Plant responses to long term elevated ozone in a British upland semi-natural grassland

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Abstract

A novel free-air gas concentration enrichment (FACE) experiment exposed a species rich semi-natural upland grassland to ozone at average levels of ambient + 75 ppb, + 25 ppb and + 15 ppb over a 6 year period in the North Pennines. In addition to this, high exposures lasting 4 days of up to 309 ppb occurred in June 2012. Three main factors were investigated over the growing season of 2012 in relation to exposure to elevated ozone: the flowering behaviour of 11 species; the nectar volume and composition of the hemi-parasite *Rhinanthus minor*; and the CO_2 exchange of the ecosystem.

Dactylis glomerata flower abundance significantly increased over ambient levels with exposure to + 15 ppb ozone, but not ambient + 25 ppb. *Conopodium majus* also exhibited significant increases in flower abundance, though the effect was seen in ambient + 25 ppb when compared to ambient air, but not ambient + 15 ppb. However, *Ranunculus acris* flower abundance reduced sharply in both ozone elevations. Peak flowering dates were shown to be two weeks later in two grass species, *Festuca pratensis* and *Trisetum flavescens*, when exposed to a long term background of ambient + 25 ppb, and short term exposure to a mean 140 ppb. Short term exposure to 77 ppb and 52 ppb ozone did not significantly affect nectar volume or composition in *R. minor* flowers; however there may have been an effect on sugar composition at 77 ppb exposure. There was no significant effect of long term ozone exposure at ambient + 75 ppb, ambient + 25 ppb and ambient + 15 ppb on ecosystem respiration, net ecosystem exchange (NEE), or gross primary production (GPP) relating to CO_2; however data suggest NEE and GPP may have been reduced through foliar injury relating to acute ozone exposure to an average 309 ppb. The findings have wider implications for conservation of upland grassland species diversity, and also indicate the potential for implications for the wider grassland carbon sink during peak ozone episodes.

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Acknowledgements

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I would like to thank my supervisors, Dr Sylvia Toet and Prof Mike Ashmore, for their guidance and support throughout the duration of this study.

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I would also like to acknowledge the support and encouragement of my family and friends throughout this study.

Declaration

The research in this thesis was developed by the author between April 2012 and September 2013. I declare that all the work within this thesis, apart from work by authors who have been clearly acknowledged, is the result of my own and original work.

1 Introduction

Ozone (O_3) is a gaseous compound and is a secondary gaseous tropospheric pollutant that acts as a greenhouse gas, formed through a series of photochemical reactions involving sunlight, nitrogen oxides (NO_x) and volatile organic compounds (Ashmore, 2005; Mills *et al.*, 2009). It has a strong odour, and has effects both on human health, and on vegetation (Fuhrer *et al.*, 1997; Royal Society, 2008). It is very reactive in the troposphere; Equation 1.1 outlines the reactions that lead to ozone formation and destruction in clean air; however, a photochemical reaction involving NO_2 and an inert molecule 'M'– typically N_2 – is also defined in Equation 1.2 as this also forms ozone, and this is the typical reaction in polluted air (Royal Society, 2008).

$$O_2 \rightarrow 2O$$

$$O + O_2 \rightarrow O_3$$

$$O_3 \rightarrow O_2 + O$$

$$O + O_3 \rightarrow 2O$$ **Equation 1.1**

$$NO_2 + h\upsilon \rightarrow NO + O(^3P)$$

$$O(^3P) + O_2 + M \rightarrow O_3 + M$$ **Equation 1.2**

1.1 Trends in ozone concentration

Ozone concentrations are considered using two terms: background ozone; and peak ozone. The Royal Society (2008) clearly defines both terms, with background ozone outlined as the natural and imported ozone within a regional atmosphere; while peak ozone is described as an episode where high emissions of ozone precursors coincide with ideal meteorological conditions, such as low wind (thus reducing mixing and dispersion) and high levels of sunlight, typically over 100 ppb in concentration. AQEG (2009) reported that in 2005, annual mean English levels outside of London were 23 ppb, but just 18 ppb in greater London, showing that concentrations tend to be higher away from urban areas; this is due to elevated levels of NO_x where agricultural land use is prevalent.

Concentrations of NO_x, VOCs and carbon monoxide (CO) have all increased in the last century, and anthropogenic activities such as transport, land use change and industrial activity are responsible (Logan, 1985); Figure 1.1 outlines the contribution to these emissions by source in 2000. Thus, tropospheric ozone has increased with the rise in its precursor compounds. However, due to a number of emissions controls in the late 20th century, atmospheric concentrations of these compounds have reduced, with European NO_x levels having dropped by 30% in 2005 when compared to 1990 levels (EEA, 2007). Yet, ozone is continuing to cause concern as a result of rising precursor emissions on a global scale, and northern hemispheric background concentrations have increased by 5 ppb in the 20-30 years previous to 2006 (AQEG, 2009). Moreover, background concentrations are projected to increase to 75 ppb by 2100 in Europe (Sitch *et al.*, 2007).

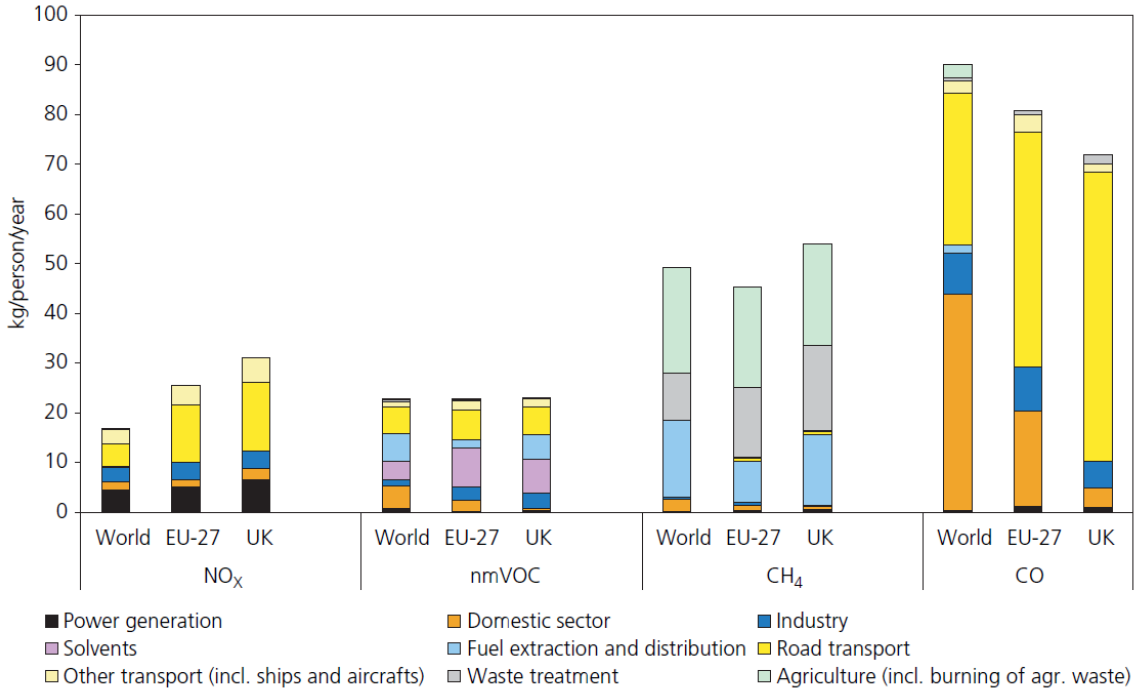

Figure 1.1 – Per capita emissions of ozone precursor compounds for the world, EU-27, and the UK in 2000 (Royal Society, 2008).

Additional to the multi-year trends above, there are annual trends, and Figure 1.2 illustrates the typical annual trend in background ozone in the UK. Background ozone is highest in spring, from March-May, and lowest in November-January. However, as depicted by the grey bars, peak episodes typically occur from late spring to early autumn, when climatic conditions are ideal.

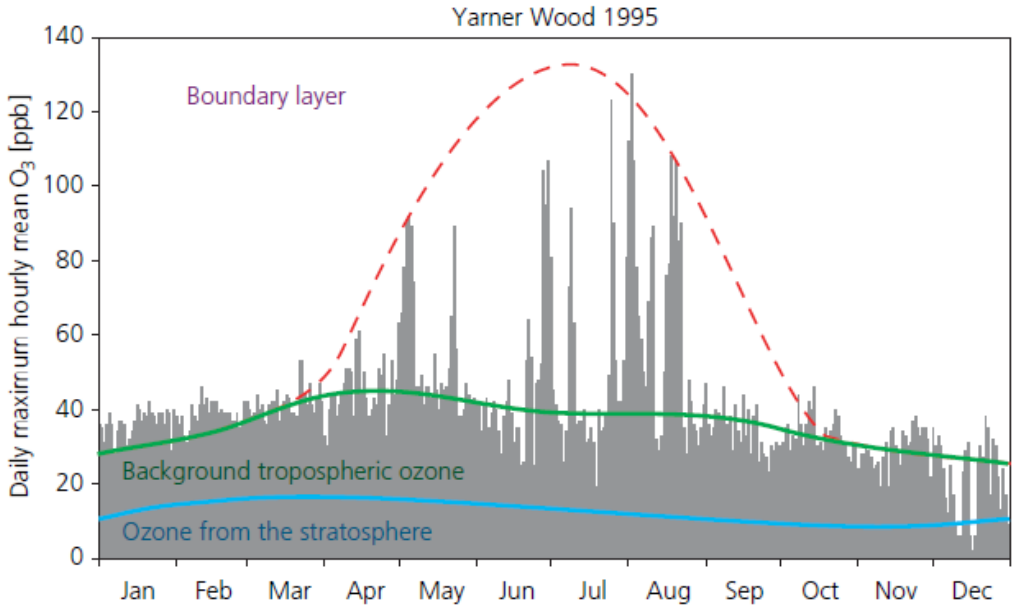

Figure 1.2 – Seasonal variations in surface ozone concentration over one year in Southern England. The chart shows daily peak hourly values (grey bars), and an indication of ozone source apportioned to tropospheric (green line) and stratospheric ozone (blue line) (Royal Society, 2008).

Figure 1.3 further explores the temporal trends in background ozone between March and July by comparing concentrations on a spatial scale across the UK, with levels in March-May exceeding those in May-July. This further supports the notion that the highest background concentrations are found in rural areas, with upland areas such as the Scottish Highlands, North Pennines, Lake District, mid Wales and Dartmoor showing the highest concentrations.

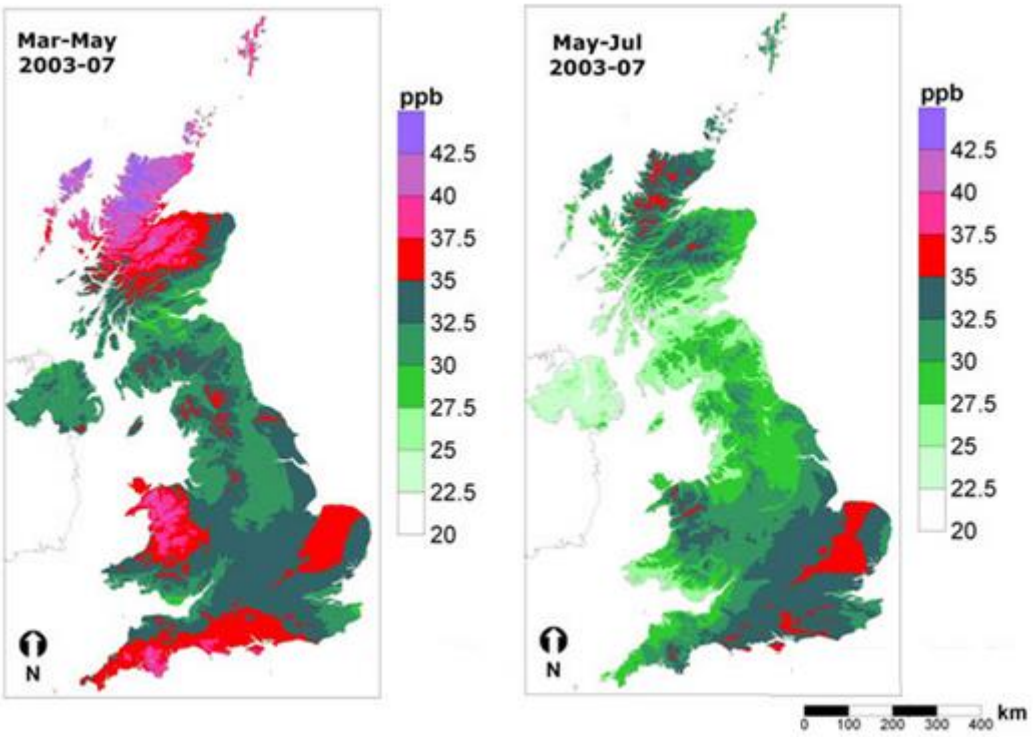

Figure 1.3 – Distribution of mean seasonal ozone concentrations across the UK for the periods March-May and May-July, averaged from 2003-2007 data (RoTAP, 2012).

1.2 Effects of ozone on vegetation

With background concentrations highest in rural areas, ozone has been identified in the literature as the most important rural air pollutant as a result of its potential to affect human health and vegetation, with the effects on the latter first identified in the 1950s (Ashmore, 2005). Ozone is known to enter a plant via the stomata; pores that open and close to regulate the exchange of gases such as CO_2 and H_2O. Once inside the plant, ozone can react in both the gas and liquid phases, creating potentially damaging reactive oxygen species and oxidative stress within the plant (Lyons and Barnes, 1998). The importance of the stomata and stomatal conductance was corroborated by Volin *et al.* (1998), who found that grasses within the C_3 group of plants with higher stomatal conductance show higher oxidative stress than species within the C_4 group.

Vegetation can show the effects of ozone stress in a number of ways, both in the short and long term. Short term effects are often visible, with flecks, stipples, and discolouration occurring to the leaves of species (Skelly *et al.*, 1999). Figure 1.4 shows an example of such acute ozone stress on *Trifolium pratense*; high levels of ozone exposure for a short time result

in the paler, almost brown patches to the tips of leaves. This is reported by Langebartels *et al.* (2002) to be a result of a kind of hypersensitive response leading to cell death and subsequent leaf senescence. Effects in the longer term that have previously been reported include damage to stomatal guard cells, leading to alterations in stomatal conductance, growth reduction and changes in resource allocation (e.g. Fuhrer and Booker, 2003; Bassin *et al.*, 2007).

Figure 1.4 – Example of visible ozone injury to a *Trifolium pratense* leaf in a natural grassland sward, visible as the pale brown lesions across the leaves, taken in June 2012 at High Keenley Fell, Northumberland.

Ozone exposure is discussed with the use of a number of measures in literature, with 8 hourly mean concentrations used frequently to display peak episodes in ozone. However, daily and seasonal means give better representations of longer term trends. In addition to these, an index was developed in response to the need for a critical ozone level to be identified relating to vegetation effects; the AOT40 – defined as the accumulated exposure over 40 ppb during daylight hours (Fuhrer *et al.*, 1997; Royal Society, 2008). A combination of these measures will be referenced throughout this discussion.

Effects of ozone have been seen in a wide range of plant species. Trees are a group that have been widely studied. Fuhrer *et al.* (1997) summarised that effects included negative effects on photosynthetic rate and carbon allocation, and that effects can be not only within the same

growing season, but also in the year after exposure, and also cumulative. A recent review of the effects on *Pinus sylvestris* found that mature trees were more sensitive to ozone exposure than younger trees (Huttunen and Manninen, 2013).

Arable crops have received a lot of research attention due to the potential for reduced yields. In a literature review, Fuhrer and Booker (2003) also identified changes in the nutritional quality of harvested crops, including reductions in the oil content of oil seed rape and increases in protein content in wheat grains. A recent Indian study found that ozone exposure reduced leaf area, photosynthesis and seed yield in mustard crops (Singh *et al.*, 2013). Another recent study reported reductions in growth and yield in *Beta vulgaris* crops grown in elevated ozone, and in the presence and absence of elevated CO_2 (Kumari *et al.*, 2013). Reductions in yield and quality of yield as a result of elevated ozone have wider implications, such as the need to increase fertiliser input, or increase land area under cultivation, to maintain current yield levels and quality.

Species that make up natural ecosystems have also been studied, though not as intensively as productive systems. *Calluna vulgaris*, abundant in British moorlands, became more susceptible to frost following elevated ozone exposure, and displayed higher levels of damage and mortality during winter (Foot *et al.*, 1997). Additionally, previous ozone exposure at 70 ppb for 7 hours per day over 28 days led to reduced survival in simulated winter conditions in *Ilex aquifolium* (Ranford and Reiling, 2007a); plus higher leaf loss rates and lower leaf production in the seasons following ozone exposure (Ranford and Reiling, 2007b).

Grasslands are a major habitat in the UK, covering over 50% of the land area (Wilkins, 2005). However, highly valued, semi-natural grassland managed as upland hay meadows are diminishing, with just 1000 Ha remaining in the UK – approximately 40% of these are in the North Pennines region (North Pennines AONB Partnership, 2012). High aesthetic value is placed upon them by conservation groups, as their inherent species richness allows for a constantly changing floral show through the spring and summer seasons. The species richness of these sites – particularly the forb and legume fractions – also makes such upland hay meadows vital for pollinators and thus is important to restore and retain (Forup and Memmott, 2005).

Elevated ozone has been shown to influence both the productivity and diversity of grasslands. Fuhrer and Booker (2003) reported a reduction in pasture forage quality in response to

elevated ozone. Other studies suggest cumulative effects of ozone on clover biomass after several years of exposure (Montes *et al.*, 1982). Ashmore (2005) reported that a number of studies showed that ozone effects were greater in the first exposure year when studying grasslands, with acclimation to ozone levels apparent in subsequent years. In a review of the literature, Bassin *et al.* (2007) reported that the effect of ozone upon grassland communities depends upon the sensitivity of constituent species, and that competitive interactions can cause some grassland species to increase in abundance, while other species display reductions as a result of altered resource availability.

Much research has been published into the effects of ozone on individual grassland species, with species proving to be both positively and negatively affected by elevated ozone as well as being resistant to change. A meta-analysis by Hayes *et al.* (2007) summarised the biomass responses of 83 grassland species, and reported that one third of species showed reductions in above-ground biomass of 10% or more at exposures of 15 ppm-h AOT40, while another 15 species displayed increased biomass levels of at least 5% in response to exposures of 15 ppm-h AOT40. The remaining 41 species proved unaffected by ozone (Hayes *et al.*, 2007), thus showing the range in responses. Wedlich *et al.* (2012) reported changes in species and functional group above-ground biomass following a 3 year field exposure study that found that forb biomass was significantly reduced, while also reporting reductions in biomass of *Ranunculus* species and *Rhinanthus minor* where ambient background ozone was elevated by 4 and 10 ppb through the growing season. Hayes *et al.* (2010) reported increased senescence in a 2 year study, an effect also seen in peak episode exposures. Additionally, the study found a negative cumulative effect of background ozone elevated to 35 ppb on both the above-ground biomass of the grass *Anthoxanthum odoratum*, and of the entire community (Hayes *et al.*, 2010).

1.3 Previous free air gas concentration enrichment (FACE) studies

Studies relating to the effects of ozone upon a plant community have largely been undertaken in open top chamber (OTC) experimental set ups. Many involve pots of varying sizes being sown with seed mixtures best representing the community being studied, though some OTCs are located in situ within natural communities. Pot-based experiments do not reciprocate the environmental factors in the way natural communities would, such as soil water content and

wind. Moreover, the microclimate within an OTC is typically different to a field situation, and this has been proven to historically bias results (Erbs and Fangmeier, 2005). One major disadvantage to OTC experiments is that, as a result of different microclimatic conditions occurring within OTC environments compared to open air, rates of ozone uptake in plants may not reciprocate field conditions, as proven by Nussbaum and Fuhrer (2000).

As an alternative to OTC systems, FACE was first developed by Harper *et al.* (1973). An advantage of FACE systems is that they allow the study of ecosystems *in situ* and without disturbance, with larger study plots available and while being exposed to naturally occurring environmental conditions. While there are fewer limitations to FACE systems, there are only a relatively small number of experiments involving ozone, and some issues have been highlighted such as the lack of statistical power in results due to the lack of treatment replicates, and the exposure to gradients in environmental conditions (as opposed to homogenous conditions experienced within controlled OTCs or glasshouses) (Erbs and Fangmeier, 2005). Nevertheless, there are a small number of FACE systems to draw knowledge from, such as SoyFACE (Soybean Free Air Concentration Enrichment), which was established in Illinois, USA, from 2001-2009 and studied the combination effects of ozone, carbon dioxide, drought stress and temperature on soybean and maize crops (Eastburn *et al.*, 2010; Morgan *et al.*, 2006). Experiments based at the University of Kuopio, Finland, have studied the effects of ozone on young poplar and silver birch trees using FACE experimental setups (Blande *et al.*, 2007; Oksanen, 2001), while the KROFEX (Kranzberg Ozone Fumigation Experiment), based in Germany, fumigated a stand of mature spruce and beech trees using a FACE system in 2000 and 2001 (Werner and Fabian, 2002). Another FACE system – AspenFACE, was established in 1997 and operated until 2010, with the aim of studying effects of ozone and CO_2 on poplar (Sitch *et al.*, 2007; Darbah *et al.*, 2011).

Two previous studies have focused upon the effects of ozone on grasslands. One was situated in the Swiss Alps at an altitude of 2000 m. Established in 1998 to study the effect of ozone exposure in combination with other factors (e.g. Volk *et al.*, 2006; Bassin *et al.*, 2007). However, it utilised grassland monoliths brought onto the study site from elsewhere, rather than the natural vegetation of the area, and grazing was simulated using a cutting regime, potentially altering the species and nutrient dynamics.

A second experiment, also established in Switzerland, is similar to the field site used in the present study in that it was an upland semi-natural grassland managed for species richness

(Chapter 2). The study site yielded a low to medium productivity, species-rich grassland that had been used as a pasture for 30 years (Volk *et al.*, 2006) and had an established annual regime of one summer hay cut, followed by sheep grazing. The field study represents the longest established FACE system yet in grassland; with fumigation operating for five consecutive growing seasons from 1999-2003.

The study site at High Keenley Fell incorporates a mesotrophic upland grassland situated in Upper Allendale, within the North Pennines in Northumberland (grid reference NY 7922 5586). It lies at an altitude of approximately 360 m above sea level, and was managed under the Natural England Higher Level Stewardship scheme for the maintenance and restoration of species-rich, semi-natural grassland (Natural England, 2012). The specific management plan for this site, previously outlined by Wedlich (2009), involves Scottish Blackface sheep (a large breed typically weighing over 70 kg), grazing the meadow from September until mid-spring the following year. These are removed from the site 8 weeks before the desired hay cut date. The autumn and winter grazing achieves a sward height of 2-10 cm during October and November, and by the time the livestock are moved the following year, the sward has been opened up. The hay is cut once a year, usually in early-mid August, though this is weather dependent. Once the hay is mechanically cut to a sward height of approximately 5 cm, the hay is left *in situ* to air dry, and is mechanically turned every few days before being removed to be used as winter feed for livestock. No reseeding, ploughing, or sub-surface cultivation is allowed. One farmyard manure application is allowed per year, with no other input permitted.

Research has been undertaken at High Keenley Fell since 2007, and ceased in August 2012. Previous research into the effects of ozone on species composition and above-ground biomass reported significant shifts in species composition with a decrease in the forb portion of the sward after three years of fumigation (Wedlich, 2009; Wedlich *et al.*, 2012). Research has also been undertaken into the effects of ozone upon flower abundance (three years' data), and timing for selected species (one years' data) (Rintoul, 2013). Measurements of ecosystem CO_2 exchange were taken in 2008 but are not yet published, and preliminary below-ground assessments have indicated mycorrhizal colonisation (Rintoul, 2013). Some plots have been subject to simulated nitrogen deposition in addition to elevated ozone, where elevated levels of nitrogen were seen to exacerbate the negative impact of ozone in a grass species above-ground biomass (Wyness *et al.*, 2011).

There have been no subsequent collections of data at the site relating to the effect of ozone on ecosystem CO_2 fluxes, and this is an area of research requiring further work according to Volk *et al.* (2011) following research undertaken in the Swiss alpine site. Additionally, research into the effect on flower timing needs to be extended, as there is no known research into the effects of ozone on timing in a field setting. Finally, there is nothing in literature relating to the effects of ozone upon flower nectar, and with knowledge of physiological effects of ozone on plants, this is an area with potential for research.

1.4 Aims and content of thesis

This study investigated the effects of previously elevated tropospheric ozone upon the semi natural grassland community at High Keenley Fell, and examined a number of different plant responses to ozone over the 2012 growing season. Effects on flower abundance and timing were examined. The composition of nectar within a single grassland species' nectar secretion was also considered. Furthermore, the effect of ozone on ecosystem CO_2 exchange of the grassland community was investigated. The general aims of this study are outlined below; the chapter to which the aim refers to is supplied in parentheses, and are as follows:

1. Establish whether there are effects of previous ozone exposure on flower abundance and timing (Chapter 3);
2. Determine whether previous exposure to ozone affects nectar production and quality of the hemiparasite *Rhinanthus minor*, relating to changes in nectar volume and composition (Chapter 4);
3. Investigate whether there is an effect of previous ozone exposure upon ecosystem CO_2 exchange, and relate this to temperature and soil water content (Chapter 5).

2 Field site at High Keenley Fell

2.1 Field Ozone Fumigation

The experimental area was set out in 2007 on level ground at the base of a NE-facing slope, and comprised three replicate treatments. Each transect was laid out as follows. A 6 m pipe running across the slope emitted ozone at a controlled rate aimed to elevate ambient ozone concentrations at 10 m downwind of release by 30 ppb at the vegetation level. Ozone release was dependent upon wind speeds being between 0.3 m s^{-1} and 3 m s^{-1} and the direction being from the SW (180-270°). Consequently, fumigation was not achieved constantly; and as a result the elevation of ambient ozone levels was targeted to achieve a long term elevation of +15 ppb at 10 m downwind.

