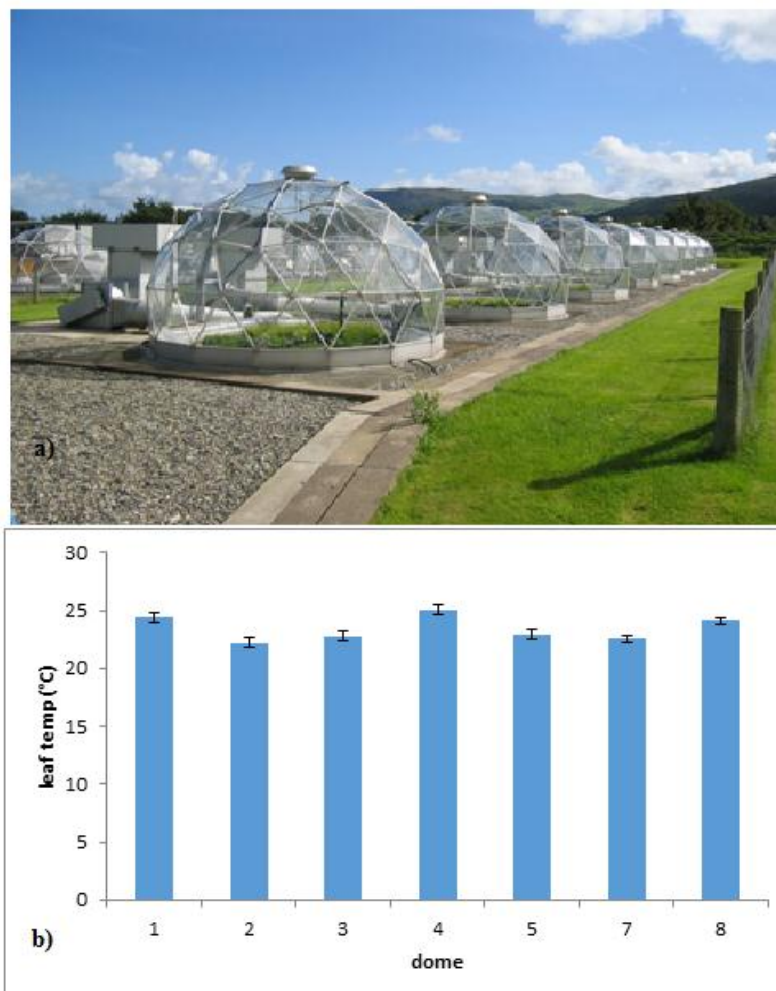


Figure A1: (a) The Ozone exposure solardome facility at CEH Bangor (Henfaes Farm, Abergwyngregyn, near Bangor, Wales, UK) (b) mean leaf temperature recorded over the growing season for the 7 solardomes used in the experiment detailed in Chapter 2. For experimental purposes the solardomes are numbered consecutively 1 to 8, with no. 8 being the solardome in the foreground of the photograph. The ozone treatments were randomly allocated to the solardomes as indicated in Tables 2.1, 4.3 and 5.1 in the main text. Although the climatic conditions are not routinely recorded in each solardome, the fan speeds are matched across solardomes ensuring that there are the same number of air changes per minute in each solardome. To provide support for matching climatic conditions in each solardome, Figure 1b contains the mean leaf temperature recorded between 10:00-16:00h by the porometer (model AP4, Delta T Devices, Cambridge, UK), for 60 measurements made in each solardome over the growing season during the experiment detailed in Chapter 2 (data combined for measurements on Merviot and Crusader). There was no significant linear relationship between mean leaf temperature and either solardome number ( $p=0.97$ ) or seasonal mean ozone concentration ( $p=0.30$ , Linear regression, R software).