Each replicate had an independent ozone generator (Model OZ2000, Clearwater, USA), creating ozone from pure oxygen on site, and these were controlled in real time through continuous monitoring in ambient air at 10 m downwind of release at intervals of 12 minutes. All generator systems were computer controlled, and could check for malfunctions within the system that could cause abnormally high ozone release – in this event, the system was meant to cease generating ozone at source.

The experimental layout is illustrated in Figure 2.1 below, and was established, controlled and maintained by the Centre for Ecology and Hydrology (Edinburgh branch) and Newcastle University. Sampling subplots were marked out in 2007 at 2.5 m, 5 m and 10 m downwind of the ozone release pipe for flowering analysis, though in this study only the latter two were sampled. Separate plots were also established at the same locations downwind for CO_2 flux measurements. The control area shown in Figure 2.1 show the area exposed to ambient ozone only, and the positioning of sampling subplots established within this area for each transect: a total of 3 treatments for flowering studies, and 4 for CO_2 flux studies. While the ozone exposure system was in operation, target exposure concentrations were 75 ppb above ambient at 2.5 m, 25 ppb above ambient at 5 m and 15 ppb above ambient at 10 m.

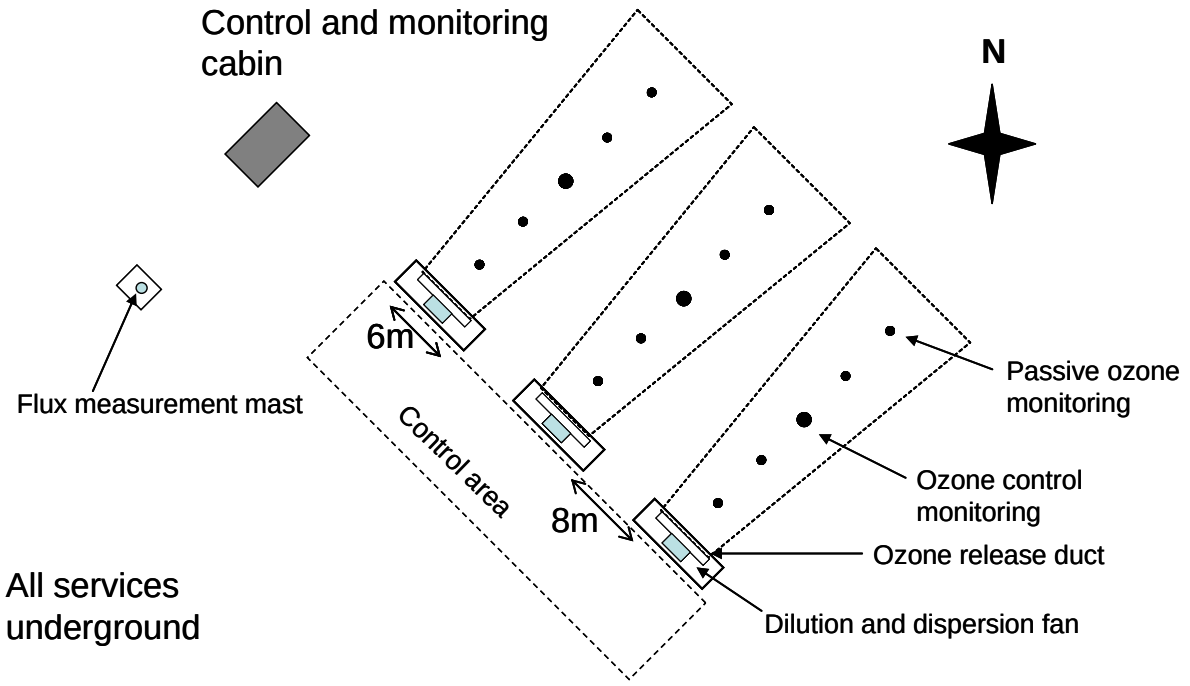

Figure 2.1 – Schematic diagram of experimental set up at High Keenley Fell. The diagram shows the layout of the three experimental transects in relation to each other, and the control cabin and flux mast. Transect 1 lies closest to the cabin.

As this is a study into the effects of previous exposure to elevated ozone, Figures 2.2 and 2.3 focused upon the annual ozone trends on site from 2008 – 2011. Figure 2.2 shows the variation in annual ozone fumigation trends at each treatment position, while Figure 2.3 displays variation in fumigation split by transect. Overall, in the years 2008 and 2009 the site was exposed to low levels of ozone enrichment, with 2010 and 2011 higher. There was a marked spatial variation in ozone fumigation, with an east – west gradient apparent where concentrations were highest at transect 1 and lowest at transect 3.

The 2012 growing season saw very little exposure, and Figure 2.4 outlines the measured ambient levels for the fumigation period. A brief exposure lasting no more than four days in total occurred in mid to late June; however within this period there was an extremely high ozone exposure lasting up to 57 hours: Transect 1 received up to 538 ppb at 2.5 m; 194 ppb at 5 m downwind; and 96 ppb at 10 m; however the effect was variable between transects (Figure 2.5). The fumigation and monitoring systems were taken offline on 19th July 2012, and monitoring ended at this point.

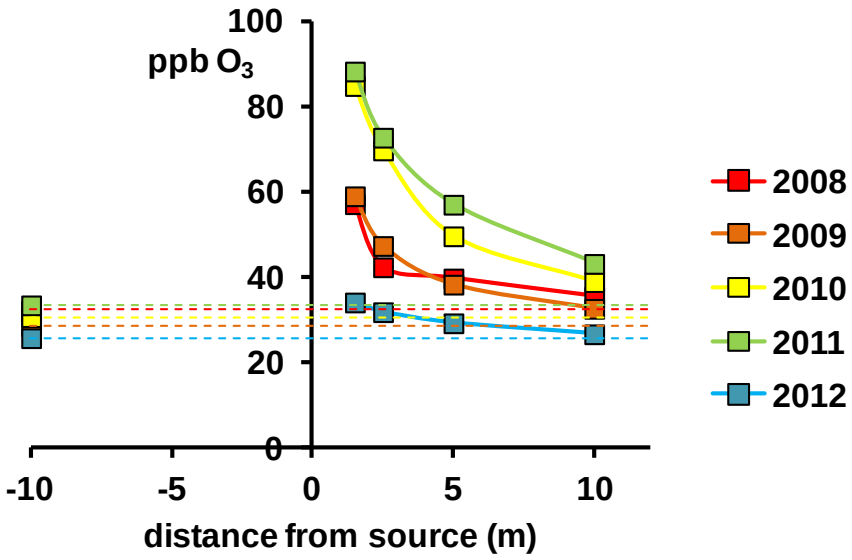

Figure 2.2 – Mean annual ozone concentration achieved by fumigation from 2008 – 2012, averaged from March-September fumigation, and split by year. Dashed lines represent ambient ozone concentrations (Defra, 2013).

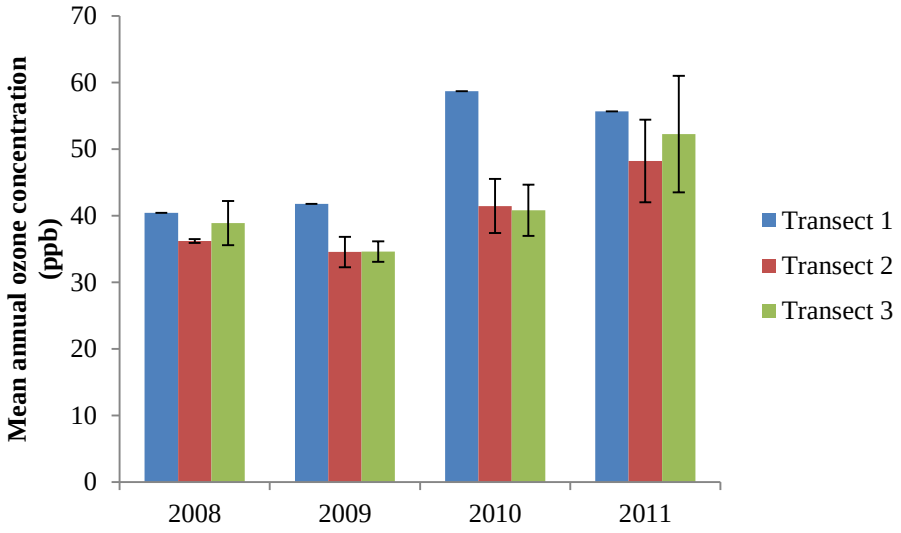

Figure 2.3 – Annual mean ozone concentration achieved at 5 m downwind of release by fumigation from 2008 – 2011 split by transect. Error bars represent standard error between replicate samples within each transect (n = 3).

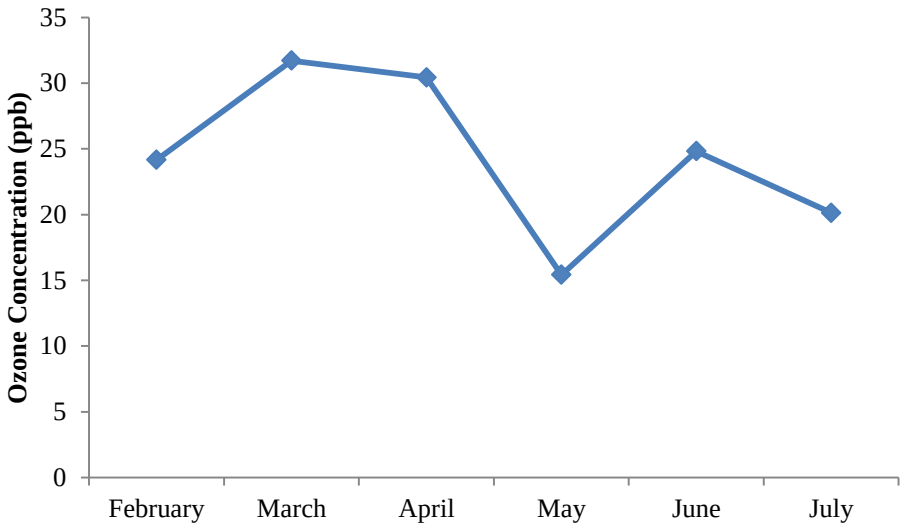

Figure 2.4 – Monthly mean ambient ozone concentration measured at High Keenley Fell, February – July 2012.

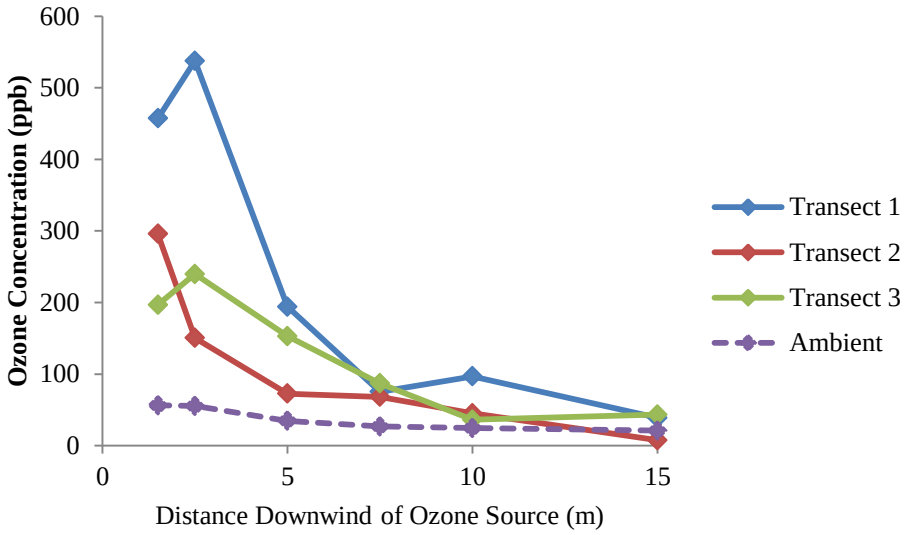

Figure 2.5 – Estimated mean ozone exposure at High Keenley Fell for the fumigation that occurred during the period of 13 June to 19 July 2012, which totalled no more than 4 consecutive days. Ambient concentrations are included as a reference to levels experienced pre- or post-high exposure.

Due to the partial monitoring that took place in 2012, Figure 2.6 includes the monthly variation in ozone at each treatment for the full year of 2010. The ambient ozone peak occurred at the field site in March and April in both 2010 and 2012, which is typically prior to the peak growing season of grassland plants, and varies due to climatology. Moreover, this corroborates with the annual trend monitored in south England and previously shown in

Figure 1.3. Additionally, mean exposure by transect suggests that wind speed and direction strongly influences ozone concentration at the field site, with a predominantly south-westerly wind leading to higher concentrations in transect 1, the western most transect (Figure 2.3).

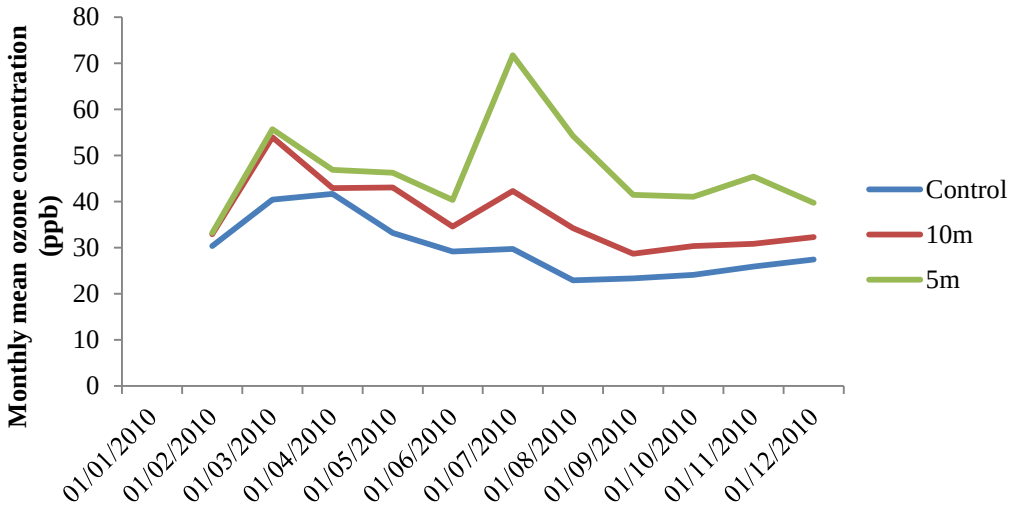

Figure 2.6 – Monthly mean ozone concentration at High Keenley Fell in 2010, split by treatment.

2.2 Climatic Conditions

Climate data were collected from the micrometeorological station established at High Keenley Fell, and is presented for 19th June-8th August 2012 in Table 2.1 and Figures 2.7 and 2.8 (Coyle, 2013). At the field site, July was the warmest month, with the warmest day the 23rd July. The coldest month during the study period was August, with the coldest day falling on the 7th August. Rainfall data were not presented for August, as measurements ceased on the 8th August.

Table 2.1 – Monthly records from High Keenley Fell for daily mean temperature, daily mean PAR (measured between 09:30 and 00:00 daily), and total monthly rainfall for the 3 months comprising the sampling period of 2012 (Coyle, 2013).

Month	Mean Daily Temperature (°C)	Mean maximum Temperature (°C)	Mean minimum Temperature (°C)	Mean daily PAR (µmol m^{-2} s^{-1})	Total Rainfall (mm)
June	11.4	14.4	9.2	303.1	109.2
July	12.9	16.7	9.9	429.8	52.6
August	9.9	13.1	7.4	290	-

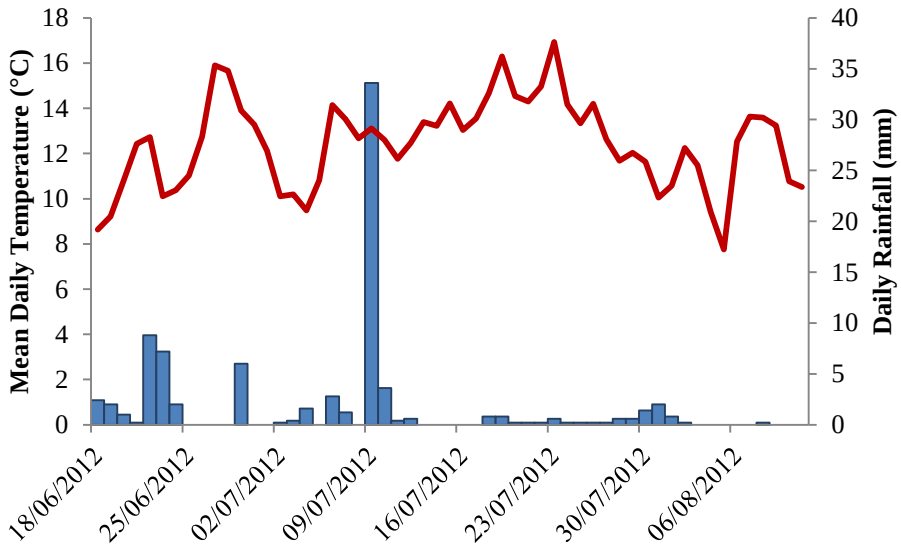

Figure 2.7 – Mean daily air temperatures (red line) and rainfall (blue bars) at High Keenley Fell for the sampling period of this study (Coyle, 2013).

2.3 Soil profile

Soil samples were collected and analysed in April 2011. Methods for sampling and analysis are outlined by Rintoul (2013). The concentrations of a number of variables are shown in Table 2.2 (below), and are provided to characterise the soil at the different ozone treatments across the High Keenley Fell field site. With all nutrient variables there was a spatial gradient following the slope of the field site, with highest values for nitrate, ammonium and phosphate all occurring in control plots – at the relative slope head – followed by 5 m and 10 m plots

downwind of the ozone release. Loss on ignition also followed this trend; however bulk density was lowest in control plots, and highest in 10 m plots. Bulk density was within a range deemed ideal for plant growth in clayey soils (USDA, 2008). Soil pH was the only variable that did not follow the spatial gradient, with pH highest at 5 m downwind, and lowest at 10 m.

While large differences were apparent in nitrate concentrations prior to ozone exposure in 2007, the statistical significance was lost by 2011. A statistically significant difference developed in loss on ignition between 2007 and April 2011 (P = 0.012; Rintoul, 2013), suggesting an effect relating to above ground changes caused by ozone exposure.

Table 2.2 – Profile of variables measured in soil in plots at High Keenley Fell in April 2011, split by ozone treatment. Values stated relate to soil at 0-10 cm depth (Rintoul, 2013). n = 12.

Soil Variable	**Control**	**10 m**	**5 m**
Soil pH	5.74	5.72	5.84
Loss on ignition (%)	18.2	14.83	15.13
Bulk density (g cm^{-3})	0.43	0.49	0.47
NO_3-N (mg kg^{-1})	0.97	0.39	0.45
NH_4-N (mg kg^{-1})	22.26	20.13	20.9
PO_4-P (mg kg^{-1})	15.96	6.09	7.26

3 Effects of ozone upon the flowering behaviour of flora within an upland grassland community

3.1 Introduction

3.1.1 Effect of ozone on flowering and subsequent implications

While there is an increasing amount of research into the effects of ozone on grassland productivity through the measurement of above-ground biomass (*e.g.* Fuhrer and Booker, 2003; Timonen *et al.*, 2004; Volk *et al.*, 2006; Bassin *et al.*, 2009; Mills *et al.*, 2011; Hooper *et al.*, 2012), there is far less known about the impacts upon flowering. Hayes *et al.* (2012) highlight flowering as a critical life-cycle stage in plants. It is therefore important to ascertain how ozone could alter the timing of flowering, seed set, and subsequent effects on pollination foraging.

Flower abundance is a relatively easy factor to study, yet few papers have focused on how ozone affects this. However, findings in the few papers on the subject suggest that ozone does affect abundance. One review of over 120 articles found that, while elevated ozone reduced seed weight both per seed and per plant, by 18% and 27% respectively, it did not affect either the flower number, or weight of flowers in a range of crop and grassland species (Leisner and Ainsworth, 2012). A study using pot-grown simulated communities in solardomes found mixed responses in forb flower abundance: while *Lotus corniculatus* showed no ozone-induced change in flower abundance, *Campanula rotundifolia* displayed a 40% reduction (Hayes *et al.*, 2012). This agrees with a pot-sown study in Finland, where *C. rotundifolia* again showed dramatic reductions in flower abundance (Ramö *et al.*, 2007). Other studies have also found that ozone significantly reduces flower abundance or biomass in legumes as well as forbs (Franzaring *et al.*, 2000; Gimeno *et al.*, 2004). Most studies are conducted either on single species in pots, or simulated communities created by either sowing a species mixture, or engineering a community by using specified numbers of plug plants. As a consequence, there is a lack of field-based study in natural communities, particularly those managed for high species diversity.

The flowering phenology of grassland species is altered by various factors. It is already known that phenological processes are affected by temperature, photoperiod, rainfall, nutrient

availability and exposure to elevated CO_2, and these are widely reported (*e.g.* Rusterholz and Erhardt, 1998; Simpson *et al.*, 1999; Menzel, 2002; Keatley and Hudson, 2007; Springer and Ward, 2007). However, there is some evidence that ozone also plays a part in altering flower timing: a solardome study of three forb species planted in pots replicating natural communities found that one species' flowering peaked 5 days earlier when exposed to elevated ozone when compared to ambient air (Hayes *et al.*, 2012). This mixed phenological response shows there is no single effect of ozone on flower timing, and there is a lack of research in this area – especially field-based – to build strong conclusions from. Thus, more research is imperative.

3.1.2 Aim and objectives

There is a lack of research into the effects of ozone on species within whole, natural grassland communities, and in field conditions. Furthermore, there is very little known on the carry-over effects on grassland species relating to previous ozone exposure. Thus, this study aimed to investigate whether there are effects on the flowering behaviour of semi-natural grassland species caused by previous long-term ozone exposure at High Keenley Fell. To this end, the objectives of this study were:

1. To define the effect of previous ozone exposure upon flower abundance in selected species;
2. To evaluate whether there is an effect of ozone exposure on the timing of flowering for selected species.

3.2 Methods

3.2.1 Species selection for sampling

As this study was a continuation of a field project that started in 2007, with flowering data recorded from 2009, there were data relating to the effects of ozone upon all species found within the flowering plots. This study aimed to concentrate on a smaller number of species that had previously shown either significant or borderline significant flower responses to ozone, to determine whether effects persisted from one year to the next. Previous responses to ozone at High Keenley Fell were used in part to select species, and were reported by Rintoul

(2013). Species were also selected based on a grassland species indicator score developed within the 'Hay Time' project by North Pennines AONB Partnership (2012). These indicator scores are based on whether species are deemed to be "positive" indicators, in that they show a good quality meadow; or "negative" indicators, in that they are evidence of agricultural improvement. A range of positive and negative indicator species were studied, with an emphasis on positive indicator species. The species selected are listed in Table 3.1.

Table 3.1 – Grassland species selected for flowering study. Indicator scores are positive and negative; the higher the number, the better the species. Previous ozone responses relate to flowering response, and are noted as positive (+), or negative (-).

Species	Indicator Score	Previous Ozone Response
Grasses		
Anthoxanthum odoratum	+1	+
Dactylis glomerata	-1	+
Festuca pratensis	+1	-
Holcus lanatus	0	-
Lolium perenne	-1	-
Trisetum flavescens	+2	+
Forbs		
Conopodium majus	+2	+
Ranunculus acris	+1	-
Ranunculus bulbosus	+2	-
Rhinanthus minor	+2	-
Rumex acetosa	0	-
Stellaria graminea	+2	-*
Legumes		
Trifolium pratense	+1	-
Trifolium repens	-1	+**

*n.b. * - significant only in 2010; ** - effect not significant.*

3.2.2 Field sampling

Flower sampling was undertaken in established sampling plots at control, 5 m downwind of ozone fumigation, and 10 m downwind of fumigation. Figure 3.1 illustrates the sampling plot layout. Sampling plots were not protected in any way, so were fully exposed to climatic conditions and grazing by sheep that were present on the site from late summer until early spring. The sampling plots were managed by a single hay cut in August. All sampling was undertaken prior to this harvest.

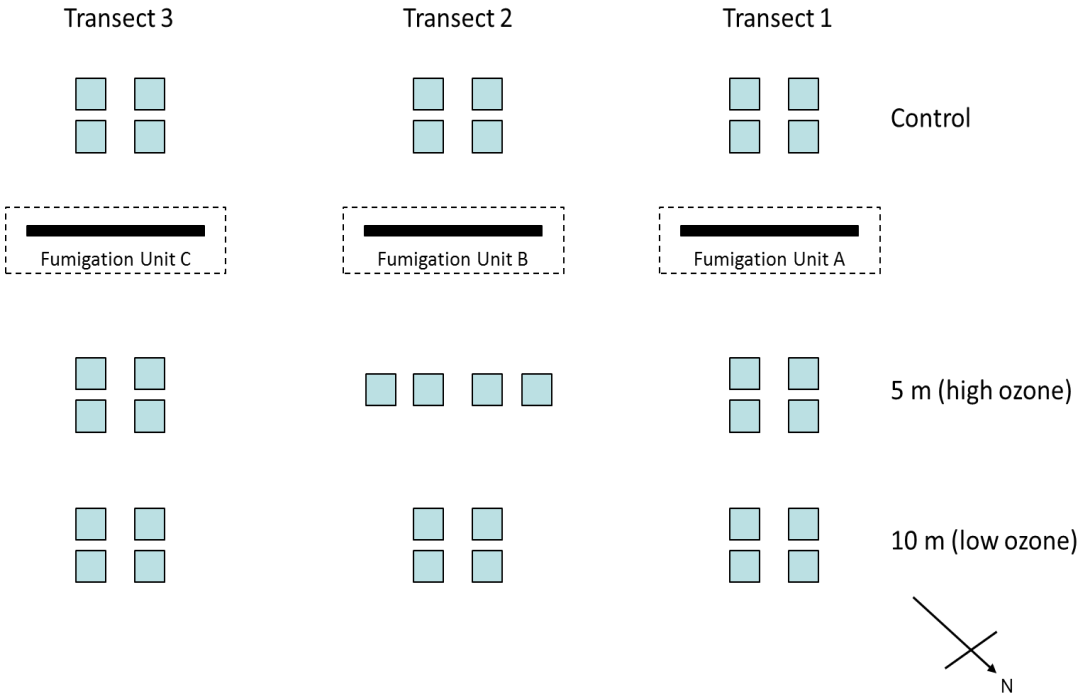

Figure 3.1 – Sampling plot layout for flowering count plots. Each blue box represents a 1 m^2 plot that has been established, and sampled, since 2007.

Field sampling occurred on five occasions during 2012, typically spaced two weeks apart: in mid-June; early July; mid-July; late July; and early August. Flower counts normally took two days; however at times inclement weather conditions meant that the counting was spread over three days. As weather conditions were liable to change suddenly, and flowers could partially or fully close during rainfall or later on in the day, data were collected from plots in a stratified nature to ensure weather conditions did not create bias as a result of, for instance, one treatment or one transect being sampled at once. This meant that roughly half of the subplots were sampled per day across all three transects.

When sampling subplots, a 1 m^2 quadrat was laid over the subplot. While care was taken to not include vegetation from outside of the plot, where flowers were found to be within the plot while the plant was rooted outside they were still included in the flower count.

3.2.3 Definition of a flower

A range of forbs, grasses and legumes, with differing flowering head types, were studied. Thus, there were several definitions of a single flower required. Firstly, a flower would only be counted if it was fully, or largely open, due to the previously mentioned matter of abiotic factors altering a flower's state. Figure 3.2 below identifies what flowers would be counted and what would not.

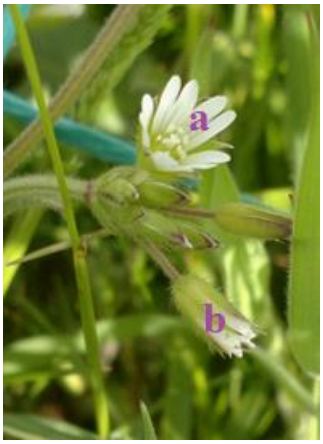

Figure 3.2 – Photograph of flowering heads of *Stellaria graminea* during sampling at High Keenley Fell. *a* denotes a flower that is predominantly open so therefore would be counted as one flower; *b* denotes a flower that is not yet sufficiently open so would not be counted.

While in some cases, such as *Ranunculus acris*, flowering heads consist of a single floret, other cases such as *Conopodium majus* have flowering heads composing numerous small, individual florets. Grass flowering heads are also mainly composed of large numbers of individual florets, so to retain accuracy and consistency in field-based sampling, flowering heads were defined in a number of ways. Grass flowering heads were typically classed as one flowering head per stem, for instance, though this was defined for each species, rather than for functional group, so as to account for morphological differences between species. Table 3.2 (below) identifies how flowering heads for each species were counted, with Figures 3.3 to 3.8 illustrating each classification of inflorescence.

Table 3.2 – Summary of the classification of a single flower for each species studied, after Rintoul (2013). *a* = entire inflorescence counted as one flower due to no clear branching between florets; *b* = numerous florets branch off the inflorescence axis, and are counted as one flower; *c* = numerous spikelets comprising one or more florets along one stem, thus single stem is one flower; *d* = numerous florets are organised into a compound umbel which comprises the flowering head. One floret is counted as one flower; *e* = single, easy-to-distinguish florets that branch off the main stem. One floret is one flower; *f* = numerous floret stems spiral around the inflorescence axis. Each floret stem is counted as one flower.

Species	**Classification of a single flower**
Anthoxanthum odoratum	*a*
Dactylis glomerata	*b*
Festuca pratensis	*b*
Holcus lanatus	*b*
Lolium perenne	*c*
Trisetum flavescens	*b*
Conopodium majus	*d*
Ranunculus acris	*e*
Ranunculus bulbosus	*e*
Rhinanthus minor	*e*
Rumex acetosa	*f*
Stellaria graminea	*e*
Trifolium pratense	*e*
Trifolium repens	*e*

Figure 3.3 – *Anthoxanthum odoratum* flowering heads (Farmer, 2002). As an example of inflorescence category *a*, each inflorescence is one flower. In this photograph there are 3 flowers.

Figure 3.4 – *Holcus lanatus* in flower (Dover, 2010). Shown as an example of inflorescence category *b*, each main stem is one flower. In this photograph there is 1 flower in the foreground.

Figure 3.5 – *Lolium perenne* in flower (Penn State University, 2013). Shown as an example of inflorescence category *c*, each stem is one flower. In this photograph there is 1 flower in the foreground.

Figure 3.6 – *Conopodium majus* flowering stem (Harrison, 2006). As an example of inflorescence category *d*, each cluster of florets counts as one flower. In this photograph there are 6 flowers.

Figure 3.7 – *Ranunculus species* flower at High Keenley Fell. As an example of inflorescence category *e*, each flower is counted as one flower. In the photograph foreground there is 1 flower.

Figure 3.8 – *Rumex acetosa* flower stem recently emerged at High Keenley Fell, used as an example of inflorescence category *f*. In this photograph there are approximately 3 flower spikes.

3.2.4 Data analysis

To ensure robust statistical analysis of data, any species that was absent in 20% or more of sampling subplots was removed from subsequent analysis, to avoid inconsistent coverage of the subplots skewing analyses. Thus, *Ranunculus bulbosus*, *Trifolium pratense* and *T. repens* were all removed from analysis, leaving 11 species to be investigated. Table 3.2 below outlines species constancy between subplots.

Table 3.2 – Number of subplots where a species is present at one or more counts over the season, reported as % constancy between plots. Any species with a constancy of <80% was removed from subsequent analysis.

Species	Constancy between plots
Anthoxanthum odoratum	100%
Dactylis glomerata	97%
Festuca pratensis	100%
Holcus lanatus	100%
Lolium perenne	100%
Trisetum flavescens	100%
Conopodium majus	86%
Ranunculus acris	100%
Ranunculus bulbosus	47%
Rhinanthus minor	81%
Rumex acetosa	94%
Stellaria graminea	97%
Trifolium pratense	69%
Trifolium repens	64%

3.2.4.1 Flower abundance

Statistical analysis for flower abundance was initially conducted on both peak flowering data, and average flowering data, as peak is favourable for species with short flowering seasons, while average is a better factor for species with longer flowering seasons. However, as the responses were so similar, and the majority of species had longer – rather than shorter – flowering seasons, average flowers were used as the factor for subsequent analysis.

Data were tested for normal distribution using the Kolmogorov-Smirnov test of normality. Where distribution was non-normal, data were transformed to improve distribution (Table 3.3). Statistical analysis was conducted using a nested ANOVA design to remove the effect of transect variation on any potential ozone effects, with a modified Tukey HSD test applied *post-hoc*, following Zar (1984). The Levene test was applied to ensure homogeneity of

variance: where there was a significant test statistic, the significance of the ANOVA was shifted, e.g. a result falling between P = 0.001-0.01 would become P = 0.01-0.05. Statistical analysis was undertaken using SPSS version 21.

Table 3.3 – Species within study and data transformation applied (where necessary) to achieve normality within data.

Species	Transformation applied
Anthoxanthum odoratum	none
Dactylis glomerata	$\sqrt{(x + 1)}$
Festuca pratensis	$\text{Log}(x + 1)$
Holcus lanatus	none
Lolium perenne	$\text{Log}(x + 1)$
Trisetum flavescens	$\text{Log}(x + 1)$
Conopodium majus	$\sqrt{(x + 1)}$
Ranunculus acris	$\sqrt{(x + 1)}$
Rhinanthus minor	$\sqrt{(x + 1)}$
Rumex acetosa	$\sqrt{(x + 1)}$
Stellaria graminea	none

3.2.4.2 Flower timing

Because *Conopodium majus* peaked early in the season and only appeared in two of five flower counts, it was not possible to analyse whether or not there were changes in the timing, so this species was not analysed further. Thus, ten species were analysed for flower timing.

To investigate whether there were carry-over effects of ozone on the timing of flowering, the peak flowering date was identified, before each species within each plot was coded, from 1-5, based on when peak flowering occurred, with 1 corresponding to the first flowering count in June, and 5 corresponding to the last flowering count in August. These converted data were then analysed using Chi-square analysis. Due to small sample sizes, Fisher's exact test was applied to each species to compute a more robust test statistic, and this test statistic was taken as the result, along with the likelihood ratio, and Cramér's V value, to better understand any changes in timing (Field, 2009, Zar, 1984).

3.3 Results

3.3.1 Flower abundance

The statistical analysis outcomes for all eleven species are reported in Table 3.4, below. Only significant treatment results will be discussed herein. Three species investigated proved to be significantly affected by previous exposure to elevated ozone. Two species – *Dactylis glomerata* and *Conopodium majus* were positively affected by ozone, resulting in a higher abundance of flowering heads. *D. glomerata* flowers were significantly more abundant in 10 m subplots than in 5 m or control subplots, with a four-fold increase in flower abundance between 10 m and control plots. This suggests a slight increase in background ozone has the largest carry-over effect on flowering abundance. *C. majus* responded differently, with flower abundance in 5 m plots significantly higher than in control plots and exhibited a thirteen-fold increase between the two treatments (Figure 3.10).

Ranunculus acris exhibited the only negative carry-over effect on flower abundance, with significantly highest abundance reported in control plots, and lowest abundance in 5 m plots. There was an 85% reduction in flower abundance between control to 5 m, and a reduction of 45% between control and 10 m (Figure 3.11).

Table 3.4 – ANOVA test results for the effect of ozone treatment (F_1) and the effect of transect within treatment (F_2), upon flower abundance of grasses and forbs. Bold values denote indicate significance at $P < 0.05$. *Post hoc* analysis is also included for significant ($P < 0.05$) treatment effects. + - P = 0.05-0.1; * - P = 0.01-0.05; ** - P = 0.001-0.01; *** - $P <$ 0.001.

Species	***F*$_1$**	***F*$_2$**	***Post hoc***
Anthoxanthum odoratum	0.234	0.799	
Dactylis glomerata	**5.852***	**3.954****	10 m > 5 m, control
Festuca pratensis	0.187	**3.602****	
Holcus lanatus	0.217	**7.679*****	
Lolium perenne	1.136	**5.771****	
Trisetum flavescens	1.939	2.377+	
Conopodium majus	**47.46*****	0.721	5 m, 10 m > control
Ranunculus acris	**7.551***	**5.618****	control > 10 m > 5 m
Rhinanthus minor	0.721	0.949	
Rumex acetosa	2.914	1.337	
Stellaria graminea	0.993	**5.461****	

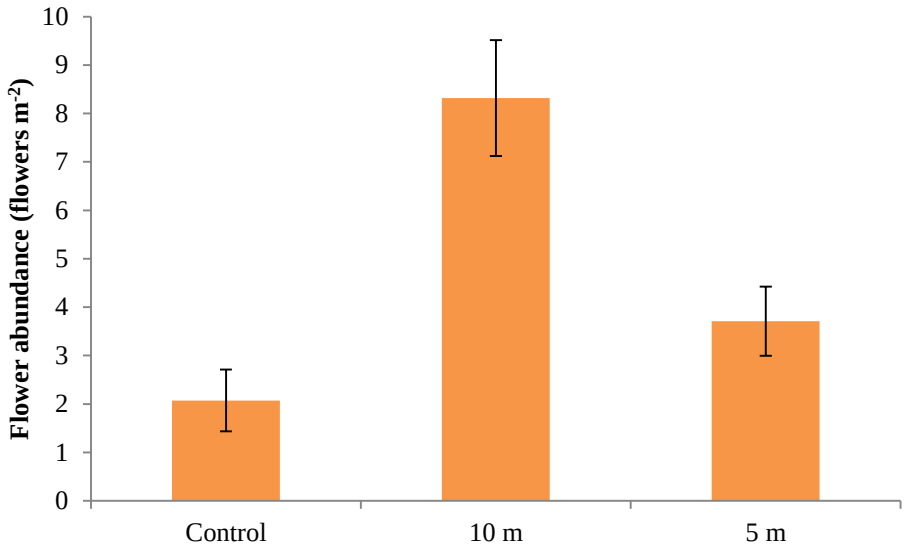

Figure 3.9 – Flower abundance split by ozone treatment for the species *Dactylis glomerata*. Error bars represent standard error. n = 12.

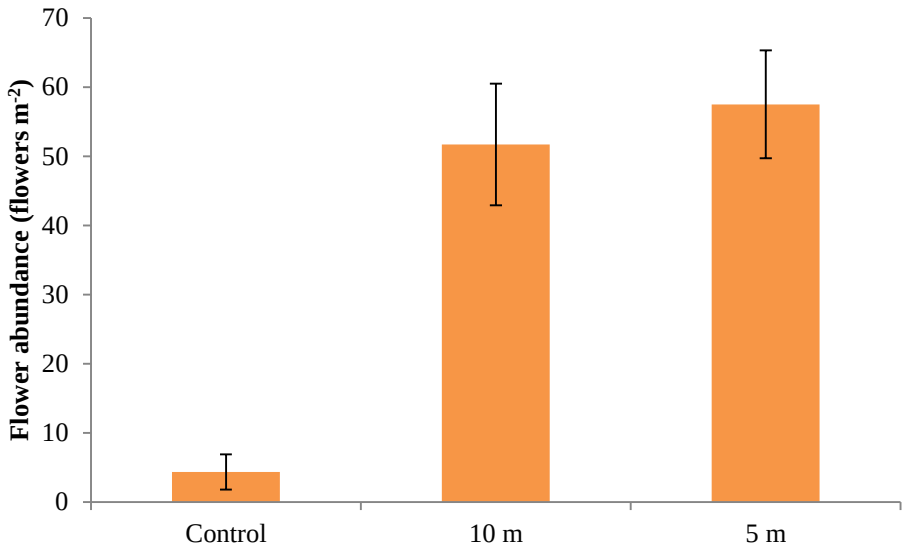

Figure 3.10 – Flower abundance split by ozone treatment for the species *Conopodium majus*. Error bars represent standard error. *n* = 12.

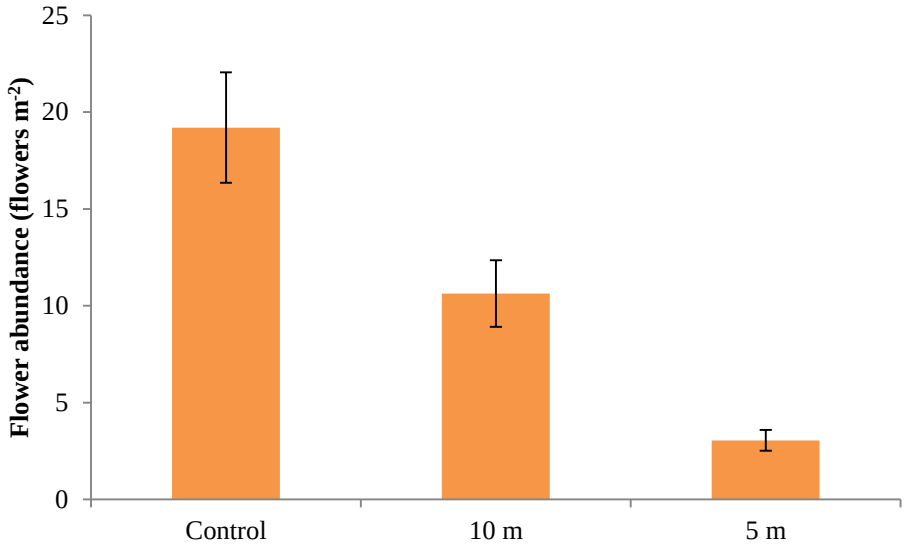

Figure 3.11 – Flower abundance split by ozone treatment for the species *Ranunculus acris*. Error bars represent standard error. *n* = 12.

Two of these species also displayed significant effects of transect within treatment – *D. glomerata*, and *R. acris*; and these differences are explored in Figures 3.12 and 3.13. In the case of *D. glomerata*, there was a large difference in flower abundance between Transect 1 and Transects 2 and 3, and the averaged trend for all three transects is reflected in Transects 1

and 2; however Transect 3 displayed a lower mean flower abundance at 5 m than in the control plots. *R. acris* showed variation between all three transects, though Transects 1 and 3 show the same flower abundance pattern as the average in response to ozone; however Transect 2 abundance was very similar in control and 10 m subplots.

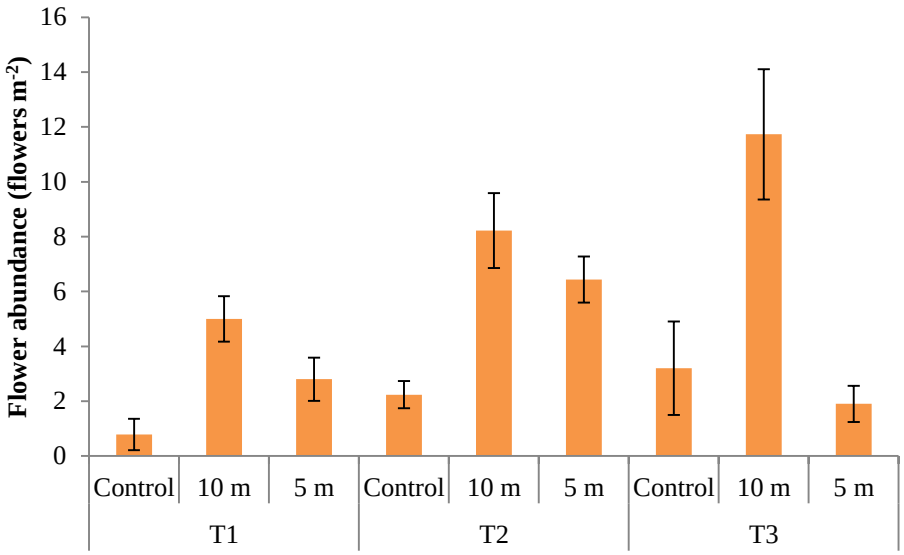

Figure 3.12 – Flower abundance for the species *Dactylis glomerata* arranged by treatment within transect. Error bars represent standard error. $n = 4$.

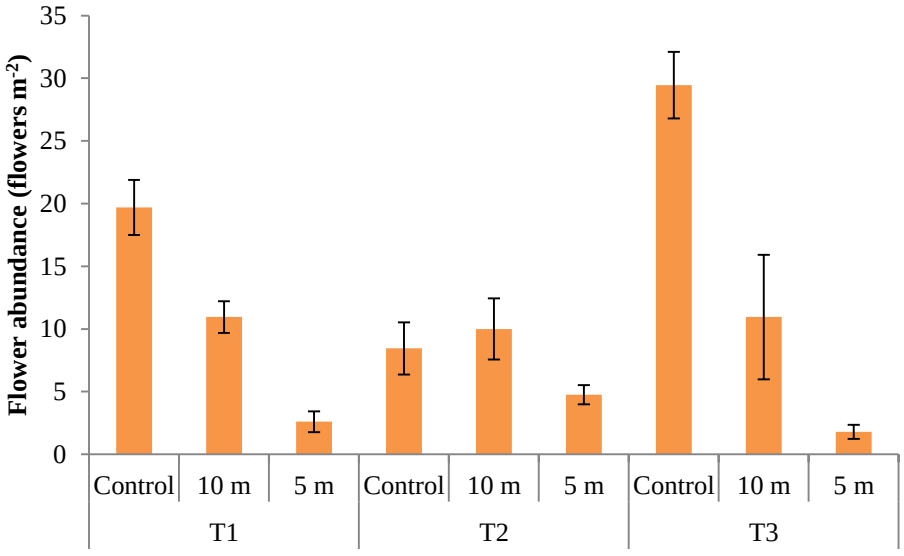

Figure 3.13 – Flower abundance for the species *Ranunculus acris* arranged by treatment within transect. Error bars represent standard error. $n = 4$.

3.3.2 Flower timing

Of ten species studied for changes in flower timing, two showed statistically significant changes relating to previous ozone exposure: *Festuca pratensis* and *Trisetum flavescens*. Table 3.5 (below) summarises the chi-square analysis for all studied species. In both cases, the Fisher's exact test provided a significant result. There was some correlation relating to species and ozone treatment in changing timing of peak flowering (shown by the Cramér's V); however a stronger correlation was exhibited for *F. pratensis*.

Table 3.5 – Chi-square test statistics for all studied species. Bold values indicate significant results at $P < 0.05$. + - $P = 0.05$-0.1; * - $P = 0.01$-0.05; ** - $P = 0.001$-0.01.

Species	Fisher's Exact Test	Cramér's V
Anthoxanthum odoratum	3.791	0.316
Dactylis glomerata	3.595	0.223
Festuca pratensis	**18.14****	**0.524****
Holcus lanatus	4.945	0.372
Lolium perenne	11.025	0.421+
Trisetum flavescens	**13.428****	**0.433***
Ranunculus acris	2.6	0.19
Rhinanthus minor	5.495	0.323
Rumex acetosa	5.333	0.306
Stellaria graminea	8.611	0.372

Figures 3.14 and 3.15 illustrate the trend in flower abundance for the two significant species across the study period. It is evident that, in both cases, flowering peaked two weeks later in the 5 m ozone treatment than in 10 m or control plots. *F. pratensis* typically peaked on the 3rd July 2012, but at 5 m peaked on the 17th July. *T. flavescens* flowering peaked on the 17th July in control and 10 m plots, but peaked on the 30th July in 5 m plots.

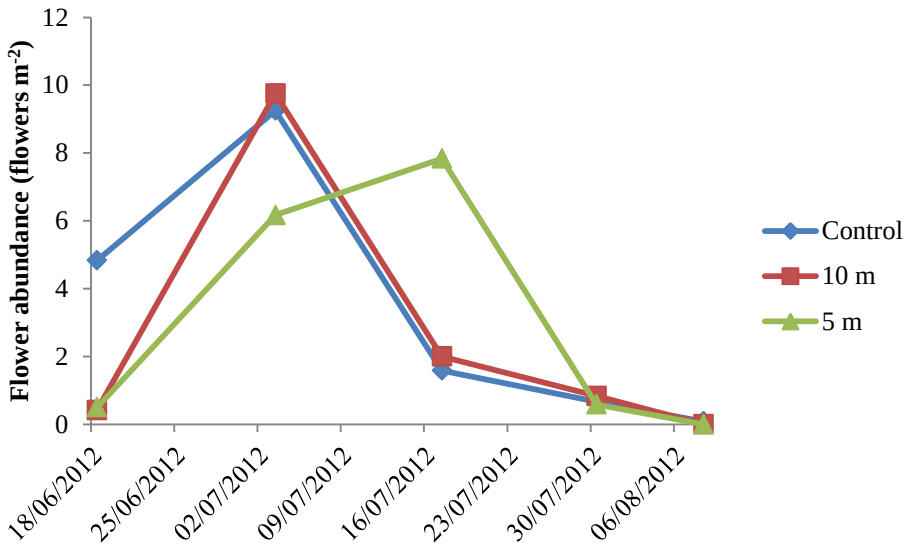

Figure 3.14 – Flower abundance of *Festuca pratensis* at five points across the 2012 growing season, split by ozone treatment. *n* = 12.

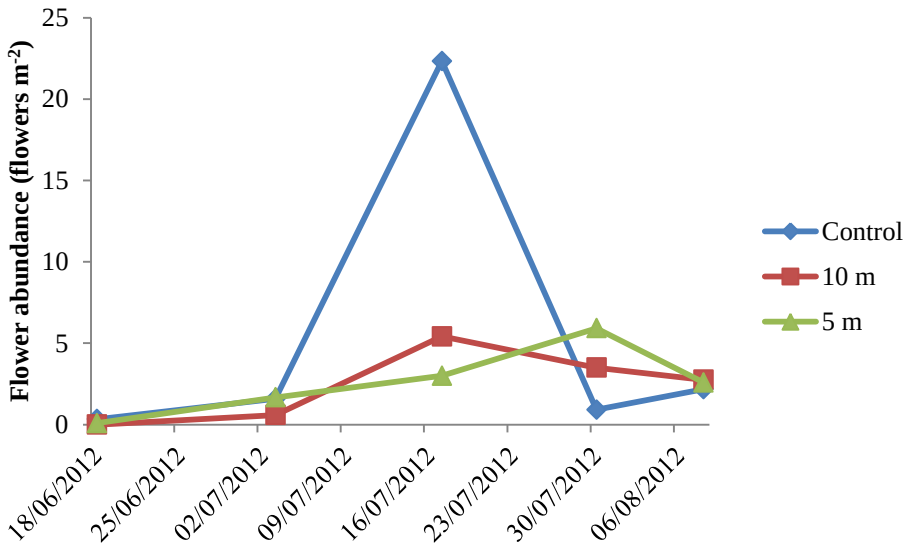

Figure 3.15 – Flower abundance of *Trisetum flavescens* at five points across the 2012 growing season, split by ozone treatment. *n* = 12.