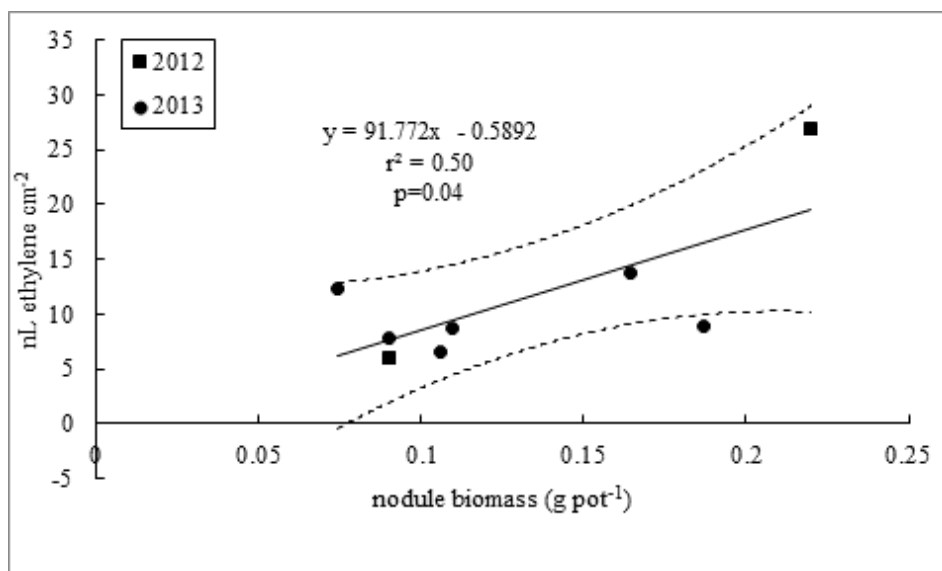


Figure A2: When average N-fixation data for the experiments detailed in Chapters 2 and 4 are considered, there was a significant positive relationship between N-fixation and nodule biomass for the two experiments, with root nodule biomass accounting for 50% of the variation in N-fixation rate. However, due to the N-fixation assay providing a “snapshot” of N-fixation activity, nodule biomass being a time-integrated response, and differences between the two experiments (including duration and the fact that the second experiment contained ryegrass in addition to clover), it would be contentious to speculate on the on effects of ozone on N-fixation on a UK scale. The relationship also seems to be driven by a single data point. Although the acetylene reduction assay can provide a proxy measure for N-fixation, it is generally considered a less-robust measure of N-fixation than other measures such as the N-difference method and total N content (Hardarson & Danso, 1993). On balance, mapping effects on N-fixation based on the results of the ARA, on a UK-wide basis, would require a number of large assumptions at this stage (Linear regression, R software).

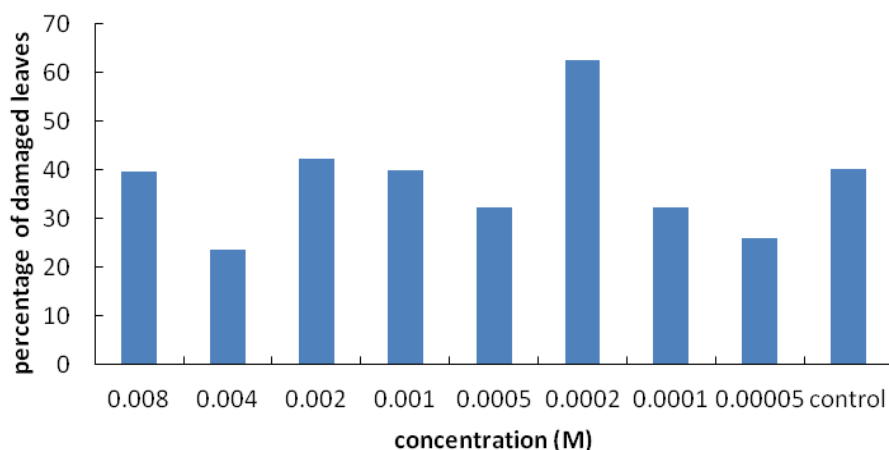


Figure A3: Effect of Methyl Jasmonate treatment on ozone injury rate in *T. repens* after 2 weekly sprayings. Values represent mean of 3 plants in a single pot. Plants were exposed to treatment 6 in chapter (weekly mean = 70ppb). After regression analysis, no effect of concentration on injury was found ( $r^2=0.15$ ;  $p=0.70$ ). A concentration of 500  $\mu\text{M}$  was decided as the best concentration to use in the experiment detailed in chapter 5 due to its intermediate strength and likelihood of effects, relatively low injury rate, and closeness to similar experiments reported in the literature.

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