Chi-square analysis contingency tables for *F. pratensis* (Table 3.6) and *T. flavescens* (Table 3.7) are below, showing how many sample subplots peaked within each ozone treatment on each sampling date. In the case of *F. pratensis*, no subplots peaked during the first count in June at 5 m, with main peaks occurring at control and 10 m in the second count at the start of July compared to mid-July in 5 m plots. This suggests the delay in peak flowering was

evident at the start of sampling. *T. flavescens* showed a more mixed response, with all control plots peaking in mid-July and half of 10 m plots peaking in mid-July. 5 m plots showed a peak spread across two sample periods, with 4 plots peaking in each of the mid- and late July samples. Based on the whole population, *F. pratensis* proved to be an overall earlier flowering grass than *T. flavescens*.

Table 3.6 – Chi-square contingency table for *Festuca pratensis* showing number of plots per treatment and total peaking on each flower count date.

Treatment	**18/6/12**	**3/7/12**	**17/7/12**	**30/7/12**	**9/8/12**	***Total***
Control	2	8	1	1	0	12
10 m	1	9	2	0	0	12
5 m	0	2	10	0	0	12
Total	3	19	13	1	0	36

Table 3.7 – Chi-square contingency table for *Trisetum flavescens* showing number of plots per treatment and total peaking on each flower count date.

Treatment	**18/6/12**	**3/7/12**	**17/7/12**	**30/7/12**	**9/8/12**	***Total***
Control	0	0	12	0	0	12
10 m	0	0	6	4	2	12
5 m	0	1	4	4	3	12
Total	0	1	22	8	5	36

3.4 Discussion

3.4.1 Flower abundance: peak *vs.* average abundance

There are merits to using both peak and average flower abundance as factors to analyse the effect of ozone exposure on grassland species. Average abundance gives a good representation of a species over the whole sampling period; however peak abundance is best suited to species with short flowering seasons. One shortfall of peak abundance over average abundance is that the true peak in abundance may be missed if it falls between flower counts – to use peak flower abundance as a truly accurate measure would require daily data

collection over several weeks, and sometimes months, and this is often unrealistic logistically.

While peak flower abundance results were not presented in this chapter, the statistically significant responses seen in average flower abundance were mirrored in peak abundance responses: *Dactylis glomerata* and *Conopodium majus* were both significant to $P < 0.05$; and *Ranunculus acris* was significant at $P < 0.1$.

3.4.2 Effects of ozone upon average flower abundance

Of the eleven species investigated, eight showed no effect of previous ozone exposure on flower abundance. Grass species unaffected were *Anthoxanthum odoratum*, *Festuca pratensis*, *Holcus lanatus*, *Lolium perenne*, and *Trisetum flavescens*. Forb species showing no response were *Rhinanthus minor*, *Rumex acetosa*, and *Stellaria graminea*. In contrast, three species did show significant responses: *D. glomerata* was the only grass, and showed a strong, positive ozone effect; the forb *C. majus* also showed a strong positive response. The only negative response was displayed by the forb *R. acris*, which showed large reductions in flower abundance associated with previous ozone exposure.

The result for *D. glomerata* is in agreement with ozone effects during exposure at High Keenley Fell: Rintoul (2013) reported the same significant, positive trend in 2011; however is in contradiction with a pot-based solardome study, which found no carry-over effect on flower abundance following 20 weeks of elevated background ozone exposure (Hayes *et al.*, 2011). Therefore, this suggests that there is indeed either a carry-over effect of elevated ozone, or effect of a peak episode at High Keenley Fell for this species, and that it benefits from a strong, positive response, even after background ozone returns to normal.

The effects on *C. majus* also correspond with the positive effects seen in the previous years at High Keenley Fell; however, whereas in previous years 10 m showed the highest abundance, in 2012 5 m showed the highest abundance. There are no flowering effects of ozone reported in literature for this species, but in this experiment the increase in abundance for this species where ozone fumigation previously occurred is marked. An interesting point to note is that this species is a long-lived polycarpic perennial, and in a plot-based study, findings relating to seed germination and establishment success indicates that this species has a long life cycle, taking at least 4 years from germination before flowering first occurs (Thompson and Baster, 1992); therefore the apparent stress response of ozone is a long-term process that started

earlier in the fumigation experiment, and will continue to be seen beyond the end of the experiment if the management at High Keenley Fell remains similar to previous years. It is interesting to note how this effect has occurred in parallel to the increasing abundance of the competitive *D. glomerata*, indicating the two species do not compete for space or nutrients.

The negative response from *R. acris*, leading to reduced flower abundance where ozone was elevated in the past, is also in agreement with responses reported in 2011. The *post hoc* response changed slightly in 2012, with control abundance significantly higher than at 10 m, and 10 m abundance is significantly higher than at 5 m. This is in agreement with the only other study of flower abundance published relating to *R. acris*: Rämö *et al.* (2007) reported reductions in flower abundance where ozone was elevated in OTC experiments; however there was no research into whether there were carry-over effects.

There were a number of species that showed significant responses to ozone in 2011 that related to flower abundance, yet did not show a significant response in 2012. These were *Festuca pratensis*, *Holcus lanatus*, *Lolium perenne* and *Rumex acetosa*: *F. pratensis*, *H. lanatus* and *R. acetosa* showed diminished abundance, especially at 5 m. However, *L. perenne* gave a mixed response where abundance was highest in control plots, followed by 5 m and 10 m plots. This suggests a spatial gradient in the field is the effect rather than ozone. As all four species populations showed high constancy across all plots, this suggests that these species respond significantly to long term elevated background ozone, where concentrations were elevated by 10 ppb at 10 m, and 25 ppb at 5 m, thus suggesting forecast ozone levels for 2050 will have significant effects. However, the exposure regime of 2012 clearly has no such effect.

Some of the effects on flower abundance mirror the effect of ozone on species above-ground biomass reported for 2011 by Rintoul (2013): *D. glomerata* biomass was highest at 10 m, followed by 5 m and control; while *R. acris* biomass was highest in control plots, followed by 10 m and 5 m plots. *C. majus* biomass is difficult to analyse as by the harvest, plants remain as dried umbel stems with very little biomass; however the biomass was highest in 10 m plots, followed by 5 m and control – fully reflecting 2011 flower abundance effects. This suggests flower abundance is related to above-ground biomass, and that effects of ozone seen in biomass lead to ozone responses in flower abundance. Thus, it is unlikely that there are changes in resource allocation in these species in response to ozone exposure, something

reported by Timonen *et al.* (2004). This would be an interesting and valuable extension to the present study.

3.4.3 Flower timing

Ten species were investigated through chi-square analysis, and all bar two – *Festuca pratensis* and *Trisetum flavescens* – showed no significant change in flower timing as a result of ozone exposure. A lack of tangible change in flowering phenology for most species suggests resilience of hormonal signalling pathways responsible for triggering flowering during the peak exposure episode in 2012; it also suggests that 80% of species studied show no carry-over response from long-term exposure ending in 2011. However, the two species affected displayed delays in peak flowering of around two weeks at 5 m downwind of release.

The effect seen in 2012 may be a result of the extreme peak ozone release in mid- to late June, as anecdotal evidence from the field site at the time and in subsequent weeks showed that some forb species displayed senescence to unopened flower buds at 5 m downwind. This suggests that early flowering heads grown by grass species could also have been injured by the high ozone exposure and could therefore be showing their response through delayed peak flowering; however this was not investigated in more depth at the time and would require further study to assess precisely what caused this delay in peak flowering.

Two species assessed for changes in flower timing were also investigated in 2010: *Rhinanthus minor* and the *Ranunculaceae* family (Rintoul, 2013). Neither displayed changes in peak flowering through the 2010 growing season, based upon weekly flower counts, and through combining this data from a year with a consistent, long term background ozone elevation with the data from 2012, there is confidence in suggesting the timing of flowering of both species is unaffected by ozone exposure.

Flower timing responses related to elevated ozone have been reported previously: Hayes *et al.* (2012) found in a pot-based solardome study that the forb *Lotus corniculatus* exhibited a flowering peak six days earlier than in ambient ozone; however two other forbs showed no effect. Another study, focusing on carry-over responses in pot-based communities found no change in the flower timing of *D. glomerata*, therefore agreeing with the present study's findings (Hayes *et al.*, 2011). The reported study also found that *Leontodon hispidus* seemed to complete the flower reproductive cycle quicker while not showing changes in flower timing. This potential acceleration of the reproductive cycle may suggest underlying changes

in flower timing that were not identified during the High Keenley Fell study. Moreover, there are studies that outline the implications on pollinators when flowering occurs earlier (e.g. Kudo *et al.*, 2004), but there seems to be little known of the implications of delayed flowering in grass species.

3.4.4 Study shortcomings

While flower abundance per subplot has been a useful measure of flowering responses to elevated ozone exposure, the measure is heavily influenced by shifts in species above-ground biomass. Thus, a way to overcome this would be to measure flower abundance by the number of flowers per plant within a subplot, as by doing this, the original measure can be retained, but with an added level of detail to the measurements. This would allow for a study that investigated whether there was a physiological response occurring within plants as a result of nutrient allocation changes, which this current study does not offer. Additionally, as the study only looked to flowers, any potential effects of ozone on reproduction could only be postulated on the basis of changes in flower abundance. Thus, work would need to be extended to investigate the number of seeds or fruit per flower in seed-reproducing species, and the subsequent viability of these seeds.

Regarding flower timing, even though five counts were undertaken, there could still have been more measurements to gain a higher resolution to the observed changes in flower timing – currently, it can only be suggested that delays were up to two weeks; however by increasing count frequency, this could be more specific. Additionally, measuring rates of bud burst – defined by Prozherina *et al*. (2003) as the number of open buds in relation to the total number of buds per plant – could prove useful in flowers as it would provide a second measure for changes in flower timing.

4 Effects of ozone upon nectar volume and quality of the hemi-parasitic grassland species *Rhinanthus minor*

4.1 Introduction

It is estimated that 78% of all temperate flowering plants are pollinated by animals (Ollerton *et al.*, 2011). Without this animal-plant interaction, many plants would be unable to set seed and successfully reproduce; and clonal propagation would not compensate for the subsequently reduced fecundity (Kearns *et al.*, 1998). To encourage pollination, plants offer two rewards to potential pollinators: pollen and nectar (Grünfeld *et al.*, 1989). De Groot (1953) identified that pollen is virtually the only source of ten essential amino acids required by honey bees, while nectar was described by Nicolson (2007) as a rich food that was easily utilised, and in attractive packaging; both authors highlight that the sugars within nectar provide the required energy for foraging, therefore utilising insects as pollination vectors.

4.1.1 Nectar sugar chemistry

There are three sugars that dominate nectar sugar composition: the disaccharide sucrose; and its component monosaccharides fructose and glucose (Herrera *et al.*, 2006; Nicolson and Thornburg, 2007). Other sugars are often present in small amounts – among others, lactose, maltose, mannitol and sorbitol were all identified in a large study analysing the nectar of Mediterranean flora (Petanidou, 2005). An early, extensive study by Percival (1961) involving 889 species concluded that by using the concentrations of sucrose and a sum of fructose and glucose (also known as hexose), all nectars could be classified into three groups: sucrose-dominant, balanced (with equal proportions of sucrose and hexose), and hexose-dominant. This was expanded upon by Baker and Baker (1982), who defined a nectar sugar ratio by concentration of sucrose to hexose sugars (Equation 4.1), and distinguished four classifications of nectar (Table 4.1).

$$\boldsymbol{Sucrose - hexose\ ratio} = \frac{Sucrose\ [g\ L^{-1}]}{\sum Fructose + glucose\ [g\ L^{-1}]} \qquad \textbf{Equation 4.1}$$

Table 4.1 – Nectar sugar classes defined by Baker and Baker (1982) relating to nectar sugar ratio and associated sucrose proportion (from Nicolson and Thornburg, 2007)

Sugar class	Sugar Ratio	% Sucrose
Sucrose-dominant	> 1.0	51 – 100
Sucrose-rich	0.5 – 1.0	34 – 50
Hexose-rich	0.1 – 0.5	10 – 33
Hexose-dominant	< 0.1	0 – 9

While these classifications are cited widely in literature, Nicolson and Thornburg (2007) highlighted that the system is flawed, stating that the transition from hexose-rich to sucrose-rich nectar occurs at 33% sucrose when it should occur at 50%. They also suggest that balanced nectar should have a ratio of 1.0, indicating balanced proportions of sucrose and hexose rather than treating the hexose sugars separately. As a result of the flaw, they suggested attention be paid also to the percentages of the overall sugar content, and these are commonly published alongside sugar ratios (*e.g.* Morrant *et al.*, 2010; Petanidou, 2005; Wolff, 2006).

Utilising nectar ratios, a theory was developed by Baker and Baker (1990) in which sucrose-rich nectar was preferred by pollinators such as moths, butterflies and long-tongued bees, and hexose-rich nectar was preferred by short-tongued bees, flies and passerine birds. However, this theory was contradicted somewhat by Wolff (2006) who found that nectar volume, and not nectar quality, was the most important factor for pollinators when choosing their food source.

While numerous studies have documented inter-species variation in nectar sugar composition (*e.g.* Percival, 1961, Wolff, 2006), few have looked at variation within the same plant species. Percival (1965) and Wykes (1952) are two studies that suggest that plants within a species are constant in nectar chemistry. Yet a study involving *Ipomopsis aggregata* found large within-species variation (Pleasants, 1983). Another study, involving *Helleborus foetidus* found large variations in nectar sugar concentrations not only within a small spatial scale, but between flowers on the same plant (Herrera *et al.*, 2006). Figure 4.1 illustrates this example of variation.

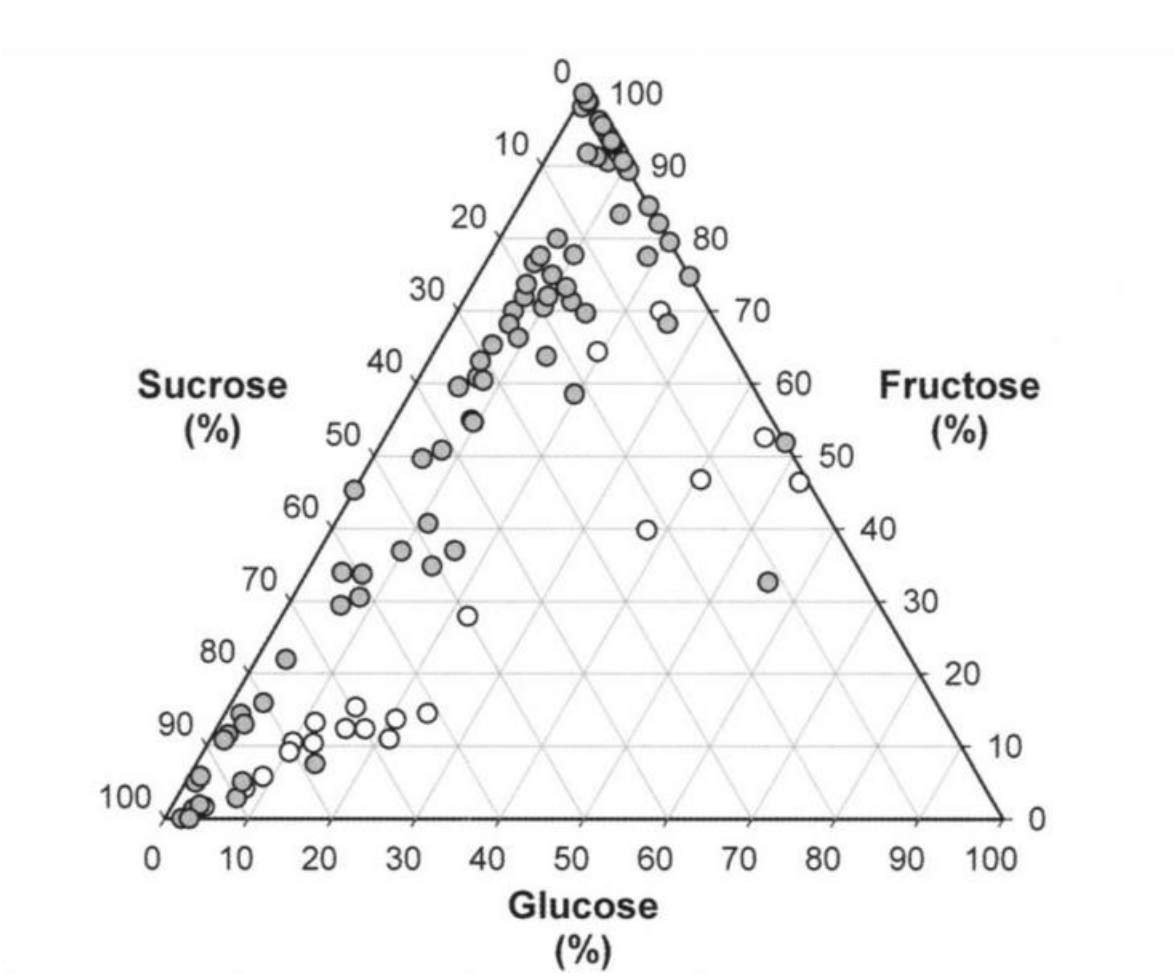

Figure 4.1 – Nectar sugar composition variation in *Helleborus foetidus* shown across a ternary diagram defined by axes corresponding to % fructose, % glucose and % sucrose. Each point shows the proportional sugar composition of nectar from a single nectary. Open circles denote a March sample; dark circles denote a May sample (Herrera *et al.*, 2006). n = 125.

4.1.2 Effect of pollutants on nectar chemistry

Little research has been conducted on the effects of pollutants upon plant nectar chemistry. No work has been published on the effects of ozone, but there has been work studying the effects of elevated CO_2 showing a mixed response. A greenhouse study exposed five species deemed important nectar sources for butterflies to CO_2 concentrations of 350 ppm and 660 ppm, and found that nectar volume was not affected in *Trifolium pratense* or *Lotus corniculatus*, but reduced volumes at 660 ppm in forbs such as *Scabiosa columbaria* and *Centaurea jacea* (Rusterholz and Erhardt, 1998). The same study also found that elevated CO_2 had no effect on sugar composition, but reduced total sugar concentration. A review of previous studies found that some species display higher nectar volumes with elevated CO_2,

while others show reduced volumes and others did not change, and suggested morphological changes within the corolla when exposed to elevated CO_2 were responsible where change occurred (Davis, 2003).

4.1.3 Species selection for study

Rhinanthus minor, or yellow-rattle, is a hemiparasitic annual plant widespread in grasslands throughout the United Kingdom, and common at the High Keenley Fell field site. It attaches roots to other grassland species and abstracts carbon, nitrogen, and other minerals from host plants via penetration of the xylem (Riopel and Timko, 1995). While the species has a host range in excess of 50 species across grasses, forbs and legumes, a single plant typically attaches to only 4 host species (Gibson and Watkinson, 1988). *R. minor* typically selects grasses and legumes as hosts, as forbs can respond with defence mechanisms – in a 2006 study, *Leucantheum vulgare* was shown to encapsulate *R. minor* haustoria attempting to penetrate the plant, blocking the attempt at parasitism (Cameron *et al.*, 2006). The same study showed that no such cellular-level defence mechanism is seen in grasses or legumes.

As a result of this parasitism, *R. minor* is regularly used in the process of grassland restoration, and is deemed a cheap and environmentally-friendly method (Hejcman *et al.*, 2011). By attaching to grasses and legumes that can overwhelm a grassland sward, *R. minor* reduces the above-ground biomass of such dominant species, while not compensating for the loss in its own growth (Cameron *et al.*, 2005).

R. minor has already been the subject of studies into changes in above-ground biomass, flower abundance and timing at High Keenley Fell. Wedlich *et al.* (2012) reported significant reductions in biomass in 2009 following three years of ozone exposure, with highest biomass in control plots and lowest biomass in 5 m plots. Additionally, Rintoul (2013) reported significant reductions in flower abundance in 2009, but no change in flower timing. These previous findings at High Keenley Fell, combined with the importance of the species in the conservation context made this an ideal study subject. Thus, this species was selected to be investigated in this study of nectar.

4.1.4 Aim and objectives

While experimental work has identified changes in forb nectar production in response to elevated CO_2 (Rusterholz and Erhardt, 1998), there is little known of the effects of elevated ozone upon floral nectar production or quality. To this end, the aim of this study was to

develop an understanding of the effects of exposure to a peak episode of high ozone in the 2012 growing season on the nectar of the hemi-parasitic species *Rhinanthus minor*. The following objectives were addressed by this study:

1. To determine the effect of ozone upon volume of nectar produced by individual floral nectaries;
2. To determine the effect of ozone upon the concentrations, and overall proportions, of individual nectar sugars;
3. To evaluate whether ozone alters the sucrose-hexose nectar sugar ratio.

4.2 Materials and methods

4.2.1 Flower sampling

Flowers from *R. minor* plants were collected from High Keenley Fell between 09:30 and 10:10 on the morning of 12th July 2012. Flowers were collected on the stem from a control area (upwind of the ozone release pipes), and then again at approximately 7.5 m and 10 m downwind, ± 1 m, from three transects. Each sample aimed to contain 10 young individual flowers. Once collected, flowering stems with flowers still attached were collected in polythene bags and stored in a cool bag for transfer to a laboratory for further analysis.

4.2.2 Nectar Volume

Three hours after the flowers were sampled, nectar was collected from each flower using one 10 µL microcapillary tube (Hurschmann Laborgerate) per flower in laboratories at Newcastle University. Nectar volume was then determined by measuring the column length within the microcapillary tube; and using this in Equation 4.2:

$$\boldsymbol{Nectar\ volume}\ [\mu\boldsymbol{L}] = nectar\ column\ [ml] \times 0.3125\ [\mu L\ mm^{-1}] \qquad \textbf{Equation 4.2}$$

Any evidence of nectar robbing – defined by Maloofe and Inouye (2000) as a process by which visitors remove nectar from a flower via a hole bitten or pierced through the corolla – was noted. These samples were then stored at -20°C in preparation for further analysis, a storage method identified by Morrant *et al.* (2008) as being suitable for samples to be analysed for nectar sugars.

4.2.3 Nectar sugar analysis

Once defrosted, nectar was removed from microcapillary tubes using a squeegee and centrifuged for one minute. A volume of 1 µL of nectar from each sample was then diluted with nanopure water by a factor of 1:2040. This was then placed in a vortex for 5 seconds to sufficiently mix, before 30 µL was transferred to glass vials and stored at -20°C. Determination of concentrations of specific sugars (fructose, glucose, mannitol, sorbitol and sucrose) was conducted using high performance liquid chromatography (HPLC). A volume of 20 µL of each sample was injected via a rheodyne valve onto a Carbopac PA-100 column (Dionex, Sunnyvale, California, USA). The calibration standards contained each sugar at a concentration of 10 ppm. Sample components were eluted isocratically from the column using 100 mM NaOH, flowing at 1 mL min^{-1}. The elution profiles were recorded using pulsed amperometric detection (ED40 electrochemical detector, Dionex), and were analysed using the Chromeleon software package (Thermofisher Scientific). During analysis, sorbitol was detected within nanopure water blanks and the decision was made to remove this sugar from subsequent analysis.

4.2.4 Data Analysis

Nectar volume was presented as µL nectar per flower. Values for individual sugar concentrations were converted from µmol/L to g/L of nectar, and then presented as % of total sugar (Equation 4.3); a common representation of nectar composition in literature (Canto and Herrera, 2012; Barnes *et al.*, 1996; Nicolson and Thornburg, 2006; Petanidou *et al.*, 2006; Wolff, 2006).

$$\boldsymbol{M_{sugar}}[\mu g\ \mu L^{-1}] = \frac{\rho_{sugar}[\mu mol\ \mu L^{-1}] \times M_{sugar}[g\ mol^{-1}]}{1\ x\ 10^{6}} \qquad \textbf{Equation 4.3}$$

n.b. ρ_{sugar} = concentration of nectar sugar detected within sample;

M_{sugar} [g mol^{-1}] = molecular mass of nectar sugar

M_{sugar} [µg μL^{-1}] = concentration of nectar sugar in nectar

The percentage [sugar] of total sugars data have been presented in graphs. Actual concentrations have been presented as an appendix, as these are also reported by some (Grünfeld *et al.*, 1989; Rusterholz and Erhardt, 1998). Individual sugars were analysed for changes in percentage of total sugar relating to ozone exposure. Nectar sucrose/hexose ratio

was also calculated for changes relating to ozone exposure. Where nectar samples had only one of three sugars detected, sucrose-hexose ratios were not, following Wolff (2006).

Data were prepared for, and subsequently analysed as previously outlined in Chapter 3.2.4.1. Table 4.2 reports the transformations applied to data to achieve normality.

Table 4.2 – Variables within study and data transformation applied to achieve normality within data.

Variable	Units	Transformation applied
Nectar Volume	µL	$\text{Log}(x + 1)^{[1]}$
Fructose	% of total sugar	None
Glucose	% of total sugar	$\text{Ln}(x + 1)^{[2]}$
Mannitol	% of total sugar	$\text{Ln}(x + 1)^{[2]}$
Sucrose	% of total sugar	Arc sine ($\sqrt{x}$)
Total Sugar	g L^{-1}	$\text{Ln}(x + 1)^{[2]}$
Sucrose/Hexose Ratio		$\sqrt{(x)}^{[1]}$

n.b. [1] – after Wolff (2006)

[2] – after Petanidou *et al.* (2006)

Following nectar collection and analysis, a number of flowers either had no nectar to analyse, or no sugars identified through HPLC analysis, and were subsequently removed from the analysis. This resulted in unequal sample size between ozone treatments and transects (Table 4.3).

A total of 3 flowers – two at 10 m in transect 2, and one at 10 m in transect 3 – showed evidence of nectar robbing, though only one presented no nectar and was removed from the study.

Table 4.3 – Number of samples (*n*) used in the nectar volume analysis, tabulated by transect and ozone treatment.

Treatment	Transect			*Total*
	1	**2**	**3**	
Control	7	9	4	***20***
10 m	7	4	3	***14***
7.5 m	9	5	3	***17***
Total	***23***	***18***	***10***	***51***

4.3 Results

4.3.1 Nectar volume

There were only very small differences of 1% in nectar volume between ozone treatments and control, with flowers from the control area secreting on average just 0.036 µL per flower more than flowers collected from elevated ozone treatments, for which both means were 2.813 µL per flower, though standard error varied slightly at ± 0.27 µL and ± 0.31 µL at 10 m and 7.5 m respectively. There was no significant effect of ozone treatment upon nectar volume (F = 0.06; P > 0.05), nor any significant effect of transect within treatment (F = 1.454; P > 0.05).

4.3.2 Individual nectar sugars

There were no significant effects of ozone treatment upon individual nectar sugar concentrations expressed as % of total sugars (Table 4.4). Only sucrose displayed a positive trend; the proportion of total sugar increased when ozone had previously been elevated. Fructose and mannitol showed declining proportions of total sugar with increasing prior ozone exposure, and glucose showed a mixed response, peaking at 7.5 m, followed by control and 10 m, which were similar in concentration (Figure 4.2). While there was no significant treatment effect for any individual sugars, fructose, glucose and sucrose showed significant transect effects within treatment (Table 4.4).

Due to the significant transect effects within ozone treatments, the sugar content trends were analysed further. Fructose exhibited three trends over the transects: Transect 1 showed an

increase in fructose with increasing ozone; Transect 2 showed the opposite and reflected the overall trend; and Transect 3 displayed highest fructose proportions at 10 m and the lowest at 7.5 m (Figure 4.3a). Glucose was reduced with higher ozone exposures in Transects 1 and 3, but the trend in Transect 2 was the opposite; none of these trends mirrored that seen in treatment plots in Figure 4.2a. Sucrose responses in Transects 2 and 3 were mixed; however Transect 1 proportions peaked at 10 m, with lowest proportions in control flowers (Figure 4.3c). As with glucose, no transect reflected the main trend seen in Figure 4.2c; however this may be a result of Transect 3 driving the average treatment trends, with the lowest sample population. Similarly, Transect 1 trends were not reflected in average treatment trends, even though the highest ozone exposure occurred in this transect.

There was no significant effect upon total nectar sugar concentration (Table 4.4); however concentrations were highest in control flowers, followed by the 7.5 m and 10 m treatments (Figure 4.2e).

Table 4.4 – ANOVA test results for four nectar sugars detected within nectar of *R. minor*, represented as % total sugar, plus total sugar. F_1 – treatment F value; F_2 – transect within treatment F value. Bold values indicate significant results at $P < 0.05$. * - $P = 0.01$-0.05.

Nectar Sugar	F_1	F_2
% Fructose	1.485	**2.454***
% Glucose	0.031	**3.263***
% Mannitol	1.059	0.884
% Sucrose	0.754	**2.805***
Total Sugar (g L^{-1})	1.467	1.220

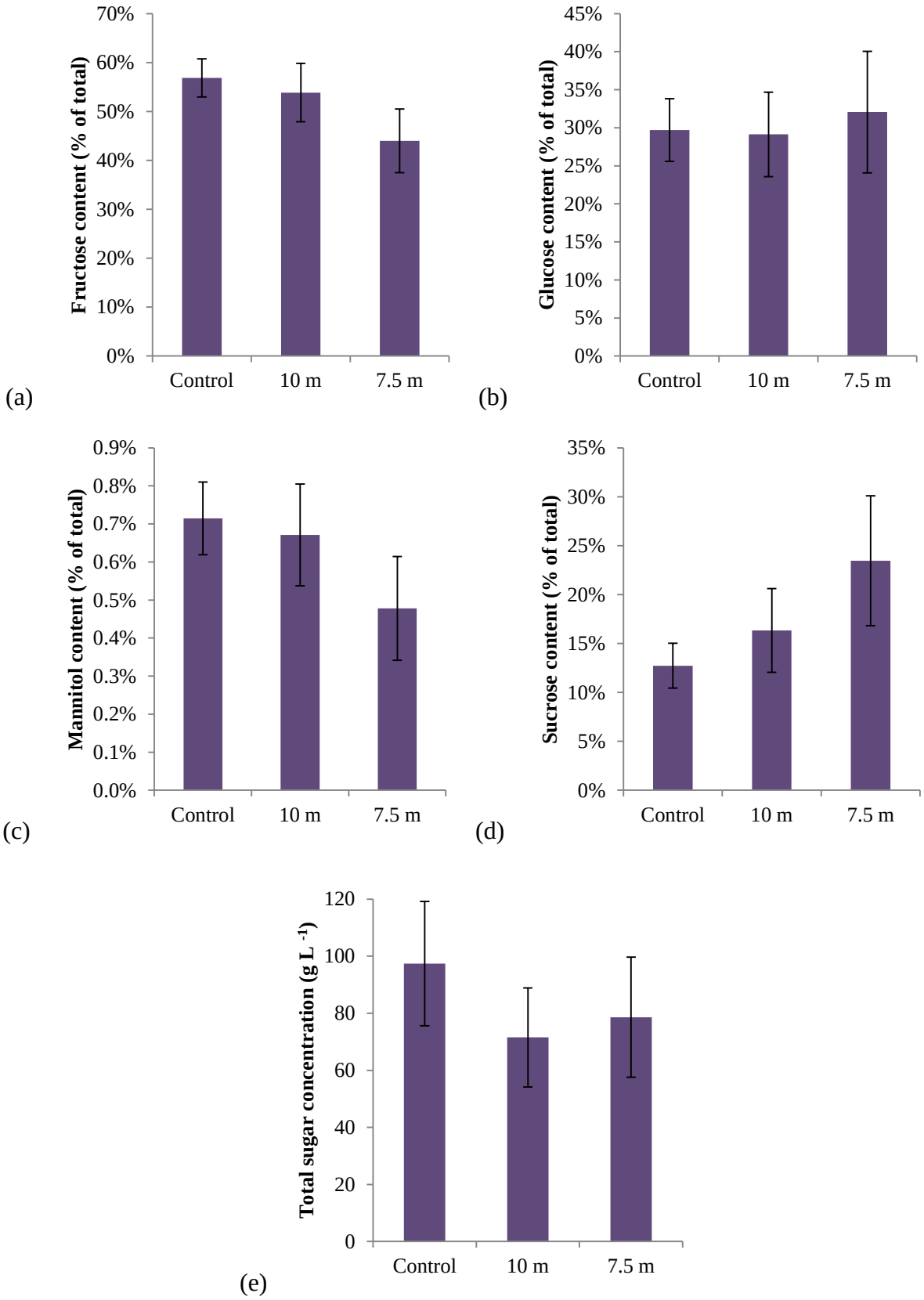

Figure 4.2 – Mean content of (a) fructose, (b) glucose, (c) mannitol, (d) sucrose as a percentage of total sugar content, and (e) concentration of total sugar in nectar of *R. minor*, arranged by ozone treatment. Error bars represent standard error. *n* = 20; 17; 14.

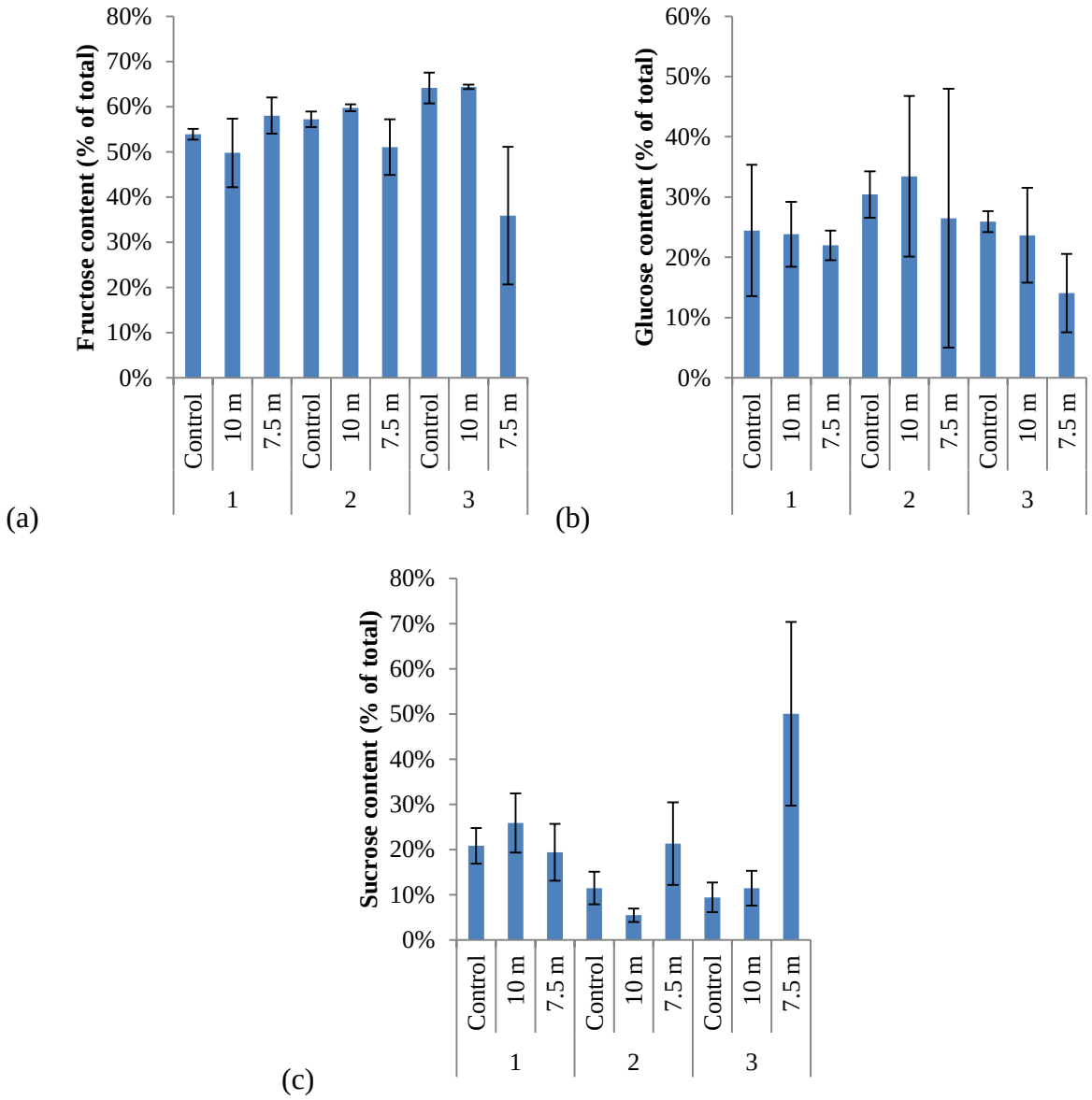

Figure 4.3 – Mean content of (a) fructose, (b) glucose and (c) sucrose as a percentage of total sugar in *R. minor* nectar, arranged by ozone treatment within transect. Error bars represent standard error. n = 23; 18; 10.

4.3.3 Nectar sugar ratio

There was no significant effect of ozone treatment upon sucrose-hexose ratio (F = 0.873; P = 0.457), or effect of transect within treatment (F = 1.384; P = 0.248). The mean ratio value across all three treatments was 0.26, which shows the nectar of *Rhinanthus minor* flowers was rich in hexose. The ratio tended to be lowest in nectar from control flowers corresponding to more hexose-rich nectar, followed by 10 m and 7.5 m which were similar and show a relatively more balanced proportion of hexose and sucrose (Figure 4.3).

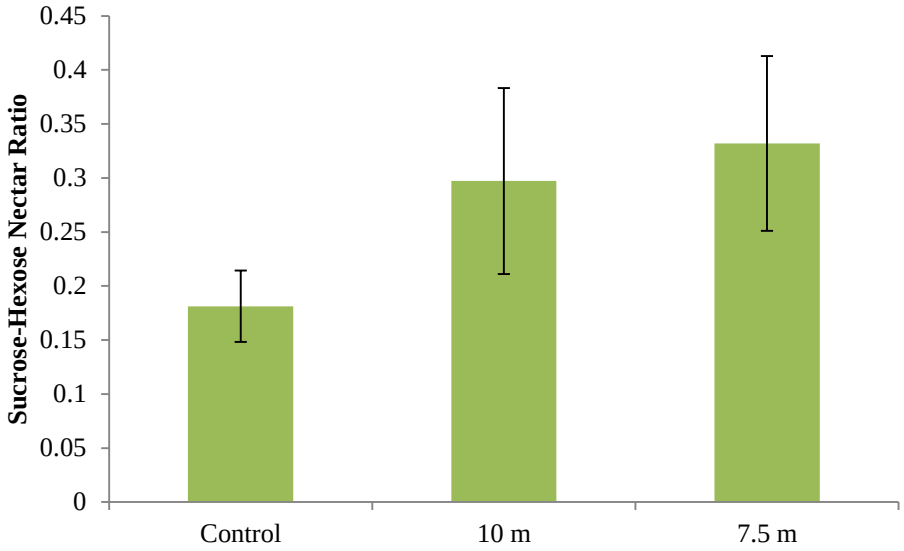

Figure 4.4 – Mean sucrose-hexose sugar ratio within nectar of *R. minor*, arranged by ozone treatment. Error bars represent standard error. *n* = 18; 15; 11.

4.4 Discussion

4.4.1 Nectar volume

With a small variation between individual flowers reported, this may suggest that the variation seen may be a result of microclimatic conditions within the sward, and location of the flower on the plant. Studies have shown that increased humidity, and recent rainfall both result in higher nectar volumes when sampling in the field (Wyatt *et al.*, 1992), with humidity causing an immediate nectary response (Bertsch, 1983). Anecdotal evidence showed that on the morning of sampling, there was a high quantity of dew on plants at the field site, and the damp nature of the ground indicated recent rainfall, which may suggest high background humidity within the sward. The suggestion that nectar production varies depending on position of the flower on a plant is reported by Zimmerman (1988), but is contradicted in an orchid study that showed there was no significant variance dependent upon the height of the flower on the plant (Westwood *et al.*, 2011). A study into apple flower nectaries found that some cultivars displayed higher nectar volume within the canopy when compared to being external to the tree canopy, though this was inconsistent (Campbell *et al.*, 1991). Thus, there

may be variation in nectar volume dependent upon the position of the flower on the plant and within the canopy, though this cannot be confirmed.

4.4.2 Individual nectar sugars

There were significant effects of transect within treatment for three sugars: fructose, glucose and sucrose. These significant transect effects seemed to result from a strong influence from Transect 3, with the lowest ozone exposure and sample populations, and the most likely – yet insignificant – ozone effects displaying shifts in sugar proportions. Conversely, the highest ozone exposure was in Transect 1, where there was no apparent shift.

These mixed responses and trends in nectar sugar proportions could mostly be explained by natural factors such as those previously mentioned for nectar volume, such as the effect of local humidity. Additionally, the natural process of nectar production and development creates changes in the nectar composition: Nicolson (2002) described how sucrose hydrolysis draws water into the nectar from the plant, and such a water influx can cause changes that result in a nectar that previously had 30% sucrose nectar becoming 20% hexose nectar, while nectar volume increases. Beside flower age, another mechanism that alters proportion of sucrose is microbial contamination, (Nicolson and Thornburg, 2007), which may come from the environment or be transported by pollinators. However, the incidence of this may increase with the age of the flower. Also, extrafloral nectar may be exposed to drying elements of the environment, which reportedly increases concentrations of fructose and glucose in nectar (Koptur, 1994).

Therefore, much of the variability could be explained by a combination of several factors. Throughout the growing season, volumetric soil water content was highest in Transect 1, and lowest in Transect 2 (data not shown), suggesting that it may be a major factor in Transect 1 trends, but less important in Transects 2 and 3, where possible ozone trends were indeterminate and strong respectively. However, other factors may also contribute to the variability, such as flower age, and especially in the case of Transect 3, small sample population.

There was no significant effect of ozone exposure on the total sugar concentration, nor was there a transect effect (Table 4.5). The large error bars on Figure 4.2e signal large natural variation between individual flowers in all treatments. The variability between ozone treatments may be a signal that differences in microclimate (e.g. humidity) could be

responsible, though without climatic variable measurements taken during sampling this cannot be explored further.

4.4.3 Nectar sugar ratio

Findings show that nectar collected from *R. minor* plants at High Keenley Fell was generally hexose-rich. This is in contradiction with one previous report, which reported *R. minor* as having a sucrose-rich nectar (Percival, 1961). The finding is however, in agreement with a study of nectar composition of two *Rhinanthus* species growing in the Netherlands, where nectar was found to be hexose-rich in two populations of *R. minor* (Kwak *et al.*, 1983). Large variation in ratio was recorded between transects, with Transect 1 having as many individual flowers deemed sucrose-rich (8/22) as hexose-rich. Transect 2 had the highest incidence of hexose-dominant flowers (10/15), and Transect 3 flowers were almost entirely hexose-rich (5/8). No flowers were deemed to be sucrose-dominant. This wide variation is in agreement with Herrera *et al*. (2006) and Pleasants (1983), but in contradiction to the long held assumption of minimal within-species variation suggested by Percival (1965) and Wykes (1952).

The variation in nectar sugar ratios was not significantly related to ozone exposure (P = 0.457; Table 4.6). This suggests that, when plants were exposed to elevated ozone during an episode of high tropospheric concentrations there was no subsequent effect upon the ratio of sucrose to hexose sugars.

There are physiological mechanisms that could account for the variation within the population, which were mostly covered relating to nectar sugar concentrations. However, sucrose-rich flowers, such as those found in Transect 1, could be young flowers as hydrolysis and microbial contamination may not have yet occurred. Nicolson and Thornburg (2007) also suggest that larger variations in ratio within a species population may show that more than natural hydrolysis is responsible. Herrera *et al.* (2006) corroborate this with their finding of changes in nectar composition in late season flowers (taken in May) when compared to early season flowers (sampled in March).

Transect 1 displayed a proportionately high number of sucrose-rich flowers, while Transect 3 has a high proportion of hexose-rich flowers, where potential ozone effects were observed. This initially suggests that there is a possible effect of ozone upon hexose-rich *R. minor* flowers in this population. However, this could also indicate that flowers sampled in Transect

3 were emerging, or had emerged, during the high ozone episode while Transect 1 flowers had not; this was as a result of low numbers of flowers along Transect 3 which did not allow for the selection of specific flowers. This suggests Transect 3 flowers were exposed to elevated ozone and Transect 1 flowers were not.

4.4.4 Study shortcomings

Although almost 90 flowers were taken from the field for analysis, only 51 flowers contained any nectar to analyse. This meant that there were two shortcomings to this study. Firstly, the aim of having 9 samples per treatment for a robust analysis was not met. Secondly, there was an issue of unequal sample populations within treatments as shown in Table 4.3. To improve upon this, sampling would ideally take place earlier in the species' flowering season when flowers are more abundant, as this would allow for younger flowers to be selected for sampling, rather than flowers across all stages of the life cycle. Sampling within higher ozone exposures could also be conducted to better develop knowledge of ozone effects. It would also allow for a consistent sample population across all treatments, therefore improving robustness of analysis. Additionally, time would have been taken to study environmental variables known to impact upon nectar production, such as soil water content and relative humidity. Finally, if possible, microbial culturing of the nectar samples would take place to identify whether contamination of the nectar had occurred, as this would give a clearer picture of effects.

5 Effects of ozone on ecosystem CO_2 exchange in an upland grassland community

5.1 Introduction

Terrestrial vegetation is both a source and sink for CO_2 in the cycling of carbon, and on a global scale is estimated to be responsible for the assimilation of 27 g C m^{-2} y^{-1} (Schimel *et al.*, 2001). In England and Wales, most of the land surface has been losing soil organic content at a mean rate of -0.6% per year between 1978 and 2003, with the highest stores and losses occurring in upland regions (Figure 5.1; Bellamy *et al.*, 2005). It is imperative that the mechanisms behind this are understood.

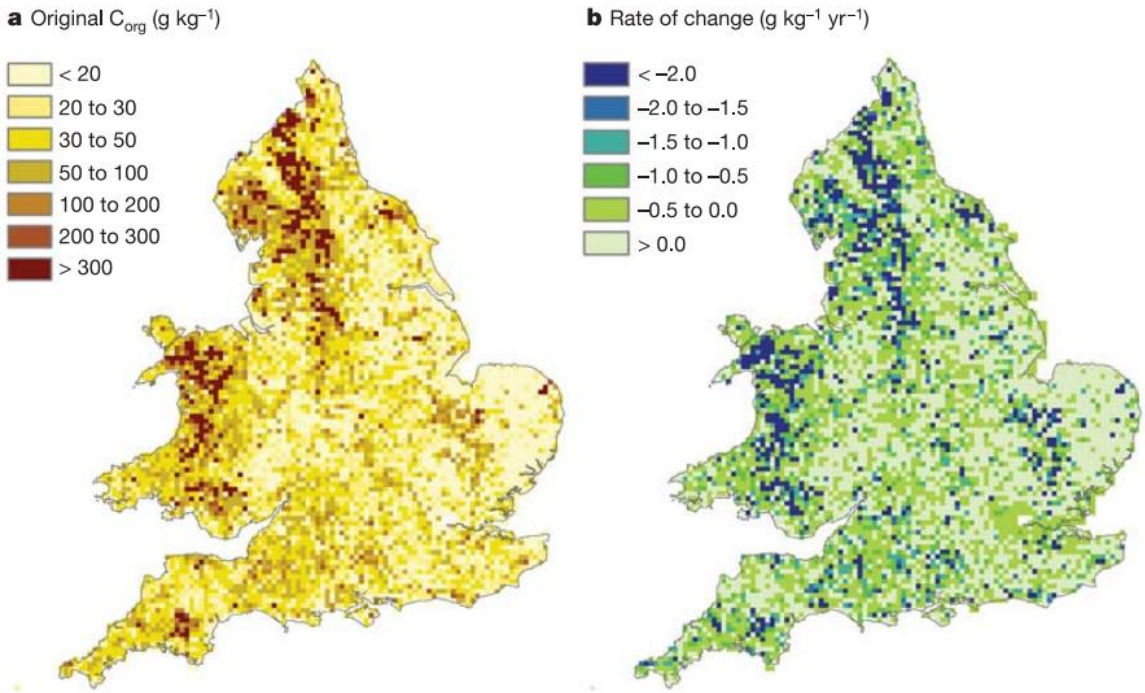

Figure 5.1 – Changes in the soil organic carbon content of soils across England and Wales, 1978-2003. **a** – soil organic carbon measures in original samplings; **b** – rates of change in soil organic content calculated over the study period. Not all sites were resampled; the associated rates of change were calculated based on original organic carbon contents (Bellamy *et al.*, 2005).

5.1.1 Carbon cycling in grassland ecosystems

Grasslands cover 37% of Earth's terrestrial area (discounting Greenland and Antarctica), and sequester between 0.01-0.3 Gt C y^{-1} (Lal *et al.*, 2004; O'Mara, 2012). On a localised scale, 40% of Western Europe's agricultural land is grassland, and the net carbon sink of these grasslands has been estimated to be between 0.57 and 104 g C $^{m-2}$ y^{-1} when including sequestration (Soussana *et al.*, 2007; Schulze *et al.*, 2010).

Numerous factors are already known to affect carbon cycling between the atmosphere and land surfaces, including land use change, rainfall, temperature, fire, and pestilence (Jackson *et al.*, 2002; Kurz *et al.*, 2008; Le Quéré *et al.*, 2009). Factors found to specifically reduce carbon assimilation in temperate grassland systems include invasion by woody vegetation – previously thought to increase sequestration (Jackson *et al.*, 2002), and grazing intensity, which can release stored carbon through under- and over-grazing (O'Mara, 2012).

Increasing atmospheric concentrations of CO_2 and NO_2 have been shown to increase ecosystem carbon storage through fertilisation effects: Ainsworth and Long (2004) reported a an average 28% increase in C_3 plant photosynthetic carbon assimilation in a meta-analysis of 12 FACE experiments with CO_2 concentrations ranging from 475-600 ppm; grasses assimilated on average 37% more C when exposed to 550-600 ppm. Agricultural land fertilised with nitrogen – either through deposition or application – may store more carbon (Schlesinger, 2009). However, Schlesinger (2000) suggests such gains are countered through CO_2 emissions related to fertiliser production and increased litter decomposition.

Climate warming has also already been proved to impact upon CO_2 fluxes in grasslands. De Boeck *et al.* (2007) reported that when air temperature was increased by 3°C in sun-lit climate controlled chambers ecosystem respiration was not altered; but photosynthesis was reduced – reportedly due to water stress. The study also investigated varying levels of species richness in relation to CO_2 exchange in a warming climate, and found that management practices to increase species richness would not alleviate the effects of a warming climate on grassland CO_2 GPP.

Studies have also looked into specifically improving the grassland carbon sink. Tilman *et al.* (2006) reported that, by increasing species diversity and through introducing deep-rooted species, carbon assimilation and storage can increase. Likewise, Conant *et al.* (2001) found that sowing grass and legume species can increase carbon assimilation by 6%, while managing grazing intensity both by increasing, or decreasing as necessary, can improve the

sink by up to 24%. Installing at least one form of improvement method such as those mentioned enhanced carbon assimilation in 74% of studied grasslands.

5.1.2 Effect of ozone on ecosystem CO_2 exchange

Ecosystem CO_2 exchange is defined by the measure of a number of CO_2 flux variables focused upon the uptake and release of CO_2 in the system. Net ecosystem exchange (NEE) is defined by Wofsy *et al.* (1993) as the net CO_2 exchange between the atmosphere and the vegetation and soil. Ecosystem respiration (R_{eco}) is the measure of CO_2 respired by plant vegetative matter and microbes in the soil (Hu *et al.*, 2008), and is dominated by root and soil microbial respiration (Valentini *et al.*, 2000). Finally, gross primary production (GPP) is the balance between ecosystem respiration and net ecosystem exchange (Albergel *et al.*, 2010). While GPP shows the balance between the uptake and release considered in these two variables, Hu *et al.* (2008) suggests that R_{eco} is the most important factor in determining spatial and temporal variations in CO_2 balance. Clear diurnal variations in ecosystem CO_2 exchange have been reported, where photosynthesis dominates daytime exchanges, and respiration night-time exchanges; and also seasonal variations, with a US deciduous forest ecosystem study showing that GPP is negative (showing net uptake) from mid-April to October, and positive (showing net release) during the winter months when leaves have dropped (Baldocchi *et al.*, 2001).

Elevated tropospheric ozone has already been proven to reduce carbon fixation and root allocation in plant species (Reich, 1987; Laurence *et al.*, 1994), and it was suggested in the late 1990s that antagonistic effects of ozone – driven by mixed responses to ozone exposure at species level – could potentially cancel out the aforementioned positive effects of elevated CO_2 and N deposition (Chapelka and Samuelson, 1998; Volin *et al.*, 1998), a finding confirmed by Ollinger *et al.* (2002) and Andersen (2003). Volin *et al.* (1998) attributed the effects in grass species to reduced photosynthetic activity and relative growth rate, driven by exposure to 95 nmol mol^{-1} ozone over 101 days.

Modelling studies have reported the combined effects of elevated ozone on a number of ecosystems, with one US study concluding that the conterminous 48 states exhibited reductions in CO_2 exchange ranging from 2.6-6.8% as a result of elevations in ozone that occurred between 1989 and 1993 (Felzer *et al.*, 2004).

Much of the experimental work into the effect of ozone on CO_2 exchange has been undertaken on Northern hemisphere forests, where Ollinger *et al.* (2002) found that rising tropospheric ozone had slowed the positive impact increasing CO_2 was having on North American forests acting as carbon sinks. The study concluded that small biomass reductions due to slower woody growth equated to larger reductions in carbon sequestration.

Few studies have focused upon the effect of ozone on grassland CO_2 exchange. However, one FACE study based in the Swiss Alps and working on grassland monoliths found that both GPP and ecosystem respiration reduced by similar amounts under elevated background ozone conditions (Volk *et al.*, 2011). Another study, investigating meadow ecosystems in OTCs in Finland reported reductions in ecosystem respiration of CO_2 in the second and third years of a three year exposure to ozone concentrations of 40-50 ppb, with the reduction apportioned to visible foliar injury and reductions in above-ground growth (Kanerva *et al.*, 2007).

5.1.3 Aim and objectives

The aim of this study was to investigate whether there is an effect of ozone on ecosystem CO_2 exchange of the grassland at High Keenley Fell. The following objectives were developed, and addressed by this study:

1. To determine the effect of ozone on grassland net ecosystem exchange (NEE), ecosystem respiration (R_{eco}), and gross primary production (GPP) over the growing season after 6 years of ozone fumigation;
2. To assess the effect of environmental variables on NEE, R_{eco}, and GPP across the field site during the growing season.

5.2 Materials and methods

5.2.1 Ecosystem CO_2 exchange measurements

Measurements of ecosystem CO_2 exchange were undertaken at three ozone treatments – 2.5 m, 5 m, and 10 m downwind – and were also conducted in control plots of the experimental grassland site at High Keenley Fell. Two subplots were present at each ozone treatment in each of the three transects (Figure 5.2). Three 20 cm diameter PVC rings of 15 cm height were randomly located in the two subplots in 2008. The rings were approximately 10 cm into

the soil, with approximately 5 cm exposed above the soil, and they provided permanent locations for CO_2 flux sampling. This allowed for direct comparison of CO_2 flux measurements both throughout the 2012 growing season, and to measurements previously taken in 2008. Like the flowering plots, the area within these rings was exposed to climatic field conditions and livestock grazing.

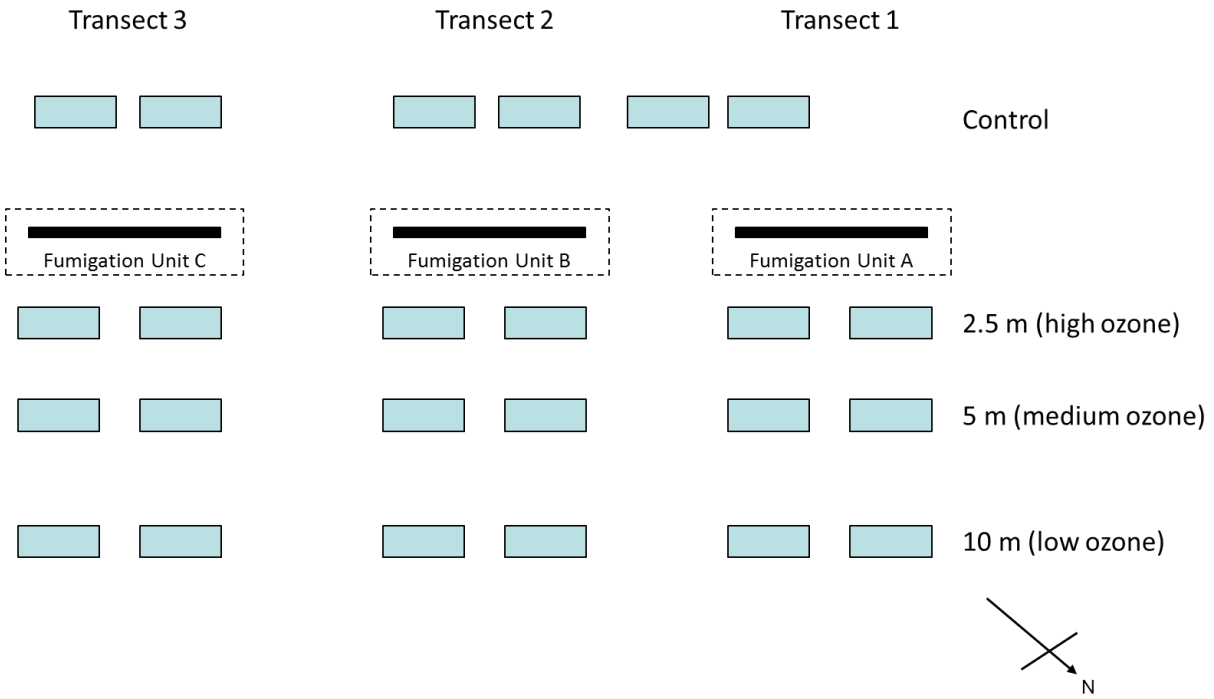

Figure 5.2 – Sampling plot layout for CO_2 flux plots. Each blue box represents a 2 x 1 m plot that has been established, and sampled, since 2008.

CO_2 flux measurements were taken using a portable infra-red gas analyser (IRGA; LiCOR Biosciences, LI-8100, Automated Soil CO_2 Flux System, Lincoln, Nebraska), and a purpose-built transparent Perspex chamber (outer diameter = 20 cm, inner diameter = 19.4 cm, height = 25 cm), with a LiCOR-type transparent vent on the top. The chamber created a gas tight seal with the PVC rings in the soil through a rubber inner tube being attached to the chamber base. CO_2 fluxes were measured in light and dark conditions by measuring the CO_2 concentration of the headspace every second over the course of 3 minutes, with a 3 minute break in between measurements to allow the headspace in the chamber to reach ambient outside air conditions again. Dark measurements – to determine R_{eco} – were facilitated through the use of a fabric cloche, created from light reflective material to the outside to reduce radiation-related temperature increases over the measurement period.

Measurements were taken on three occasions in 2012: mid-June, mid-July and early August prior to the biomass harvest. They took place over two days, and were conducted in a stratified manner, working along each transect between treatments and measuring one mesocosm ring at a time so a sample of the site could be completed in a session, thus reducing the chance of variation in weather affecting results. Prior to each ring measurement, the chamber offset caused by the ring in the ground was measured three times in each ring to allow accurate volume calculations.

In addition to the CO_2 flux measurements, several environmental variables were also measured in or near each ring. Volumetric soil moisture content was determined through the use of a theta probe (0-6 cm depth; HH2 Moisture Meter, Delta-T Devices, Cambridge, England), taking three sub-replicate measurements around each ring to reduce disturbance within rings; air and soil temperature (5 cm depth) through using a Minitherm thermometer (Hanna Instruments, HI 8751, Leighton Buzzard, Bedfordshire); and photosynthetically active radiation (PAR) was measured using a PAR meter (SKP200) with a PAR special sensor (SKP210; Skye Instruments, Llandrindod Wells), measured during the light measurements at t + 0, 30, 60, 120 and 180 seconds, and away from the ring to prevent shadows influencing measurements. Finally, canopy height was measured through the use of a 1 m long non-flexible ruler at three points within the ring.

5.2.2 Determining ecosystem CO_2 fluxes

CO_2 flux rates were calculated using LI-8100 File Viewer 3.1 software (version 2), carrying out a linear regression over time. Measurements were cropped to 50 second intervals of the overall 3-min measuring period for light measurements, and 80 second intervals for dark measurements. The first 15 seconds (in the case of light measurements), and first 30 seconds (in dark measurements) were discarded due to the initial mixing of air in the headspace after the chamber has been fitted to the ring. Following the 50/80 second measurement intervals, the rest of the measurement period was discarded as, after a period of time, the linear relationship between time and CO_2 would be lost due to changes in CO_2 concentrations or air temperature within the chamber. The CO_2 fluxes were corrected for the larger volume considering the gas volume within the ring above the soil surface (Equation 5.1). The slope (in ppm s^{-1}) of the subsequent linear regression was then used by the program to calculate a flux value from the slope in μmol m^{-2} s^{-1}, which was converted to mg C-CO_2 m^2 h^{-1} by multiplying with the molecular weight of C and 3600 , and dividing by 1000 (Equation 5.2).

Figure 5.3 (below) shows a typical linear decrease of the CO_2 concentration in the headspace over time in light conditions. The CO_2 flux data were exported into Microsoft Excel spreadsheets.

$$CO_2\ flux\ (mg\ CO_2C\ m^2\ \ h^{-1}) = \frac{CO_2\ flux\ (\mu mol\ m^{-2}\ s^{-1}) \times A_r\ [C]}{1000} \times 3600$$ **Equation 5.1**

$$CO_2\ flux\ (\mu mol\ m^{-2}\ s^{-1})[corrected\ for\ ring] = Flux\ (ppm\ m^2\ \ h^{-1}) \times \frac{V_{[chamber]}\ (mL) + V_{[ring]}(mL)}{V_{[chamber]}\ (mL)}$$

Equation 5.2

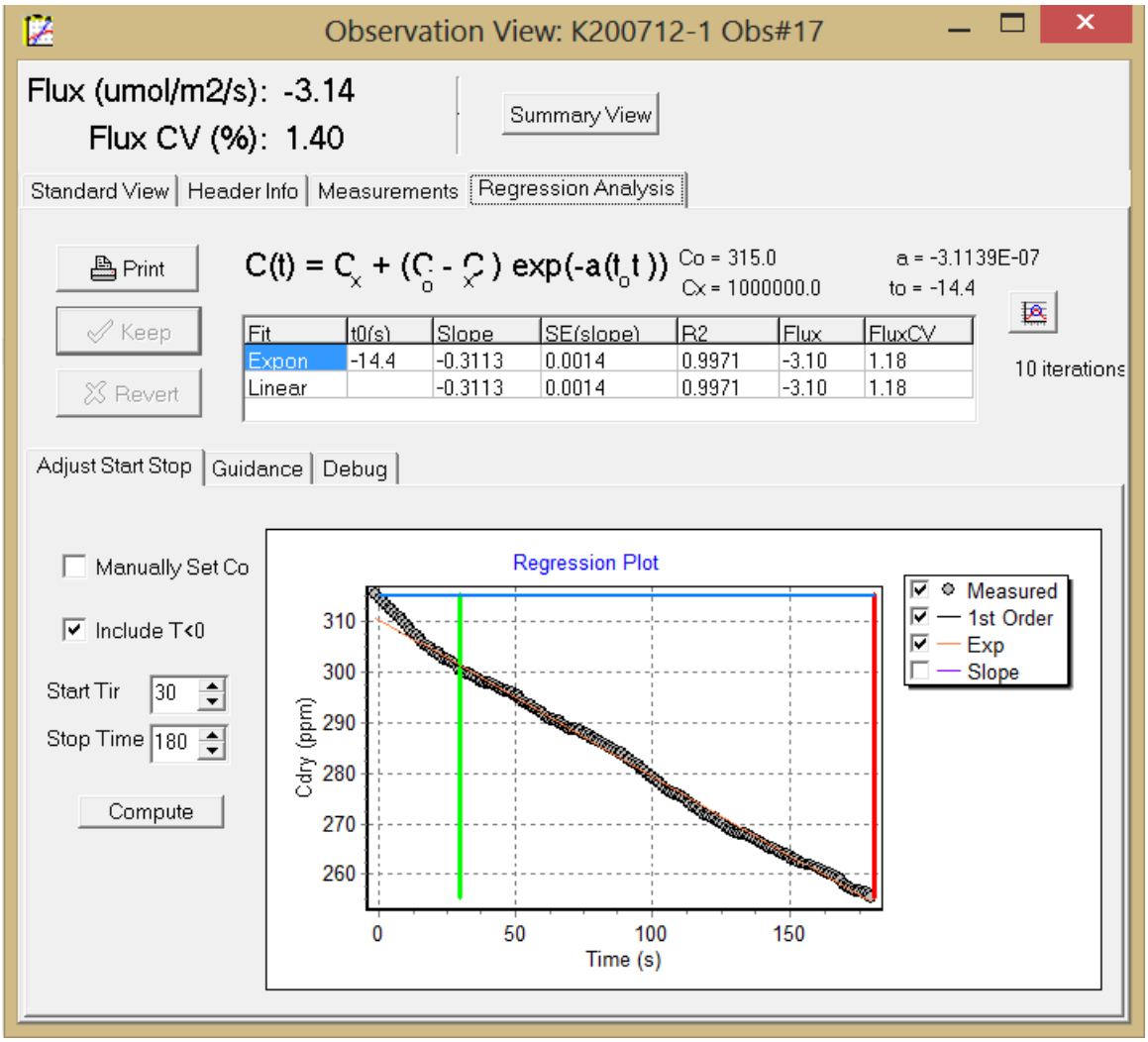

Figure 5.3 – Example IRGA file output displaying CO_2 flux during a light measurement, as viewed in LiCOR LI-8100 File Viewer 3.1.

NEE was determined in light conditions, and R_{eco} was determined in dark conditions. The GPP was calculated as the difference between NEE and R_{eco} (Equation 5.3). A positive GPP value represents a net flux of CO_2 into the atmosphere; a negative value represents a net flux from the atmosphere.

$$GPP = NEE - R_{eco} \qquad \textbf{Equation 5.3}$$

5.2.3 Data analysis

Statistical analysis was conducted to determine any ozone effect on NEE, R_{eco}, and GPP by using a nested design ANOVA, previously outlined in Chapter 3. Linear regressions were utilised to determine any correlation between flux measurements and volumetric soil water content, canopy height, air temperature, soil temperature, and PAR, and were conducted using graph-building functions in Microsoft Excel 2010.

5.3 Results

5.3.1 Climate conditions during CO_2 flux measurements

Table 5.1 presents average air and soil temperature, photosynthetically active radiation (PAR) and volumetric soil water content (SWC) for the duration of each flux measurement period. Overall, July was the coldest month, and August the warmest month. Soil temperature was coldest in July, which was also the month with the highest SWC; August was the warmest and driest month in relation to soil. Finally, PAR was at its highest in August, and lowest in July.

Table 5.1 – Average climatic conditions for each measurement month, split by transect.

		Air Temperature (°C)	Soil Temperature at 5 cm depth (°C)	PAR (µmol m^{-2} s^{-1})	Volumetric SWC at 0-6 cm depth (%)
June	**T1**	17.0	13.2	949	51.0
	T2	18.3	13.5	1198	48.0
	T3	17.8	13.3	1212	52.3
July	**T1**	16.0	13.6	581	55.5
	T2	15.6	13.5	692	54.7
	T3	15.9	13.8	653	56.4
August	**T1**	18.0	15.2	1147	48.9
	T2	18.8	14.6	1187	48.2
	T3	18.9	15.4	1094	49.4

5.3.2 Ecosystem respiration (R_{eco})

There was no significant effect of ozone on R_{eco} in the field-based mesocosms at High Keenley Fell during any of the three measurement periods (Table 5.2). The highest R_{eco} was recorded in August, with an average 295 mg CO_2-C m^{-2} h^{-1} across all treatments, while the lowest was in July, which was almost 30% lower at 203 mg CO_2-C m^{-2} h^{-1} (Figure 5.4). When combining all three months to create a growing season average, mean R_{eco} measurements were 242, 266, 254, and 238 mg CO_2-C m^{-2} h^{-1} at control, 10 m, 5 m, and 2.5 m respectively (data not shown).

There was a significant transect effect within treatment in June and August (Table 5.2). These two months displayed a consistent trend where R_{eco} was lower in Transect 1 than in Transects 2 and 3 – a trend that was apparent across all ozone treatments (Figure 5.5). *Post hoc* differences determined that, in June, Transect 1 was significantly lower than Transects 2 and 3 (F = 10.802; P < 0.001); in August the *post hoc* response was the same (F = 6.504; P = 0.004).

Table 5.2 – Nested ANOVA test results for the effect of ozone exposure on R_{eco}. Bold values indicate significant results at $P < 0.1$. ***** - $P = 0.01$-0.05; ****** - $P = 0.001$-0.01.

Measurement Month	***Treatment* *F***	***Transect(Treatment)* *F***
June	0.044	**3.540****
July	1.513	1.506
August	0.662	**2.614***

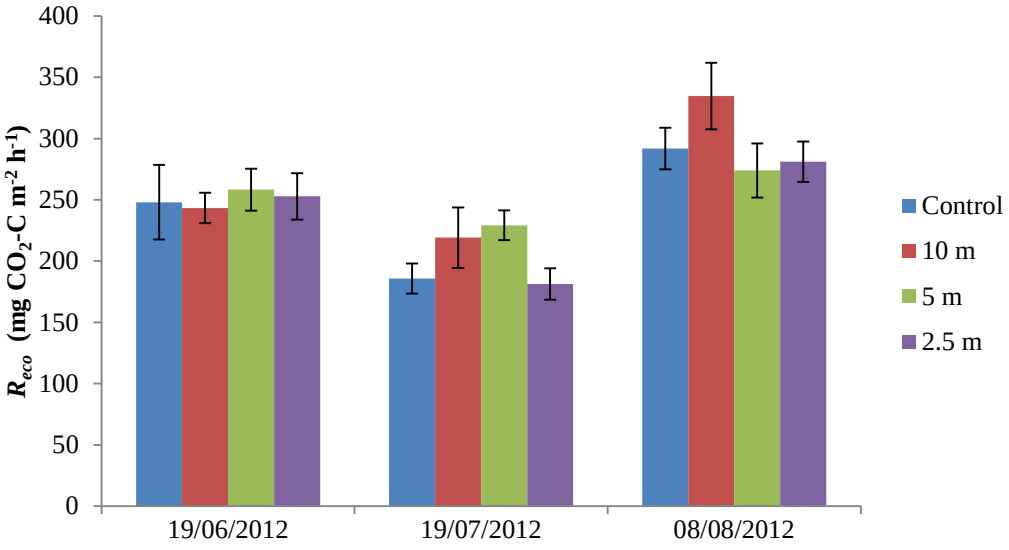

Figure 5.4 – Mean grassland R_{eco} rates on three dates at High Keenley Fell, split by ozone treatment. Error bars represent standard error. $n = 9$.

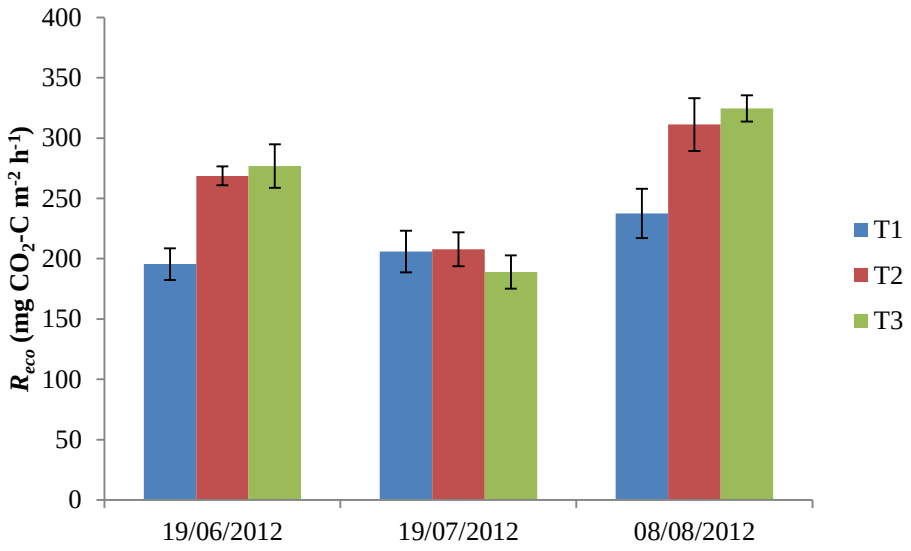

Figure 5.5 – Mean grassland R_{eco} rates on three dates at High Keenley Fell, split by transect. Error bars represent standard error. n = 12.

5.3.3 Net Ecosystem Exchange (NEE)

There was no significant effect of ozone treatment, nor transect within treatment, on NEE measured in the High Keenley Fell cores (Table 5.3). NEE was lower in August than earlier in the growing season, measuring an average -180 mg CO_2-C m^{-2} h^{-1} compared to the June peak of -267 mg CO_2-C m^{-2} h^{-1}(Figure 5.6). There was a consistent non-significant reduction in NEE at 2.5 m when compared to control: this was 10% in June; 17% in July; and 20% in August. Growing season average NEE values were -244, -244, -235, and -207 mg CO_2-C m^{-2} h^{-1} in control, 10 m, 5 m and 2.5 m plots respectively, with a 15% reduction from control to 2.5 m (data not shown).

There was no significant variation in NEE between transects in any month during the growing period (data not shown).

Table 5.3 – ANOVA test results for the effect of ozone exposure on NEE. Bold values indicate significant results at $P < 0.1$.

Measurement	***Treatment***	***Transect(Treatment)***
Month	***F***	***F***
June	0.255	0.545
July	0.646	1.506
August	0.593	0.689

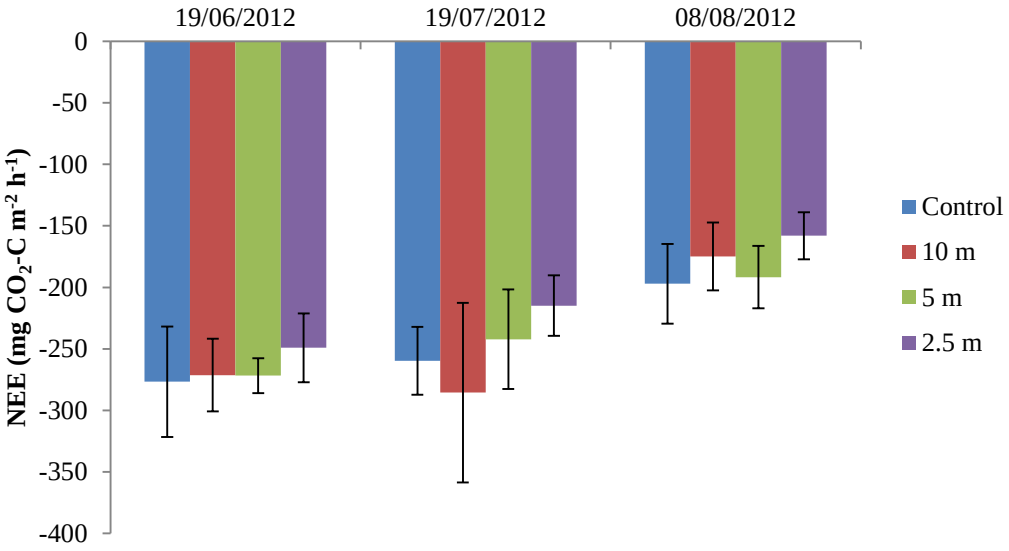

Figure 5.6 – Mean NEE rates on three dates at High Keenley Fell, split by ozone treatment. Error bars represent standard error. n = 9.

5.3.4 Gross Primary Production (GPP)

Consistent with previous findings relating to R_{eco} and NEE, there was no significant treatment effect of ozone exposure on GPP (Table 5.4). There was a non-significant reduction in GPP between control and 2.5 m plots, with reductions of 4%, 11% and 10% in June, July and August respectively (Figure 5.7). GPP measurements across the three dates were similar, though the average GPP across all treatments were lower in July than the other two months, at -454 mg CO_2-C m^{-2} h^{-1}. Growing season average GPP values were -486, -510, -489 and -446mg CO_2-C m^{-2} h^{-1} in control, 10 m, 5 m and 2.5 m plots respectively, and the drop in GPP from control to 2.5 m was 8% for the growing season (data not shown).

There was a borderline significant transect within treatment effect in June only (Table 5.4). Both June and August display a similar trend, where GPP was lowest in transect 1, and the highest in transect 3; however in July the opposite was true (Figure 5.8). Similar to NEE, this suggests a spatial gradient in GPP across the field site. GPP in June was significantly lower in transect 1 than in transects 2 and 3 (F = 5.283; P = 0.01).

Table 5.4 – ANOVA test results for the effect of ozone exposure on GPP. Bold values indicate significant results at $P < 0.1$. ~ - $P = 0.05$-0.1.

Measurement Month	***Treatment F***	***Transect(Treatment) F***
June	0.061	**2.232~**
July	1.306	0.662
August	0.541	1.734

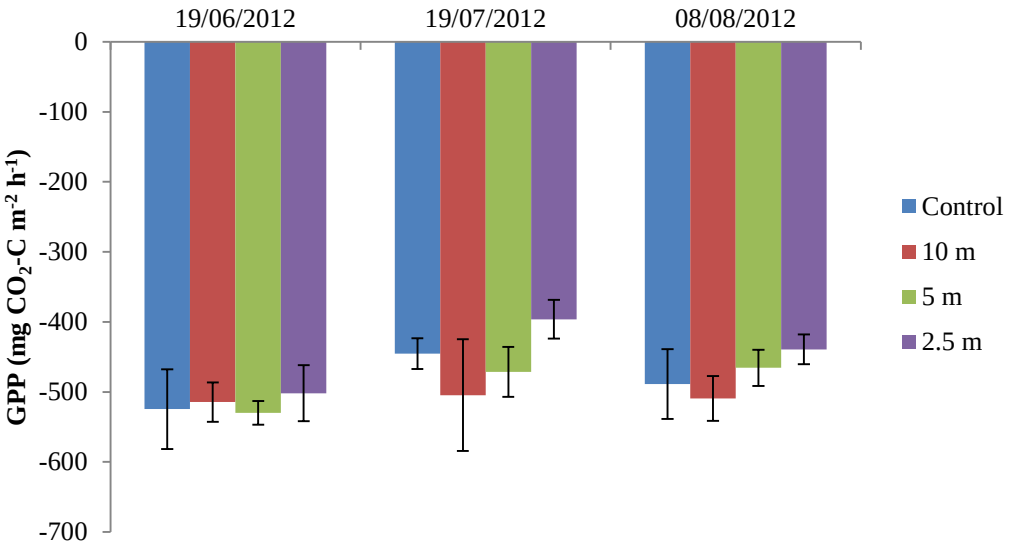

Figure 5.7 – Mean GPP rates on three dates at High Keenley Fell, split by ozone treatment. Error bars represent standard error. $n = 9$.

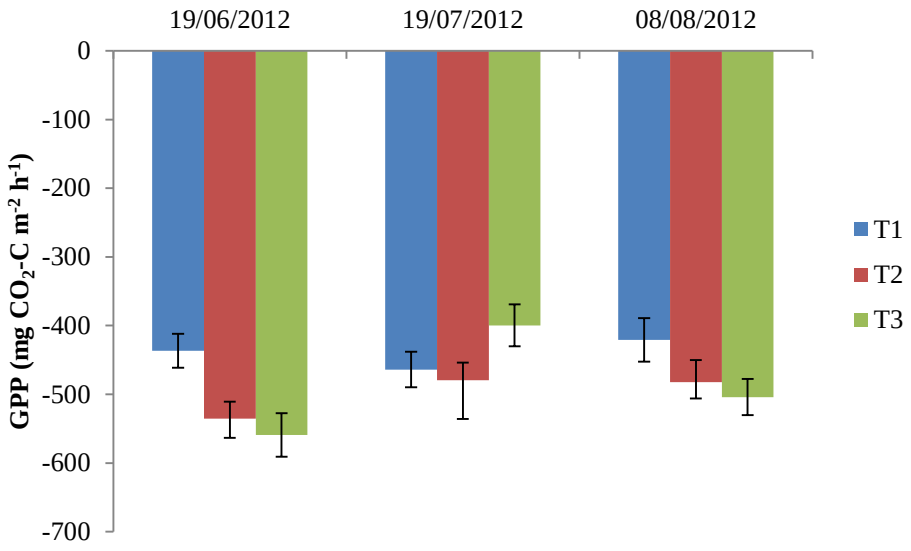

Figure 5.8 – Mean GPP rates on three dates at High Keenley Fell, split by transect. Error bars represent standard error. n = 12.

5.3.5 Effect of other environmental factors on CO_2 fluxes

Five environmental factors were investigated for effects upon R_{eco}, NEE and GPP, and the results of regression analysis are shown in Table 5.5 with the respective R values. Canopy height only correlated significantly with R_{eco} in August, with ecosystem respiration increasing with increasing canopy height (Figure 5.9). CO_2 fluxes did not significantly correlate with soil water content. R_{eco} and GPP were positively correlated with PAR in June; however this may be also be driven by air temperature (Figure 5.10 and Figure 5.11). R_{eco} also positively correlated with air temperature in both June and August (Figure 5.12; 5.13). GPP was also positively correlated with air temperature in August (Figure 5.14). Finally, NEE decreased significantly with soil temperature in August, (Figure 5.15).

Table 5.5 – Results for regression analysis relating environmental variables to CO_2 exchange measures split by month. Significant *F* ratios at $P < 0.1$ are highlighted. ~ - $P = 0.05$-0.1; ***** - $P = 0.01$-0.05; ****** - $P = 0.001$-0.01; ******* - $P < 0.001$. R values signal positive (+) or negative (-) trend in correlation.

		R_{eco}		NEE		GPP	
Variable	**Month**	***F***	**R**	***F***	**R**	***F***	**R**
Canopy Height	**June**	0.022	+0.03	0.233	+0.08	0.225	+0.08
	July	0.156	-0.07	2.23	+0.26	1.56	+0.21
	August	**5.397***	**+0.37**	0.239	-0.08	1.53	+0.21
SWC (%)	**June**	0.043	+0.03	0.037	+0.03	0.073	+0.05
	July	0.76	-0.15	0.314	-0.1	0.736	-0.15
	August	0.22	-0.08	0.003	-0.01	0.155	-0.07
PAR	**June**	**6.664***	**+0.164**	0.192	+0.07	**2.878~**	**+0.28**
	July	1.287	+0.19	0.024	+0.03	0.321	+0.09
	August	0.029	-0.01	2.763	+0.27	1.262	+0.19
Air Temperature	**June**	**16.412*****	**+0.41**	0.098	-0.05	2.56	+0.26
	July	0.62	+0.13	0.023	+0.03	0.188	+0.23
	August	**11.243****	**+0.49**	0.112	-0.05	**3.93~**	**+0.32**
Soil Temperature	**June**	0.472	+0.11	2.477	-0.26	0.762	-0.15
	July	0.115	-0.05	0.445	-0.11	0.576	-0.11
	August	0.109	+0.05	**6.171***	**-0.39**	4.462	-0.26

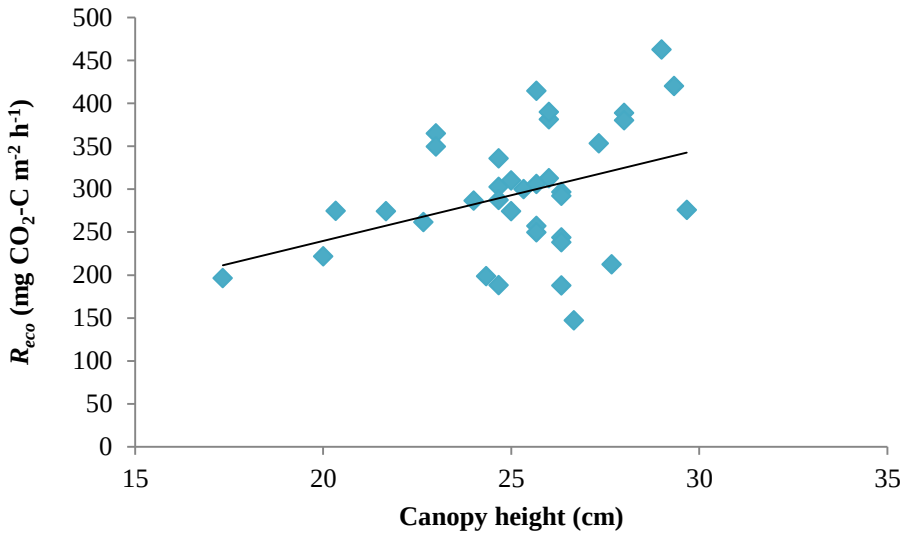

Figure 5.9 – Ecosystem respiration (R_{eco}) as a function of canopy height across all ozone treatments in August 2012, with linear regression. n = 36.

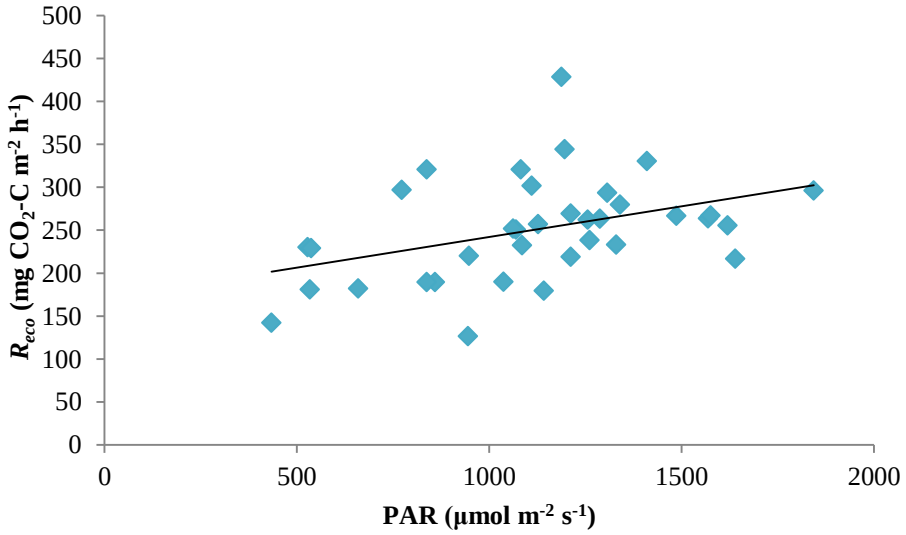

Figure 5.10 – Ecosystem respiration (R_{eco}) as a function of photosynthetically active radiation (PAR) across all ozone treatments in June 2012, with linear regression. n = 36.

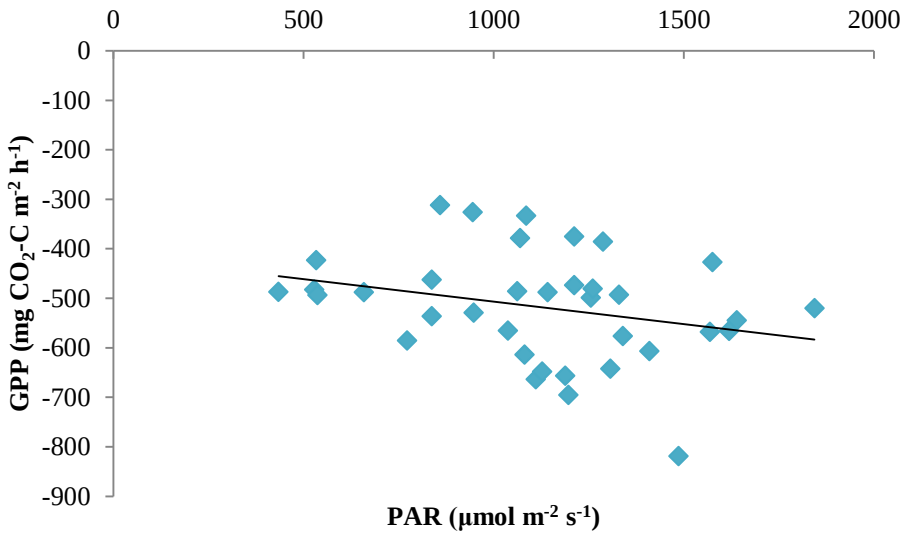

Figure 5.11 – Gross primary production (GPP) as a function of photosynthetically active radiation (PAR) across all ozone treatments in June 2012, with linear regression. n = 36.

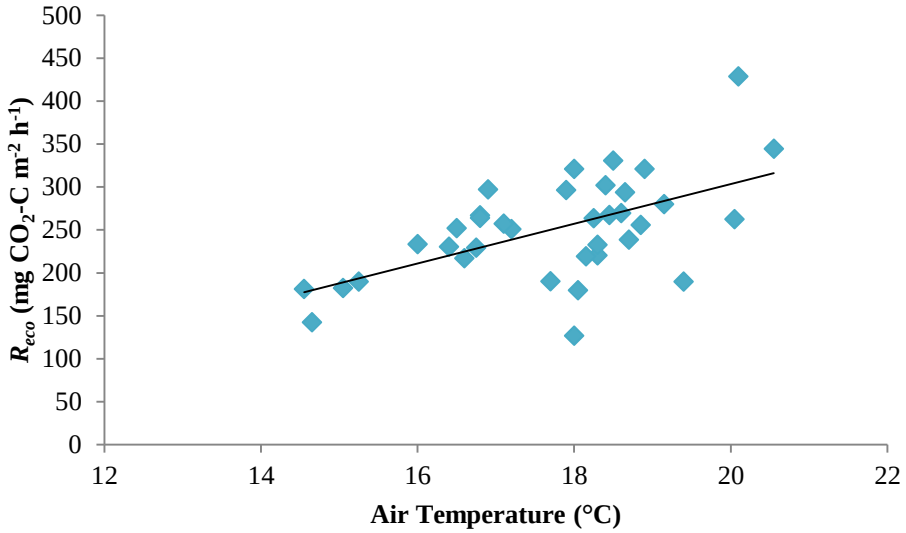

Figure 5.12 – Ecosystem respiration (R_{eco}) as a function of air temperature across all ozone treatments in June 2012, with linear regression. n = 36.

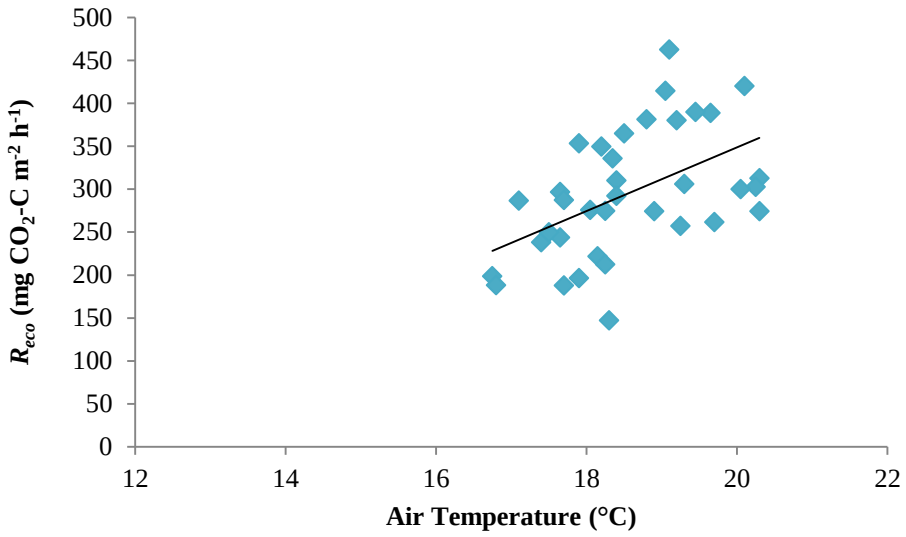

Figure 5.13 – Ecosystem respiration (R_{eco}) as a function of air temperature across all ozone treatments in August 2012, with linear regression. n = 36.

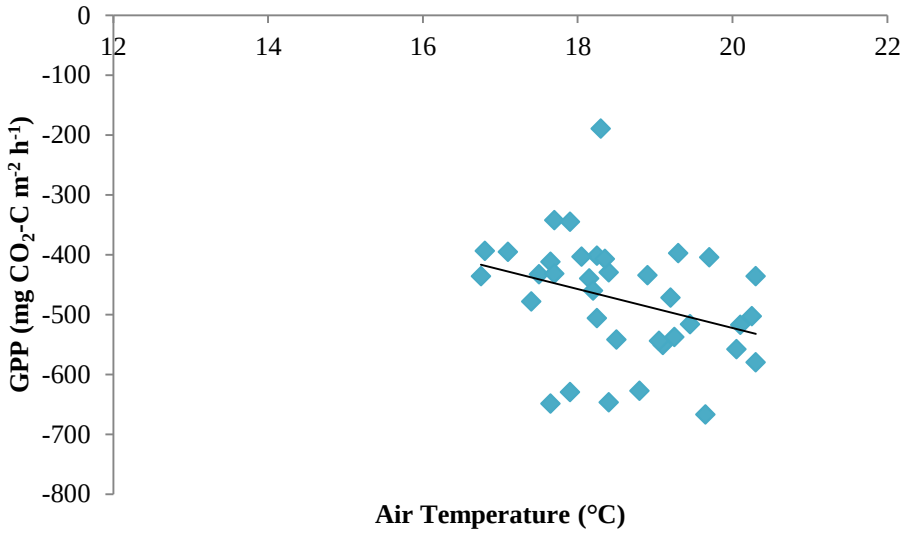

Figure 5.14 – Gross primary production (GPP) as a function of air temperature across all ozone treatments in August 2012, with linear regression. n = 36.

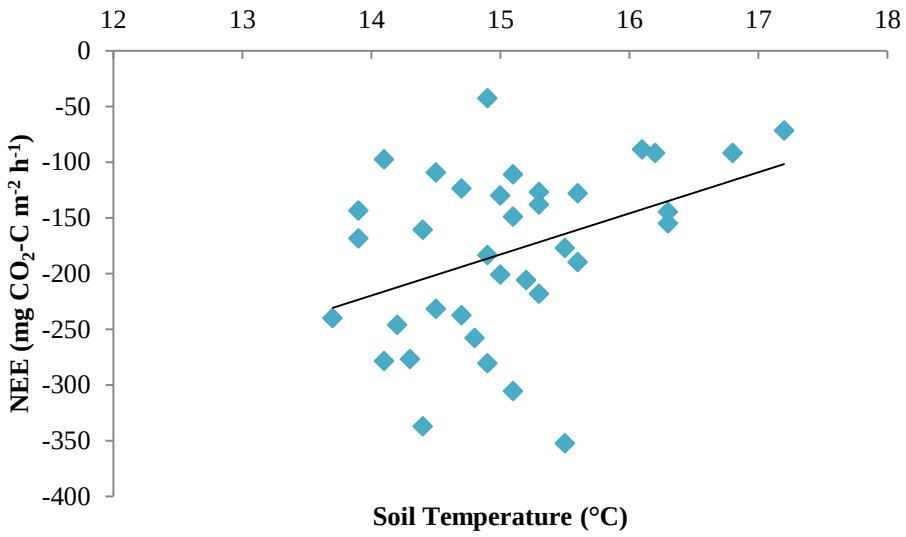

Figure 5.15 – Net ecosystem exchange (NEE) as a function of soil temperature across all ozone treatments in August 2012, with linear regression. n = 36.

5.4 Discussion

In 2012, the High Keenley Fell grassland was a net sink for CO_2, achieving an average daily gross primary production (GPP) rate of -2.3 g CO_2-C m^{-2} across all control plots between 18/6/12 and 8/8/12 when based on light and dark periods of 16.6 hours, and 7.4 hours respectively. However, this may be diminished by peak ozone episodes as suggested by GPP measurements at the 2.5 m treatment. The fact that the grassland is a net sink over summer months is consistent with findings in an alpine grassland, and American prairie, though production was comparatively lower at High Keenley Fell (Wagai *et al.*, 1998; Volk *et al.*, 2011).

In this section, effects of ozone on ecosystem respiration, and net ecosystem exchange and GPP will be discussed, with the effects of other environmental variables, and findings from 2008 measurements discussed to provide further context.

5.4.1 Ecosystem respiration

There was no significant effect of ozone treatment upon R_{eco} (Figure 5.4). This is consistent with findings from the analysis of CO_2 flux measurements from 2008, when there was no

significant effect of ozone in June, July or September (Figure 5.16). The findings are also in agreement with Volk *et al.* (2011).

R_{eco} in 2012 was on average much lower than in 2008: growing season averages (June-August) for 2012 were on average over 40% lower than 2008 measurements (June-September).

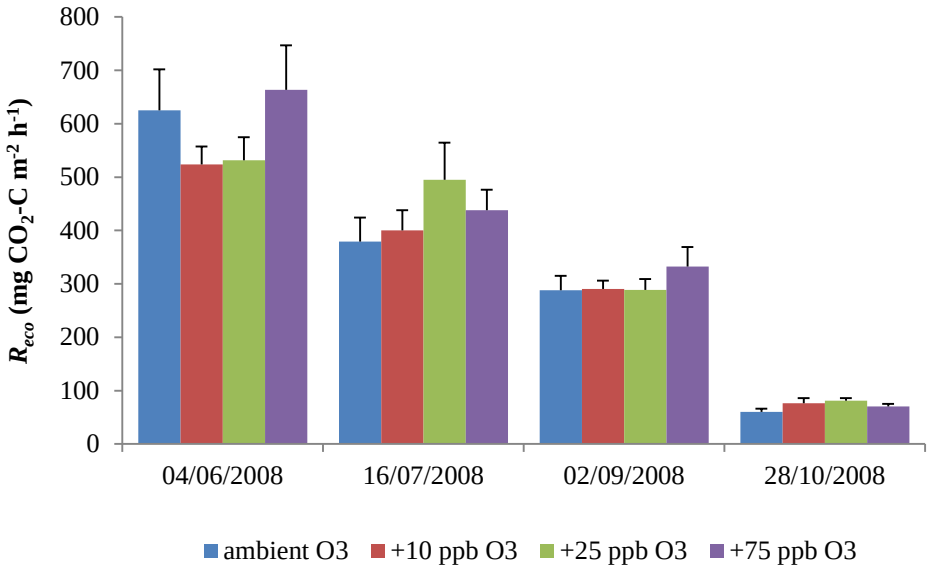

Figure 5.16 – Mean grassland R_{eco} rates on three dates at High Keenley Fell, split by ozone treatment in 2008. Error bars represent standard error. n = 9.

There were significant transect effects within treatment (Figure 5.5), suggesting spatial variation in ecosystem respiration across the field site. An environmental gradient is probably responsible. Extreme rainfall led to higher soil water content (SWC) in 2012 when compared to the 2008 growing season (Figure 5.17). Variation was also seen between transects, with transect 2 wetter than the others, suggesting SWC could have driven the transect effect (Figure 5.18). Davidson *et al.* (1998) reported reduced CO_2 fluxes when soils were waterlogged, therefore a relationship was anticipated. However, the consistently high SWC measured across the site and season may not have had enough variation to deliver impacts. Volk *et al.* (2011) found that R_{eco} was strongly associated with both soil temperature and volumetric SWC, with a suggestion that SWC is the driving factor when in drought conditions (SWC < 30%) – a factor that was not an issue at High Keenley Fell, where mean SWC was above 45% throughout the season.

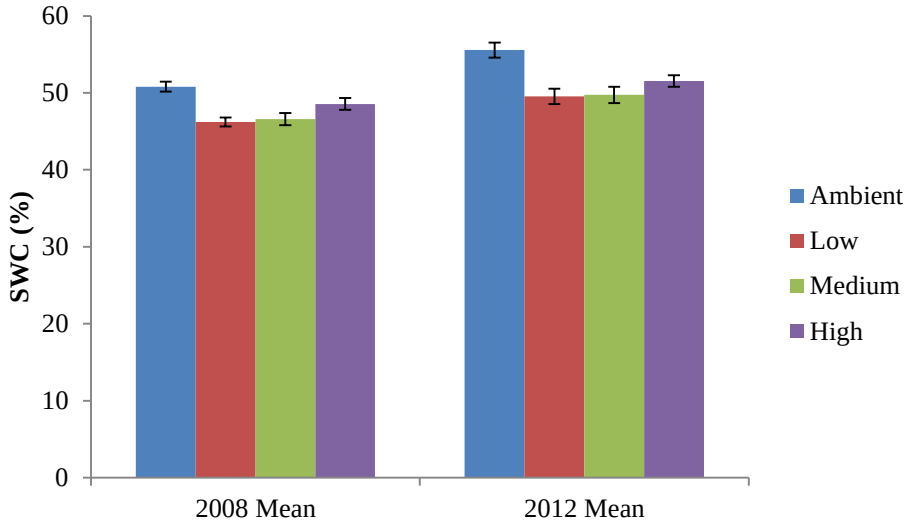

Figure 5.17 – Volumetric soil water content (SWC) (0-6 cm) at High Keenley Fell averaged across the 2008 (June-September) and 2012 (June-August) growing seasons, split by treatment. Error bars represent standard error. *n* = 9.

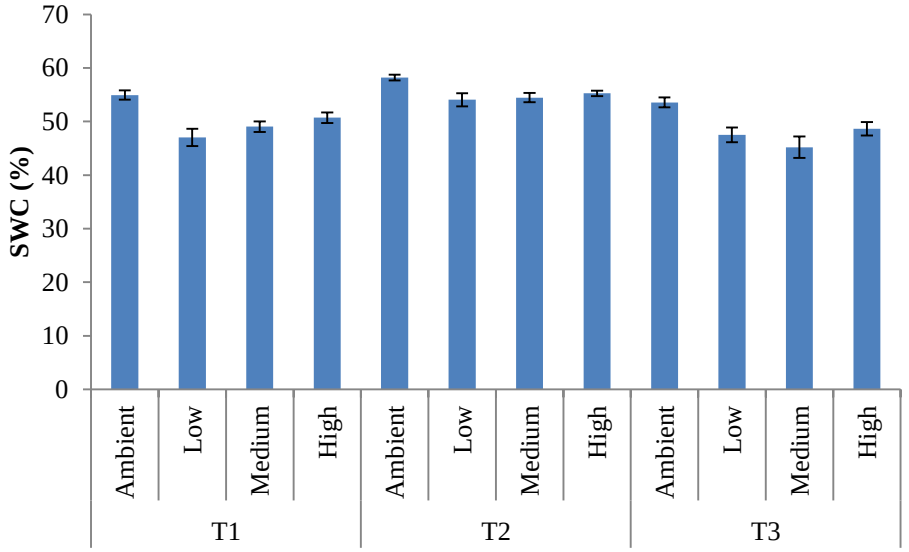

Figure 5.18 – Volumetric soil water content (SWC) (0-6 cm) at High Keenley Fell averaged across the 2012 growing season, split by treatment within each transect. Error bars represent standard error. *n* = 3.

Canopy height, photosynthetically active radiation (PAR) and air temperature were all positively correlated with R_{eco}. The impact of canopy height is easily explained by higher canopy heights suggesting larger leaf areas within mesocosms, thus implying a higher

potential for respiration within the mesocosm. PAR and air temperature are positively correlated (F = 43.44; P = <0.001; R = +0.539; data not shown), thus it is expected for both to positively correlate with R_{eco}. The findings corroborate with a grassland study in the Yellowstone National Park, which found PAR to be a driver of seasonal variation in flux when combined with soil moisture and temperature, and of diurnal variation when combined with air and soil temperature (Risch and Frank, 2010). However, soil temperature and SWC did not correlate with R_{eco} in this study. An Austrian alpine meadow study also found that PAR is a strong driver of NEE, but not R_{eco} as the factor was measured during darkness hours (Koch *et al.*, 2008), though these findings are not consistent with the present study.

5.4.2 Net ecosystem exchange and gross primary production

There was no significant effect of ozone exposure on NEE or GPP, though in both cases there was a general downward trend in both fluxes as ozone was elevated, with a marked, but non-significant reduction between ambient and 2.5 m (+75 ppb long term ozone elevation; Figures 5.6-5.7). As there was no such trend affecting R_{eco}, it is suggested that the trend in GPP is driven by the trend in NEE. Moreover, this trend was not seen in 2008 NEE or GPP measurements, suggesting this is an effect of ozone (Figures 5.19-5.20). Between control and 2.5 m plots, NEE was reduced by 14% and GPP by 9% as a growing season average, suggesting the extreme exposure in June caused foliar injury that led to reduced photosynthetic activity (Andersen, 2003; Volin *et al.*, 1998). An alternative explanation for the changes in NEE and GPP could be a cumulative impact of long-term low level O_3 elevation. In 2011, there was a significant effect of ozone on total above-ground biomass (F = 5.536; P = 0.003; data not shown), and there was a significant reduction in biomass between control and 2.5 m plots, suggesting these reductions in NEE and GPP could also be a result of long term exposure to elevated background ozone, which was historically much higher than experienced in 2012 (Figure 2.2).

The non-significant trend in GPP reduction in 2012 is consistent with findings in literature. Volk *et al.* (2011) found that ozone significantly altered GPP at concentrations of 70 ppb as a 6 month April-October mean, reducing GPP compared to control by 8%, and this was apportioned to photosynthetic system damage. Additionally, Kanerva *et al.* (2007) found in a three year experiment that ozone significantly reduced GPP at seasonal concentrations of approximately 45 ppb in the second (-10%) and third (-16%) years of the study. These findings from separate studies suggest that ozone was indeed having an effect on GPP at

High Keenley Fell for the reasons previously suggested. There are no known papers that allow for direct comparison of NEE rates in grasslands, therefore these suggestions cannot be backed up.

Overall, 2012 NEE and GPP trends were similar to 2008 trends (Figures 5.19-5.20); however NEE in 2012 was almost 50% lower, and GPP 46% lower on average when comparing growing season means. This suggests extreme climatic conditions impacted upon 2012 measurements: SWC has already been shown to be higher in 2012 (Figure 5.18); soil temperature in June 2012 was over 2°C lower than in early June 2008 (data not shown); though PAR was consistently higher in 2012 (data not shown). This suggests the lower soil temperature in June 2012, combined with higher SWC, was driving the diminished CO_2 exchanges.

While there was no effect of transect on NEE rates, there was a significant response in GPP (Figure 5.8), implying GPP was driven by environmental factors. Only soil temperature proved to be a significant driver in 2012, reducing NEE as soil temperature increased (Table 5.5); this finding was corroborated by Koch *et al.* (2008).

Regarding GPP, Transect 1 was significantly higher than the other transects in June 2012, indicating a spatial gradient across the site, though this could be a result of the high variation in ozone exposure between transects (Figure 2.5). However, climatic factors could also be driving this, as PAR and air temperature both proved to be positively correlated. The positive relationship between GPP and air temperature has already been corroborated (Raich and Schlesinger, 1992), as has the relationship between GPP and PAR (Risch and Frank, 2010). This suggests these were likely to be drivers in GPP, but the variation has not been entirely explained in this study.

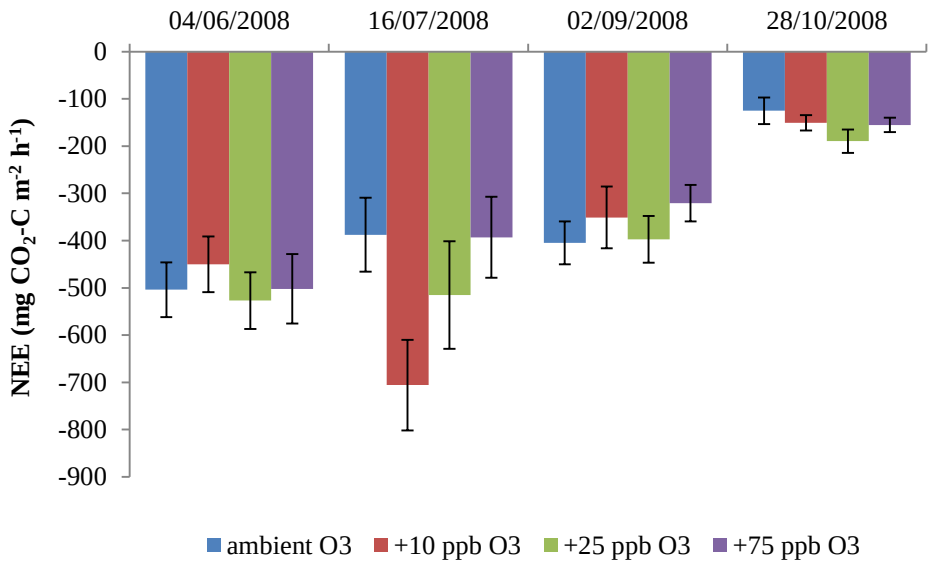

Figure 5.19 – Mean grassland NEE rates on three dates at High Keenley Fell, split by ozone treatment in 2008. Error bars represent standard error. *n* = 9.

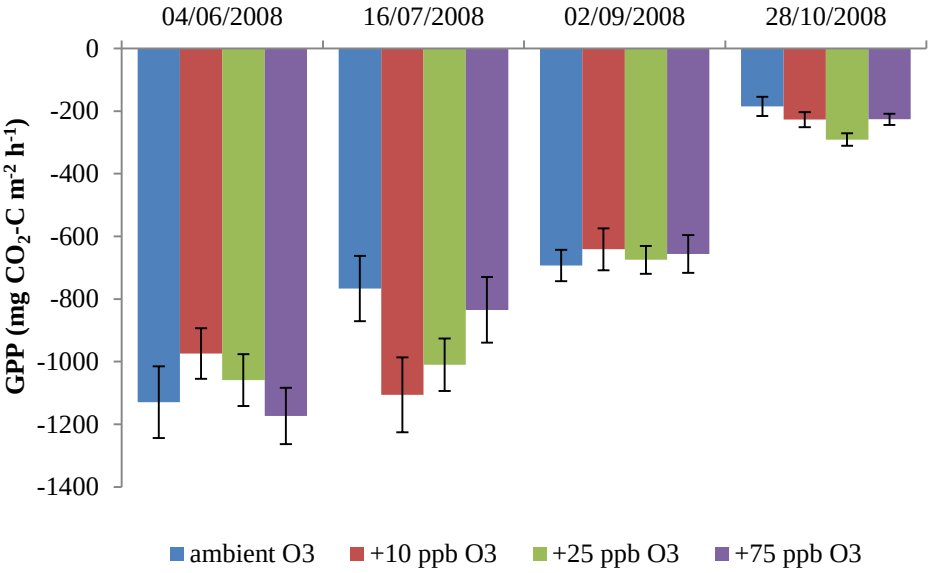

Figure 5.20 – Mean grassland GPP rates on three dates at High Keenley Fell, split by ozone treatment in 2008. Error bars represent standard error. *n* = 9.

5.4.3 Study Shortcomings

Wider issues relating to the experimental set up and ozone exposure will be discussed in Section 6. There are however shortcomings pertaining to just this study, such as the inability to calculate an annual CO_2 flux for grassland at High Keenley Fell. The study only contained

three measurements, spread between June, July, and August 2012, when similar studies used measurements over 5 or 6 months – Volk *et al.* (2011) measured from April to October; while Kanerva *et al.* (2007) measured from 15th May-10th September. If a longer measurement period had been employed in this study, then early growing season CO_2 fluxes would have been identified, as well as allowing for measurements of CO_2 fluxes post-hay harvest. Ideally, late autumn/winter measurements would also have taken place weather-permitting, to gain an understanding of how the ozone exposure had affected autumn/winter fluxes while plants are less active.

Another shortcoming of this study was the impact of extreme rainfall coupled with drainage failure in the field site, which is further discussed in Section 6. When undertaking measurements in July and August, it was apparent in some control pots that soil was saturated, as there was standing water. Davidson *et al.* (1998) reported reduced GPP with saturated soils, and while there was no significant effect when analysing field soil water content (SWC), there would likely be an effect if SWC values had been measured in a wider range.

6 General Conclusions

6.1 Summary of key findings

This present study had three main aims, each covered by a chapter within this thesis. This study aimed to establish whether ozone exposure led to changes in: (i) flower abundance and timing in selected species; (ii) *Rhinanthus minor* nectar volume and composition; and (iii) ecosystem CO_2 exchange. Main findings are summarised below.

The 2012 field campaign at High Keenley Fell presented the following key findings:

1. Flower abundance in *Dactylis glomerata* and *Conopodium majus* responded positively to elevated ozone, with the former exhibiting highest abundance in the lower ozone elevation of 15 ppb, and the latter responding best to the highest exposure of 25 ppb;
2. Flower abundance in *Ranunculus acris* declined significantly under elevated ozone exposure, with marked effects at both exposure levels;
3. Peak flowering was delayed by up to two weeks by long term elevation of ozone by 25 ppb in *Festuca pratensis* and *Trisetum flavescens*;
4. Nectar volume, and concentrations of sugars within nectar of *Rhinanthus minor* were unaffected by peak ozone exposure;
5. Ecosystem respiration, net ecosystem exchange and gross primary production were not significantly affected after 6 years of ozone exposure at 15, 25, or 75 ppb;
6. Variations in ecosystem respiration were driven by canopy height, photosynthetically active radiation and air temperature;
7. Variations in net ecosystem exchange were driven by soil temperature;
8. Variations in gross primary production were driven by photosynthetically active radiation and air temperature.

Shortcomings related to the experimental set up are addressed in the following section, followed by the wider implications of the key findings. Finally, suggestions for further work are discussed in the context of each research chapter.

6.2 Shortcomings within the study

While the novel nature of this research has provided some significant and intriguing findings, there were some limitations to the experiment overall. Shortcomings pertaining to specific sections of the research reported herein are covered in their respective chapters.

One main shortcoming to this study was the variability in ozone exposure at High Keenley Fell. Ozone fumigation commenced in 2007, with target fumigations for 2007-09 roughly half of targets for 2010 and 2011 – the previously reported value of +15 ppb at 10 m. The exposure design relied upon specific wind directions that meant up until 2011, fumigation only occurred roughly 50% of the time the system was online. In 2012 there was very little fumigation as a result of confounding climate and equipment issues, other than one extreme release of ozone in mid to late June 2012, where concentrations were on average 309 ppb at 2.5 m, 140 ppb at 5 m, and 59 ppb at 10 m downwind, with high variation between transects. Thus, the effects seen in ozone treatments reported in this study cannot be apportioned to specific ozone exposure values, as it is not known whether the responses reported from 2012 are as a result of an acute exposure brought by the extreme ozone release, a carry-over response from five years of previous exposure to elevated ozone, a cumulative effect of previous exposure, or a combination of all three. However, what can be drawn from the findings in this study is that, where species have responded to ozone exposure and is consistent with responses reported in previous study years, then there is confidence in concluding that the effect is a result of ozone exposure.

Another shortcoming to this study was the inclement nature of the climate at High Keenley Fell in 2012. Extreme weather led to serious failures in the experimental equipment as a result of power supply and connectivity issues, and also led to the major failure of drain systems installed below the field post-war which led to an outlet channel being mechanically dug by the landowner to release soil water. Severe waterlogging occurred not only across the site, but with a gradient in the experimental treatments and transects, as shown in Figure 6.1. The wettest transect was Transect 1, closely followed by Transect 3, which was close to a natural downslope channel where surface water remained for much of the summer. Control plots were markedly wetter than any other, explained by the plots being in the most upslope position along the transect. Numerous papers have reported how waterlogging affects grassland species: in one study *Dactylis glomerata* and *Lolium perenne* both displayed below-mean leaf biomass when grown in wet conditions (Turner *et al.*, 2012); in another

study *D. glomerata*, *Holcus lanatus* and *Rumex acetosa* all reported diminished above ground biomass when exposed to waterlogged conditions, and in most cases was lower than when grown in drought conditions (Jung *et al.*, 2009). Thus, reduced growth as a result of waterlogging may have affected flower abundance, confounding ozone responses.

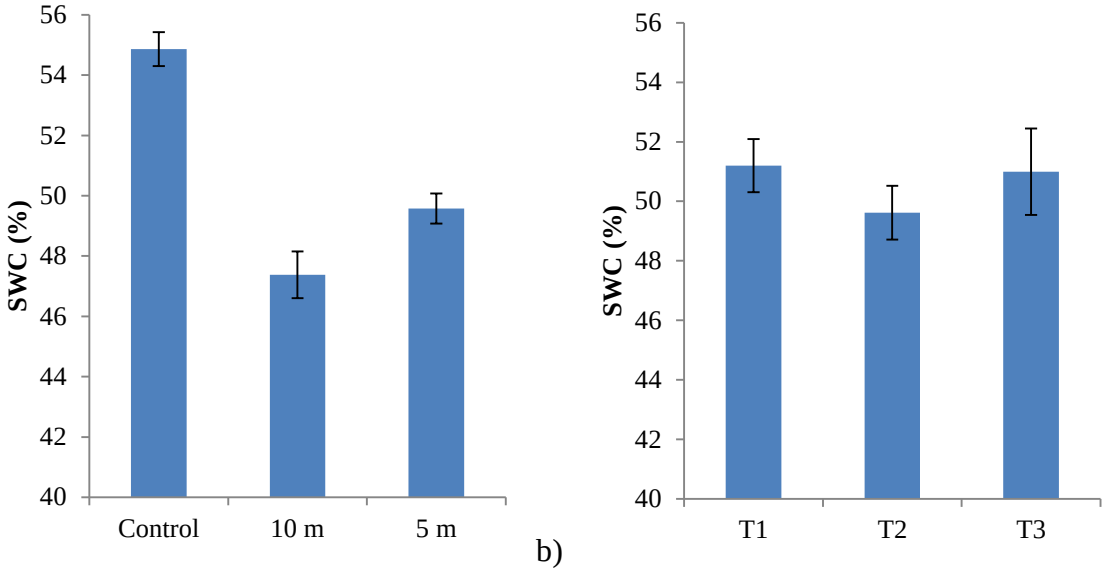

Figure 6.1 – Average volumetric soil water content in flower sampling plots from 19 June – 8 August, split by a) treatment, and b) transect. Error bars represent standard error. Mean of five measurements. n = 9 (a); 12 (b).

One final shortcoming to the overall study is the frequency of sampling. In the case of flower abundance and timing studies, the frequency was increased from 3 in previous years to 5, split by roughly fortnightly intervals in an effort to improve data quality. However, in the case of *Rhinanthus minor* nectar sampling, only one sample was taken towards the end of the flowering season. Ecosystem CO_2 exchange was also measured just three times, in line with the first, third and fifth flower counts. All three studies would benefit from more frequent sampling: flower abundance could be done weekly, or twice-weekly, to get a high resolution to abundance and timing data throughout the season. Likewise, Ecosystem CO_2 exchange could be measured throughout the growing season at more frequent intervals than this study allowed. The study of *R. minor* nectar would also benefit from more than one sampling that had occurred earlier in the season, so that a comparison of early and late season nectar composition could be drawn.

6.3 Wider implications of findings

6.3.1 Flower abundance

Dactylis glomerata, a grass species that responded positively to elevations of 15 ppb in background ozone over the course of 6 years of fumigation, is classed as an invasive species in North American grasslands, and is seen as an exploitative competitor (MacDougall and Turkington, 2004). In a study investigating the effect of removal of *D. glomerata* in a North American oak savannah, the same authors found in a separate study that removal increased species richness, with uncommon exotic grasses reappearing after one year as they regenerated from the system seed bank, with a longer term shift away from perennial grasses and towards perennial forbs. However, these effects were only seen in species already present in the savannah (MacDougall and Turkington, 2005). Thus, if background ozone increased by 15 ppb in the coming decades, *D. glomerata* may become more dominant than it currently is, with these findings suggesting that both species richness and diversity would be reduced by the dominant behaviour of this one species.

However, there are also positive factors in increasing abundance of *D. glomerata*. It is commonly included in seed mixtures for grazing pastures and grasslands managed and cut for hay, as it has a high forage quality and protein content, and shade tolerance, making it useful for sowing in orchards (Pontes *et al.*, 2007). Moreover, MacDougall and Turkington (2004) reported the positive impacts of the species in savannah settings: through the dominance shown by this species, they noted that it is very effective at reducing invasive woody growth in such ecosystems, thus retaining savannah systems and preventing natural succession. Therefore, the increasing dominance shown by the species also has the potential to be a positive factor in conservation and agricultural contexts as background ozone concentrations rise.

Ranunculus acris is a perennial forb, and, while producing fruit and seed following flowering each year, it generally reproduces via rhizomal offshoots in the autumn, with only small amounts of seed reproduction (Sarukhan and Harper, 1973). The effect of background ozone elevated by 15 ppb and 25 ppb reducing the abundance of flowers reported in this study is likely to reduce the fruit and seed production of *R. acris*; however due to the dominant reproductive behaviour being via offshoots, this is unlikely to affect the fecundity and reproduction of this species. The effect of *D. glomerata* and other competitive species

becoming more dominant within a grassland system as ozone increases is likely to have a stronger impact through competition stress than reduced flower abundance.

Conopodium majus is the only species significantly affected in this study that has a culinary use, as the bulbs grown below ground, known as pignuts, are a delicacy foraged in the wild. The large increases in flower abundance where ozone was elevated by 15 or 25 ppb indicate that the plant has reproduced more successfully in elevated ozone conditions over the 6 year study. Thus, this suggests that the incidence of pignuts would increase, creating the potential for the species to be collected on a small-scale commercial basis by foragers.

6.3.2 Flower timing

Festuca pratensis is cultivated as a major seed crop, with Northern European harvests typically occurring in late July following seed maturation (Havstad, 1998). Therefore, if peak flowering is delayed by two weeks by a long term exposure to background elevations of 25 ppb, then this would lead to a delay in harvesting seed crops and the potential for crop quality to be spoiled pre-harvest. Additionally, delayed flowering and seed development could also impact upon the seed fecundity and species reproduction rate in natural settings and as the species only reproduces by seed and is not self-fertile (Rognli *et al.*, 2000). This would have the potential to significantly affect populations in the long term.

Trisetum flavescens is common in established pastures and has been proved to be a source of Vitamin D_3, and effective as a remedy to Vitamin D deficiency in animals (Morris and Levack, 1982). However, while the vitamin is not thought to be at toxic concentrations, the species has been linked to calcinogenic symptoms in livestock, even though the grass is highly palatable to the livestock that are sickened by it (Hubbard, 1984; Mello, 2005). Thus, a delay in the flowering potentially brought on by long term exposure to background ozone elevated by 25 ppb could, like in *F. pratensis*, reduce seed fecundity and therefore reproduction and biomass. This would prove positive in situations where *T. flavescens* is unwelcome in a pasture due to its properties; however could prove negative if the species is cultivated as a feed to alleviate Vitamin D deficiency.

However, the response seen in flower timing could be due to the peak exposure of approximately 140 ppb in mid-June, rather than long term exposure to elevated background ozone. Thus, the question is raised as to the real importance of such findings, as episodes in

excess of 140 ppb are very rare, though not unheard of in rural settings – Swiss alpine regions have received spikes in excess of 250 ppb (Davies and Schuepbach, 1994).

6.3.3 *Rhinanthus minor* nectar volume and sugar composition

Assuming that there is a possible effect of ozone upon the sugar composition of *R. minor* nectar, any changes in composition could have consequences for pollinators. Ollerton *et al.* (2007) suggested that parasitic plants co-evolved within the same niche as their host; however do not share pollinators. Thus, a change in nectar composition potentially makes the nectar less attractive or unsuitable for the pollinators specific to *R. minor*, which could reduce the availability and diversity of food and reward available to pollinators. This would then lead to less plant pollination and seed production, and a diminished food source for insectivorous upland bird species.

6.3.4 Ecosystem CO_2 exchange

The non-significant reduction in NEE and GPP at 2.5 m when compared to control plots are likely an effect of peak episode ozone exposure. Such negative responses have been reported to be as a result of damage to the plant photosynthetic system, thereby reducing C entering the soil and subsequently respired from the soil (Andersen, 2003; Volk *et al.*, 2011). This response therefore has implications on ecosystem CO_2 exchange on a wide scale, as it suggests that a peak ozone episode may cause a lingering reduction in GPP as a result of ozone injury, therefore reducing the carbon sink effect of grasslands. However, the peak ozone episode at High Keenley Fell of approximately 309 ppb would be unlikely to be matched in natural conditions.

6.4 Opportunities for further study

6.4.1 Carry-over effects of ozone on flowering behaviour of grassland flora

Further study into flowering behaviour could focus on several areas, one being the way in which abiotic factors interact with ozone exposure in a field setting. While work has been published looking at the combination effects of water stress and ozone on flowering in pot-based experiments (Hayes *et al.*, 2012), there is little based on natural communities or in field settings. Another worthwhile extension would be to study the number of flowers per plant,

with the aim of finding whether resource allocation changes are occurring under ozone stress as suggested by Hayes *et al.* (2011). Additionally, extending the study to investigate seed production per flower and germination success rate as carry-over effects of previous exposure would be of interest to the wider conservation community, especially when considering annuals important to the species composition such as *Rhinanthus minor*.

A major extension to the study, which would require a lot of research effort, would be to investigate the combination effects of competition and ozone upon the flowering behaviour. While this would require the integration of above-ground biomass and full species flower counts in the field, any findings could be backed up by the findings of pot-based studies that pair species thought to have competitive interactions, with the aim of attempting to map how an entire grassland community might change in the face of inter-species competition and previously elevated background ozone.

When regarding flower timing for further study, a key extension to the study would be to study the changes in more detail, by not only increasing the frequency of counts to 2-3 per week – perhaps even daily if manpower can be achieved; but by also extending count periods to investigate changes in first and last flowering dates. Additionally, timing studies could also look at the first fruit/seed set for species that reproduce by seed. This would give a full and clear phenological record for species, and will allow for further investigation into such changes by having more than one endpoint based upon peak flowering.

6.4.2 Effect of ozone upon nectar of *Rhinanthus minor*

There are many factors that potentially naturally alter the nectar composition and volume of *Rhinanthus minor* flowers, which require further investigation to better understand the variability seen in nectar composition.

Firstly, a study could be undertaken to gain an understanding of how nectar composition changes with the flower age. Another study could investigate how composition varies across the flowering season, as there may be a significant effect seen at another part of the plant's season. The effect of soil moisture, humidity, and radiation could also be investigated, to provide a well-rounded understanding of the nectar chemistry relating to *R. minor*. Following this work, a new ozone fumigation study could be undertaken, exposing plants to elevated ozone for the duration of the plant's life cycle, to see whether elevated background ozone has an effect upon the nectar.

Additional to the studies of the effect of factors upon secreted nectar, a study could be designed to investigate whether elevated background ozone has a morphological impact upon the nectary, akin to the study conducted by Davis (2003) into the effects of elevated CO_2 on the nectary.

6.4.3 Effect of ozone upon ecosystem CO_2 exchange

This study focused upon one year's data, with some comparison to measurements taken in 2008. As the field site is no longer in commission, any extensions to this study would need to focus upon pot- or core-based studies. Thus, one major extension to the study of ecosystem CO_2 exchange would be to study the stomatal conductance of species within each mesocosm. Previous studies have shown that ozone exposure alters stomatal closure under water stress conditions in both arable crops and grassland species such as *Dactylis glomerata* (Hayes *et al.*, 2012; Pleijel *et al.*, 2007), though in different systems to that studied at High Keenley Fell. Another study looking at the effects of ozone on stomatal conductance found that poplar tree guard cells took longer to react to elevated CO_2, and that photosynthesis was reduced in elevated ozone, regardless of CO_2 concentration (Dumont *et al.*, 2013). Thus, this would be a valuable extension to the study of ecosystem CO_2 fluxes.

A third extension to this study would be to investigate the combination effects of elevated ozone and nitrogen (N) deposition on carbon flux. Studies in a subalpine grassland using monoliths showed sensitivity to N deposition, but not ozone or a combination of N x ozone (Bassin *et al.*, 2013; Volk *et al.*, 2011). However, they reported shifts in responses over time as a result of N deposition causing species composition shifts, but no change in productivity relating to this. These results suggest interesting interactions could occur between the two variables, and that an *in situ* study looking to this for British upland grasslands could prove beneficial.

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